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Targeted Deletion of CD44v7 Exon Leads to Decreased Endothelial Cell Injury but Not Tumor Cell Killing Mediated by Interleukin-2-activated Cytolytic Lymphocytes*

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In the current study, we investigated the nature and role of CD44 variant isoforms involved in endothelial cell (EC) injury and tumor cell cytotoxicity mediated by IL-2-activated killer (LAK) cells. Treatment of CD44 wild-type lymphocytes with IL-2 led to increased gene expression of CD44 v6 and v7 variant isoforms and to significant induction of vascular leak syndrome (VLS). CD44v6-v7 knockout (KO) and CD44v7 KO mice showed markedly reduced levels of IL-2-induced VLS. The decreased VLS in CD44v6-v7 KO and CD44v7 KO mice did not result from differential activation and expansion of $CD8^+$ T cells, NK, and NK-T cells or from altered degree of perivascular lymphocytic infiltration in the lungs. LAK cells from CD44v7 KO mice showed a significant decrease in their ability to adhere to and mediate lysis of EC but not lysis of P815 tumor cells *in vitro*. CD44v7-mediated lysis of EC by LAK cells was dependent on the activity of phosphatidylinositol 3-kinase and tyrosine kinases. Interestingly, IL-2-activated LAK cells expressing $CD44^{hi}$ but not $CD44^{lo}$ were responsible for EC lysis. Furthermore, lysis of EC targets could be blocked by addition of soluble or enzymatic cleavage of CD44v6-v7-binding glycosaminoglycans. Finally, anti-CD44v7 mAbs caused a significant reduction in the adherence to and killing of EC and led to suppression of IL-2-induced VLS. Together, this study suggests that the expression of CD44v7 on LAK cells plays a specific role in EC injury and that it may be possible to reduce EC injury but not tumor cell killing by specifically targeting CD44v7.

Endothelial cell (EC)[1] injury is a widely occurring pathological condition seen during a variety of infections, autoimmunity, transplantation, and graft-*versus*-host disease and following immunotherapy with cytokines and immunotoxins (1–6). It is becoming increasingly clear that EC injury results from cytotoxicity of EC mediated by immune effector lymphocytes (7–10). Interleukin-2 (IL-2) therapy has been shown to be effective against human melanomas and renal cell carcinomas (11–13). It is also being tested to treat AIDS and other viral infections (14). However, IL-2 therapy is also accompanied by severe life-threatening toxicity characterized by EC injury leading to capillary or vascular leak syndrome (VLS) (11, 15, 16).

CD44 is a widely distributed cell surface glycoprotein expressed by a variety of lymphoid and non-lymphoid cells. CD44 is involved in a number of processes including lymphocyte migration and extravasation, lymph node homing, and lymphocyte activation (17, 18). CD44 is coupled to at least two tyrosine kinases, suggesting that it can directly act as a signaling molecule (19). Studies from our laboratory demonstrated that ligation of CD44 triggers lytic activity in activated $CD8^+$ cytotoxic T lymphocytes as well as in double-negative T cells that accumulate in *lpr/lpr* mice (20–22). Other studies showed that treatment with anti-CD44 mAbs increases the lytic activity of natural killer (NK) cells (23, 24). In addition, reports suggest that CD44 plays an important role in adherence of lymphokine-activated killer (LAK) cells to tumor targets (25). Initial studies from our laboratory examining the role of CD44 in IL-2-induced VLS revealed that the induction of VLS in CD44 KO mice was significantly reduced when compared with CD44 wild-type (WT) mice (10). Together, these observations suggested that activation through CD44 can enhance the effector functions of T lymphocytes and NK cells and that this may lead to the increased EC injury leading to VLS as well as effective killing of tumor cells. However, the precise nature of CD44 isoforms involved in EC injury and the cytotoxicity of tumor cells is not clear.

The standard form of CD44, designated CD44s, is the most common form and has a size of 85–95 kDa (26). In addition to CD44s, a number of larger CD44 isoforms, ranging in size from 80 to 250 kDa, develop as a result of alternative splicing of variant exons (27). It has been suggested that expression of specific isoforms may play a role in the regulation of the immune response as well as in the development of autoimmune disorders. For example, CD44v7 has been shown to be involved

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[1] The abbreviations used are: EC, endothelial cell; VLS, vascular leak syndrome; NK, natural killer; LAK, lymphokine-activated killer; GFP, green fluorescent protein; HA, hyaluronic acid; CFSE, carboxyfluorescein diacetate succinimidyl ester; rTRU, relative turbidity reducing unit(s); IL, interleukin; mAb, monoclonal antibody; KO, knockout; WT, wild-type; PBS, phosphate-buffered saline; FCS, fetal calf serum; PI, phosphatidylinositol; FACS, fluorescence-activated cell sorting; PE, phycoerythrin; ConA, concanavalin A; FITC, fluorescein isothiocyanate; RT, reverse transcriptase; TNF, tumor necrosis factor; GST, glutathione *S*-transferase.

TABLE I
Sequences, amplicon sizes, and annealing temperatures of RT-PCR primers

Gene	Primer name	Sequence (5′–3′)	Length	T_m
			bp	*°C*
β-Actin	BA U	AAGGCCAACCGTGAAAAGATGACC	427	60
	BA L	ACCGCTCGTTGCCAATAGTGATGA		
Perforin	Perf U	GCTCCGGCTCCTTCCCAGTG	514	60
	Perf L	GCCGTGATAAAGTGCGTGCCATAG		
TNF-α	TNF A U	CAGGGGCCACCACGATCTTC	516	60
	TNF A L	TTGCCCCGCTTACAGTTCCTCTTT		
FasL	FasL U	GGGCTCCTCCAGGGGTCAGTT	435	58
	FasL L	GAGCGGTTCCATATGTGTCTTCC		
CD44	CD44 U	GCACCCCAGAAGGCTACATTTT	328[a]	57
	CD44 L	TTCTGCCCACACCTTCTCCTACTA		
CD44v3	v3 U	TACGGAGTCAAATACCAACC	Variable[b]	46
	v3 L	ATAAAATCTTCATCATCATCAAT	Variable	45
CD44v6	v6 L	CCCTTCGTCACATGGGAGTCTTC	Variable	57
CD44v5	v5 U	GGACCCCGGAACCACAGC	Variable	56
CD44v7	v7 U	TTCGGCCCACAACAACCA	Variable	55
	v7 L	GATGTGAGATTGGGTCGAAGAAAT	Variable	55
CD44v8	v8 U	GCCTACTGCAGCTCCAAATA	Variable	55

[a] CD44S (standard form). These primers also amplify CD44 isoforms containing alternatively spliced exons, but the standard form is of lower molecular weight and more abundant, and is therefore preferentially amplified by PCR.
[b] Multiple PCR products of variable size were generated when using variable exon-specific primers in conjunction with conserved region primers because of the inclusion of other alternatively spliced exons. See Fig. 1 for a depiction of the PCR strategy.

in the production of cytokines by B cells and to play a role in the interaction of T cells with antigen-presenting cells, and progenitor cells with stromal cells (17, 28). Other isoforms of CD44, such as CD44v6 and CD44v9, were shown to be involved in B cell adhesion to stromal cells (29). Treatment with anti-CD44v7 antibodies can inhibit the production of IL-2 and interferon-γ by $CD8^+$ cells (28). In addition, studies have shown that trinitrobenzene sulfonate-induced colitis can be prevented and cured by treatment with anti-CD44v7 mAbs (30). Therefore, it is possible that specific CD44 variant isoforms play a role in mediating EC damage seen in disorders such as VLS.

In the current study, we examined whether specific CD44 isoforms play a role in mediating IL-2-induced VLS and tumor cytotoxicity. To this end, we screened splenocytes from mice treated with high doses of IL-2 for the expression of various CD44 isoforms and used CD44 variant knockout (KO) mice to examine whether the absence of specific isoforms would lead to reduced level of VLS. The results from this study demonstrated that CD44v7 plays an important role in the induction of vascular leak and EC damage following IL-2 treatment and that antibodies directed against CD44v7 can block IL-2-induced EC damage and VLS. Interestingly, CD44v7 did not play a role in the cytotoxicity of P815 tumor cells mediated by LAK cells. Together, the information in this study suggests that strategies designed to target the expression of CD44v7 may help to prevent EC damage seen in a variety of disorders.

MATERIALS AND METHODS

Mice—Adult female C57BL/6 (CD44 WT) mice were purchased from the National Institutes of Health (Bethesda, MD). CD44 KO mice represent those in which the entire CD44 gene is deleted; therefore, such mice fail to express all CD44 isoforms (10, 31). CD44v7 KO and CD44v6-v7 mice are those with targeted deletion of CD44v7 or CD44v6 and -v7 exon products, respectively. Such mice, therefore, fail to express CD44 isoforms bearing the variant exon v7 or v6 and v7, respectively (30). All these strains were on C57BL/6 background. The CD44 KO, CD44v6-v7 KO, and CD44v7 KO mice were bred in the animal facilities at the Medical College of Virginia Campus, Virginia Commonwealth University, and screened for the CD44, CD44v6-v7, and CD44v7 deletion, respectively. TIE2-GFP mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice express green fluorescent protein (GFP) under the control of the endothelial-specific receptor tyrosine kinase (TIE2) promoter (32).

Cell Lines—TME (an endothelial cell line; $H\text{-}2^K$), SVC3H (a fibroblast cell line; $H\text{-}2^K$), and P815 (a mastocytoma) were maintained *in vitro* by serial passage in RPMI 1640 containing 10% FCS as described (33).

RNA Isolation and RT-PCR Analysis—Total RNA was isolated from single cell suspension of splenocytes using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentration was determined spectrophotometrically, and the integrity of the each preparation was verified by agarose gel electrophoresis. cDNA was synthesized by reverse transcription of 50 ng of total RNA using the SensiScript RT Kit (Qiagen). All PCR reactions were prepared using MasterAmp PCR Premix F (Epicenter Technologies, Madison, WI) according to the recommendations from the manufacturer, and using Platinum *Taq*DNA polymerase (Invitrogen, Carlsbad, CA). A half-nested RT-PCR approach was used to amplify CD44 isoforms, as endogenous isoform expression is too low to be detected directly by RT-PCR (data not shown). An initial amplification using primers CD44 U and CD44 L, homologous to the 5′ and 3′ standard regions of the CD44 gene, was used to amplify both CD44 standard form and all isoforms. The products of this reaction were used as template in a second reaction using a CD44 isoform-specific primer in conjunction with the appropriate CD44 standard region primer (CD44 L and CD44 isoform U). The primers used are described in Table I.

Cloning and DNA Sequencing—PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen). After overnight incubation at 37 °C on LB plates containing ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), positive (white) colonies were subcultured in 300 μl of LB_{amp100} medium at 37 °C for 4 h. Two μl of each culture was used as template in a PCR reaction to confirm the presence of an insert. Cultures containing CD44 isoform products were grown overnight in 3 ml of LB_{amp100} medium. Plasmids were isolated using the Spin Miniprep Kit (Qiagen). Plasmids were sequenced using the M13 Reverse and M13 Forward (−40) primers, and using ABI BigDye terminator (version 2) chemistry (Applied Biosystems, Foster City, CA).

