ORIGINAL ARTICLE

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Adjuvant IL-15 does not enhance the efficacy of tumor cell lysate-pulsed dendritic cell vaccines for active immunotherapy of T cell lymphoma

Abstract There has been a recent interest in using IL-15 to enhance antitumor activity in several models because of its ability to stimulate $CD8^+$ T cell expansion, inhibit apoptosis and promote memory T cell survival and maintenance. Previously, we reported that C6VL tumor lysate-pulsed dendritic cell vaccines significantly enhanced the survival of tumor-bearing mice by stimulating a potent tumor-specific $CD8^+$ T cell response. In this study, we determined whether IL-15 used as immunologic adjuvant would augment vaccine-primed $CD8^+$ T cell immunity against C6VL and further improve the survival of tumor-bearing mice. We report that IL-15 given after C6VL lysate-pulsed dendritic cell vaccines stimulated local and systemic expansion of NK, NKT and $CD8^+$ $CD44^{hi}$ T cells. IL-15 did not, however, augment innate or cellular responses against the tumor. T cells from mice infused with IL-15 following vaccination did not secrete increased levels of tumor-specific TNF-α or IFN-γ or have enhanced C6VL-specific CTL activity compared to T cells from recipients of the vaccine alone. Lastly, IL-15 did not enhance the survival of tumor-bearing vaccinated mice. Thus, while activated- and memory-phenotype $CD8^+$ T cells were dramatically expanded by IL-15 infusion, vaccine-primed $CD8^+$ T cells specific for C6VL were not significantly expanded. This is the first account of using IL-15 as an adjuvant in a therapeutic model of active immunotherapy where there was not a preexisting pool of tumor-specific $CD8^+$ T cells. Our results contrast the recent studies where IL-15 was successfully used to augment tumor-reactivity of adoptively transferred transgenic $CD8^+$ T cells. This suggests that the adjuvant potential of IL-15 may be greatest in settings where it can augment the number and activity of preexisting tumor-specific $CD8^+$ T cells.

Keywords Dendritic cells · Tumor immunity · Vaccination · Interleukin-15

Abbreviations NK: Natural killer · NKT: Natural killer T cells · C6VL-DC: DC pulsed with C6VL lysate · MBL-2-DC: DC pulsed with MBL lysate · DLN: Draining lymph node · CLLN: Contralateral lymph node · h: Hour · d: Day

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Introduction

Interleukin-15 was identified and purified based on its abilities to bind the IL-2 receptor β-chain and to stimulate the proliferation of IL-2-dependent cell lines and antigen-specific T cell clones, including cytolytic effector cells [11]. It has since been shown that while IL-2 and IL-15 share many functions, especially in innate immunity, they can play distinct and contrasting roles in T cell-mediated immune responses [40]. While systemic administration of IL-2 can act as a potent adjuvant to tumor vaccinations and adoptive T cell transfer immunotherapy strategies [1, 34], it also limits the magnitude and duration of T cell responses through elimination or suppression of responding T cells [23, 41] and interference with $CD8^+$ memory T cells survival [21, 46]. High dose IL-2 can also cause severe, dose-limiting toxicities in patients [39]. In contrast, IL-15 rescues T cells from IL-2-mediated activation-induced cell death [23] and

promotes memory cell generation and maintenance [2, 4, 13, 32, 46]. IL-15 is also essential for the development and activation of NK and NKT cells in vitro [8, 15]. In murine tumor models, IL-15 exhibits a superior therapeutic index [26] and a stronger target-specific accumulation with more rapid clearance from the circulation [20] compared to IL-2. Thus, IL-15 may be an attractive alternative to IL-2 for adjuvant use in immunotherapy.

Three primary goals in the generation of effective immunotherapeutic approaches for tumors are to induce effective tumor-specific cell-mediated responses, promote the survival of antigen-specific T cells, and to induce long-lived T cell memory against the tumor [5]. There are few published reports assessing the effects of IL-15 on CD8^{+} T cell responses in vivo [7, 16, 43, 46]. However, collective in vitro and in vivo evidence from various groups suggests that IL-15 positively influences the initiation, clonal expansion, contraction and maintenance phases of antigen-specific CD8^{+} T cell immunity [38]. Two groups have recently reported the use of IL-15 in adoptive transfer models of tumor therapy. Brentjens et al. [3] showed that genetically targeted T cells expanded in IL-15 prior to transfer uniquely persisted in tumor-bearing hosts and cleared disseminated B cell tumors. Secondly, Klebanoff et al. [19] demonstrated that systemic administration of IL-15 in addition to T cell stimulation with an altered peptide ligand following adoptive transfer of antigen-specific transgenic CD8^{+} T cells improved their in vivo tumor activity. While there have been few accounts of IL-15 use in active immunotherapy protocols, the results have supported the hypothesis that IL-15 has strong adjuvant potential. IL-15 augmented the primary antigen-specific CD8^{+} T cell response when delivered systemically following peptide-pulsed dendritic cell (DC) vaccine [35], and stimulated long-lived memory CD8^{+} responses when delivered in a vaccinia vector with an HIV antigen [28]. However, IL-15 in combination with active immunotherapy against tumor has not been reported in a therapeutic model, where there was no preexisting pool of tumor-reactive CD8^{+} T cells.

