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ORIGINAL ARTICLE

Role of CD44 in lymphokine-activated killer cell-mediated killing of melanoma

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Abstract In the current study, we examined the potential significance of CD44 expression on lymphokine-activated killer (LAK) cells in their interaction and killing of melanoma cells. Stimulation of splenocytes with IL-2 led to a significant increase in the expression of CD44 on T cells, NK cells, and NKT cells. Treatment of melanoma-bearing CD44 WT mice with IL-2 led to a significant reduction in the local tumor growth while treatment of melanoma-bearing CD44 KO mice with IL-2 was ineffective at controlling tumor growth. Furthermore, the ability of splenocytes from IL-2-treated CD44 KO mice to kill melanoma tumor targets was significantly reduced when compared to the anti-tumor activity of splenocytes from IL-2-treated CD44 WT mice. The importance of CD44 expression on the LAK cells was further confirmed by the observation that adoptively transferred CD44 WT LAK cells were significantly more effective than CD44 KO LAK cells at controlling tumor growth in vivo. Next, the significance of the increased expression of CD44 in tumor killing was examined and showed that following stimulation with IL-2, distinct populations of cells with low ($CD44^{lo}$) or elevated ($CD44^{hi}$) expression of CD44 are generated and that the $CD44^{hi}$ cells are responsible for killing of the melanoma cells. The reduced killing activity of the CD44 KO LAK cells did not result from reduced activation or expression of effector molecules but was due, at least in part, to a reduced ability to adhere to B16F10 tumor cells.

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Introduction

It is estimated that approximately 50,000 new cases of malignant melanomas are diagnosed each year in the United States [1]. Due to the widespread growth of the metastatic lesions, surgical treatment is usually ineffective and many of these tumors are resistant to current chemotherapeutic agents. Therefore, there is great need for finding new adjuvant therapies. Melanoma is a good candidate for immunotherapy considering that it has been observed that patients can become naturally immunized against autologous tumors [2]. In addition, tumor-infiltrating lymphocytes can be isolated and expanded from many melanoma patients. In fact, it was shown that tumor-specific lymphocytes could be isolated from and expanded from 78% of tested individuals [3]. Together, these studies provide promising data suggesting that it may be possible to mount an effective immune response against melanoma in certain patients. The use of high-dose IL-2 therapy in the treatment of malignant melanoma has shown limited success [4–6]. Unfortunately, high-dose IL-2-therapy only leads to a slight increase in survival and can have severe life-threatening side effects, which significantly hamper its usefulness. More recently, investigators have been exploring the efficacy of adoptively transferring IL-2-stimulated lymphocytes into melanoma patients in conjunction with high-dose IL-2 treatment [3]. This approach initially showed significant promise, increasing the response rate from 24% in patients treated with high-dose IL-2 alone to 33% in patients treated with the combination of adoptively transferred IL-2-stimulated lymphocytes and high-dose IL-2 treatment. Although some progress has been

made in the treatment of malignant melanoma using immunotherapy, significantly improved strategies are needed to more successfully treat this disease.

CD44 is a widely distributed cell surface glycoprotein expressed by a number of lymphoid and non-lymphoid tissues. CD44 exist as a number of isoforms, which differ in their molecular weights, ranging from 85 to 260 kDa [7, 8]. Evidence suggests that CD44 is directly involved in lymphocyte activation. For example, lymphocytes can be triggered to proliferate by binding of mAbs specific for CD44 [9]. This proliferative response was comparable to that seen when the cells were stimulated with anti-CD3 mAbs and was IL-2 dependent. These reactions led to phosphorylation of proteins of identical molecular mass as proteins phosphorylated by anti-CD3 stimulation and could be blocked with tyrosine kinase inhibitors. In addition to T lymphocytes, NK cell activation is also affected by CD44 binding. Binding of CD44 significantly increased NK cell lysis of NK-sensitive targets [10]. This activity was shown to be monocyte independent, indicating a direct effect on the NK cell population. CD44 has also been implicated in the lysis of a number of tumor cells. One of the initial observations came from studies that demonstrated that CTL, NK/LAK cells, and cytotoxic double-negative (DN) T cells that accumulate in *lpr/lpr* mice express high levels of CD44 and when activated through CD44 mediate efficient lysis of targets cells [11, 12]. In addition, CD44 can act as a receptor mediating cell lysis. This was demonstrated using "redirected" lysis of target cells, in which a CTL clone incubated with mAbs against adhesion molecules was tested for toxicity against Ag-negative FcR^+ target cells [11].

Recent work from our laboratory described a distinct interaction between CD44v7 on IL-2-activated lymphocytes and specific glycosaminoglycans on endothelial cells. Furthermore, we demonstrated that following exposure to IL-2, there is a significant increase in the expression of CD44 isoforms and that lymphocytes expressing high levels of CD44 have potent cytolytic activity. Interestingly, melanomas are particularly sensitive to IL-2 therapy, while other tumors seem to be more resistant. This suggests that there may be a unique interaction between IL-2-stimulated cytotoxic lymphocytes and melanomas. In the current study, we examined the role of CD44 in the interaction between IL-2-activated lymphocytes and melanomas.

Materials and methods

Mice

Adult female C57BL/6 (CD44 WT) mice were purchased from the National Institutes of Health (Bethesda, MD). CD44 knockout (CD44 KO) mice on C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). CD44 knockout (KO) mice represent those in which exon 1 and part of intron 1 were disrupted and therefore such mice fail to express all CD44 isoforms [13, 14]. The CD44 WT and CD44 were bred in the animal facilities at Mercer University School of Medicine's Animal facility and screened for CD44 expression.

Cell lines

The murine melanoma B16F10, the murine leukemia EL-4 and the murine pancreatic cancer Panc-02 were purchased from ATCC (Manassas, VA). B16F10 and EL-4 were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 1 mM glutamine, and 40 μg/ml gentamicin sulfate. Panc-02 was maintained in DMEM supplemented with 10% fetal calf serum, 10 mM HEPES, 1 mM glutamine, and 40 μg/ml gentamicin sulfate.