Antibodies—PE- or FITC-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD16 (Fc block), anti-CD19, anti-CD25, anti-CD44, Mac-3, and anti-NK1.1 mAbs were purchased from Pharmingen (San Diego, CA). Anti-CD44v7 mAbs (LN7.1) were prepared as previously described (34). Briefly, CD44v6-v7 KO mice were immunized with 100–200 μg of CD44v4-v10/glutathione *S*-transferase (GST) fusion protein, three times over a 12-day period. Two days following the last immunization, the cells from the regional lymph nodes were fused using the X63-Ag8 cell line. The epitope recognized by LN7.1 was mapped and was shown to recognize the following peptide located within the CD44v7 region: QEDVSWTDFFDPISHP (30). The mAbs were purified from cell culture supernatants using protein G-Sepharose columns (Amersham Biosciences), and isotypes of the mAbs were determined using commercially available kits (Serotec, Roche Molecular Biochemicals) (30). Isotype control mAbs (mouse IgG1) were purchased from Pharmingen.

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

Flow Cytometric Analysis of Cell Surface Markers and Cell Sorting—Splenocytes from mice treated with high dose IL-2, as described below, or splenocytes stimulated with IL-2 *in vitro* were analyzed for the expression of various cell surface markers. Nonspecific staining was

blocked by incubation of the cells with Fc block (Pharmingen) for 15 min. The expression of various cell surface markers were determined by staining the cells with FITC- or PE-conjugated mAbs for 30 min on ice, followed by washing three times. The cells (5000) were analyzed by flow cytometry. In experiments in which CD44 cell expressing low ($CD44^{lo}$) and high ($CD44^{hi}$) levels of CD44 were separated, nonspecific staining was blocked by incubation of the cells with Fc block (Pharmingen) for 15 min. Next, the cells were stained with PE-conjugated anti-CD44 mAbs (IM7) for 30 min on ice and washed twice with PBS. The $CD44^{lo}$ and $CD44^{hi}$ cells were separated using an Epics 753 cell sorter (Beckman Coulter, Fullerton, CA).

Quantification of Vascular Leak Syndrome—Vascular leak was studied by measuring the extravasation of Evan's blue, which, when given intravenously, binds to plasma proteins, particularly albumin, and following extravasation can be detected in various organs as described previously (10, 35, 36). Vascular leak was induced by injection of IL-2 as previously described (9, 10). Groups of five mice were injected intraperitoneally with 75,000 units of rIL-2 or PBS as a control, three times a day for 3 days. On day 4, the mice received one injection and 2 h later were injected intravenously with 0.1 ml of 1% Evan's blue in PBS. After 2 h, the mice were exsanguinated under anesthesia, and the heart was perfused with heparin in PBS as described previously (37). The lungs were harvested and placed in formamide at 37 °C overnight. The Evan's blue in the organs was quantified by measuring the absorbance of the supernatant at 650 nm with a spectrophotometer. In experiments examining the effect of anti-CD44v7 mAbs on IL-2-induced VLS, mice were treated with isotype control mAbs or anti-CD44v7 mAbs (100 μg/mouse daily). In adoptive transfer experiments, CD44 WT and CD44v7 KO mice were injected intravenously with splenocytes (5×10^7) isolated from CD44 WT or CD44v7 KO mice (10). Next, the recipient mice received IL-2 treatment (75,000 units, three times a day for 3 days) or PBS as described above, and on day 4, the mice were studied for VLS induction. The VLS seen in IL-2-treated mice was expressed as percentage of increase in extravasation when compared with that of PBS-treated controls and was calculated as: [(optical density of dye in the lungs of IL-2-treated mice) − (optical density of dye in the lungs of PBS-treated controls)]/(optical density of dye in the lungs of PBS-treated control) × 100. Each mouse was individually analyzed for vascular leak, and data from five mice were expressed as mean ± S.E. percentage increase in VLS in IL-2 treated mice when compared with that seen in PBS-treated controls (9, 10).

Histological Analysis—Groups of three mice were injected with IL-2 or PBS as described earlier, and on day 4, the lungs were fixed in 10% formalin solution. The organs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, as described (9, 10). Perivascular infiltration was scaled by counting the number of lymphocytes infiltrating the vessel and averaging the minimum and maximum range for each group. Four samples per mouse were analyzed, and a minimum of four mice were included.

Mitogen Stimulation and Cell Proliferation—Spleen cells (5×10^5) from CD44 WT, CD44v6-v7 KO, or CD44v7 KO mice were cultured for 48 h in the presence of ConA (5 μg/ml), IL-2 (100 units/ml), or hyaluronic acid (HA; 0–0.5 mg/ml) in 0.2 ml of medium containing 10% FCS. The cells were pulsed with 2 μCi of [^{3}H]thymidine during the final 8 h of culture. The cells were then harvested using a cell harvester, and the labeled DNA was determined using a liquid scintillation counter.

Generation of LAK Cells—Spleens were harvested from CD44 WT and CD44v7 KO mice and were prepared into a single cell suspension using a laboratory homogenizer (Stomacher, Tekmar, Cincinnati, OH). Contaminating erythrocytes were removed by resuspending the cells in 3 ml of red blood cell lysing buffer (Sigma) for 5 min and then washing three times in RPMI containing 10% FBS. After the third wash, the viable spleen cells were quantified by trypan blue dye exclusion and counting using a hemocytometer. The splenocytes were adjusted to 5×10^6/ml and were cultured *in vitro* for 48 h with 1000 units/ml IL-2 in RPMI containing 10% FBS. The cells were harvested, and viable cells were purified by density gradient centrifugation using Ficoll-Hypaque (Sigma). Such cells will be referred to as LAK cells. For generation of LAK cells *in vivo*, CD44 WT and CD44v7 KO mice were treated with IL-2 as described above. On day 4, the spleens from IL-2-treated mice were harvested and prepared into a single-cell suspension, as described above. LAK cells generated *in vitro* and *in vivo* were tested for cytotoxicity against P815 tumor, SVC3H fibroblast, or TME EC targets using the ^{51}Cr release assay. 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 effector to target ratios and incubated for 4 h at 37 °C. In these experiments the LAK cells are defined as the effector cells that mediate lysis of the $^{51}NaCrO_4$-labeled EC or tumor cells, which are defined as the target cells. In experiments examining the effect of anti-CD44v7 mAbs on the LAK activity, soluble anti-CD44v7 mAbs or isotype control mAbs were added to the LAK cells 1 h prior to the addition of the EC targets. In experiments examining the effects of soluble glycosaminoglycans on LAK activity, the various glycosaminoglycans including chondroitin sulfate A, B, and C, heparin, hyaluronate, and hyaluronate (Sigma) were added to the cultures at the initiation of the 4-h assay. In experiments examining the effects of wortmannin (Sigma) and herbimycin (Sigma) on LAK activity, the inhibitors were added at the initiation of the 4-h assay. 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 γ counter.

HA Binding Studies—HA was bound to Covalink NH plates using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce) (29). Briefly, 100 μl of HA (100 μg/ml) mixed with 50 μl of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 50 μl of HCl (0.4 mM) were added to individual wells of a 96-well plate and incubated for 2 h at room temperature. The plates were washed and stored at 4 °C before use in adhesion assays. To determine the level of adherence following stimulation with IL-2, splenocytes from untreated and IL-2-treated lymphocytes were labeled for 4 h with [^{3}H]thymidine (5 μCi/ml). The labeled splenocytes (10^5/well) were added to each well and incubated for 1 h at 37 °C. Non-adherent cells were removed by gently pipetting with PBS. The percentage of cells adhering to the plate was determined using the following formula: [(counts/min (cpm) of cells adhering to HA-coated plates − cpm of cells adhering to non-coated plates)/total cpm (non-adherent cells plated on non-coated plates)] × 100%.

Enzymatic Treatment—To examine the effect of enzymatic cleavage of glycosaminoglycans on LAK activity, chondroitinase AC (0.02 units/ml), chondroitinase B (0.02 units/ml), chondroitinase C (0.02 units/ml), heparinase (0.02 units/ml), or hyaluronidase (100 rTRU/ml) was added at the initiation of the 4-h ^{51}Cr release assay. The enzymes were purchased from Sigma. In assays in which EC and LAK cells were pretreated with hyaluronidase (100 rTRU/ml), the cells were treated for 1 h at 37 °C, after which time the cells were washed with RPMI 1640 medium containing 10% FCS and tested in the ^{51}Cr release assay.

Cell Adhesion Assay—EC were plated at 1×10^4/well and cultured at 37 °C overnight in a 96-well flat-bottomed plate. LAK cells generated *in vitro* as described earlier were independently cultured for 16 h in the presence of 5 μCi of [^{3}H]thymidine. The LAK cells were washed and added to the empty wells (total [^{3}H]thymidine activity) or wells containing EC cultures and incubated for 2 h at 37 °C. In experiments examining the effect of anti-CD44v7 mAbs on the LAK cell adhesion to EC, soluble anti-CD44v7 mAbs or isotype control mAbs were added to the LAK cells 1 h prior to culture on EC-coated plates. 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. Adherent cells were quantified by measuring the [^{3}H]thymidine activity and compared with the [^{3}H]thymidine activity of cells removed from wells without EC (total activity). The percentage of LAK cells adhering to the EC was determined using the following formula: (cpm of adhering cells/cpm of total activity) × 100.

Cell Aggregation—Cell aggregation was visualized using a protocol as described by Matsumoto *et al.* (25). In experiment using TME EC, LAK cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and EC were labeled with PKH26-GL fluorescent cell linker compound (PKH, Sigma-Aldrich). In experiments using GFP-positive EC isolated from TIE2-GFP mice, LAK cells were labeled with PKH and mixed with EC that constitutively express GFP. EC (1×10^6/ml) and LAK cells (4×10^6/ml) were mixed together in equal volumes (total volume of 1 ml) and centrifuged for 10 min at 500 rpm. The resulting pellets were cultured for 10 min at 37 °C to facilitate cell aggregation. To stop cell aggregation, the pellets were resuspended in ice-cold PBS. Cell aggregation was visualized using a fluorescent microscope (Nikon, Melville, NY) by overlaying images of red fluorescence (PKH) with images of green fluorescence (CFSE or GFP). In experiments using GFP and PKH, PKH-positive cells are depicted as yellow following overlay of the images. Quantification of cell aggregation was performed by determining the percentage of EC conjugated to at least one LAK cell in 10 random fields. The results were depicted as percentage of cell aggregation and was calculated as: (number of endothelial cells per field conjugated to at least one LAK cell)/(total number of endothelial cells per field) × 100.

