

Effect of Age on Pulmonary Metastases and Immunotherapy in Young and Middle-aged Mice

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Background: We used B16-F10 (B16) melanoma tumor cells and syngeneic C57BL/6 (B6) mice as a study model for pulmonary metastases, to better understand whether or not there exist differences in tumorigenicity and in the effectiveness of immunotherapy as a function of host age (1-, 3-, 12- and 24-month-old).

Methods: Intravenous injection of B16 melanoma cells were administered to B6 mice of different ages with/without interleukin (IL)-2 and IL-12 daily treatment. Tumor growth, splenocyte function, serum cytokines (IL-10, interferon- γ , vascular endothelial growth factor) and survival were compared.

Results: The study showed that, without IL-2 and IL-12 treatment, middle-aged mice suffering from pulmonary metastases had fewer pulmonary metastases and better survival than younger mice suffering from pulmonary metastases. Three days' IL-2 plus IL-12 treatment could not prolong mice survival, but prolonged treatment significantly improved the survival of both the younger and older tumor-bearing mice, especially the older mice, despite the fact that the younger mice had a better serum cytokine and splenocyte cellular immune response to cytokine treatment.

Conclusion: The young B6 mice that suffered from B16 pulmonary metastases had a poorer prognosis than the middle-aged mice. Short-term IL-2 plus IL-12 treatment is ineffective in prolonging survival, and a longer duration of treatment is needed. This kind of immunotherapy was effective in both the young and middle-aged mice, but it was more effective in the middle-aged mice. [*J Chin Med Assoc* 2007;70(3):94–102]

Key Words: age, immunotherapy, pulmonary metastases

Introduction

Although the incidence of cancer increases in the elderly, some tumors appear to grow more slowly in older patients compared to younger patients. For example, lung cancer was found to be more frequently in an advanced stage and with a worse prognosis in younger patients than in older patients.^{1–5} The underlying mechanism of this clinical finding is unknown.

B16-F10 (B16) melanoma is a melanoma cell line derived from C57BL/6 (B6) mice. The use of pulmonary metastasis models with B16 melanoma and B6 mice is a well-documented method to study ways of enhancing anti-tumor immunity.^{6,7} Cytokines, such as interleukin (IL)-2 and IL-12, have been found to

be involved in cellular immunity, and play an important role in anti-tumor immunity.^{8–12} We have also found that IL-2 plus IL-12 can restore the cytotoxic function of immunosuppressed lymphocytes isolated from malignant pleural effusion, and IL-2 plus IL-12 can also enhance B6 mice immunogenicity against the pulmonary metastases of B16 melanoma.^{6,10} This work used B6 mice and an intravenous injection (IV) of B16 melanoma tumor cells as a model to study whether or not B16 melanoma tumor cells had higher tumorigenicity in young mice than in middle-aged mice, and whether or not immunotherapy had different efficacy in young and middle-aged tumor-bearing mice. The results showed that the younger mice suffered from more severe pulmonary metastases and poorer survival

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than the middle-aged mice after IV of B16 melanoma cells. An IV of IL-2 plus IL-12 prolonged the survival of both the younger and older tumor-bearing mice, but cure was not possible.

Methods

Mice

Permission was obtained from the experimental animal protocol review board of our institution. Female C57BL/6 (B6) mice, 3–4 weeks old, were purchased from the Veterinary Resource Center, National Science Council, Taiwan. The mice were used for experiments at the age of 1 month as young mice, or raised up to 24 months old and used at different ages. The 12-month-old mice were used as middle-aged mice.

Melanoma cell preparation

The tumor cell line used in the study was the B16-F10 (B16) melanoma cell line. The cells were maintained *in vitro* in RPMI-1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island Biological Co., Grand Island, NY, USA), 2 mM glutamine (GIBCO), 50 µg/mL streptomycin and 500 U/mL penicillin.

Cytokines

Recombinant human IL-2 and mice IL-12 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Daily intraperitoneal injections (IPs) of IL-2 2,000 IU/mouse and IL-12 100 µg/mouse were given at different durations of time for immunotherapy. For the *in vitro* study, IL-2 300 IU/mL and IL-12 10 ng/mL were used for the splenocyte culture and stimulation test.

Pulmonary metastasis model

For the pulmonary metastasis model, IV of single cell suspensions of 5×10^5 B16 tumor cells were administered into the lateral tail vein of each mouse. Within 21 days after tumor injection, grossly visible metastases were present on the surface of the lungs of the normal mice injected with tumor cells. These pulmonary metastases were confirmed by histologic examinations.