RNA isolation, RT-PCR and real-time PCR analysis

Total RNA was isolated from single-cell suspension of splenocytes using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentration and integrity were determined spectrophotometrically. cDNA was synthesized by reverse transcription of 50 ng total RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Carlsbad, CA). All PCRs were prepared using Gotaq colorless master mix MasterAmp (Promega, Madison, WI) according to the manufacturer's recommendations. Real-time PCR was performed using a SYBR Green PCR kit (Applied Biosystems, Carlsbad, CA). Amplications were performed and monitored using an ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA). The gene-specific primers for perforin, Fas ligand, TNF-α, and β-actin have been previously described [15]. In addition, the following primers were used: Fas primers 5′-GAGAATTGC TGAAGACATGACAATCC-3′ and 5′-GTAGTTTTCACT CCAGACATTGTCC-3′ and IFN-γ primers 5′-TGCATC TTGGCTTTGCAGCTCTTCCTCATGGC-3′ and 5′-TG GACCTGTGGGTTGTTGACCTCAAACTTGGC-3′. The threshold cycle (ΔC_T) method was used for relative quantification of gene transcription in relation to expression of the internal standard β-actin. Fold changes of mRNA levels in IL-2-stimulated splenocytes relative to unstimulated splenocytes were determined using the $2^{-\Delta\Delta Ct}$ method [16].

Antibodies

Fluorescence-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD16 (Fc block), anti-CD44, anti-CD69, and anti-NK1.1 mAbs were purchased from PharMingen (San

Diego, CA). Anti-CD44v7 mAbs were prepared as previously described [17], and isotype control mAbs (mouse IgG1) were purchased from PharMingen (San Diego, CA).

Interleukins

Recombinant IL-2 was provided by the NCI Biological Resources Branch (Rockville, MD).

Flow cytometric analysis of cell surface markers

Splenic cells were analyzed for the expression of various cell surface markers. Non-specific staining was blocked by incubation of the cells with Fc block for 15 min. The expression of various cell surface markers was determined by staining the cells with fluorescently conjugated mAbs for 30 min on ice, followed by washing three times. The cells (10,000) were analyzed by flow cytometry.

Generation of LAK cells

For in vitro generation of LAK cells, splenocytes (5×10^6/ml) from CD44 WT and CD44 KO mice were cultured for 48 h with 1,000 U/ml of IL-2. The cells were harvested, and the viable cells were purified by density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO). For generation of LAK cells in vivo, CD44 WT and CD44 KO mice were treated with IL-2 as described below. On day 4, the spleens from IL-2-treated mice were harvested and prepared into a single-cell suspension. LAK cells generated in vitro and in vivo were tested for cytotoxicity against B16F10 tumor. Briefly, 1×10^6 target cells were labeled with $^{51}NaCrO_4$ at 37°C for 1 h. The LAK cells were plated in triplicate into 96-well round-bottomed plates at varying E/T ratios and incubated for 4 h at 37°C. Spontaneous release was determined by culturing the target cells alone, and the total release was determined by incubating the target cells with 1% triton X 100. The supernatants were harvested after 4 h, and the radioactivity was measured using a gamma counter.

Cell adhesion assay

B16F10 tumor cells were plated at 1×10^4/well and cultured at 37°C overnight in a 96-well flat-bottomed plate. CD44 WT and CD44 KO LAK cells generated in vitro as described earlier were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, Eugene, OR). The LAK cells were washed and added to wells containing EC cultures and incubated for 2 h at 37°C. LAK cells not adhering to EC cells were removed by gently washing the cultures with medium. Washing was performed by removing the initial medium followed by two washing steps, which consisted of gentle pipetting with 100 µl of medium. The percentage of adherent cells was quantified by measuring the level of fluorescence remaining following washing of the cells compared to the fluorescence in non-washed wells (total fluorescence). Fluorescence was quantified using a Tecan Infinite M1000 multimode plate reader (Männedorf, Switzerland). The percentage of LAK cells adhering to the EC was determined using the following formula: (fluorescence of adhering cells/total fluorescence) × 100%.

Assessment of CD44 localization

LAK cells were generated from CD44 WT mice as described above. LAK cells (5×10^6/ml) were mixed with B16F10 (1×10^6/ml) in a volume of 2 ml. The mixed cells were spun down at 400 rpm for 5 min and then incubated at 37°C for 15 min. The mixed cells were resuspended gently, and 60 µl was incubated on a Polyprep microscope slide (Sigma-Aldrich, St. Louis, MO) for 15 min at 37°C. The slides were washed twice with PBS and then fixed with 4% paraformaldehyde, 0.1% PBS. Next, the slides were washed twice and stained with Cy5-conjugated anti-CD44 (IM7) for 1 h and analyzed using a confocal microscope.

Quantification of the effect of CD44 on B16F10 tumor growth and metastasis in vivo

To examine the effects of CD44 on local tumor growth following IL-2 treatment, groups of CD44 WT or CD44 KO mice were injected s.c. with 3×10^5 B16F10 melanoma cells. In addition, experiments were conducted to examine the role of CD44 on B16F10 metastasis in IL-2-treated mice. To this end, CD44 WT and CD44 KO mice were injected i.v. with 3×10^5 B16F10 melanoma cells. In experiments examining local growth mice were treated on days 7–9, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). The tumor volume was observed, recorded, and calculated using the following equation: tumor volume = length × width^2 × 0.52. In experiments examining the effects on metastatic growth mice were treated on days 3–5, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). The level of metastatic nodules was determined on day 11 by directly quantifying the number of metastatic nodules located in the left lobe of the lungs.

Adoptive transfer of LAK cells

In adoptive transfer experiments, splenocytes from CD44 WT and CD44 KO mice were stimulated in vitro for 48 h with IL-2. After which, the splenocytes (5×10^6) were

injected i.v. into B16F10 tumor-bearing CD44 WT mice on days 2, 4, and 6 following tumor injection. The level of metastatic lesions in the lungs was determined on day 7.

Statistical analysis

ANOVA and Student's t test were used to determine statistical significance, and $P < 0.05$ was considered to be statistically significant.

Results

IL-2 stimulation leads to increased levels of CD44 on LAK cells

Experiments were conducted to examine the effect of IL-2 stimulation on the expression levels of CD44 on LAK cells. More specifically, we examined the levels of CD44 on the various spleen cell populations including CD3+ T cells, CD4+ T cells, CD8+ T cells, NK cells, and NKT cells (Fig. 1). Splenocytes were cultured for 48 h in the presence or absence of IL-2 (1,000 units/ml). The cells were harvested and stained with fluorescently labeled mAbs specific for phenotypic markers as well as for CD44. The results from this experiment showed that stimulation with IL-2 led to a general increase in CD44 expression in CD3+ T cells. However, this increase was primarily seen in $CD8^+$ cells as little increase in CD44 expression was seen in the $CD4^+$ populations. In addition, IL-2 stimulations led to a significant increase in CD44 expression in NK and NKT cells. Together, these results suggest that stimulation of splenocytes with IL-2 leads to a significant increase in the expression of CD44 primarily on the $CD8^+$ T cells, NK, and NKT cell populations.