EC Isolation—EC were isolated from TIE2-GFP mice using methods described by Marelli-Berg *et al.* (38). Briefly, lungs were isolated from

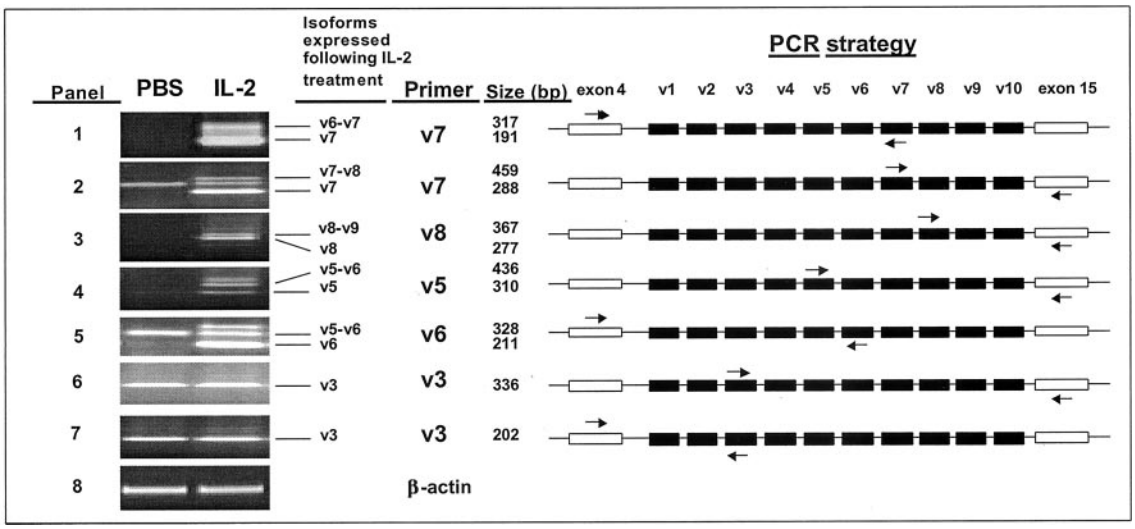

FIG. 1. **RT-PCR-based characterization of CD44 isoform expression in IL-2-activated splenocytes.** RNA isolated from splenocytes of PBS-treated and IL-2-treated mice was subjected to RT-PCR analysis using the PCR strategy depicted above and the primer sets listed in Table I. PCR products were resolved on 1.2% agarose gels. The alternatively spliced exons comprising the PCR products from each reaction, confirmed by sequence analysis, are listed to the *right* of the gel images along with the length of each product in base pairs. The results of each PCR reaction, containing a pool of alternatively spliced isoforms, was cloned into the TOPO-TA vector, and positive colonies screened by PCR. Colonies containing CD44 isoforms were then sequenced in both directions using SP6 and T7 primers.

TIE2-GFP mice, mechanically dissected, and digested in collagenase (0.5 mg/ml) for 1 h at 37 °C. The cells were washed twice in PBS containing 2.5% FCS and incubated with murine serum to block Fc receptors. The cells were cultured with rat anti-mouse CD31 and CD105 mAbs as well as biotinylated isolectin B4 for 30 min on ice. Next, the cells were washed and cultured with goat anti-rat IgG-conjugated microbeads and streptavidin-conjugated microbeads for 15 min on ice. The cells were then passed through a MACS separation unit, and the cells magnetically retained were eluted. The purity of EC was confirmed by analyzing the cells for GFP using a flow cytometer, and the EC preparation was found to be >95% GFP-positive.

Statistical Analysis—Analysis of variation and Student's t test were used to determine statistical significance, and $p < 0.05$ was considered to be statistically significant.

RESULTS

Up-regulation of CD44v7 and CD44v6-v7 Variant Isoforms after IL-2 Treatment in Vivo—To determine whether IL-2 treatment led to the differential regulation of CD44 variant isoform expression, spleen cells from CD44 WT mice injected with IL-2 or PBS, as described under "Materials and Methods," were harvested on day 4 and analyzed for the expression of various CD44 isoforms. The results showed that treatment with IL-2 *in vivo* led to the expression of a number of CD44 variant isoforms (Fig. 1). Most notable was the dramatic induction of isoforms containing alternatively spliced exons v6 and v7 (compare PBS *versus* IL-2; see Fig. 1, *panels 1*, *2*, *4*, and *5*). Amplification using a v7 reverse primer and CD44 conserved region forward primer (Fig. 1, *panel 1*) did not yield a product in PBS-treated sample. However, in IL-2-treated samples, products of 191 and 317 bp were generated. Cloning and sequence analysis of these products revealed them to be isoforms containing v7 alone and v6-v7, respectively. Similarly, amplification using a v7 forward primer and CD44 conserved region reverse primer also yielded no product in the PBS-treated sample. Cloning and sequence analysis revealed that the faint band seen in the PBS lane (Fig. 1, *panel 2*) is a nonspecific artifact, resulting from the half-nested amplification strategy used. Two products of 288 and 459 bp were generated in the IL-2-treated samples, which, following cloning and sequencing, were revealed to contain v7 alone and v7-v8, respectively. CD44v6 isoforms were amplified using a v6 reverse primer and CD44 conserved region forward primer (Fig. 1, *panel 5*). A 328-bp product was amplified in the PBS-treated sample, which was found to contain exons v6 and v5. Amplification of the IL-2-treated sample resulted in products of 211, 328, and 410 bp. Sequencing of the 211-and 328-bp products revealed them to be v6 alone and v6-v5, respectively. The expression of v6 and v5 was confirmed by amplification using a v5-specific forward primer and CD44 conserved region reverse primer (Fig. 1, *panel 4*). No products were amplified in the PBS-treated sample; however, bands of 310 and 436 bp were amplified from IL-2-treated samples. Sequencing of these products confirmed them to be v5 alone and v5-v6. In addition, IL-2 treatment resulted in up-regulation of isoforms containing v8 and v8-v9 (Fig. 1, *panel 3*). Isoforms containing v3 were found to be constitutively expressed, and were not affected by IL-2 treatment (Fig. 1, *panels 6* and *7*).

Decreased VLS in CD44v7 KO and CD44v6-v7 KO Mice—To investigate the role of CD44v7 and CD44v6 isoforms in IL-2-induced VLS, CD44 WT, CD44 KO, CD44v7 KO, and CD44v6-v7 KO mice were injected with 75,000 units of IL-2 three times daily for 3 days and once on day 4. On the last day, the mice were injected with 1% Evan's blue and VLS was studied by determining the extravasation of the dye in the lungs. Fig. 2 shows a representative experiment in which the CD44 WT mice displayed significant VLS following IL-2 treatment and CD44 KO mice showed marked decrease in VLS as reported previously (10). Interestingly, the CD44v7 KO and CD44v6-v7 KO mice also exhibited significantly lower levels of VLS in the lungs. The decreased VLS seen in CD44v7 KO mice was similar to that observed in CD44 KO and CD44v6-v7 KO mice, suggesting that the product of the v7 exon may play a specific role in the induction of IL-2-induced VLS. Inasmuch as, in an earlier study we had extensively investigated the IL-2-induced VLS in CD44 KO mice (10), all subsequent experiments were confined to CD44v7 KO and CD44v6-v7 KO mice.

Histological Analysis of Lungs from IL-2-treated Mice—Previous studies demonstrated that IL-2 treatment triggered marked infiltration of lymphocytes and macrophages in the lungs (9, 10). Histological studies were therefore conducted to determine whether the reduced levels of VLS seen in CD44v7 KO and CD44v6-v7 KO mice were caused by an alteration in the migration of mononuclear cells into the lungs. Mice were injected with PBS or IL-2, as described in Fig. 2. On day 4, the

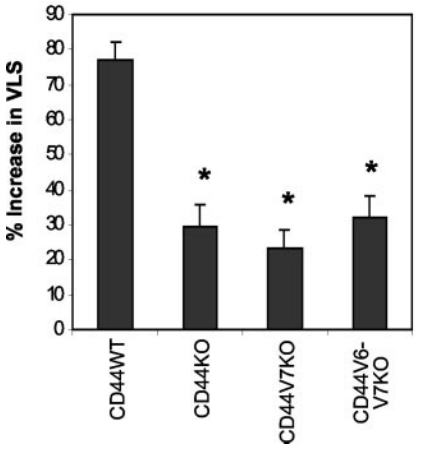

FIG. 2. **VLS induction following IL-2 treatment *in vivo*.** CD44 WT, CD44 KO, CD44v7 KO, and CD44v6-v7 KO mice were injected intraperitoneally with 75,000 units of IL-2 alone 3 times daily for 3 days and once on day 4 or with PBS. Two hours after the last injection with IL-2, the mice were injected with 1% Evans blue, and VLS was studied by determining the extravasation of Evans blue dye in the lungs. The *vertical bars* represent the percentage of increase in VLS ± S.E. following IL-2 treatment compared with that in the PBS-treated controls as described in "Materials and Methods." *Asterisks* indicate statistically significant difference when compared with the CD44 WT controls, $p < 0.05$.

lungs were harvested and stained with hematoxylin and eosin (Fig. 3, *A–D*). The lungs from the PBS-treated mice did not show any perivascular infiltration. In contrast, the IL-2-treated CD44 WT mice exhibited significant perivascular infiltration consisting mainly of lymphocytes around the pulmonary vein. In comparison, the lungs from CD44v7 KO and CD44v6-v7 KO mice treated with IL-2 showed similar levels of perivascular infiltration as the IL-2-treated CD44 WT mice (Fig. 3*E*). These data suggested that the decrease in IL-2-induced VLS seen in CD44v7 KO and CD44v6-v7 KO mice was not the result of alterations in the ability of mononuclear cells to infiltrate into the lungs.

The Effect of IL-2 Treatment in Vivo on the Expansion of Effector Cell Populations in CD44 WT, CD44v6-v7 KO, and CD44v7 KO Mice—We examined whether the reduction in the level of VLS seen in CD44v7 KO and CD44v6-v7 KO mice when compared with CD44 WT mice resulted from altered expansion of the effector cells. To this end, CD44 WT, CD44v7 KO, and CD44v6-v7 KO mice were treated with IL-2, as described above. After the final injection of IL-2 on day 4, spleens were harvested, cellularity was determined and the cells were stained for the expression of CD4, CD8, and combined CD3 and NK1.1. IL-2 treatment caused marked and somewhat similar increase in total cellularity of the spleen, in all groups of mice, when compared with the PBS-treated controls (Table II). Furthermore, IL-2 treatment led to an increase in the percentage and total number of $CD8^+$ and a consequent decrease in the percentage of $CD4^+$ cells (Table II). Similar results were seen in the CD44v7 KO and CD44v6-v7 KO mice, indicating that the reduction in VLS seen in these mice was not the result of an alteration in the expansion of $CD8^+$ cells *in vivo*. When the effect of IL-2 treatment on NK ($CD3^-NK1.1^+$) and NK-T ($CD3^+NK1.1^+$) cells was investigated, it was observed that IL-2 caused a similar increase in the percentage and total number (Fig. 4, Table II) of NK and NK-T cells in CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice. Together these results suggested that treatment with IL-2 led to the expansion of $CD8^+$ T cells, NK cells, and NK-T cells and that this expansion was not altered in CD44v7 KO or CD44v6-v7 KO mice when compared with CD44 WT mice.