In vitro cytotoxicity assay

An overnight (18 hours) ^{51}Cr release assay was performed, using B16 and P815 cells as targets. The effector cells were splenocytes isolated from sacrificed mice. Target cells were labeled with $\text{Na}_2^{51}\text{CrO}_4$ (Amersham, Buckinghamshire, England) for 90 minutes. Different volumes of effector cells and 50 µL of ^{51}Cr -labeled

target cells (5×10^3) were assigned at effector:target cell ratios of 100:1, 30:1 and 10:1 to the wells of round-bottomed microtiter plates (Nunc, Roskilde, Denmark) in triplicate, and were incubated overnight at 37°C in a humidified 5% CO_2 incubator. After incubation, the supernatant was harvested, and radioactivity was counted using a gamma scintillation counter. The spontaneous ^{51}Cr release was determined by the incubation of target cells without the effectors, and maximal release was determined by counting 50 µL of the ^{51}Cr -labeled target cells. The percentage of cytotoxicity was determined using the following formula (where cpm = counts per minute):

$$\% \text{ of cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

One lytic unit (LU) was defined as the number of effectors required to give 30% lysis (specific or net % lysis) of 5×10^3 target cells. The cell line, P815, a murine mastocytoma cell line, was used as the control tumor target in the cytotoxicity assay.

Determination of proliferative response

Cultured splenocytes at 0.1 mL were dispensed into 96-well flat-bottom microplates in triplicate. ^3H -thymidine at 2 µCi/mL was added to each well, and the cells were incubated overnight at 37°C in a humidified 5% CO_2 incubator. The incorporation of ^3H -thymidine was determined using a β -plate counter after the incubated cells were harvested using a cell harvester. The results were expressed as CPM \pm SEM (mean counts per minute \pm standard error of the mean).

Enzyme-linked immunosorbent assay (ELISA) analysis of cytokine levels

The blood samples collected from direct cardiac punctures in the sacrificed mice of the various groups were centrifuged at 4°C, and the supernatants were collected and stored at -70°C in microcentrifuge tubes. IL-10, interferon- γ (IFN- γ) and vascular endothelial growth factor (VEGF) were determined in duplicate using a solid-phase ELISA method that employed the quantitative “sandwich” enzyme immunoassay technique (Quantikine; R&D Systems, Inc.). Positive and negative controls were included in the assay.

Statistical analysis

The 2-tailed Mann-Whitney *U* test was used to evaluate the statistical difference in the number of pulmonary

metastases seen on the surface of the lungs of the tumor-bearing mice, cytolytic and proliferative activity of cultured splenocytes, and cytokine levels of the serum from sacrificed mice. Scheffe's *post hoc* test was performed for multiple comparisons. For survival analysis in the pulmonary metastasis model, the Kaplan-Meier method and log-rank test were used.

Results

There was poorer survival in young tumor-bearing mice compared to middle-aged tumor-bearing mice, and survival could not be prolonged with 3-day treatment of IL-2 plus IL-12. Twenty-nine female mice were classified into 4 groups, including 6 young mice that received B16 melanoma IV on day 0, 6 young mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 2, 9 middle-aged mice that received B16 melanoma IV on day 0, and 8 middle-aged mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 2. Survival analysis (Figure 1) showed that middle-aged mice suffering from pulmonary metastases without cytokine treatment had better survival than young mice suffering from pulmonary metastases without cytokine treatment (median survival 25 *vs.* 20 days, $p=0.0003$). Three days of treatment with IL-2 plus IL-12 could not prolong mice survival (median survival was 20 days in both groups of young mice and 25 days in both groups of middle-aged mice).

Eleven days of IL-2 plus IL-12 treatment prolonged mice survival

Fifty-eight female mice were classified into 4 groups, including 14 young mice that received B16 melanoma IV on day 0, 15 young mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 10, 15 middle-aged mice that received B16 melanoma IV on day 0, and 14 middle-aged mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 10. Eight mice were sacrificed on day 5 and another 8 mice were sacrificed on day 9 for microscopic examination and serum collection. Eighteen mice were sacrificed on day 14 in order to count the number of pulmonary metastases on the surface of the lungs and to perform histologic examination. The remaining mice were allowed to survive until their death, for survival analysis.