Targeted deletion of CD44 leads to enhanced tumor growth in IL-2-treated mice

Next, we examined the effect of deletion of CD44 on the growth of B16F10 melanoma following treatment with IL-2. To this end, mice were injected s.c. with B16F10 melanoma cells. On days 7–9, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). Tumor growth was monitored (Fig. 2a) and demonstrated that IL-2 treatment was effective at controlling the local growth of the B16F10 melanoma in CD44 WT mice. In contrast, treatment of tumor-bearing CD44 KO mice with IL-2 was not able to control tumor growth. Separate experiments were conducted to examine whether deletion of CD44 had an effect on controlling B16F10 metastasis to the lungs in IL-2-treated mice. CD44 WT and CD44 KO mice were injected i.v. with 3×10^5 B16F10 melanoma cells. On days 3–5, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). On day 11, the number of metastatic nodules in the lungs of was determined (Fig. 2b). The results demonstrated that treatment of CD44 WT mice with IL-2 led to a significant reduction in the number of metastatic nodules compared to PBS injected mice. In contrast, IL-2 treatment of CD44 KO mice did not lead to a significant reduction in metastatic nodules in the lungs compared to PBS-treated CD44 KO mice.

CD44 deletion leads reduced killing of B16F10 melanoma by IL-2-activated splenocytes

Splenocytes from PBS (control) or IL-2-treated CD44 WT and CD44 KO tumor-bearing mice were tested for their ability to kill B16F10 melanoma cells in a 4-h chromium release assay (Fig. 2c). The results showed that splenocytes from IL-2-treated CD44 WT mice were able to mediate significantly more lysis of the melanoma targets, when compared to splenocytes from IL-2-treated CD44 KO mice. In addition, we tested the ability of IL-2-activated splenocytes from CD44 WT and CD44 KO mice to kill a mouse erythroleukemia (EL-4) and a mouse pancreatic (Panc-02) cancer cell line (Fig. 2d). The results demonstrated that stimulation with IL-2 led to significant anti-tumor activity. However, in contrast to killing of B16F10, deletion of CD44 had no significant effect on the ability of IL-2-activated spleen cells to kill EL-4 or Panc-02. These results suggest that CD44 expression plays a unique role in the lysis of melanoma cells following IL-2 treatment.

Adoptive transfer of IL-2-stimulated CD44 WT splenocytes leads to reduced B16F10 metastasis

Next, we examined whether adoptive transfer of IL-2-stimulated splenocytes from CD44 WT or CD44 KO mice would have any effect on the level of B16F10 metastatic lung nodules. To this end, splenocytes from CD44 WT and CD44 KO mice were stimulated in vitro for 48 h with IL-2. After which, the splenocytes (5×10^6) were injected into B16F10 tumor-bearing CD44 WT mice on days 2, 4, and 6 following tumor injection. The level of metastatic lesions in the lungs was determined on day 7 and showed that transfer of IL-2-stimulated splenocytes from CD44 WT mice led to a significant reduction in the level of metastatic lesions when compared to control mice (Fig. 3). However, the injection of tumor-bearing mice with IL-2-stimulated splenocytes from CD44 KO mice was less effective. Together, these results suggest that the use of adoptively transferred IL-2-stimulated splenocytes may ultimately be efficacious for the treatment of metastatic melanoma and that CD44 may play an important role in this process.

