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ORIGINAL ARTICLE

Local Interleukin-2 Immunotherapy of Breast Cancer: Benefit and Risk in a Spontaneous Mouse Model

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Abstract

Earlier, naturally arising mammary cancer in BLRB female mice was shown to reproduce some key pathological characteristics of the familial set of human breast cancer. Then we advanced a novel 3S–paradigm of anticancer research that helped to develop selection criteria and to estimate benefit/risk of local interleukin-2 (IL-2) effects in this spontaneous mouse model. In this paper, the efficacy of single and triple local IL-2 doses is compared using properly selected murine BLRB females based on our previously published data. Only BLRB females bearing spontaneous mammary tumors without subclinical period were used. The tumor growth rate and recipient survival of single and triple IL-2 applications were compared with corresponding parameter values of untreated control. Tumor growth rate was decreased in both experimental groups versus control parameter values. Single IL-2 application resulted in a significant prolongation of the average survival time while triple application caused acute tumor rejection in some females decreasing the survival time of the rest of the recipients. As a result, proper treatment protocol in accurately selected females allowed increasing the complete response rate to 14% in spontaneous mouse model of breast cancer. In conclusion, our approaches may demonstrate the principle methodology developing preselection procedure for breast cancer patients for local IL-2 therapy application.

Keywords Breast cancer · Interleukin-2 · 3S–paradigm · Spontaneous mouse model · BLRB

Introduction

Stimulating anticancer response for therapeutic benefit in cancer patient is a favorable goal of oncology research. Interleukin-2 (IL-2) is a well-known cytokine; the feasibility of IL-2 immunotherapy to cause tumor rejection has been studied for more than three decades [1]. We believe that the IL-2 therapy of breast cancer (BC) has only been of limited use as the results were ambiguous both in BC clinics (long time ago [2, 3] and currently [4, 5]) and in the experimental research [6, 7]. The dual role of IL-2 as the main regulator of immune responses has been demonstrated as this cytokine promotes the development of either tolerance or antitumor immunity in both cancer patients and tumor-bearing mice [8, 9]. Moreover, IL-2 was shown a long time ago to both promote and inhibit hormone dependent but not hormone independent BC cells depending on the IL-2 dose in vitro and in vivo [10]. In addition, carcinoma cell lines from some clinical BC specimens were shown to express receptors for IL-2 and proliferate in reply to the cytokine [11]. These spectra of contradictory IL-2 characteristics add complexity to the use of extensive basic knowledge about this cytokine in order to obtain a real clinical benefit for the BC patient. The success or the failure of IL-2 therapy for an individual patient may be achieved as a result of a delicate balance in the interaction of various immune cell populations caused by exogenous IL-2 application [8, 9]. Therefore, the development of a strategy to enhance the effectiveness of IL-2 against BC is of current interest [4, 7, 9].

Earlier we demonstrated that the spontaneous BLRB mouse model (naturally arising mammary cancer (MC) in old mice) reproduces some key pathological and immunologic characteristics of the familial set of human BC [12, 13]. The conventional spontaneous mouse model of the human BC has a number of advantages (age related comorbidities are of prognostic value similarly to human BC [14]) compared to traditional transplanted models and modern genetically

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engineered ones, since the latter does not fully reproduce the pathogenesis of the BC as a multifactorial disease [13, 15]. However, the spontaneous tumor model has several limitations: it is restricted by the sample size available at the moment of the experiment and has an expressed heterogeneity of the female tumor-bearing population in a number of both host and mammary tumor characteristics. Previously, we developed new criteria for evaluating the effectiveness of IL-2 therapy in the spontaneous murine MC models [13] and showed that the IL-2 effects, in addition to the regimen, depends both on the physiological characteristics of the tumor-bearing female mice (hormonal status, age, immune status) and tumor growth characteristics (the duration of a subclinical period, the growth rate after clinical manifestation, and the tumor size at the therapy start) [16–18]. The presence of a subclinical period (more than two weeks before tumor clinical manifestation, i.e. tumor diameter near 4–5 mm, preinvasive step) and application in the advanced stage (tumor exceeds 10 mm) limit the therapeutic efficacy of IL-2 when used after clinical manifestation of MC [17]. However, local IL-2 treatment started before clinical manifestation of the tumor shortens the subclinical period [16]. The aim of the current study was to compare the efficacy of two different regimens of IL-2 (single and triple local applications) using mammary tumor-bearing mice *specifically selected for immunotherapy* (i.e. standardized as much as possible for a spontaneous model): only virgin BLRB females of a similar age, bearing tumors without subclinical period and with mean tumor diameter not exceeding 6 mm at the start of the therapy.