The overnight splenocyte proliferation assay (without adding mitogen or cytokine into the culture medium) performed on the splenocytes retrieved on days 5 and 9 from the sacrificed mice showed that the proliferative activity of the splenocytes from the tumor-bearing mice was lower than that of the splenocytes from the normal control mice. The mice treated with IL-2 and IL-12 had a splenocyte proliferative response similar to that of the normal control mice that were sacrificed on day 5 (day-5 mice), but this response was reduced again in the splenocytes from the mice sacrificed on day 9 (day-9 mice) (Figure 2).

The cytolytic activity of the splenocytes from the sacrificed normal mice and from the tumor-bearing

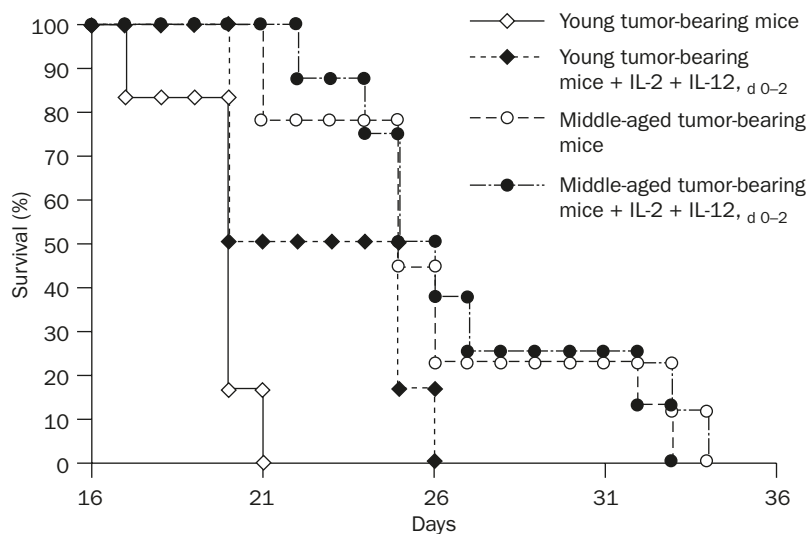


Figure 1. The survival curve of B6 mice with/without 3 days of interleukin (IL)-2 and IL-12 treatment. The overall survival showed that middle-aged mice suffering from pulmonary metastases without cytokine treatment had better survival than young mice suffering from pulmonary metastases without cytokine treatment (median survival 25 *vs.* 20 days, $p=0.0003$). Three days of IL-2 plus IL-12 treatment could not prolong mice survival.

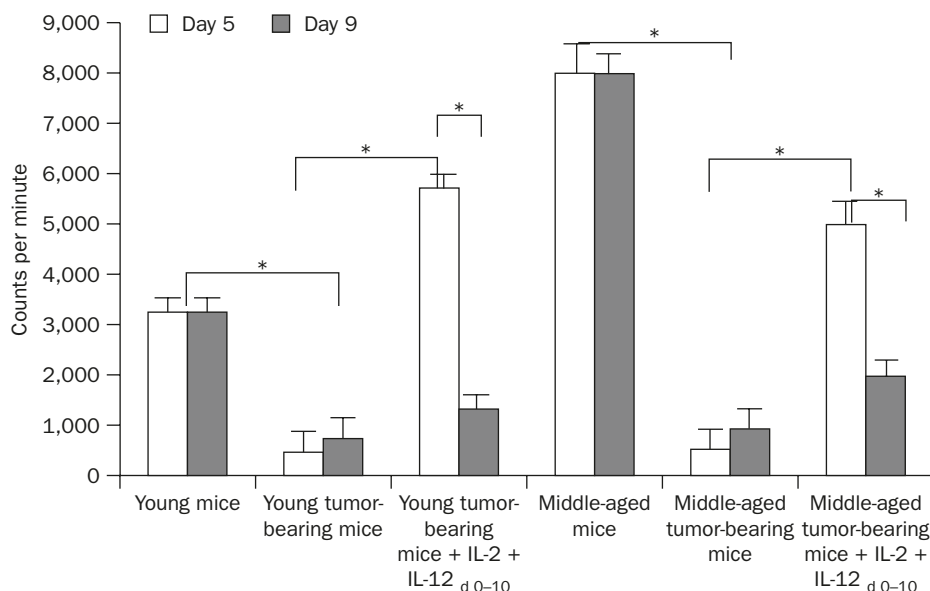


Figure 2. Overnight proliferation assay of splenocytes retrieved from each group of mice sacrificed on days 5 and 9. The proliferative activity of splenocytes from tumor-bearing mice was lower than that of normal control mice; interleukin (IL)-2- and IL-12-treated tumor-bearing mice had a proliferative response similar to the normal control mice on day 5, but that decreased again on day 9. * $p < 0.05$.