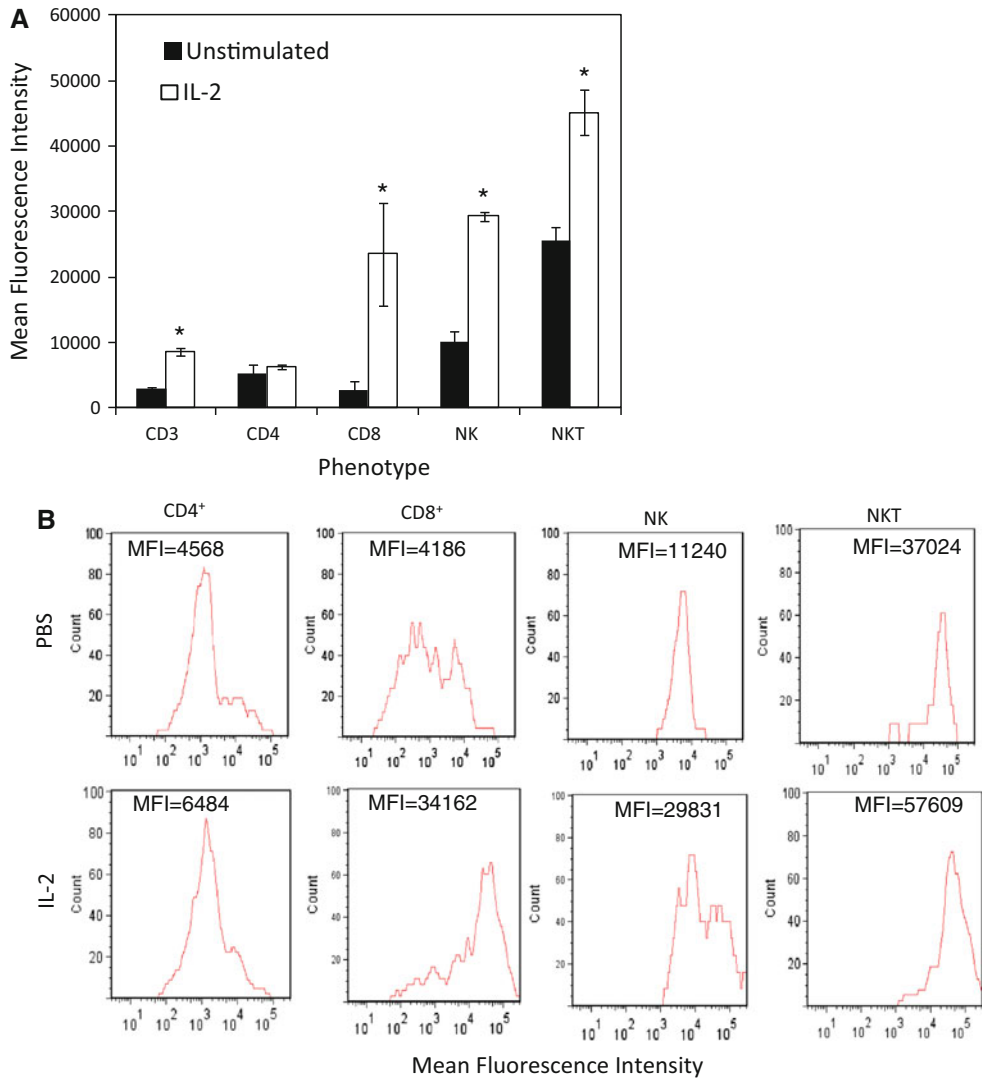

Fig. 1 IL-2 stimulation leads to increased levels of CD44 on LAK cells. Splenocytes from CD44 WT mice were cultured for 48 h with IL-2 (1,000 U/ml) or in medium alone (unstimulated). Next, cells were stained with FITC-conjugated anti-CD3 mAbs, PE-conjugated anti-NK1.1 mAbs, and Cy5-conjugated anti-CD44 mAbs. In addition, cells were stained with FITC-conjugated anti-CD4 mAbs, PE-conjugated anti-CD8 mAbs, and Cy5-conjugated anti-CD44 mAbs. $CD3^+$, $NK1.1^+$, $CD4^+$, and $CD8^+$, and $CD3^+$ $NK1.1^+$ (NKT) cells were gated, and the level of CD44 expression on these populations was determined by FACS analysis. **a** The data are depicted as mean fluorescence intensity (MFI) ± SD from individual experiments. More than 1,000 events were analyzed per gated population. **b** Data from representative experiment depicting MFI of unstimulated or IL-2-stimulated lymphocytes

The reduced lytic activity of LAK cells from CD44 KO mice is not due to alterations in activation or expression of cytolytic effector molecules

Next, we examined the possibility that the reduced IL-2-induced tumor killing seen in CD44 KO mice resulted from decreased responsiveness to activation stimuli and/or due to a reduction in the expression of effector molecules involved in the lytic activity of LAK cells. To this end, splenocytes were cultured in the presence of IL-2 for 48 h and the proliferative responsiveness was measured (Fig. 4a). The results showed that splenocytes from the CD44 KO mice responded to IL-2 to the same extent as cells from CD44 WT mice. These results suggested that the reduced lysis of B16F10 seen in the CD44 KO mice was not due to a defect in the activation and proliferation of T cells from these mice.

Increased levels of FasL, perforin, and TNF-α play an important role in the cytolytic activity of lymphocytes. Therefore, we examined the expression of FasL, perforin, and TNF-α mRNA in splenocytes following culture with PBS (control) or IL-2 in vitro using RT-PCR (Fig. 4b) as

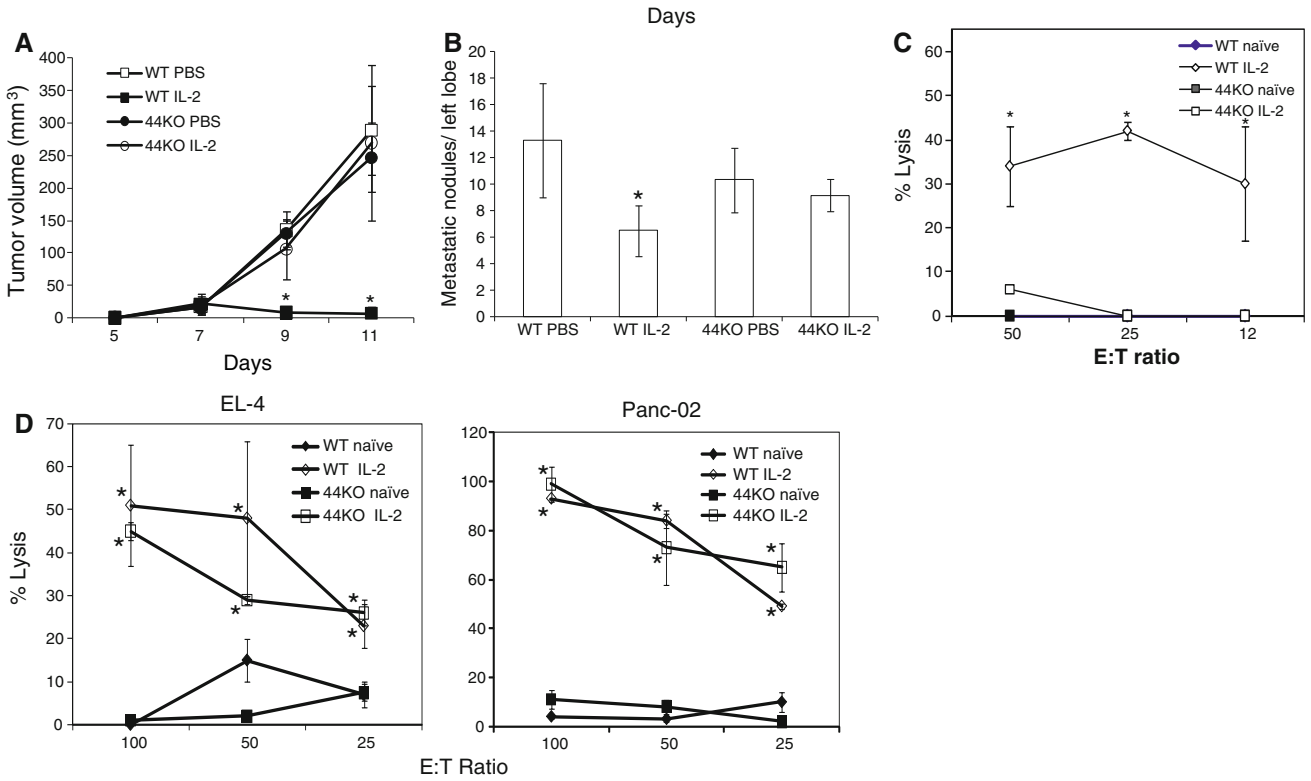

Fig. 2 Targeted deletion of CD44 leads to enhanced tumor growth and reduced LAK activity in IL-2-treated mice. To examine the effects of CD44 on local tumor growth following IL-2 treatment groups of CD44 WT or CD44 KO mice were injected s.c. with 3×10^5 B16F10 melanoma cells. On days 7–9, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). Tumor growth was monitored through day 11. The data depict the tumor volumes in groups of five mice per treatment group (**a**). In addition, experiments were conducted to examine the role of CD44 on B16F10 metastasis in IL-2-treated mice. To this end, CD44 WT and CD44 KO mice were injected i.v. with 3×10^5 B16F10 melanoma cells. On days 3–5, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). The level of metastatic nodules was determined on day 11 by directly quantifying the number of metastatic nodules located in the *left* lobe of the lungs (**b**). The data depict the number of metastatic nodules in the *left* lobe of the lungs from two independent experiments each with three mice per treatment group ($n = 7$ per treatment group). Asterisk indicates statistically significant difference when compared to control (PBS-treated) mice. Experiments were performed to examine the effect of CD44 deletion on LAK cell-mediated killing of B16F10 (**c**). CD44 WT and CD44 KO mice were injected i.v. with 3×10^5 B16F10 melanoma cells. On days 7–9, groups of mice were injected with PBS or IL-2 (75,000 U/mouse 3 times daily for 3 days). On day 11, the spleen cells were isolated and tested for their ability to kill B16F10 melanoma cells in a 4-h chromium release assay. Finally, the effect of CD44 deletion on the ability of IL-2-activated spleen cells to kill EL-4 and Panc-02 tumor cells was determined (**d**). The data indicate the mean percentage of cytotoxicity of triplicate cultures ±SD. *Asterisk* indicates statistically significant difference when compared to cytotoxicity of naïve spleen cells