We previously demonstrated that DC pulsed with tumor cell lysates stimulated CD8^{+} T cell mediated clearance of the murine T cell lymphoma, C6VL [10], resulting in ∼40% survival of tumor bearing mice. We hypothesized that using IL-15 as an immunologic adjuvant would augment vaccine stimulated CD8^{+} T cell immunity against C6VL, and result in higher levels of survival among vaccine recipients. While IL-15 stimulated local and systemic expansion of NK, NKT and CD8^{+} CD44^{hi} CD62L^{+/−} T cells, it did not increase innate or cellular responses against the tumor. Mice infused with IL-15 following active immunization with C6VL lysate-pulsed DC (C6VL-DC) did not have augmented NK lytic activity, tumor-specific secretion of Th1-type cytokines or C6VL-specific CTL activity compared to recipients of the vaccine alone. Correspondingly, the delivery of IL-15 did not enhance the survival of tumor-bearing vaccinated mice. Thus, while IL-15 dramatically expanded activated- and memory-phenotype CD8^{+} T cells following C6VL-DC vaccines, vaccine-primed CD8^{+} T cells with specificity for C6VL were not significantly or preferentially expanded, and the therapeutic efficacy of DC-based vaccine therapy was not enhanced. These results, along with reports of success in IL-15 use with adoptive transfer tumor therapy, suggest that IL-15 will be most useful as an immunologic adjuvant in settings where there is a preexisting population of tumor-specific CD8^{+} T cells rather than using it to augment primary CD8^{+} T cell responses stimulated by active immunotherapy in a tumor-bearing host.

Materials and methods

Mice

Female C57Bl/6 mice (H-2[b]) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 6 weeks of age. The animals were maintained under specific pathogen free conditions according to the guidelines in the *Animal Component of Research Protocol* implemented in VA research centers. The mice were between 7 weeks and 9 weeks of age when vaccine protocols were initiated.

Media and cytokines

AIM-V serum-free media (Invitrogen; Carlsbad, CA, USA) containing 50 μM 2-ME was used to generate DC-based vaccines as described below. C6VL for tumor challenges and all tumor lines used for in vitro assays were grown in complete media (CM), consisting of RPMI 1640 supplemented with 10% FBS and 50 μM 2-ME. Murine rGM-CSF (sp. act. ≥ 5.0×10^{6} U/mg) and murine rIL-4 (6.1×10^{8} U/mg) were purified from over expressing cell lines generated in our laboratory. Human rIL-2 (18.0×10^{6} U/mg) was obtained from Chiron Corp. (Emeryville, CA, USA). Human rIL-15 (2.39×10^{8} U/mg; endotoxin levels < 2.65 EU/mg protein) was kindly provided by Amgen Corporation (Seattle, WA, USA).

Cell lines

C6VL (H-2[b]) is a radiation-induced T cell thymoma of C57Bl/Ka background and expresses TCR-$\alpha\beta$, Thy 1.2, CD3, CD4, and H-2D[b] [27]. C6VL does not express MHC Class II or Fas [29]. MBL-2 (H-2[b]) is a T cell lymphoma cell line of C57Bl/6 origin (provided by I. L. Weissman, Stanford, CA, USA) that was used as a control T cell lymphoma lysate and control tumor for in vitro restimulation. YAC-1 (H-2[b]) is a Maloney murine leukemia virus-induced murine T cell lymphoma line of A/Sn origin that is susceptible to NK-mediated lysis [17, 18] and was purchased from the American Type Culture Collection (Manassas, VA, USA).