We demonstrated that both IL-2 regimens used caused tumor growth rate delay. Single IL-2 treated recipients survived longer on average but no females were cured; while in the triple treated group some females were cured but the survival of the rest of the group was shortened.

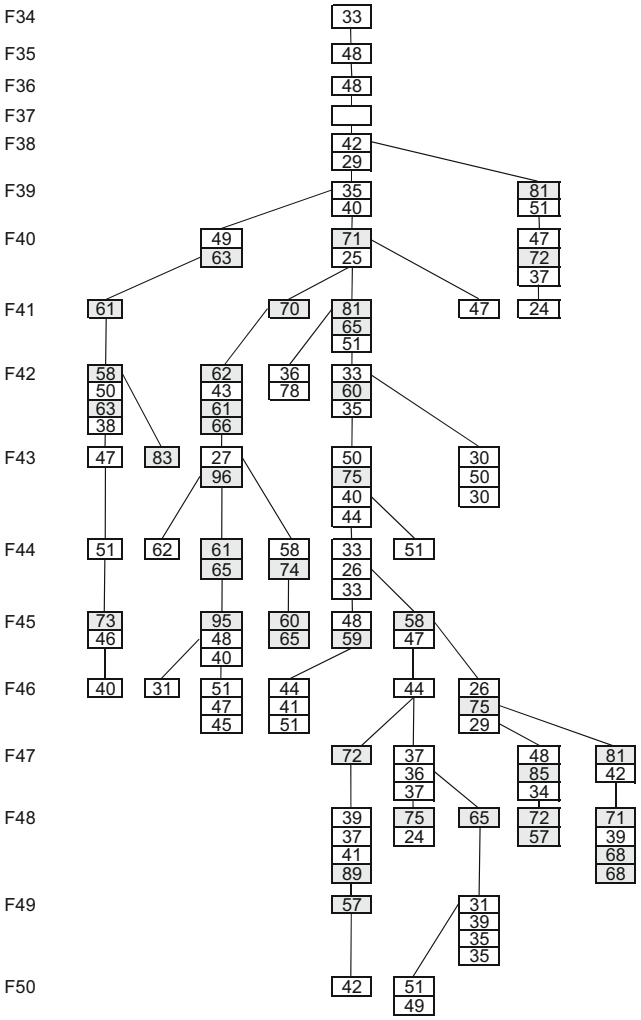

Fig. 1 Fragment of ancestor pedigree of BLRB breeding nucleus for 104 breeding females. Each figure shows time of early (<52 weeks, open square) or late (≥52 weeks, grey square) mammary cancer onset in weeks. The proportion of early and late onset daughters among early-onset mothers was estimated as 65% and 35%, respectively ($p < 0.05$, Fisher's criterion)

Materials and Methods

BLRB Mouse Model Original inbred mouse strain BLRB-Rb(8.17)1Iem (further, BLRB http://www.informatics.jax.org/inbred_strains/mouse/docs/BLRB.shtml) was maintained in a thoroughly controlled conventional (non-SPF, specific pathogen free) environment at the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences. BLRB females were characterized by a high incidence of the spontaneous (naturally arising) mammary cancer (MC). Each female was individually marked and followed as a patient during the whole life (Fig. 1). Females were examined daily for the health monitoring and weekly for the detection of subcutaneous mammary tumors.

MC usually occurred in bred and virgin BLRB females with ageing sometimes in association with ovarian/uterine lesions, similarly to the population of women with a family history of the BC [19]. A fragment of ancestor pedigree of the BLRB breeding nucleus is presented to show that murine age of MC onset in weeks is similar to the human age of BC onset in years. The vast majority of bred females got mammary cancer (the average age of 52 ± 2 weeks, Fig. 1 [13]). 63% of females got MC earlier than 52 weeks (early onset), the rest fell to a later onset subgroup. The BLRB females with early MC onset (<52 weeks of age) were predisposed to give birth to early-onset daughters [13] similarly to human data where early-onset BC (before the age of 50 years) in a family member is a well-known risk indicator for cancer among first-degree relatives [19].