well as real-time RT-PCR (Fig. 4c). Splenocytes from IL-2-treated CD44 WT and CD44 KO mice were found to express increased levels of FasL when compared to PBS-treated mice. Such an increase was similar in both groups of mice. IL-2 treatment caused a significant increase in the levels of perforin and IFN-γ mRNA in splenocytes from both the CD44 WT and CD44 KO mice. IL-2 stimulation had little effect on Fas and TNF-α mRNA in both groups of mice when compared to their PBS-treated counterparts. These results suggested that the reduced IL-2-induced lytic activity against B16F10 seen in the CD44 KO mice did not result from reduced expression of cytolytic effector molecules.

Involvement of CD44 in the adhesion between IL-2-activated LAK cells and B16F10 melanoma

CD44 has been reported to play an important role in the adhesion of effector cells to target cells [18]. Therefore, we examined the possibility that the absence of CD44 on IL-2-activated lymphocytes leads to a reduction in their adherence to B16F10 melanoma. To this end, the effect CD44 expression on the adherence of LAK cells to B16F10 was examined using an adhesion assay. The results showed that compared to unactivated splenocytes from CD44 WT mice, there was significant increase in adhesion between B16F10 melanoma cells and IL-2-activated splenocytes from CD44

WT mice (Fig. 5a). In contrast, compared to unactivated splenocytes from CD44 KO mice, there was no significant increase in the binding of IL-2-activated splenocytes to B16F10. These results suggest that the IL-2-induced increase in CD44 expression on lymphocytes may play an important role in their ability to adhere to B16F10 melanoma tumor cells.

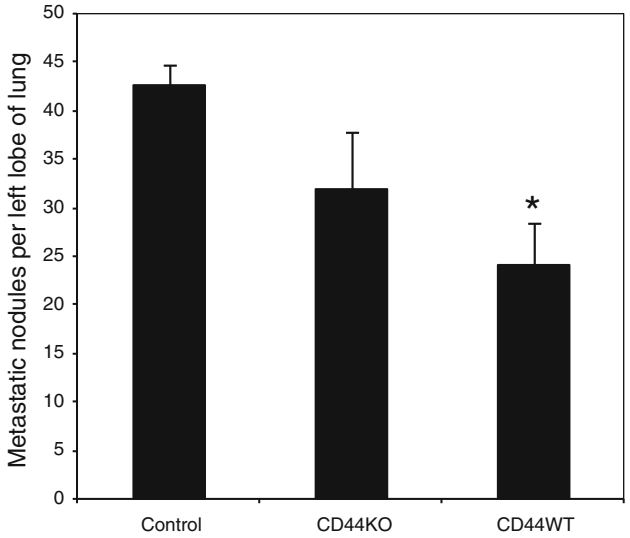

Fig. 3 Adoptive transfer of IL-2-stimulated CD44 WT splenocytes leads to reduced B16F10 metastasis. CD44 WT mice were injected with B16F10 i.v. Next, IL-2-activated splenocytes (5×10^6) from CD44 WT or CD44 KO mice were injected into B16F10 tumor-bearing CD44 WT mice on days 2, 4, and 6 following tumor injection. The level of metastatic lesions in the lungs was determined on day 7. The data depicted represent the mean number of metastatic nodules in the *left* lobe of tumor-bearing mice. *Asterisk* indicates statistically significant difference when compared to control (PBS-treated) mice

Involvement of CD44 in the formation of an immunological synapse between IL-2-stimulated lymphocytes and B16F10 melanoma

Recent reports show that an immunological synapse forms between cytolytic lymphocytes and target cells [19]. The cytotoxic synapse shares many characteristics with the synapse formed between $CD4^+$ cells and APC. More specifically, both synapses contain a peripheral region (pSMAC) that contains adhesion molecules and a central region (cSMAC) that contains signaling molecules. However, one key difference between the two synapses is that the cytotoxic synapse contains a distinct secretory domain within the pSMAC used to deliver cytotoxic agents such as perforin and granzymes [19]. Although many of the molecules involved in the formation of the cytotoxic synapse have been identified, the role of CD44 in synapse formation remains to be elucidated. Therefore, in initial experiments, we examined whether CD44 was localized at the site of cytotoxic lymphocyte–tumor cells contact. Here, splenocytes from CD44 WT mice were stimulated with IL-2 (1,000 U/ml) for 48 h. After which, the IL-2-activated splenocytes (5×10^6) were incubated with B16F10 melanoma cells (1×10^6) for 10 min at 37°C. The cells were then added to microscope slides, stained with Cy5-conjugated anti-CD44 (IM7) for 1 h and analyzed using a confocal microscope (Fig. 5b). The results showed that IL-2-stimulated splenocytes not forming a synapse with B16F10 melanoma cells had relatively uniform cell surface expression of CD44. In contrast, highly localized expression of CD44 could be seen at the contact site between IL-2-stimulated splenocytes and B16F10 melanoma cells.