mice with/without daily IL-2 and IL-12 treatment that were sacrificed on days 5 and 9, against B16 melanoma cells and P815 cells, was very low, with LU < 1 . However, when the splenocytes retrieved from the day-5 tumor-bearing mice that had been treated daily with IL-2 and IL-12 were cultured in medium containing IL-2 and IL-12 for 4 more days, their cytolytic activity against B16 cells and P815 cells was markedly increased (Figure 3). This cytolytic activity was relatively more specific to the B16 melanoma cells in those splenocytes from cytokine-treated tumor-bearing mice that were further cultured with IL-2- and IL-12-containing medium for 4 more days than the splenocytes from normal mice that were cultured with medium containing IL-2 and IL-12 (Figure 3).

A counting of the number of lung surface metastases in the mice that had been sacrificed on day 14 (day-14 mice) showed that the young tumor-bearing mice had the highest number of metastases, followed by the middle-aged tumor-bearing mice. Eleven days' treatment with IL-2 plus IL-12 reduced the number of metastases in the young tumor-bearing mice to a degree less than that in the middle-aged tumor-bearing mice without cytokine treatment. Middle-aged tumor-bearing mice treated with IL-2 plus IL-12 had the least number of metastases (Table 1). In addition to the difference in the number of lung surface metastases, the size of the metastatic lesions was smaller in the middle-aged mice than in the young mice, as well as in the cytokine-treated tumor-bearing mice compared to those without cytokine treatment.

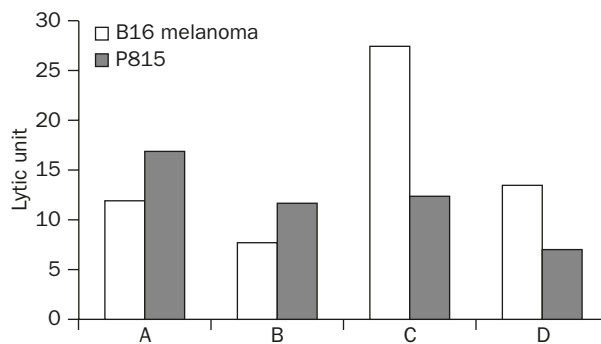


Figure 3. Cytolytic activity of cultured splenocytes against B16 cells and P815 cells. A = splenocytes retrieved from young normal mice and cultured with interleukin (IL)-2- and IL-12-containing medium for 4 days. B = splenocytes retrieved from middle-aged normal mice and cultured with IL-2- and IL-12-containing medium for 4 days. C = splenocytes retrieved from young tumor-bearing mice that had been treated daily with intraperitoneal injections (IPs) of IL-2 and IL-12 and sacrificed on day 5, then cultured with IL-2- and IL-12-containing medium for 4 more days. D = splenocytes retrieved from middle-aged tumor-bearing mice that had been treated daily with IPs of IL-2 and IL-12 and sacrificed on day 5, then cultured with IL-2- and IL-12-containing medium for 4 more days. When splenocytes isolated from the day-5 tumor-bearing mice that had been treated daily with IPs of IL-2 and IL-12 continued to be cultured in medium containing IL-2 and IL-12 for 4 more days, their cytolytic activity against B16 cells and P815 cells was markedly increased.

Regarding survival, the middle-aged tumor-bearing mice without cytokine treatment consistently survived longer than the young tumor-bearing mice without cytokine treatment ($p = 0.0006$), as in part 1 of the

Table 1. Number of lung surface metastases in day-14 sacrificed mice*

Group (number of mice)	Mean \pm SEM	Median (range)	<i>p</i>
Young tumor-bearing mice (4)	> 200	> 200	
Young tumor-bearing mice + IL-2 + IL-12 _{d 0-10} (5)	20.9 \pm 2.4	19 (7-39)	< 0.001 [†]
Middle-aged tumor-bearing mice (5)	31.6 \pm 3.2	26 (18-64)	< 0.001 [†]
Middle-aged tumor-bearing mice + IL-2 + IL-12 _{d 0-10} (4)	6.7 \pm 1.4	6 (0-16)	< 0.001 [†]
			< 0.011 [†]
			< 0.001 [†]
			< 0.001 [‡]
			< 0.001 [§]

*Three investigators blindly and independently counted the number of lung surface metastases in these mice; [†]when compared with young tumor-bearing mice; [‡]when compared with young tumor-bearing mice + IL-2 + IL-12_{d 0-10}; [§]when compared with middle-aged tumor-bearing mice. Scheffe's post hoc test showed there was significant difference between these groups. SEM = standard error of the mean; IL = interleukin; d = days.