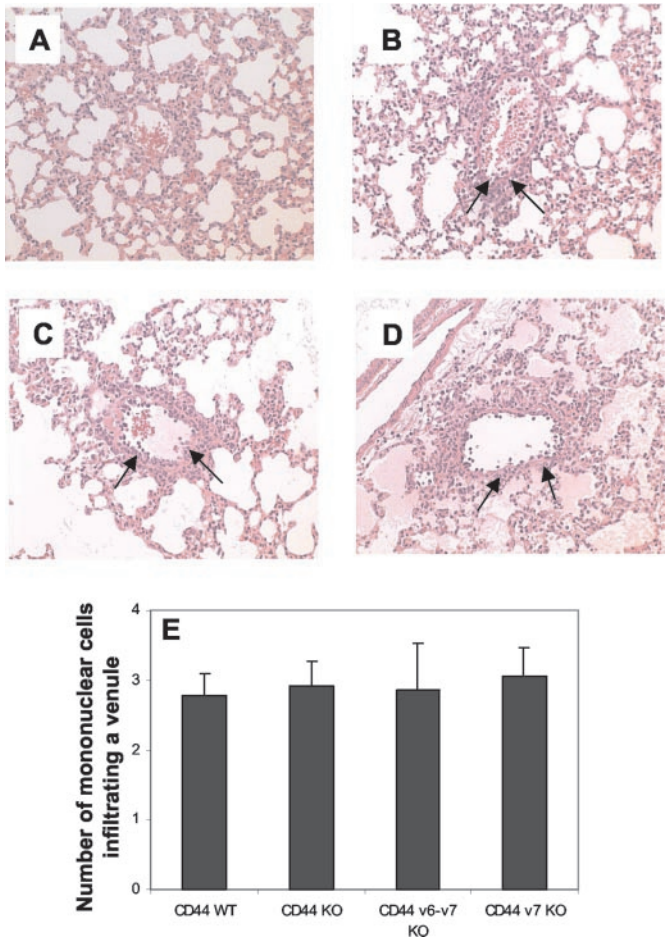

FIG. 3. **Histological studies of lungs following IL-2 administration.** Lungs from CD44 WT mice treated with PBS (*A*) and lungs from CD44 WT (*B*), CD44v6-v7 KO (*C*), and CD44v7 KO (*D*) mice treated with IL-2 were harvested, preserved in 10% formalin solution, sectioned, and stained with hematoxylin and eosin. *Arrows* indicate perivascular infiltration, consisting mostly of lymphocytes. *E*, The level of perivascular infiltration was determined by counting the number of cells infiltrating a venule. The data depict the mean ± S.E. of sections from four individual mice.

IL-2-activated Killer Cell Activity against Endothelial Cell (EC) Targets Is Reduced in CD44v6-v7 and CD44v7 KO Mice in Vitro and in Vivo—IL-2-activated cytotoxic cells can kill EC and induce VLS (33). Therefore, we compared the ability of splenocytes from IL-2-treated CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice to lyse TME, a well characterized endothelial cell line. To this end, CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice were treated with IL-2 for 4 days, as described above. The splenocytes were harvested and tested for cytotoxicity against EC targets using a standard chromium release assay (Fig. 5*A*). The results demonstrated that splenocytes from CD44 WT mice caused significant lysis of EC. However, the lysis of the EC was greatly diminished in the cultures containing the CD44v6-v7 and CD44v7 KO splenocytes.

The decreased LAK activity of splenocytes from CD44v6-v7 and CD44v7 KO mice against EC was further confirmed by experiments in which splenocytes from CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice were cultured with high concentrations of IL-2 (1000 units/ml) for 48 h *in vitro* and examined for their ability to lyse TME EC, SVC3H fibroblasts, and P815 tumor cells. The results showed that IL-2-activated cytotoxic cells from CD44v6-v7 KO and CD44v7 KO mice exhibited decreased ability to kill EC when compared with the LAK cells from the CD44 WT mice (Fig. 5*B*). The lytic activity was spe-

TABLE II
Effects of IL-2 on effector cell populations in vivo

	Cellularity[a]	Splenocyte populations[b]			
		$CD4^+$	$CD8^+$	$CD3^-NK1.1^+$	$CD3^+NK1.1^+$
	cell no. $\times 10^6$*/spleen*				
CD44 WT + PBS	131.8 ± 8.2[c]	24.7 (32.6)	14.8 (19.5)	8.5 (11.2)	1.3 (1.7)
CD44 WT + IL-2	194.9 ± 2.3	18.4 (35.9)	21.6 (42.1)	29.4 (57.3)	5.6 (10.9)
CD44v7 KO + PBS	98.7 ± 3.0	28.6 (28.2)	11.2 (11.1)	8.5 (8.4)	0.7 (0.7)
CD44v7 KO + IL-2	192.2 ± 10.4	15.9 (30.5)	21.4 (41.1)	24.21 (46.5)	2.8 (5.4)
CD44v6-v7 KO + PBS	85.1 ± 5.3	24.7 (21.0)	15.0 (12.8)	11.35 (9.7)	1.2 (1.0)
CD44v6-v7 KO + IL-2	198.5 ± 3.2	15.5 (30.8)	21.2 (42.1)	26.6 (52.8)	4.2 (8.3)

[a] Mice were treated with IL-2 (75,000 units/mouse three times daily for 4 days) or the vehicle. On day 4, the spleens were harvested and the total cellularity was determined.
[b] Splenocyte subsets were determined using flow cytometry. The data represent the percentage of $CD4^+$ T cells, $CD8^+$ T cells, $CD3^-NK1.1^+$ cells, and $CD3^+NK1.1^+$ cells in the spleens obtained from 3 mice. The numbers in parentheses represent the mean total cellularity $\times 10^6$ of each cell population found in the spleens.
[c] Data represent mean ± S.E. obtained from 3 mice.

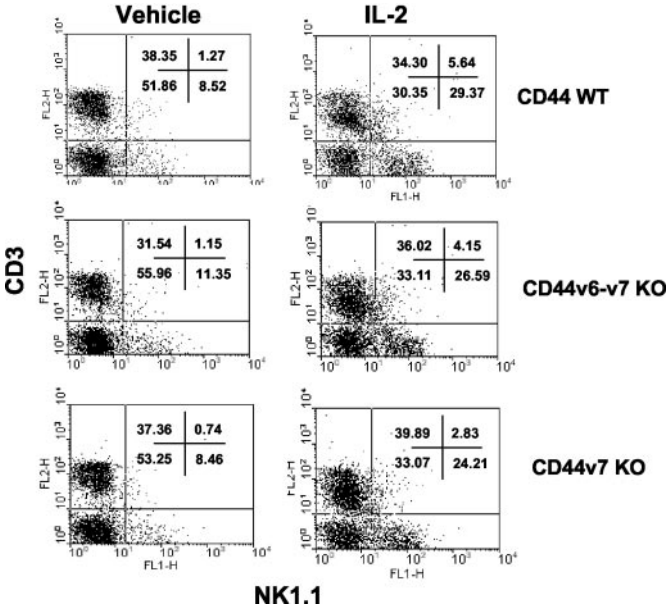

FIG. 4. **Expansion of NK and NK-T cells following IL-2 stimulation *in vivo*.** Splenocytes from PBS- and IL-2-treated CD44 WT and CD44v7 KO mice were stained with PE-conjugated anti-CD3 mAbs and FITC-conjugated anti-NK1.1 mAbs for 30 min and analyzed by a flow cytometer. The percentage of positive cells is depicted in each *quadrant*.

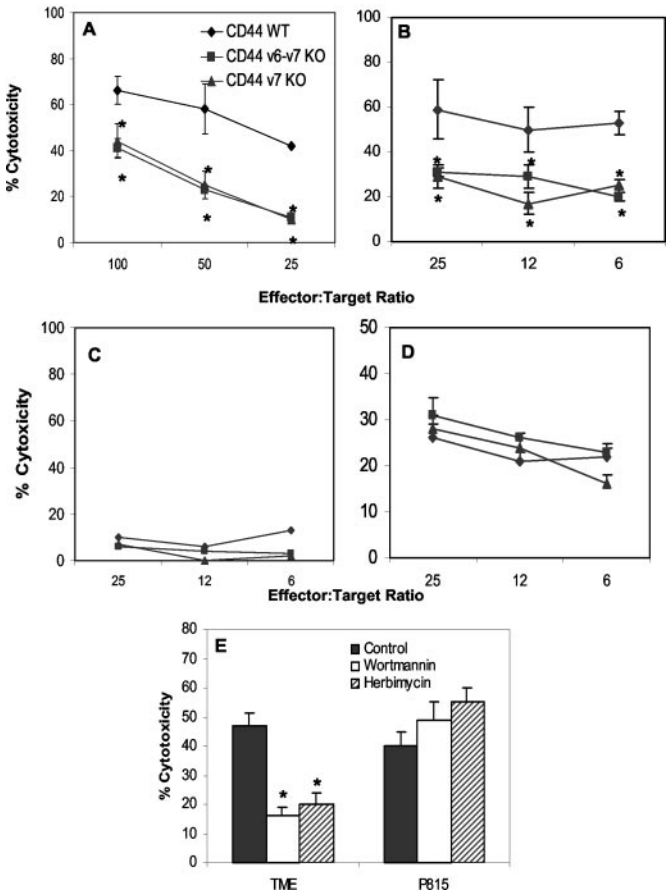

FIG. 5. **Cytolytic activity of IL-2-stimulated LAK cells.** Splenocytes from IL-2-treated CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice (*A*) or naive splenocytes from CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice cultured *in vitro* for 48 h with 1000 units/ml IL-2 (*B*) were used as effector cells and were tested for cytolytic activity against ^{51}Cr-labeled TME endothelial cell targets. Splenocytes cultured for 48 h with 1000 units/ml IL-2 were used as effectors against ^{51}Cr-labeled -SVC3H fibroblast (*C*) or -P815 (*D*) target cells. *E*, the effects of wortmannin (5 nM) and herbimycin (0.52 μM) on IL-2-induced LAK-mediated killing of EC and P815 tumor targets (50:1, effector to target ratio). LAK activity was determined using the 4-h ^{51}Cr release assay. The data indicate the mean percentage of cytotoxicity of triplicate cultures ± S.E. *Asterisk* indicates statistically significant difference when compared with the CD44 WT controls, $p < 0.05$.

cific for endothelial cells, as the LAK cells were unable to cause significant lysis of SVC3H fibroblasts bearing the same MHC molecules as the TME EC (Fig. 5*C*). Interestingly, the ability of the LAK cells from CD44v6-v7 KO and CD44v7 KO mice to lyse the P815 tumor targets was not significantly affected when compared with LAK cells from CD44 WT mice (Fig. 5*D*). Together, these results suggested that the reduction in IL-2-induced VLS in CD44v6-v7 KO and CD44v7 KO mice may be the result of reduced EC killing.