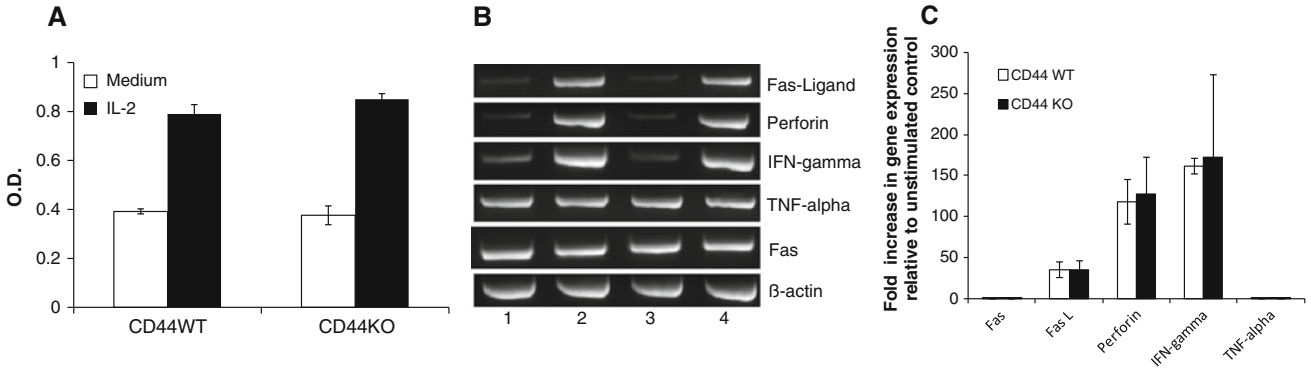

Fig. 4 The reduced lytic activity of LAK cells from CD44 KO mice is not due to alterations in activation or expression of cytolytic effector molecules. Splenocytes from CD44 WT and CD44 KO mice were cultured with IL-2 (1,000 U/ml) or in medium alone. The proliferative response was measured 48 h using the MTT test. The data represent the mean O.D. from triplicate cultures (**a**). *Asterisk* indicates statistically significant difference when compared to O.D. of spleen cells from medium treated. Detection of Fas ligand, perforin, IFN-γ, and TNF-α mRNA in splenocytes cultured in medium of IL-2 (1,000 U/ml) (**b**). Total RNA was isolated from splenocytes stimulated for 48 h with IL-2 or cultured in medium alone. mRNA was reverse transcribed and amplified by PCR with primers specific for Fas ligand, perforin, IFN-γ, TNF-α, and β-actin. A photograph of ethidium bromide-stained amplicons is depicted. *Lane 1*, CD44 WT + medium; *lane 2*, CD44 WT + IL-2; *lane 3*, CD44 KO + medium; *lane 4*, CD44 KO + IL-2. The results were further confirmed by real-time RT-PCR (**c**). Relative gene expression of cytokines are presented as fold change ($2^{-\Delta\Delta Ct}$ method was used for defining relative gene expression as described in "Materials and methods")

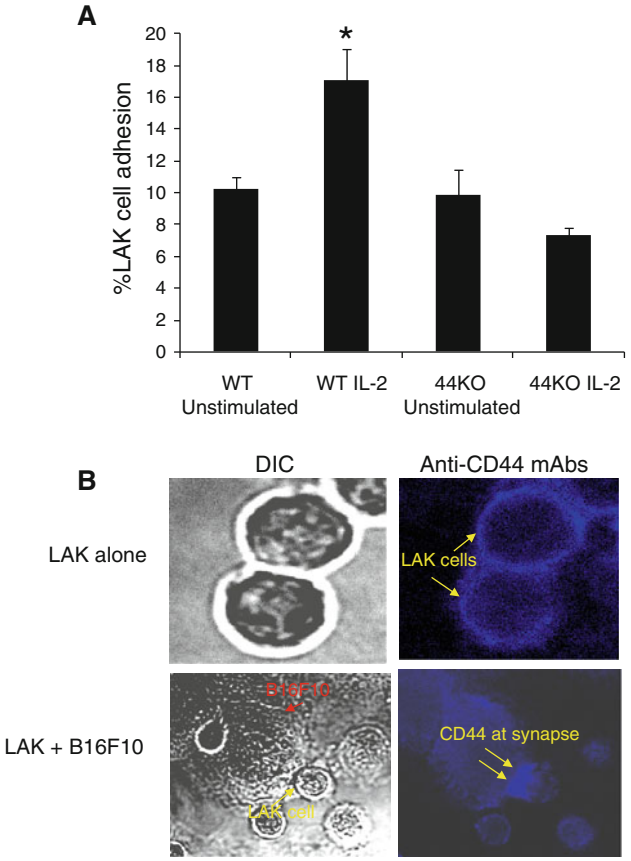

Fig. 5 Involvement of CD44 in the adhesion between IL-2-activated LAK cells and B16F10 melanoma. **a** CFSE-labeled LAK cells from CD44 WT and CD44 KO mice were cultured for 2 h at 37°C in plates containing adherent B16F10 tumor cells. LAK cells not adhering to B16F10 cells were removed by gently washing the cultures with medium. The percentage of adherent cells was quantified by measuring the level of fluorescence remaining following washing of the cells compared to the fluorescence in non-washed wells (total fluorescence). *Asterisk* indicates statistically significant difference when compared with the CD44 WT unstimulated splenocytes, $P \leq 0.05$. **b** LAK cells were mixed with B16F10 and spun down at 400 rpm for 5 min and then incubated at 37°C for 15 min. The mixed cells were resuspended gently, and 60 µl was incubated on a Polyprep microscope slide for 15 min. The slides were stained with Cy5-conjugated anti-CD44 (IM7) for 1 h and analyzed using a confocal microscope

These results suggest that CD44 may play an important role in the formation of synapses between IL-2-activated splenocytes and melanoma tumor cells.

Lysis of B16F10 following IL-2 stimulation is mediated by "CD44^{hi}" LAK cells

Further examination of CD44 expression following IL-2 stimulation revealed the generation of a CD44^{hi} and CD44^{lo} cell populations (Fig. 6a). Phenotypic characterization of the CD44^{hi} and CD44^{lo} cells showed enrichment in CD8+ CTLs and NK cells in the CD44^{hi} group compared to the CD44^{lo} population (Fig. 6b). Next, we examined whether CD44^{lo} and CD44^{hi} differed in their cytolytic activity against B16F10. To this end, splenocytes CD44 WT mice were stimulated for 48 h with IL-2 (1,000 U/ml) and stained with Cy5-conjugated pan anti-CD44 mAbs (IM7) and then sorted by FACS into CD44^{lo} and CD44^{hi} populations. Figure 6a depicts the results from a typical cell sorting experiment. As seen from the left panels, IL-2-activated splenocytes exhibited CD44^{lo} and CD44^{hi} populations. Approximately 84% of the IL-2-activated cells express low levels of CD44, while approximately 16% expressed high levels of CD44. After sorting the CD44^{lo} and CD44^{hi} cells, these subsets were reanalyzed for CD44 expression. As seen from Fig. 6a (middle and right panels), CD44^{lo} cells expressed mean fluorescent intensity (MFI) of 2959, while the CD44^{hi} cells exhibited MFI of 20019 in CD44 WT mice, thereby confirming the purity of the subpopulations. Next, the CD44^{lo} and CD44^{hi} LAK cells from CD44 WT mice were tested for their ability to kill B16F10 targets (Fig. 6c). The results showed that CD44^{hi} cells from CD44 WT mice exhibited marked cytolytic activity against B16F10 targets. In contrast, CD44^{lo} cells from CD44 WT mice had virtually no lytic activity against B16F10. These data further established that IL-2-induced expression of CD44 on LAK cells plays an important role in melanoma killing and suggest that it might be possible to further enrich for tumor-reactive LAK cells based on the levels of CD44 expression.