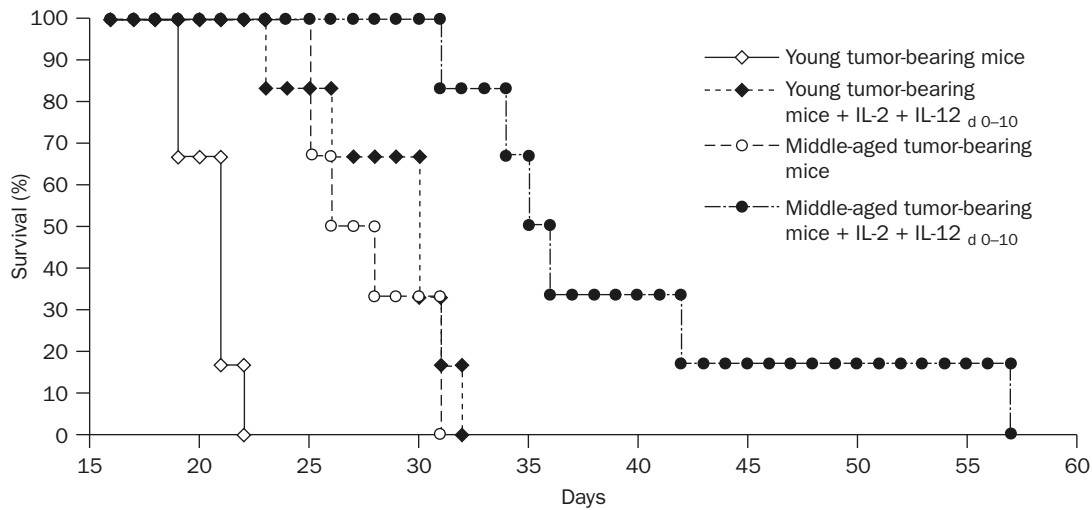


Figure 4. Eleven days of interleukin (IL)-2 plus IL-12 treatment prolonged both the young and middle-aged tumor-bearing mice's survival significantly, and the survival of the middle-aged cytokine-treated tumor-bearing mice was significantly better than that of the young cytokine-treated tumor-bearing mice. Six mice were included in each group, including young tumor-bearing mice, young tumor-bearing mice that received cytokine treatment, middle-aged tumor-bearing mice and middle-aged tumor-bearing mice that received cytokine treatment. Intravenous injection of B16 melanoma on day 0 was administered to produce tumor-bearing mice. Cytokine treatment included intraperitoneal injections of IL-2 plus IL-12 once daily from day 0 to day 10. Median survival was 21, 30, 26 and 35 days for each group, respectively. $p=0.0006$ for group 1 vs. group 2; $p=0.0006$ for group 1 vs. group 3; $p=0.0022$ for group 3 vs. group 4; $p=0.0041$ for group 2 vs. group 4.

experiment. Eleven days' treatment with IL-2 plus IL-12 significantly prolonged the survival of both the young and middle-aged tumor-bearing mice ($p=0.0006$ and 0.0022 , respectively); the cytokine-treated middle-aged tumor-bearing mice also survived significantly longer than the cytokine-treated young tumor-bearing mice ($p=0.0041$) (Figure 4).