Experimental Design For this study, we used virgin BLRB females ($n = 21$) aged 67.1 ± 1.7 weeks with spontaneous mammary tumors sized 5 ± 1 mm during the first two weeks

after MC detection (i.e. without subclinical period). Mean tumor diameter (MTD) was used as a measure of tumor size: (**a** + **b** + **h**)/3; where **a** is the maximal length, **b** is the maximal width, and **h** is the average height of a regularly shaped tumor. If several tumor localizations were present, the volume of each tumor was calculated according to the formula $V = 1/6\pi$ **abh** and all the volumes were summed up. The total diameter was calculated and used as the total MTD of all tumors for the female [13].

Lyophilized IL-2 (Chiron/Novartis, Amsterdam, the Netherlands) was dissolved *prior* to administration in a 0.1% solution of syngeneic mouse serum in 0.9% sodium chloride solution. The IL-2 solution was administered locally (peri- and intratumorally [6]) at a dosage of 10^5 IU of IL-2 in 0.5 ml *per* mouse *per* injection. The first experimental group with tumors of about 4–6 mm at the first detection were treated by a single IL-2 application (single IL-2 group, short course, $n = 6$). The second experimental group was treated twice at the first and second week after tumor detection; one-two weeks later (according to the individual tumor growth rate and the MTD not exceeding 10 mm). Females belonging to this group was additionally treated by the third injection of IL-2 (triple IL-2 group, prolonged course, $n = 8$). Intact mammary tumor-bearing mouse females with similar host and tumor characteristics were used as the control group (untreated control, $n = 7$).

The health status of the mice was estimated daily; the body mass and the MTD were individually measured weekly. The individual tumor growth rate was expressed as the MTD dynamics. Tumor growth rate and survival of IL-2 treated females were compared with untreated control parameter values to estimate the efficacy of the IL-2 therapy. Average MTD dynamic for 58 intact mammary tumor-bearing females was used as a historical control (from [17]).

Females at the end point (MTD more than 20 mm, body weight loss more than 15%, and/or refusal of food) were euthanized by cervical dislocation. Necropsy and histopathological analysis were performed as described: the tumor and surrounding tissues were fixed in 4% buffered formalin, paraffin sections were prepared and stained with hematoxylin-eosin using a standard protocol [12, 20].

The obtained individual data were stored and analyzed using the MS Office (Microsoft, WA) and STATISTICA 10 (StatSoft, OK). Data are presented as mean ± standard error of mean. The statistical significance of the differences was determined using the Mann-Whitney U-test.

All animal experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (US Department of Health and Human Services, National Institute of Health Publication No 93–23, revised 1985) and were approved by the Institutional Animal Care and Use Committee (http://www.ibch.ru/downloads/documents/553/Institutional_Policy_on_the_Use_of_Laboratory_Animals.pdf).

Results

We applied IL-2 locally to mammary tumors without subclinical period early after mammary tumor detection and compared tumor growth rate and survival in the single IL-2 treated and triple IL-2 treated groups in relation to corresponding parameter values of untreated control females. Both therapy modes resulted in the same extent of significant tumor growth delay compared to the control growth rate starting from the second week after the first IL-2 application (Fig. 2a; $p < 0.05$).

The average survival of females treated with a single IL-2 application was prolonged as compared to control survival (10.3 ± 0.8 versus 6.9 ± 0.9 weeks, respectively, Fig. 2b, $p<0.05$); but no recipients were cured. Remarkably, two of the eight females treated with prolonged IL-2 course demonstrated acute rejection of the primary spontaneous tumor (complete response). The rest of triple IL-2 treated group survived on average shorter than the single IL-2 treated group (5.8 ± 0.2 versus 10.3 ± 0.8 weeks, respectively, Fig. 2b, $p<0.01$), although, no significant differences from the control survival were detected.