Discussion

Immunotherapeutic approaches to treat malignant melanoma continue to receive considerable interests. This is in part due to the observations that spontaneous regression of malignant melanoma is much more frequent than that of any other solid tumor [20]. In addition, there is some evidence that the tumor regression may be due to the development of an effective anti-tumor immune response as tumor-infiltrating lymphocytes can be isolated and expanded from many melanoma patients. In fact, in recent study, it was shown that tumor-specific lymphocytes could be isolated from and expanded from 78% of tested individuals [3]. The discovery of melanoma-related tumor-associated antigens has sparked continued interest into investigations exploring immunotherapeutic approaches to treat melanoma including but not limited to the use of cytokines, intratumoral gene transfer of cytokines, immunization with tumor-associated peptides and/or dendritic cells, adoptive transfer of tumor-reactive lymphocytes as well as approaches to eliminate regulatory T cells [21]. Unfortunately, most of these attempts have resulted in only limited clinical successes. More recently, the use of the anti-CTLA-4 mAb

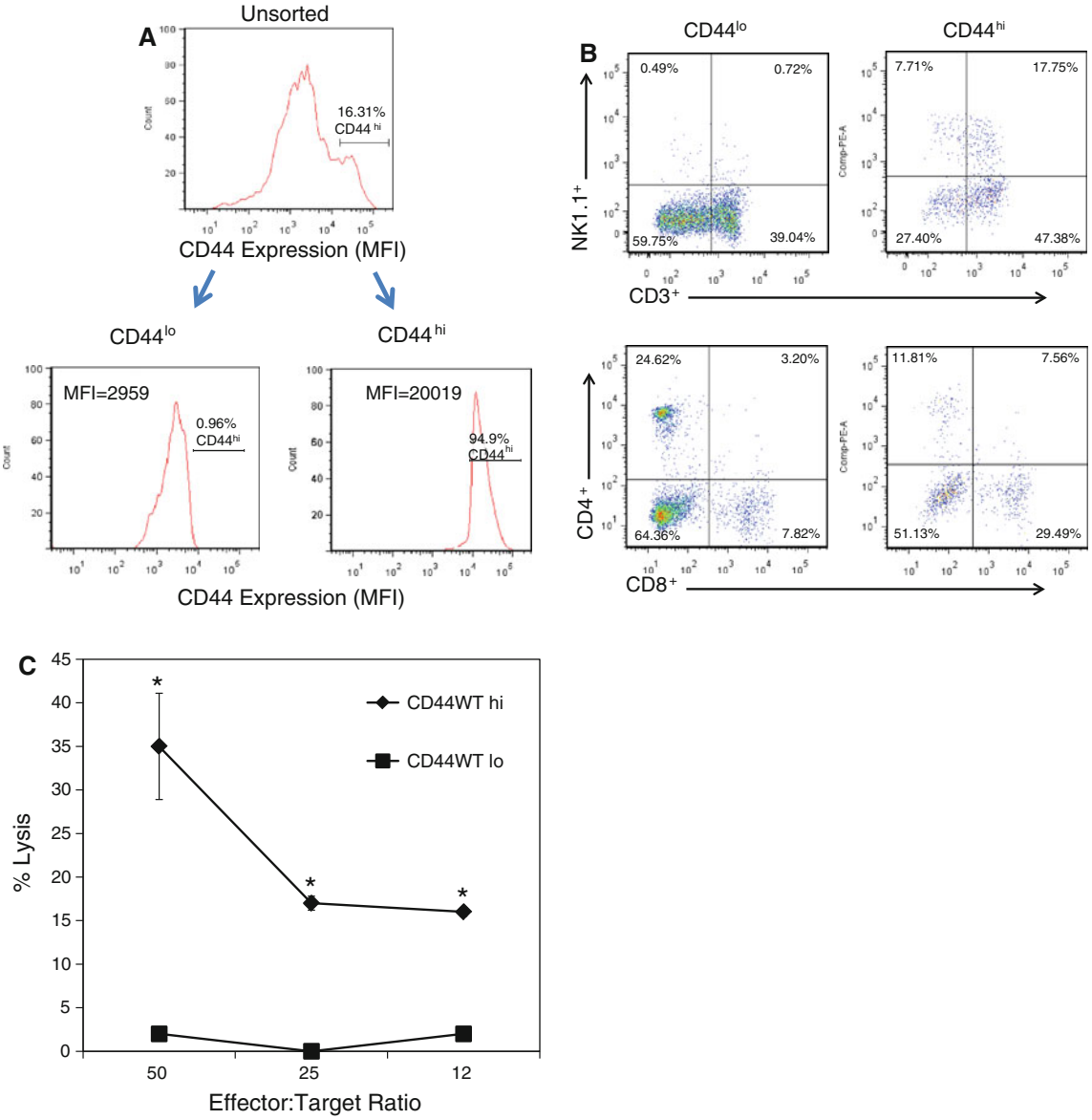

Fig. 6 IL-2 stimulation leads to the development of a highly active CD44^{hi} population enriched with NK, NKT, and CD8+ lymphocytes. Splenocytes from CD44 WT mice were stimulated with IL-2 (1,000 units/ml) for 48 h. The cells were then stained with Cy5-conjugated anti-CD44 mAbs, and the percentage of CD44^{hi} and CD44lo populations were determined (**a**). In addition, LAK cells were triple stained with Cy5-conjugated anti-CD44 mAbs along with either FITC-conjugated anti-CD3 mAbs and PE-conjugated anti-NK1.1 mAbs, or FITC-conjugated anti-CD4 mAbs and PE-conjugated anti-CD8 mAbs. The percentage of T cells (CD4$^+$ or CD8$^+$), NK (CD3$^+$NK1.1$^-$), and NKT (CD3$^+$NK1.1$^+$) cells located within the CD44^{lo} and CD44^{hi} populations was determined by FACS analysis (**b**). The cytolytic activity of CD44^{hi} and CD44^{lo} LAK cells was examined (**c**). Splenocytes from CD44 WT mice were stimulated with IL-2 (1,000 units/ml) for 48 h. The cells were then stained with Cy5-conjugated anti-CD44 mAbs and sorted according to the level of CD44 expression. *Panel A* depicts the expression levels of CD44 on IL-2-activated splenocytes before (*Unsorted*) and after sorting (*CD44^{lo}* and *CD44^{hi}*). CD44^{hi} and CD44^{lo} splenocytes from CD44 WT mice were then tested for their ability to lyse B16F10 targets. LAK activity was determined using the 4-h ^{51}Cr release assay. The data indicate the mean percentage of cytotoxicity of triplicate cultures ±SD. *Asterisk* indicates statistically significant difference when compared to cytotoxic activity of CD44 WT^{lo} LAK cells

ipilimumab has generated considerable excitement following a study demonstrating that treatment with ipilimumab improved survival in patients with unresectable stage III or IV melanoma to 10.1 months compared to 6.4 months in patients receiving gp100 vaccination [22]. Unfortunately, as with many immune-potentiating strategies, treatment with ipilimumab is associated with severe and possibly life-threatening side effects [22]. Although use of the immune system to fight cancer has made important step forward, additional treatment strategies with increased efficacy and reduced side effects are greatly needed.