Recently, it was shown that CD44-dependent cytolysis could be distinguished from natural cytotoxicity according to the sensitivity to inhibition by the PI 3-kinase inhibitor, wortmannin (24). More specifically, it was shown that CD44-dependent cytolysis was highly sensitive to wortmannin inhibition, whereas natural cytotoxicity was relatively resistant. Therefore, we examined the effect of wortmannin (5 nM) treatment on the ability of LAK cells to mediate lysis of EC as well as P815 tumor cells. The results demonstrated that the lysis of EC was significantly inhibited by wortmannin, whereas the lysis of P815 was generally unaffected (Fig. 5*E*). In addition, we examined the effect of the tyrosine kinase inhibitor, herbimycin (0.52 μM), on LAK-mediated lysis of EC and P815. Exposure of LAK cells to herbimycin effectively inhibited their ability to lyse EC but not P815 tumor cells (Fig. 5*E*). Together, these results suggest that both PI 3-kinase and tyrosine kinases play an important role in LAK cell lysis of EC and that the mechanism

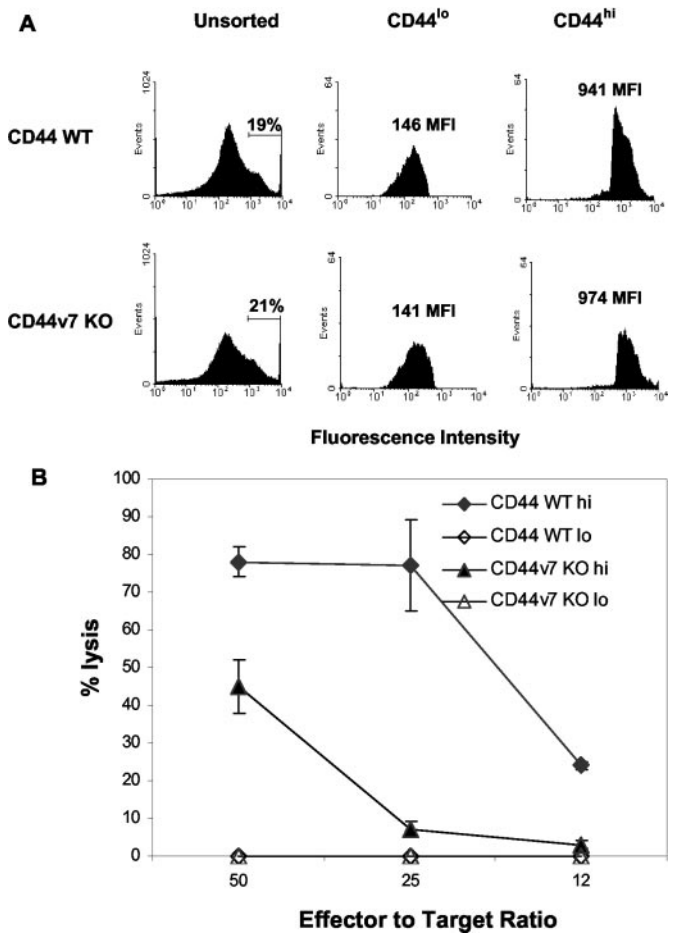

FIG. 6. **Cytolytic activity of IL-2-activated splenocytes expressing high or low levels of CD44.** Splenocytes from CD44 WT and CD44v7 KO mice were stimulated with IL-2 (1000 units/ml) for 48 h. The cells were then stained with PE-conjugated anti-CD44 mAbs and sorted according to the level of CD44 expression. *Panel A* depicts the expression levels of CD44 on IL-2-activated splenocytes before (*Unsorted*) and after sorting (*CD44^{lo}* and *CD44^{hi}*). *B*, CD44^{hi} and CD44^{lo} splenocytes from CD44 WT and CD44v7 KO mice were then tested for their ability to lyse EC targets. LAK activity was determined using the 4-h ^{51}Cr release assay. The data indicate the mean percentage of cytotoxicity of triplicate cultures ± S.E.

of EC lysis by LAK cells is distinct from the mechanism of LAK cell-mediated lysis of P815 tumor cells.

Lysis of EC following IL-2 Stimulation Is Mediated by "CD44^{hi}" LAK Cells—As shown previously (39) and in the current study (Fig. 8, *E–G*), normal splenocytes constitutively express lower levels of CD44 (CD44^{lo}) and upon stimulation with IL-2, a significant proportion of the cells up-regulate CD44 (CD44^{hi}). We examined whether the cytolytic activity against EC was mediated by CD44^{lo} and/or CD44^{hi} cells and, furthermore, whether the cytotoxicity exhibited by these subpopulations was altered in CD44V7 KO mice. To this end, splenocytes CD44 WT or CD44v7 KO mice were stimulated for 48 h with IL-2 (1000 units/ml) and stained with PE-conjugated pan-anti-CD44 mAbs (IM7) and then sorted by FACS into CD44^{lo} and CD44^{hi} populations. Fig. 6*A* 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. After sorting the CD44^{lo} and CD44^{hi} cells, these subsets were reanalyzed for CD44 expression. As seen from Fig. 6*A* (*middle* and *right panels*), CD44^{hi} cells expressed mean fluorescence intensity of 146, whereas the CD44^{hi} cells exhibited mean fluorescence intensity of 941 in CD44 WT mice, thereby confirming the purity of the subpopulations. Similar results were obtained using splenocytes from CD44v7 KO mice. Next, the CD44^{lo} and CD44^{hi} LAK cells from CD44 WT and CD44v7 KO mice were tested for their ability to kill EC cell target (Fig. 6*B*). The results showed that CD44^{hi} cells from CD44 WT mice exhibited marked cytolytic activity against EC targets. In contrast, the CD44^{hi} cells from CD44v7 KO mice had dramatically reduced lytic activity against EC. Moreover, CD44^{lo} cells from both groups of mice had virtually no lytic activity against EC. These data further established that IL-2-induced expression of CD44v7 on LAK cells plays an important role in EC killing.

Expression of CD44v7 on LAK Cells Plays an Important Role in IL-2-induced EC Damage and VLS—To further corroborate *in vivo* that CD44v7 expression on LAK cells plays a critical role in VLS, we performed experiments in which splenocytes from CD44 WT and CD44v7 KO mice were adoptively transferred into CD44 WT or CD44v7 KO recipient mice followed by IL-2 treatment (Fig. 7*A*). The results demonstrated that transfer of CD44 WT splenocytes into CD44 WT or CD44v7 KO mice led to a significant increase in IL-2-induced VLS when compared with the transfer of splenocytes from CD44v7 KO mice into CD44 WT or CD44v7 KO mice. The importance of LAK cell expression of CD44v7 was confirmed in a crisscross mixing experiment. To this end, splenocytes from CD44 WT or CD44v7 KO mice were stimulated with IL-2 for 48 h. The LAK cells were then stained with CFSE and then cultured with freshly isolated lung EC from either CD44 WT or CD44v7 KO mice. The lytic activity was determined following 4 h of culture by staining the cells with propidium iodide. Cells staining negative for CFSE were gated, and the percentage of cells positive for propidium iodide was calculated (Fig. 7*B*). The results demonstrated that the percentage of EC lysed by LAK cells from CD44 WT mice was significantly greater than the percentage of EC targets lysed by LAK cells from CD44v7 KO mice, regardless of the expression of CD44v7 on the EC targets. Together, these results suggested that the expression of CD44v7 on IL-2-activated LAK cells plays an important role in mediating EC lysis.

The Reduced Lytic Activity of LAK Cells from CD44v6-v7 KO and CD44v7 KO Mice Is Not the Result of Alterations in Activation or Expression of Cytolytic Effector Molecules—Next, we examined the possibility that the reduced IL-2-induced EC damage seen in CD44v6-v7 KO and CD44v7 KO mice resulted from decreased responsiveness to activation stimuli and/or because of 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 (Fig. 8*A*) or ConA (Fig. 8*B*) for 48 h and the proliferative responsiveness was measured. The results showed that splenocytes from the CD44v6-v7 KO and CD44v7 KO mice responded to IL-2 or ConA to the same extent as cells from CD44 WT mice. Similar results were seen when the levels of CD69, an activation marker, were measured using FACS analysis (Fig. 8*C*). These results suggested that the reduced induction of VLS seen in the CD44v6-v7 KO and CD44v7 KO mice was not the result of 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 injection of PBS (control) or IL-2 *in vivo* (Fig. 8*D*). Splenocytes from IL-2-treated CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice were found to express increased levels of FasL when compared with PBS-treated mice. Such an increase was similar in all three groups of mice. IL-2 treatment caused a modest or no significant increase in

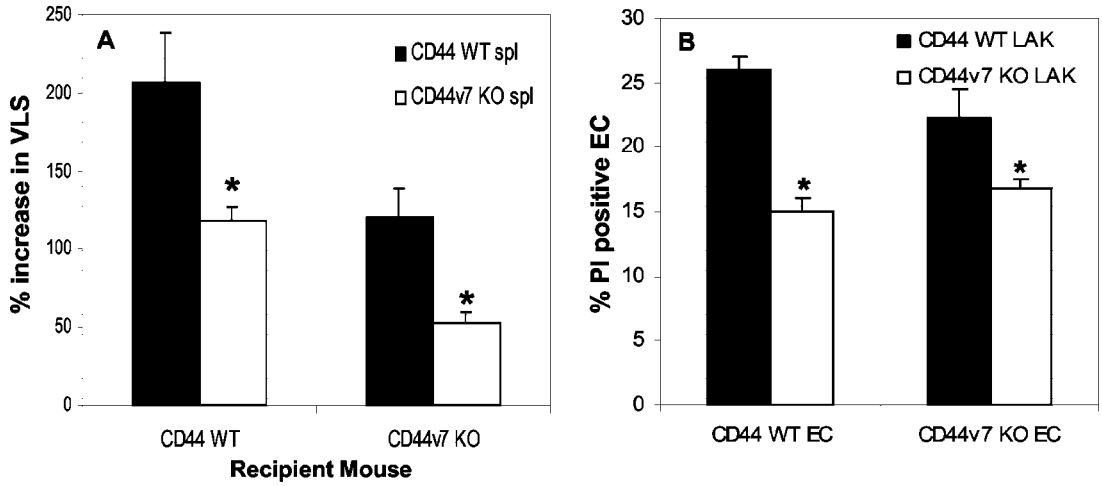

FIG. 7. **Expression of CD44v7 on LAK cells plays an important role in IL-2-induced EC damage and VLS.** *A*, splenocytes (5×10^7) from CD44 WT mice or CD44v7 KO mice were adoptively transferred into CD44 WT or CD44v7 KO recipient mice. The mice were then treated with IL-2 three times daily for 3 days and once on day 4. Two hours after the last injection of IL-2, the mice were injected with 1% Evans blue dye and VLS was studied by quantifying the extravasation of dye in the lungs. *B*, splenocytes from CD44 WT and CD44v7 KO mice were stimulated for 48 h with 1000 units OF IL-2. The LAK cells were stained with CFSE and then cultured for 4 h with freshly isolated EC from either CD44 WT or CD44v7 KO mice. The cultures were stained with propidium iodide, and the percentage of lysis of EC was assessed by determining the percentage of CFSE-negative/PI-positive cells. *Asterisk* indicates statistically significant difference when compared with the CD44 WT controls, $p < 0.05$.

the levels of perforin and TNF-α mRNA in all three groups of mice when compared with their PBS-treated counterparts. Moreover, the levels of mRNA for perforin and TNF-α were similar in these three groups of mice treated with PBS or IL-2. These results suggested that the reduced IL-2-induced VLS and decreased lytic activity against EC seen in the CD44v6-v7 KO and CD444v7 KO mice did not result from reduced expression of cytolytic effector molecules.