A fragment of the tumor growth dynamics of one individual tumor with nearly complete rejection (triple IL-2 treated group, Fig. 3a, closed triangles) is shown in comparison with the average growth rate of 58 intact spontaneous mammary tumors (Fig. 3a, dotted line, data taken from (17)). However, a local relapse occurred three weeks after the rejection of this IL-2 treated tumor; the newly appeared tumor grew progressively and finally caused the death of the recipient. The histopathological analysis of this tumor at the endpoint of the recipient survival revealed the presence of an anaplastic solid carcinoma, which gradually replaced hemorrhagic cystic adenocarcinoma (Fig. 3b). The presence of a three-week disease-free period additionally increased the individual survival of this recipient with temporal tumor rejection: the survival was 15 weeks after the first IL-2 application. So, individual survival of female treated by prolonged course (three IL-2 injections) was extended 2-folds compared with an average survival of the control group. Similar characteristics were found in the second recipient of the triple IL-2 treated group with signs of acute tumor rejection (data not shown).

Discussion

The rationale to apply IL-2 therapy to treat breast cancer (BC) locally was disclosed long time ago: locoregional immune deficiency [21], and/or functional abnormalities of cancer responsive cells in the host [22], and increased cytotoxicity of tumor associated T and NK cells after IL-2 application in vitro and in vivo [23]. Early published papers did not show promising results of local IL-2 application in the BC clinics [2], systemic application of IL-2

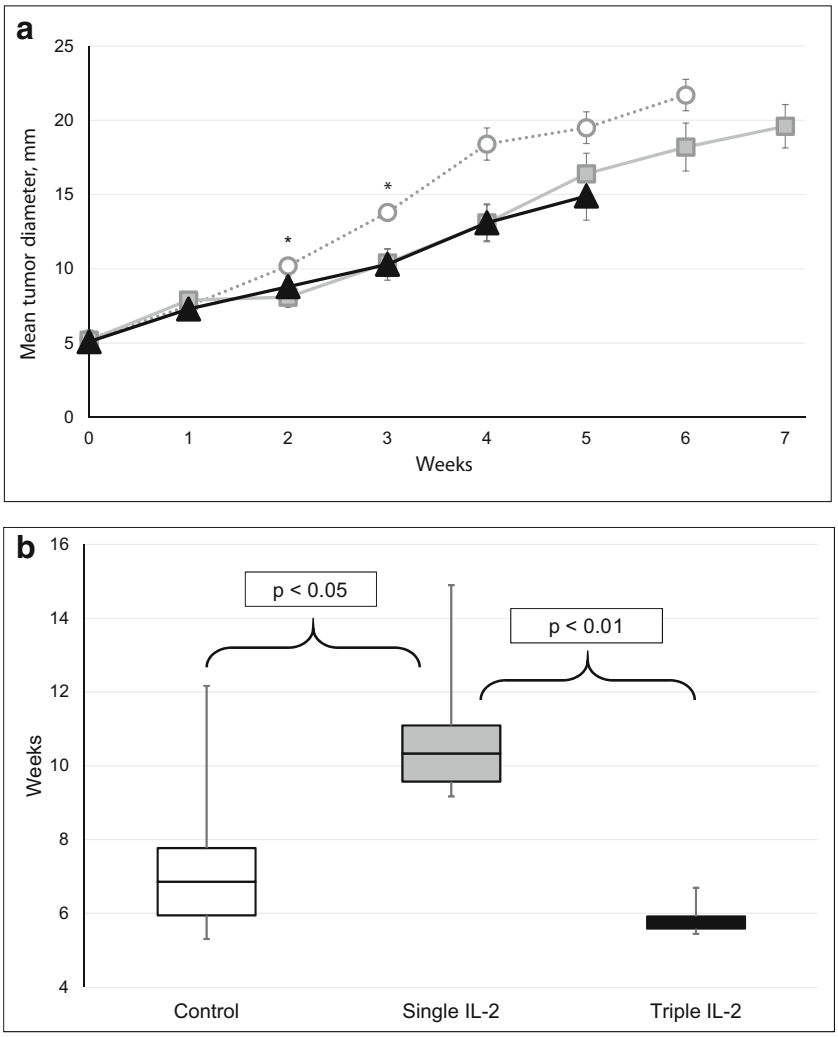

Fig. 2 Dynamics of tumor growth and average survival time of females treated with IL-2 once and triply, in comparison with the control. **a** Dynamics of tumor growth in females who received one local injection of IL-2 (grey squares) and three injections (closed triangles), compared with the dynamics of tumor growth in controls (open circles), * – $p < 0.05$. **b** The average survival time of control females (open box) and females who received a local injection of IL-2 once (grey box) and three times (closed box); boxes – standard error of mean, whiskers – 95% confidence interval; * – $p < 0.05$; ** – $p < 0.01$

was also not therapeutically effective [3]. Despite the generally successful preclinical research in mouse models shown by Vaage (for example, [24]) and others [25], beneficial IL-2 immunotherapy against human BC is rather exceptional [5].