Maintenance therapy with IL-2 and IL-12 did not increase tumor-bearing mice survival

The experiment was then extended to include long-term IL-2 plus IL-12 treatment, including 7 young mice that received B16 melanoma IV on day 0, 5 young mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 2, 10 young mice that received B16 melanoma IV on day 0 and daily

IL-2 and IL-12 IP from day 0 to day 10, then, 3 times weekly for 3 weeks, 10 young mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 10, then, every day except Sunday, for 3 weeks, 7 middle-aged mice that received B16 melanoma IV on day 0, 7 middle-aged mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 2, 11 middle-aged mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 10, then, 3 treatments weekly for 3 weeks, and 11 middle-aged mice that received B16 melanoma IV on day 0 and daily IL-2 and IL-12 IP from day 0 to day 10, then, every day except Sunday for 3 weeks. Survival analysis again showed that the middle-aged tumor-bearing mice had better survival than the young tumor-bearing mice; 3

days' treatment with IL-2 plus IL-12 did not prolong survival, as a previous study has demonstrated, but long-term maintenance therapy with IL-2 plus IL-12 did improve the mice's survival. However, this improvement in survival was not better than that of the mice that received 11 days' treatment (data not shown).

Similar experimental results with 3- and 1-month-old mice, and with 2- and 1-year-old (middle-aged) mice

The studies were extended to test 3-month-old mice, 1-year-old mice and 2-year-old mice to determine whether or not the abovementioned findings were similar between 1- and 3-month-old mice, and between 1- and 2-year-old mice. The results showed that the 2-year-old tumor-bearing mice had a borderline longer survival than the 1-year-old tumor-bearing mice ($p=0.065$), and the 3-month-old tumor bearing mice had a poorer survival than the 1- and 2-year-old mice (each group had 6 mice, $p=0.018$). Eleven days' IL-2 plus IL-12 treatment prolonged their survival significantly (each group had 5 mice) compared to the tumor-bearing mice without cytokine treatment. Another 4 mice from each group of tumor-bearing mice with/without cytokine treatment were sacrificed on day 14 in order to count the number of pulmonary metastases on the surface of the lungs. The results for the 3-month-old mice were similar to those for the 1-month-old mice mentioned above, and the results for the 2-year-old mice were similar to those for the 1-year-old mice mentioned above, in both the tumor-bearing mice and the tumor-bearing mice with cytokine treatment (data not shown).

Cytokine analysis

The serum from normal young mice had higher IL-10 and IFN- γ levels than that from normal middle-aged mice. Serum IL-10 levels were significantly elevated after the mice had suffered from pulmonary metastases for 7 days, while the change in serum IFN- γ levels was less marked. After 7 days' IP of IL-2 plus IL-12 were given daily, the normal young mice had markedly elevated serum IFN- γ , while the increase in the middle-aged normal mice was less marked. When the mice suffered from pulmonary metastases and were treated daily with IL-2 plus IL-12, the increase in serum IFN- γ was much less than that of the normal mice without pulmonary metastases and treated with IL-2 and IL-12. When normal mice were treated with IL-2 plus IL-12, the serum IL-10 levels decreased in the young mice, and showed no obvious change in the middle-aged mice. When the mice that were suffering from

pulmonary metastases were treated with IL-2 plus IL-12, their serum IL-10 levels were lower than those of both the young and middle-aged tumor-bearing mice that were not cytokine-treated. These results meant that the B6 mice had IL-10 and IFN- γ ratios that were in favor of the Th-2 pathway when in a normal condition, and that shifted even more when the mice were suffering from pulmonary metastasis. IL-2 and IL-12 treatment could drive the pathway in a Th-1 direction. All these cytokine levels and changes in level were more marked in the young mice than in the middle-aged mice (Figure 5).

In terms of VEGF, both the normal young and middle-aged mice had similar serum levels. However, when the mice suffered from pulmonary metastasis with/without IL-2 plus IL-12 treatment, the middle-aged mice had elevated VEGF levels compared to the normal middle-aged mice with/without IL-2 plus IL-12 treatment (Figure 5C). However, this elevation in the middle-aged mice's serum VEGF levels compared to the young mice's levels was statistically insignificant ($p>0.05$), either with/without pulmonary metastases or with/without IL-2 and IL-12 treatment.

Discussion

Cancer incidence increases with age. However, the growth and spread of tumors is often slow and prolonged in the elderly. Transplantable murine tumors grow and spread less readily in older mice, and can be used as models to study the effect of host age.^{7,13,14} Neovascularization is crucial for the growth of solid tumors, and alterations in host vascular response may underlie the changes in tumor growth that occur with age. It has been found that tumor growth in older animals is associated with less formation of new blood vessels, and this may explain the slower tumor growth observed in aged animals with certain experimental tumors.^{13,14} The elevated serum VEGF levels in the older tumor-bearing mice in this study probably reflect the presence of low levels of VEGF receptors that uptake VEGF in the lungs of the older mice, and thus the older tumor-bearing mice were unable to develop as many new tumor-associated vessels as in the young mice.