Previous studies from our laboratory have shown that IL-2 treatment *in vivo* leads to increased expression of CD44 (9, 10). Because the CD44v6-v7 and CD44v7 isoforms have been specifically deleted from the CD44v6-v7 KO and CD44v7 KO mice, respectively, IL-2 stimulation should still lead to up-regulation of remaining CD44 isoforms in these mice. Therefore, we examined whether the expression of CD44 was increased in CD44v6-v7 KO and CD44v7 KO mice following IL-2 treatment. CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice were treated for 4 days with IL-2 as described above. After which, the splenocytes were analyzed for the expression of CD44 using a pan antibody, IM7, which recognizes all isoforms of CD44 (Fig. 8*E*). The results showed that there was a significant increase in the expression of CD44 in splenocytes from CD44 WT, CD44v6-v7 KO, and CD44v7 KO mice following IL-2 treatment *in vivo*, which was similar in all three groups of mice.

It is possible that the increased expression of CD44 on splenocytes seen after IL-2 treatment *in vivo* may result from migration of CD44 cells into the spleen rather than direct activation of LAK cells. To address this, the effect of *in vitro* IL-2 stimulation of splenocytes on the expression of CD44 isoforms containing the CD44v6 and CD44v7 exons was determined. To this end, splenocytes from CD44 WT and CD44v7 KO mice were stimulated for 48 h with 1000 units/ml IL-2. As shown in Fig. 8*F*, stimulation with IL-2 *in vitro* caused an increase in the expression CD44 isoforms containing the v6 and v7 exons in splenocytes from CD44 WT mice. In comparison, CD44 isoforms containing the v6 exon but not the v7 exon were increased in splenocytes from CD44v7 KO mice. It should be noted that the PBS-injected control mice failed to exhibit CD44v6-v7 when directly tested (Fig. 1*A*), whereas the *in vitro* control cultures of splenocytes incubated with medium alone showed CD44v6-v7 expression. This can be explained by the fact that *in vitro* culture conditions provide fetal calf serum and other growth factors, which may activate some splenocytes to express CD44v6-v7. Fig. 8*G* depicts the level of CD44 protein expression of splenocytes from CD44 WT or CD44v7 KO mice following 48-h culture of splenocytes with or without IL-2 *in vitro*. CD44 expression was determined by staining splenocytes with anti-CD44 mAbs (IM7), which recognizes both the standard form and all isoforms of CD44, followed by flow cytometric analysis. The results showed that, following stimulation with IL-2 *in vitro*, CD44 expression increased to a similar extent in splenocytes from CD44 WT and CD44v7 KO mice, as indicated by an increase in the mean fluorescence intensity.

Adherence and Cell Aggregation between EC and IL-2-induced LAK Cells from CD44 WT and CD44v7 KO Mice in Vitro—We next examined the possibility that the absence of CD44v7 on IL-2-activated lymphocytes leads to a reduction in their adherence to EC. To quantify the adhesion, LAK cells from CD44 WT and CD44v7 KO mice were labeled with [^{3}H]thymidine and cultured with confluent layers of EC for 2 h. The percentage of cells adhering to the EC was determined as described under "Materials and Methods." The results showed that there was significant adhesion between EC and IL-2-activated splenocytes from the CD44 WT mice (Fig. 9*A*). In contrast, the IL-2-activated splenocytes from the CD44v7 KO mice showed a reduced capacity to adhere to the EC. The effect on adherence of LAK cells to EC was further demonstrated using PKH-labeled LAK cells and CFSE-labeled EC (Fig. 9*C*). In this experiment, increased numbers of LAK cells from CD44 WT mice bound to the EC when compared with the CD44v7 KO mice.

Because TME is an established cell line and inasmuch as EC from the lungs may be unique, we examined whether IL-2-activated splenocytes from CD44v7 KO mice had a reduced capacity to form conjugates with freshly isolated primary lung EC. To this end, we isolated EC from TIE2-GFP transgenic mice in which GFP is expressed only by EC. The GFP$^+$ EC were mixed with PKH-labeled LAK cells, and cell aggregation was studied. Cell aggregation was quantified by determining the percentage of endothelial cells per field conjugated to at least one LAK cell, using the formula described under "Materials and Methods." The data depicted in Fig. 9*B* represent the mean ± S.E. from 10 randomly chosen fields. Representative images of LAK cells from either CD44 WT or CD44v7 KO mice

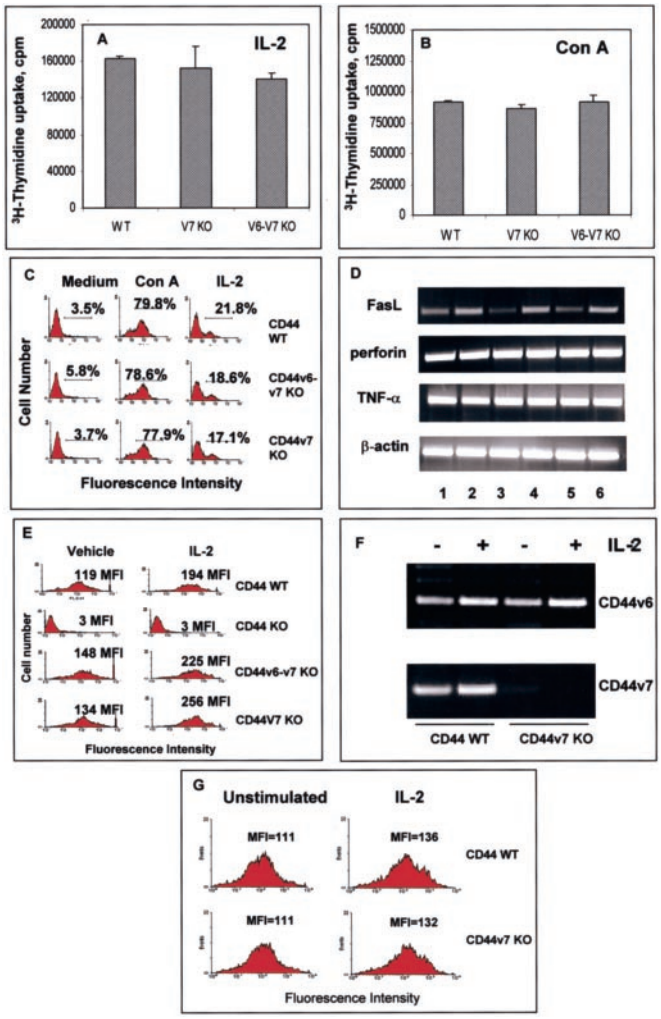

FIG. 8. **Reduced lytic activity of LAK cells from CD44v6-v7 KO and CD44v7 KO mice is not the result of alteration in proliferation/activation or expression of effector molecules such as Fas ligand, perforin, or TNF-α.** Splenocytes from CD44 WT and CD44v7 KO mice were stimulated for 48 h with interleukin-2 (1000 units/ml) (*A*) or ConA (5 μg/ml) (*B*). During the final 8 h of culture, the cells were pulsed with 2 μCi of [^{3}H]thymidine. Thymidine incorporation was determined by β-scintillation counting. The data represent mean ± S.E. of triplicate wells. *C*, activation was further assessed by examining the effect of ConA and IL-2 on CD69 expression by FACS analysis. *D*, detection of Fas ligand, perforin, and TNF-α mRNA in splenocytes from PBS- or IL-2-treated mice. Total RNA was isolated from splenocytes of PBS- or IL-2- injected mice as described in Fig. 1. mRNA was reverse transcribed and amplified by PCR with primers specific for Fas ligand, perforin, TNF-α, and β-actin. *Lane 1*, CD44 WT + PBS; *lane 2*, CD44 WT + IL-2; *lane 3*, CD44v6-v7 KO + PBS; *lane 4*, CD44v6-v7 + IL-2; *lane 5*, CD44v7 KO + PBS; *lane 6*, CD44v7 KO + IL-2. A photograph of ethidium bromide-stained amplicons is depicted. *E*, the expression of CD44 on splenocytes from CD44 WT, CD44 KO, CD44v6-v7 KO, and CD44v7 KO mice following activation with IL-2 *in vivo* was determined using FACS analysis. *F*, the expression of CD44 isoform mRNA containing the v6 or v7 exon in splenocytes unstimulated or stimulated with IL-2 (1000 units/ml) for 48 h was determined by RT-PCR analysis. *G*, the expression of CD44 on splenocytes from CD44 WT and CD44v7 KO mice following activation with IL-2 *in vitro* was determined using FACS analysis.

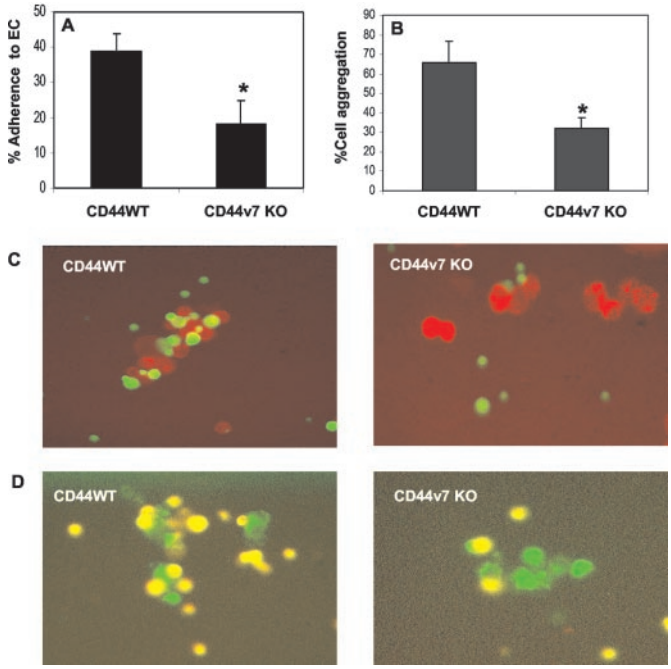

FIG. 9. **Adherence and cell aggregation between endothelial cells and IL-2-induced LAK cells from CD44 WT and CD44v7 KO mice *in vitro*.** *A*, [^{3}H]thymidine-labeled LAK cells were cultured for 2 h in plates containing TME endothelial cells. LAK cells not adhering to TME cells were removed by gently washing with medium. Adherent cells were quantified by measuring the [^{3}H]thymidine activity and compared with the [^{3}H]thymidine activity of cells removed from wells without TME cells (total activity). The percentage of LAK cells adhering to the TME was determined as described under "Materials and Methods." *Asterisk* indicates statistically significant difference when compared with the CD44 WT splenocytes, $p < 0.05$. *C*, in parallel studies, PKH-labeled LAK cells (*red*) were cultured with CFSE-labeled TME cells (*green*). Cell aggregation was visualized using a fluorescent microscope (original magnification, × 400). *B*, cell aggregation between LAK cells and freshly isolated endothelial cells was quantified by counting the percentage of endothelial cells from TIE2-GFP mice conjugated to at least one LAK cell per field. The data depicted represent the mean ± S.E. from 10 randomly chosen fields. *Asterisk* indicates statistically significant difference when compared with the CD44 WT mice, $p < 0.05$. *D*, cell aggregation was visualized by centrifuging PKH-labeled LAK cells (*yellow*) with endothelial cell (*green*) isolated from TIE2-GFP mice as described under "Materials and Methods." Cell aggregation was visualized using a fluorescent microscope (original magnification, ×400).

adhering to TIE2-GFP lung EC are shown in Fig. 9*D*. These results further confirmed that the level of cell aggregation between LAK cells and freshly isolated lung EC was significantly greater in CD44 WT mice when compared with CD44v7 KO mice.