We pay strong attention to the mouse models and the approaches used to find out the reason for this discrepancy in experimental and clinical results. Earlier, the IL-2 effect was shown in transplanted models [24, 25], where a spontaneous mammary tumor was commonly used as a donor of tumor cells that were transplanted to young healthy mice. It is important to emphasize, that newly arisen tumor blood vessels in those models evidently facilitate anti-tumor effect of cytokine in vivo; and anti-tumor response in a naturally arisen autochthonous mammary tumor is limited (shown for IL-12, [26]). Currently, only naturally arisen autochthonous mammary tumors were used in this series of our previous research (46 IL-2 treated females and 58 controls = 104 cases in [17], and 33 IL-2 treated females and 92 control = 125 cases in [16]) and in the current study (14 IL-2 treated females and 7 controls = 21 cases). And only two of 8 females that were thoroughly preselected for this research demonstrated mammary tumor rejection and remained tumor free after 3 both peri- and intratumoral IL-2 applications. So, altogether, 250 treated and intact females with naturally arisen MC were used to show the beneficial IL-2 effect for two (!) mice in a spontaneous mouse model of human BC. Our data demonstrate the importance of proclaimed preselection procedure [20] as in this paper the beneficial proportion (complete response rate) was increased from 2% (2 from 93 of IL-2 treated recipients, including unselected females) to 14% (2 from 14 of properly selected triple-treated females). By the way, proportion of beneficial recipients was comparable to 6% of complete and 10% of partial responses in metastatic melanoma patients treated by high-dose intravenous bolus IL-2 regimen [27]. Lessons from this experimental series determine which factors could influence the therapeutic effect of the local IL-2 of BC treatment [13, 16, 17].

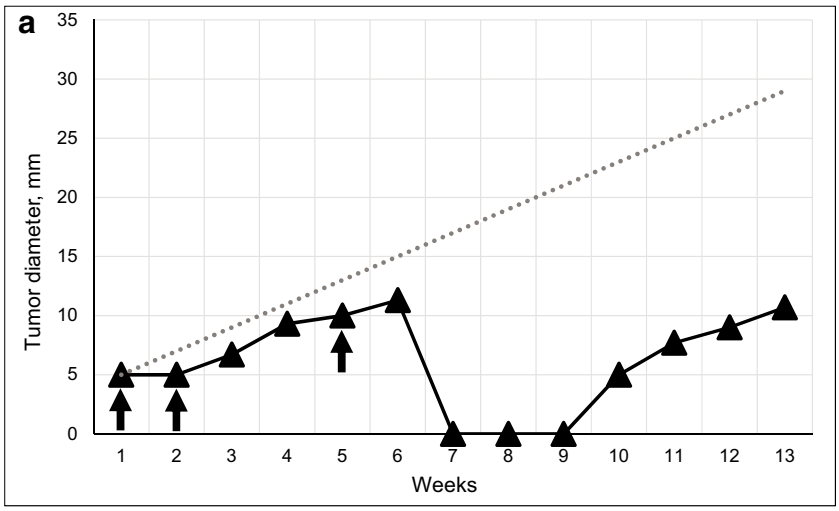

Fig. 3 Growth and histopathology of an individual tumor of a female treated with triple local IL-2 application with the rejection of the primary tumor and the appearance of a relapse. **a** Dynamics of tumor growth in female with rejection of spontaneous mammary cancer nodule, which received three peri- and intratumoral injections of IL-2 (closed triangles), compared to the typical dynamics of female mammary cancer in controls (grey line). The arrows indicate the time of IL-2 administration. **b** Anaplastic solid carcinoma (left bottom) replaces hemorrhagic cystic adenocarcinoma (upper right); HE staining

Host beneficial characteristics:

• Genotype: BLRB mammary tumors were more "sensitive" than CBRB genotype.

• Hormonal status: virgin.

• Age: around the average age at the MC diagnosis (about 50–60 weeks).

Tumor beneficial characteristics:

• Absence of subclinical period in a suddenly appeared MC with tumor pathology similar to human medullary BC with pronounced tumor leucocyte infiltrations (good prognosis for both humans and mice).