In the current study, we report a role of CD44 in IL-2-induced killing of melanoma. Furthermore, our results suggest that CD44 plays a role in MHC-unrestricted killing of melanoma as in vitro stimulation of splenocytes from naïve CD44 WT mice with IL-2 led to increased expression of CD44 and significant killing of B16F10 melanoma when compared to IL-2-activated splenocytes from naïve CD44 KO mice. It is likely that this type of approach would not directly lead to the development of immunologic memory against melanoma. However, adoptive transfer of ex vivo-activated lymphocytes may lead to an environment favorable for the development of a memory response by providing initial control of tumor growth and possibly providing cytokines necessary for the development of an adaptive anti-tumor immune response. Strategies to use HLA-unrestricted tumor killing are also favorable because of the extended time it takes to develop/expand HLA-matched T cells. It has been shown that it is often difficult to obtain TILs from patients and it can take a long time to expand these cells adequately for reintroduction into the patient. Further limiting the use of TILs is the fact that the survival time is limited in malignant melanoma patients therefore the ability to develop and expand tumor-reactive immune cells quickly is a critical parameter in the development of effective immunologically based treatments for melanoma.

In addition, this study further supports the possibility of using ex vivo-expanded IL-2-activated lymphocytes for treating malignant melanoma as adoptively transfer of ex vivo-activated splenocytes from CD44 WT mice led to a significant reduction in metastatic lesions. A number of investigators are currently examining this possibility. For example, work from the Klingemann laboratory reported on the possible use of ex vivo-expanded NK-92 for the treatment of malignant melanoma. This was first reported using SCID mouse model and more recently expanded to phase I clinical trials in patients with renal cell carcinoma and melanoma [23, 24]. In addition, the results from our study suggest that using CD44 expression as a way to further enrich for tumor-reactive activity may further increase the effectiveness of adoptively transferred LAK cells. More specifically, we demonstrated that treatment with IL-2 leads to development or expansion of a $CD44^{hi}$ population. Following IL-2 stimulation, approximately 15–20% of the splenocytes express high levels of CD44. Importantly, we demonstrated that these cells have potent anti-tumor activity when compared to the $CD44^{lo}$ population. Therefore, it may be possible to enrich for this population and selectively use them for adoptive immunotherapy for melanoma and possibly other LAK-sensitive tumors.

The exact role(s) CD44 plays in LAK-mediated killing of melanoma remains uncertain. However, in addition to evidence presented in our current study, other reports suggest that CD44 is directly involved in lymphocyte-mediated tumor killing. For example, it has been reported that CTL, NK/LAK cells, and cytotoxic double-negative (DN) T cells express high levels of CD44 and that activation through CD44-mediated efficient lysis of targets cells [11, 12]. In addition, CD44 can act as a receptor mediating cell lysis. This was demonstrated using "redirected" lysis of target cells, in which a CTL clone incubated with mAbs against adhesion molecules was tested for toxicity against Ag-negative FcR^{+} target cells [11]. Here, it was observed that only mAbs against the TCR, CD44 or L-selectin could trigger redirected lysis of target cells, while mAbs against CD8, LFA-1, CD45R, VLA-4, CD3, and Class I MHC were not effective. Further studies revealed that the CD44-mediated lysis was TCR-independent and MHC unrestricted due to the ability to kill MHC-negative targets [25]. Similar results were seen when investigating the role of CD44 in NK/LAK and DN T cell lysis of target cells [12, 26]. In addition, studies revealed that soluble CD44 fusion protein or anti-CD44 Fab fragments can block CTL lysis of endothelial cells [27]. Interestingly, in our study, deletion of CD44 had no significant effect on the ability of LAK cells to kill the mouse leukemia EL-4 or the mouse pancreatic cancer Panc-02. This suggests that, in this model, CD44 expression plays an important and unique role in IL-2-induced killing of melanoma.

In addition, our study provides evidence that CD44 expression plays an important role in the ability of LAK cells to adhere to and form an effector immune synapse between LAK cells and melanoma cells. To the best of our knowledge, this is the first report demonstrating this specific function of CD44. However, the importance of other adhesion molecules in the formation of effector immunological synapses has been widely reported. For example, a number of studies demonstrate an important role of LFA-1 (CD11a) expression by activated effector cells in the adhesion between effector cells (CTL and NK) and target cells [28–32]. Other molecules such as CD2 and Mac-1 (CD11b) also play an important role in the adherence of effector cells to target cells [33, 34]. Although evidence of a role of CD44 in the formation of an effector immunological synapse between LAK cells and melanoma tumor targets has not been previously reported, a recent study suggests a possible role of CD44 in the formation of a regulatory immune synapse between dendritic cells and T cells [35]. Taken together, these data support our findings that CD44 constitutes an important molecule in LAK cells lysis of melanoma cells possibly through a role in the formation of the effector immunological synapse.

In summary, multiple lines of evidence suggest that the use of the immune system in treating melanoma holds promise. Unfortunately, many attempts to use the immune system have only resulted in marginal response. IL-2 therapy is hampered by severe, often life-threatening side

effects such as vascular leak. The mechanisms related to immune-mediated elimination of melanoma versus the side effects initiated by IL-2 are poorly understood. Gaining insight into these specific mechanisms may ultimately lead to increased anti-tumor activity while at the same time reduction in the undesirable side effects. Here, we report a specific role of CD44 in the interaction of activated immune cells with melanoma. In a previous study, we demonstrated that IL-2 leads to differential regulation of CD44 isoform expression and that specific CD44 isoforms played an important role in the development of vascular leak. More specifically, we identified a role of CD44v7 in IL-2-induced vascular leak [18]. Currently, we are extending these finding and have initiated work on examining the role of CD44 isoforms in the interaction and killing of melanoma. We propose that the importance of CD44 in killing of melanoma by IL-2-activated lymphocytes may also be influenced by CD44 isoform expression, and the ability to characterize the involvement of specific CD44 isoforms in immune-mediated control of melanoma growth may lead to development of enhanced treatments for this cancer. Furthermore, this type of information may lead to the identification of novel CD44 ligands that may serve as new tumor targets.

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Conflict of interest The authors declare that they have no conflicts of interest.

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