The Effect of Anti-CD44v7 mAbs on LAK Cell Adherence to and Lysis of EC in Vitro and IL-2-induced VLS in Vivo—To further confirm the involvement of CD44v7 in IL-2-induced EC damage and VLS, we examined the effect of anti-CD44v7 mAbs on LAK cell adherence to and killing of EC. First, we examined the effect of anti-CD44v7 mAbs on the ability of LAK cells to adhere to EC. To this end, [^{3}H]thymidine-labeled LAK cells + soluble anti-CD44v7 mAbs or [^{3}H]thymidine-labeled LAK cells + soluble isotype control antibodies were added to plates coated with EC (Fig. 10*A*). The percentage of LAK cells adhering to the EC was determined 2 h later as described under "Materials and Methods." The results showed that anti-CD44v7 mAbs effectively blocked the adherence of LAK cells to EC. Next, we examined the effect of anti-CD44v7 mAbs on the lysis of EC by LAK cells. To this end, LAK cells were cultured with anti-CD44v7 or isotype control mAbs along with ^{51}Cr-labeled EC or P815 tumor cells and cytotoxicity was studied using the 4-h chromium release assay. The results showed that incubation with anti-CD44v7 mAbs significantly reduced LAK cell lysis of EC (Fig. 10*B*). Interestingly, incubation with anti-CD44v7 mAbs had no effect on the killing of P815 tumor cells. Finally, we examined whether treatment with anti-CD44v7 mAbs would have any effect on the level of IL-2-induced VLS (Fig. 10*C*). To this end, mice were injected for 4 days with IL-2. In addition, the mice were treated daily with isotype control mAbs

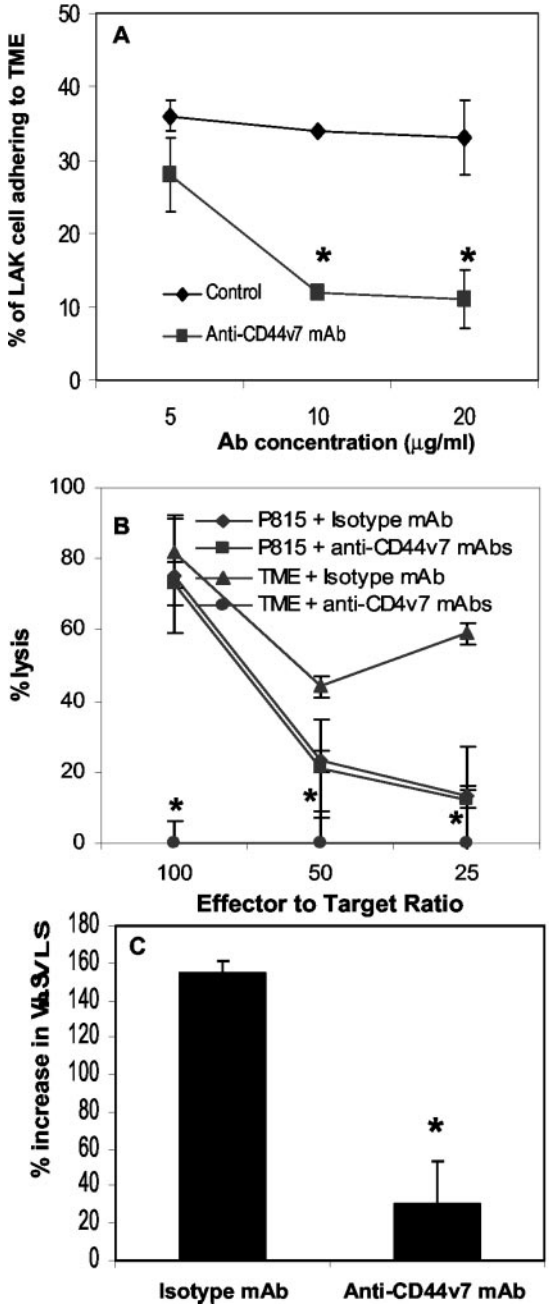

FIG. 10. **Anti-CD44v7 mAbs inhibit LAK cell adherence to and lysis of endothelial cells *in vitro* as well as the induction of IL-2-induced VLS *in vivo*.** *A*, the effect of anti-CD44v7 mAbs on the adherence of LAK cells to TME endothelial cells was determined by culturing [^{3}H]thymidine-labeled LAK cells + anti-CD44v7 mAbs or isotype control mAbs on plates coated with TME cells. The percentage of LAK cells adhering to the TME cells was determined 2 h later as described under "Materials and Methods." *B*, to examine the effect of anti-CD44v7 mAbs on the lysis of TME cells, LAK cells were cultured with anti-CD44v7 mAbs (10 μg/ml) or isotype control mAbs (10 μg/ml) and directed against ^{51}Cr-labeled TME or P815 tumor cells in a 4-h chromium release assay. The data indicate the mean percentage of cytotoxicity of triplicate cultures ± S.E. *C*, the effect of anti-CD44v7 mAbs on IL-2-induced VLS *in vivo* was determined by injecting mice for 4 days with IL-2, as described under "Materials and Methods," and treating the mice with anti-CD44v7 mAbs (100 μg/mouse intraperitoneally) or isotype control mAbs (100 μg/mouse intraperitoneally) once a day for 4 days. Vascular leak was determined as described above. *Asterisk* indicates statistically significant difference when compared with the controls, $p < 0.05$.

or anti-CD44v7 mAbs. The results from this experiment demonstrated that treatment with anti-CD44v7 mAbs significantly reduced the level of IL-2-induced VLS when compared with the

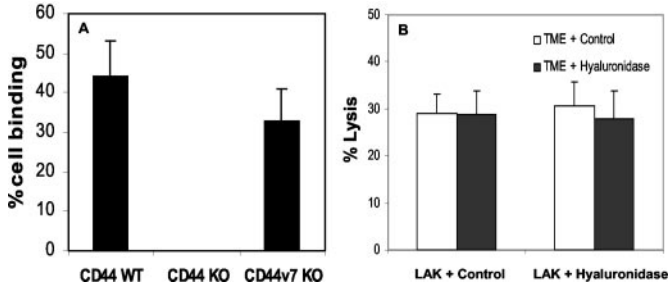

FIG. 11. **The role of CD44-HA interactions in CD44 WT and CD44v7 KO mice.** *A*, to determine the ability of IL-2-stimulated splenocytes to bind to HA-coated plates, the splenocytes were labeled for 4 h with [^{3}H]thymidine (5 μCi/ml). The labeled splenocytes (10^5/well) were added to each well of HA-coated plates and incubated for 1 h at 37 °C. Non-adherent cells were removed by gently pipetting with PBS. The percentage of cells adhering to the plate was determined using the formula described under "Materials and Methods." *B*, the effect of pretreatment of either LAK cells or EC with hyaluronidase on the LAK-mediated killing of EC cells was determined. Briefly, IL-2-stimulated splenocytes from CD44 WT mice and EC were untreated (control) or pretreated for 1 h at 37 °C with hyaluronidase (100 rTRU/ml). After treatment, the cells were washed and EC lysis was determined using the 4-h ^{51}Cr release assay. The data represent an effector to target ratio of 25 and indicate the mean percentage of cytotoxicity of triplicate cultures ± S.E.

isotype control mAb-treated group. Together, these results suggested that anti-CD44v7 mAbs effectively blocked LAK cell adherence to and lysis of EC and, furthermore, markedly reduced IL-2-induced VLS *in vivo*.

Role of CD44-HA Interactions in CD44 WT and CD44v7 KO Mice—HA is one of the major ligands for CD44. Previous reports demonstrated that activation of lymphocytes leads to increased HA binding (40, 41). It was possible that deletion of v6 and v7 exon products resulted in altered binding to HA. Therefore, we examined whether splenocytes from CD44 WT, CD44 KO, or CD44v7 KO mice exhibited altered ability to bind to HA. Following stimulation with IL-2 *in vitro*, splenocytes from CD44 WT and CD44v7 KO mice showed significant and comparable binding to HA-coated plates (Fig. 11*A*). In contrast, splenocytes from CD44 KO mice did not show any detectable binding to HA. Unactivated splenocytes, regardless of the expression of CD44, were not able to bind HA-coated plates (data not shown). In addition, we examined the effect of pretreatment of LAK cells and/or EC cells with hyaluronidase on the LAK-mediated killing of EC (Fig. 11*B*). The results showed that pretreatment of either LAK cells or EC with hyaluronidase had no significant effect on the ability of IL-2-stimulated cytotoxic cells to kill EC targets. Together, these results suggested that CD44v7 was not critical for the binding of IL-2-activated splenocytes to HA.

Lysis of Endothelial Cells by LAK Cells Is Suppressed by Addition of Soluble or Enzymatic Cleavage of CD44v7 Ligands—In this study we demonstrated that the ability of splenocytes from CD44v7 KO mice to bind HA was similar to that seen using cells from CD44 WT mice (Fig. 11*A*), thereby suggesting that the absence of CD44v7 did not alter HA binding. The expression of CD44v6-v7 has been shown to facilitate the binding to other glycosaminoglycans including chondroitin sulfate, heparin, and heparin sulfate (42). Therefore, we examined the effect of soluble chondroitin sulfate and heparin on IL-2-stimulated LAK-mediated lysis of EC and P815 tumor cells. The results showed that addition of soluble chondroitin sulfate A, B, and C and heparin caused significant inhibition in the lysis of EC (Fig. 12*A*) without causing any decrease in the lysis of P815 tumor cells (Fig. 12*B*). These data were confirmed by the fact that addition of enzymes specific for chondroitin sulfate and heparin, significantly reduced LAK cell-mediated