• Tumor growth rate after clinical manifestation: moderate, as both very fast (cystic tumor pathology) and very slowly growing tumors were less "sensitive" (poor tumor leucocyte infiltrations).

• Stage of tumor growth and IL-2 application time: first few weeks after MC diagnosis as both early subclinical (before 4 mm in size) and late advanced steps were non-sensitive to IL-2.

IL-2 treatment protocol:

• Dosage: 10^5 IU per mouse per injection seems to be better than 10^6 IU.

• Local application: both peri- and intratumoral were better than only peritumoral application.

• Number of applications (10^5 IU of IL-2/mouse): single – to enhance average survival time, and triple – to cure 2/8 individuals at the same time decreasing survival time of the rest of the treated group.

Thus, the logic of the biomedical experiment led us to a moral dilemma: what is better as a matter of principle (both in preclinical research and BC clinics), to increase the average survival of *all* ("first do not harm") or to cure only *some* with the simultaneous worsening the lifespan of the *rest* ("do not harm more, than succour")? This issue has two major concerns. From the one side, we discovered an ethical problem in biomedical research similar to the one faced by clinicians [28]. From another side, it was essential for us to find a proper

Table 1 Schematic description of individualized 3S–paradigm in comparison with standard principles in the preclinical procedure (from [37, 38])

S	Novel individualized 3S- concept	Standard paradigm
1st	*Set* of complementary non-SPF models (including spontaneous) to cover the whole spectra of human BC characteristics ➔ "Multiple level" models	"One level" models
2nd	*Stratification* (representatives from sensitive, resistant, and intermediate clusters are used to generate stratified sampling) and specific *Subgrouping* approach to analyze individualized data	Randomization and averaging
3rd	*Steps* of testing ➔ from in vitro and ex vivo (short-term cultures of tumor and immune cells) to in vivo research to study both direct and non-direct, both short-term and long-term effects during these consecutive steps of the testing	Study of direct short-term drug effect

definition of the term "curative therapy", particularly in relation to immunotherapy to treat BC. According to Frei [29], the definition of "curative chemotherapy" was clear for the majority of solid tumors except for the BC. For the latter, the term "therapy with curative intent" is currently used, as opposed to palliative treatment (for example, [30]). Recently, the European Society for Medical Oncology (ESMO) stated that the value of any new therapeutic strategy should be determined by the magnitude of its clinical benefit balanced against its cost and released a standardized validated approach to stratify the magnitude of clinical benefit [31].

We finally managed to find our own solution based on the three revolutionary findings in the biomedical area. First of all, it has been recently generally accepted that the immune system plays both host-protective and tumor-promoting roles in breast cancer initiation and progression (for example, [32]). And endogenous IL-2 apparently plays a role in this scenario: serum IL-2 level has a prognostic value in BC patients [33, 34]. Second, adverse consequences of immunostimulation were well established [35]. Third, the opposing effects of exogenous IL-2 therapy were shown by us [8, 13, 20] and others on functional [36] and even on molecular levels [9]. As a consequence, considerable variation in the risk of therapy outcomes would be expected in the IL-2 treated population; therefore, both beneficial and non-beneficial subgroups were found [8, 13, 20]. This approach led us to advance the individualized 3S–paradigm of anticancer research [37, 38] in the parallel of the "do not harm" concept in predictive, preventive, and personalized medicine [39] (Table 1).

Stratified sampling and specific subgrouping within the control and experimental groups are only possible if based on the individualized animal data. These methods allow revealing both positive and negative effect of an anti-cancer drug (or approach) in each experimental set, this was demonstrated on the example of local IL-2 immunotherapy firstly in transplanted [20], and then in spontaneous BC model [17].

Furthermore, benefit/risk outcomes of treatment in BC clinics and preclinical research should be strictly predicted before therapy application. Earlier, the clear prognostic value was shown for hematological [40] and biochemical serum parameters [41] of the host in BC clinics; we demonstrated that our model set generally reproduced these regularities [38, 42].

In conclusion, the benefit/risk estimation of given therapy mode is not an ethical, and not a financial, but a strongly scientific question; and proper mouse models and adequate approaches can facilitate to develop an appropriate preselection procedure, at least in the field of local IL-2 therapy of BC.

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