Although some studies have researched the influence of age on pulmonary metastasis, there has been no report on the effect of cytokine immunotherapy on tumor-bearing mice in different age groups, as in the present study. The results of our study show that the splenocyte proliferative response was decreased in both day-5 and day-9 young and middle-aged tumor-bearing mice compared with the splenocyte response in normal

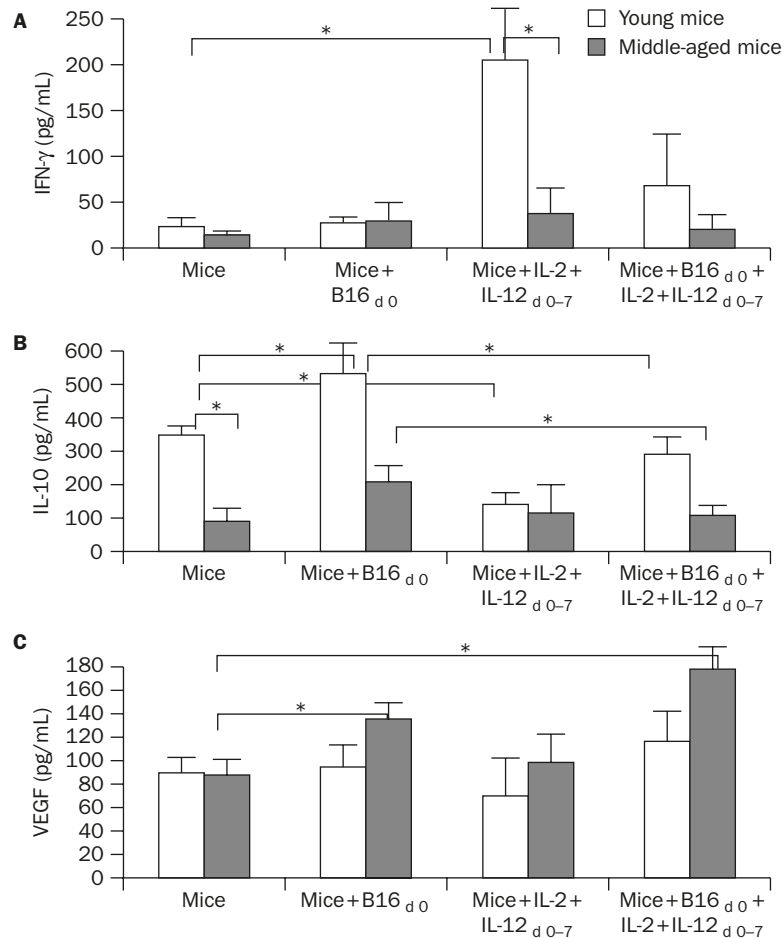


Figure 5. (A) Interferon (IFN)- γ ; (B) interleukin (IL)-10; and (C) vascular endothelial growth factor (VEGF) levels in the serum of the mice sacrificed on day 7. At least 5 mice from each group of the normal young and middle-aged mice, tumor-bearing young and middle-aged mice, normal young and middle-aged mice treated daily with IL-2 plus IL-12 from day 0 to day 7, and tumor-bearing young and middle-aged mice treated with IL-2 plus IL-12 from day 0 to day 7, were sacrificed on day 7. Their sera were collected for ELISA. The normal young mice had higher IL-10 levels than the middle-aged mice, and both groups of mice had even higher IL-10 levels when suffering from pulmonary metastases, while IFN- γ showed no obvious change. IL-2 plus IL-12 treatment in the normal young mice increased IFN- γ levels significantly. When tumor-bearing mice were treated with IL-2 plus IL-12, IL-10 levels decreased to a level less than that of the tumor-bearing mice without treatment. Serum VEGF was elevated in the middle-aged mice suffering from pulmonary metastases. * $p < 0.05$.