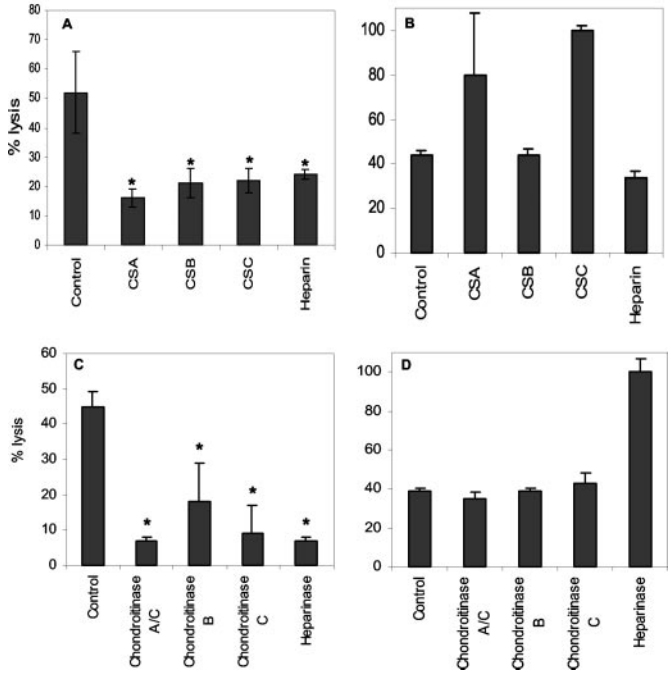

FIG. 12. **Lysis of endothelial cells by LAK cells is suppressed by addition of soluble or enzymatic cleavage of CD44v7 ligands.** To determine the effect of soluble CD44v7 ligand, LAK cells were directed against endothelial cell (*A*) or P815 (*B*) in the presence of 0.5 mg/ml various glycosaminoglycans. To determine the effect of enzymatic cleavage of CD44v7 specific glycosaminoglycans, LAK cells were cultured with endothelial cells (*C*) or P815 (*D*) in the presence of various glycosaminoglycan enzymes. The LAK activity was determined using the 4-h ^{51}Cr release assay. The data indicate the mean percentage of cytotoxicity of triplicate cultures ± S.E. *Asterisk* indicates a statistically significant decrease when compared with the controls, $p < 0.05$.

lysis of EC targets (Fig. 12*C*) without significantly decreasing the lysis of the P815 tumor cells (Fig. 12*D*). Together these data suggested that interaction between CD44v6-v7 and certain glycosaminoglycans may play a significant role in LAK cell-mediated lysis of EC.

DISCUSSION

EC damage is widely reported in a number of pathological diseases including infections, autoimmunity, transplantation, graft-*versus*-host disease, and following immunotherapy with cytokines and immunotoxins. Previous work from our laboratory implicated CD44 expressed by cytolytic lymphocytes in IL-2-induced EC injury leading to VLS (10). However, the nature of specific isoforms of CD44 involved was unclear. Therefore, the current study was designed to determine which CD44 isoforms were involved in VLS. We demonstrated that, following IL-2 administration *in vivo*, the expression of CD44v7 and CD44v6-v7 was up-regulated. In addition, we demonstrated that IL-2-induced VLS was markedly abrogated in mice lacking CD44v6-v7 and CD44v7. This abrogation was not the result of decreased activation or expansion of effector cell population but was the result of reduced lytic capabilities of lymphocytes lacking the CD44v6-v7 or CD44v7 isoforms because of reduced adhesion to EC. Furthermore, the LAK cell binding to and lysis of EC *in vitro* and IL-2-induced VLS *in vivo* was blocked by anti-CD44v7 mAbs.

CD44 has been implicated in a number of immunological processes including lymphopoiesis, lymphocyte activation, homing, and leukocyte rolling and extravasation into sites of inflammation (19). Studies have shown that interactions between CD44 and HA are important in the initial attachment and rolling of lymphocytes along the endothelium (43). However, evidence from this study suggests that CD44v7 may not be critical for the interaction with HA or for lymphocyte extravasation into the lungs. IL-2-activated splenocytes from CD44 WT or CD44v7 KO mice showed similar levels of binding to HA; furthermore, pretreatment of LAK cells or EC with hyaluronidase did not affect the ability of the LAK cells to kill EC. Finally, we noted that infiltration of lymphocytes into the lungs following IL-2 treatment was not significantly altered in CD44v6-v7 KO or CD44v7 KO mice. These data are consistent with our earlier findings that, in IL-2-induced VLS or ConA-induced hepatitis, the infiltration of mononuclear cells in the lungs or liver is not significantly altered in CD44 KO mice, which lack the entire CD44 gene (10, 44). Moreover, the binding of CD44 to HA and other ligands is tightly regulated and depends on the status of the CD44 molecule. In fact, most CD44 molecules are unable to bind HA but must be induced to bind to HA. Some factors that may be involved in inducing CD44 binding to HA include increased expression, receptor clustering, glycosylation, and sulfation (45). In addition, evidence suggests that the expression of CD44 isoforms was not necessary for binding of lymphocytes to HA on EC (43). Interestingly, it has been shown that the expression of CD44 isoforms containing v6 and v7 led to increased binding to other glycosaminoglycans including chondroitin sulfate, heparin, and heparin sulfate (42). EC express such glycosaminoglycans (46), thereby suggesting the possibility that the increased expression of CD44v6-v7 on IL-2-activated lymphocytes may lead to increased binding to EC. The consequence of such increased binding could be the induction of cytotoxicity of EC resulting in vascular leak as shown in the current study. In fact, increased CD44-mediated binding of mononuclear leukocytes to target cells has been implicated in other diseases such as inflammatory bowel disease (47).

Previous studies have demonstrated that EC also express CD44 (43, 48). Thus, we cannot entirely exclude the possibility that the expression of CD44v7 on EC plays some role in IL-2-induced VLS. Nevertheless, using adoptive transfer experiments, we noted that lymphocytes transferred from CD44 WT into CD44v7 KO mice caused higher levels of IL-2-induced VLS when compared with transfer of CD44v7 KO lymphocytes into CD44v7 KO mice. Moreover, results from crisscross mixing experiments demonstrated that expression of CD44v7 on LAK cells is important for killing either CD44 WT EC or CD44v7 KO EC. Together, these data suggested that expression of CD44v7 on IL-2-stimulated LAK cells, rather than EC, plays an important role in the induction of VLS.

We demonstrated that treatment with IL-2 *in vivo* led to the expansion of $CD8^+$ T cells, NK cells, and NK-T cells in both CD44 WT and CD44 KO mice. Additionally, we showed that splenocytes activated *in vitro* or *in vivo* with IL-2 efficiently killed EC targets while leaving fibroblast targets unharmed. The fact that both the fibroblast and EC used in this study had the same MHC would suggest that the killing of the endothelial cells was MHC-unrestricted and specific for EC. These data also suggested that the induction of vascular leak is mediated by IL-2-activated LAK cells. This is further supported by earlier studies showing that IL-2-activated LAK cells can adhere to and kill EC targets (49). In addition, the toxic side effects induced by IL-2 were significantly reduced following NK cell depletion (50). In addition, previous studies from our laboratory showed that mice deficient in FasL or perforin had markedly decreased VLS (9).

In a study by Baluna *et al.* (51), it was reported that IL-2 coupled to mouse IgG could bind directly to and cause damage to EC. However, in the current study we did not see any direct toxicity to endothelial cell cultures following exposure to IL-2

in vitro (data not shown). This apparent discrepancy could be the result of differences in experimental set-up. In the study by Baluna *et al.*, the presence of mouse IgG was necessary for the induction of IL-2-mediated EC damage. In our experiments examining the direct toxic effects of IL-2 on EC, mouse IgG was not added. In addition, studies suggest that exposure of EC to IL-2 does not affect the expression of a number of molecules involved in the adhesion between lymphocytes and EC. For example, exposure of EC to IL-2 had no direct effect on the expression of hyaluronan, P-selectin, E-selectin, ICAM-1, ICAM-2, VCAM-1, and PECAM-1 (52, 53). The effect of IL-2 on the EC expression of heparin, heparin sulfate, chondroitin sulfate, and other CD44 ligands remains to be fully elucidated. Together, these data suggest that IL-2 acts primarily on the lymphocyte population leading to increased adherence to and damage of EC.

Previously, we demonstrated that Fas ligand and perforin play an important role in the induction of vascular leak (9). This work suggested that blocking perforin or Fas ligand may reduce IL-2-induced EC damage and vascular leak, thereby facilitating tumor immunotherapy. However, because perforin and Fas ligand play an important role in lymphocyte killing of tumors, blocking perforin and/or Fas ligand may also decrease the efficacy of IL-2 therapy in the treatment of cancers. This is supported by our findings of reduced killing of YAC-1 tumor targets in *gld* and perforin KO mice (9). In contrast, in the current study, we noted that blocking CD44v7 decreased the killing of EC but not P815 tumor cells. Therefore, targeting CD44 isoforms may offer an alternate and effective approach to reducing VLS without affecting tumor killing.

We and others have shown that ligation of CD44 can signal the induction of cytolytic activity in CTL and NK cells (20, 24). However, little is known about the natural targets and/or the ligands on target cells responsible for facilitating CD44-mediated lysis. In this report we demonstrated that CD44v7 expression on LAK cells plays an important and specific role in the injury to EC, and that this injury was mediated, at least in part, by interactions with various glycosaminoglycans such as chondroitin sulfate, and heparin. Previously, Sconocchia *et al.* (24) demonstrated that PI 3-kinase and tyrosine kinase played a role in CD44-directed lysis and that CD44-directed lysis could be distinguished from natural cytotoxic activities of LAK cells by the level of sensitivity to inhibition by wortmannin. In the current study, we demonstrated that the lysis of EC but not P815 cells was inhibited by anti-CD44v7 mAbs and that lysis of EC but not P815 tumor cells was highly sensitive to treatment with the PI 3-kinase inhibitor wortmannin and the tyrosine kinase inhibitor herbimycin. Taken together, our study suggests that the mechanisms of CD44v7-mediated lysis of EC involves PI 3-kinase and tyrosine kinases and that the lysis of EC and P815 tumor cells may be mediated by different and distinct $CD44^{lo}$ and $CD44^{hi}$ pathways.

In the current study we observed that activation of splenocytes with IL-2 up-regulates CD44 expression. Interestingly, when we separated $CD44^{lo}$ and $CD44^{hi}$ cells from the activated population, only $CD44^{hi}$ but not $CD44^{lo}$ cells had the potential to kill EC. These findings are highly significant because it may be possible to isolate such cells from tumor-bearing or immunized host and be used for immunotherapy. Alternatively, it is possible to target such cells with antibodies or fusion proteins to cause their depletion *in vivo*, as a treatment modality to prevent transplant rejection, graft-*versus*-host diseases, allergies, etc.

In conclusion, this study demonstrates for the first time that CD44v7 plays a critical role in EC injury and vascular leak caused by IL-2. CD44v7 deficiency does not affect the activation, Fas ligand, and perforin expression or the migration of LAK cells. In addition, we observed that LAK cells from CD44v7 KO mice are able to kill P815 tumor targets to the same extent as LAK cells from CD44 WT mice. Therefore, by blocking the interaction between CD44v7 and the appropriate ligands on the EC, such as by using anti-CD44v7 antibodies or siRNAs directed against CD44v7, it may be possible to reduce IL-2-induced vascular damage without affecting the antitumor efficacy of IL-2 therapy. Patients with metastatic disease have poor prognosis and a 5-year survival rate of less than 10% (54). High dose IL-2 therapy has a response rate of up to 30% in patients with metastatic melanoma, renal cell carcinoma, and non-Hodgkins lymphoma (55, 56). Thus, to date, the best systemic therapy for treating metastatic disease is high dose IL-2. Our studies shed new light not only in understanding the basic mechanisms involved in the interactions between LAK cells and EC but also provide useful information in improving immunotherapy of cancer using IL-2.

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