mice (Figure 2), while daily IL-2 and IL-12 treatment could reverse this tumor-induced immunosuppressive splenocyte proliferative response in the day-5 mice. However, the stimulating effect of daily IL-2 plus IL-12 treatment on splenocytes was surpassed by the tumor-induced immunosuppressive effect on day 9, when there was greater tumor burden on the mice. This improvement in the proliferative response of the splenocytes in the day-5 tumor-bearing mice brought about by IL-2 plus IL-12 IP implies that these cytokine treatments did have some effect on the mice's lymphocyte function against tumor cells. However, cytolytic activity against B16 tumor cells was still not activated, and was very low in the splenocytes isolated from the day-5 and day-9 tumor-bearing mice that had been treated daily with IL-2 and IL-12 IP. In

contrast, the cytolytic activity of the day-5 splenocytes was markedly increased after they were cultured with medium containing IL-2 and IL-12 for 4 more days, and this cytolytic activity was relatively specific to B16 melanoma cells compared to P815 tumor cells (Figure 3), suggesting that relatively tumor-specific killer cells had been formed or primed in the spleens of the tumor-bearing mice. In addition, activated splenocytes from the young mice had higher cytolytic activity than the activated splenocytes from the middle-aged mice (Figure 3), and the recovered splenocyte proliferative response was better in the day-5 tumor-bearing young mice that had been treated with IL-2 plus IL-12 than in the day-5 tumor-bearing middle-aged mice that had been treated with IL-2 plus IL-12 (Figure 2), implying that the young mice had better cellular

immunity than the middle-aged mice when suffering from malignant disease.

The results of the serum cytokine profiles (IFN- γ and IL-10) suggest that normal mice are usually within or in favor of the Th-2 pathway (IL-10 levels higher than IFN- γ) under normal conditions, and that the situation worsens (elevation of IL-10, with the pathway driven further in the Th-2 direction) when the mice suffer from B16 pulmonary metastasis. IL-2 and IL-12 treatment could drive this pathway in the Th-1 direction (increased IFN- γ and decreased IL-10). Similar to the findings on splenocyte cellular function (proliferation and cytolytic activity), the data of these cytokine levels and their changes were more marked in the young than in the middle-aged mice.

Our findings showed that there was a poorer survival in the young tumor-bearing mice compared to the older tumor-bearing mice, and survival could not be prolonged with 3 days' treatment with IL-2 plus IL-12. However, IL-2 plus IL-12 IP for 11 days could prolong mice survival. Unfortunately, prolongation of IL-2 plus IL-12 treatment by another 3 weeks did not increase survival. The lack of efficacy of short-term cytokine treatment in prolonging tumor-bearing mice survival was most likely due to the inadequate treatment duration, which was too short to see treatment effects. In contrast, the underlying cause of the failure of long-term cytokine maintenance treatment to prolong tumor-bearing mice survival, as compared to the 11-day cytokine treatment, was probably due to the inability to completely inhibit pulmonary metastasis from the beginning, resulting in continuous tumor growth despite cytokine treatment, and the tumor growth surpassing the cytokine function and triggering profound tumor-induced immunosuppression, probably after 10 days of cytokine treatment. Thus, IL-2 plus IL-12 treatment could only delay and initially decrease tumor-induced immunosuppression. Another issue is that prolonged cytokine treatment may be very toxic to mice, especially young mice, because of the progressive decrease in the weight of many mice during the treatment. In addition, the study was extended to include 3-month-old and 2-year-old mice to demonstrate the effect of aging on host response to tumors and the immunotherapy. The results were similar between the 1- and 3-month-old mice, as well as between the 1- and 2-year-old mice.

Silagi et al¹⁵ reported that high-dose IL-2 (50,000 IU/mouse) and IFN- γ (5–15 μ g/mouse) IPs every other day could cure about half of the B6 mice that received B16 tumor IP. The main difference between their study and this one was that their study was more likely an *in vivo* tissue culture situation with the addition

of tumor cells, local high cytokine concentrations, and close interaction of tumor and immune cells within the peritoneal cavity, while this study was a distant metastasis model in which the tumor burden in the lung was high, and the cytokine levels that were used to research the pulmonary circulation or the lung were low. There was also less close interaction between the tumor and immune cells in this study. Thus, immunotherapy with IL-2 and IL-12 was found to be less useful in this study, similar to Silagi et al's finding that the effect of local treatment on a localized tumor was superior to systemic or distant treatment. However, this study model is closer to the actual clinical situation in which disseminated tumors usually cannot be cured by immunotherapy, and immunotherapy can only reduce the tumor burden temporarily.

In summary, the young mice had more severe pulmonary metastases and poorer survival than the older mice when both were challenged with intravenous tumor injections, despite the fact that the young mice had better cytokine and cellular immune function. Cytokine treatment with IL-2 and IL-12 could improve both groups' survival, but the older mice still survived longer than the younger mice.

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