Antibodies

FITC-conjugated anti-CD4 (GK1.5, Rat IgG$_{2b}$,κ), anti-NK1.1 (PK136, Mouse IgG$_{2a}$,κ), anti-CD44 (IM7, Rat IgG$_{2b}$,κ), anti-CD122 (5H4, Rat IgG$_{2a}$), PE-conjugated anti-CD8b.2 (53–5.8, Rat IgG$_1$), anti-CD4 (H129.19, Rat IgG$_{2a}$), anti-CD25 (3C7, Rat IgG$_{2b}$,κ), anti-CD44 (IM7, Rat IgG$_{2b}$,κ), anti-CD69 (H1.2F3, Hamster IgG1), anti-CD43 (1B11, Rat IgG$_{2a}$,κ), anti-CD62L (MEL-14, Rat IgG$_{2a}$,κ), and biotinylated anti-CD8b.2 (53–5.8, Rat IgG$_1$) and anti-TCR β-chain (H57-597, Hamster IgG) were used for phenotypic analysis (all from BD Biosciences, San Diego, CA, USA). Streptavidin-PerCP-Cy5.5, Fc block and all isotype antibody controls were also purchased from BD Biosciences. ELC-Ig chimera was kindly provided by Dr Timothy Springer (Harvard University, Boston, MA, USA). PE-conjugated goat antihuman IgG was purchased from Southern Biotechnology Associates (Birmingham, AL, USA).

Tumor challenge

Two days prior to tumor challenge, an aliquot of tumor cells was thawed and cultured in CM for 48 h. The cells were collected by centrifugation, washed and counted using a hemocytometer. The viability of the tumor cells prior to tumor challenge was generally >98% as determined by trypan blue exclusion. A lethal number of tumor cells in 500 μl HBSS was injected i.p. The LD$_{100}$ for C6VL was 1,000 cells. Mice received their first vaccination 1 day after receiving their tumor challenge. Groups of ten mice were used for survival analysis and were monitored for 60 days. Survival curves were constructed according to the Kaplan–Meier method [14]. Statistical differences between vaccine groups was determined using the log-rank test [30], and was significant when $P < 0.05$.

Generation of bone marrow-derived DC

Bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice, depleted of erythrocytes, and cultured for 5 days in AIM-V serum-free media containing 50 μM 2-ME and 10 ng/ml rGM-CSF and rIL-4 as previously described [10]. Nonadherent cells were enriched for DC using one of two methods. They were harvested by gentle pipetting and suspended in AIM-V to a density of 5.0×10^6 cells/ml, overlayed over media containing 14.5% (w/v) metrizamide [9] or resuspended in 11.5% Optiprep (iodixanol, Sigma, St. Louis, MO, USA) in Optiprep diluent (0.88 w/v NaCl, 1 mM EDTA, 0.5% w/v bovine serum albumin, 10 mM hepes-NaOH, pH 7.4) and placed under HBSS [36]. The cells were centrifuged at 600×g for 15 min and the cells at the interface collected, washed and analyzed by flow cytometry for expression of cell surface markers as previously described [10]. Cells from each preparation were ≥80% positive for the co-expression of MHC II, CD11c, CD54, CD80 and CD86 (data not shown). The DC preparations did not have cells that expressed CD3 or CD19.

Ag pulsing of DC, Immunizations and IL-15 administration

After purification, DCs were resuspended to 1.0×10^6 cells/ml in AIM-V media containing freeze-thaw cell lysate from either C6VL or MBL-2 (control) as previously described [10]. The DC were pulsed with lysates from three tumor cells per DC. After incubating for 18 h, the DC were collected, washed three times and suspended in HBSS at 5.0×10^6 cells/ml. 5.0×10^5 lysate-pulsed DC were injected s.c. in the right flank of tumor-bearing mice every 10 days. Mice used for lymphocyte immunophenotype analysis, CTL and cytokine secretion assays and as lymphocyte transfer donors were immunized twice. Mice monitored for survival were immunized three times. 0.5 μg rhIL-15 diluted in HBSS containing 1% naïve mouse serum was administered s.c. at the vaccine site beginning 1 day after each vaccine and continued every 12 h for five consecutive days. Mice that did not receive IL-15 were infused with only the diluent on the same schedule.

Immunophenotype of DLN, CLLN and splenocytes

Eighteen hours after the last IL-15 infusion, DC vaccine primed draining lymph node (DLN, right inguinal), contralateral lymph node (CLLN, left inguinal) and spleens were harvested from tumor-challenged mice ($n = 6$–20 per group). Tumor-challenged mice treated with diluent or IL-15 only were included as controls. Lymph nodes and spleens were processed separately into single-cell suspensions and then passed through 70-μm nylon mesh filters. Splenocytes were depleted of erythrocytes using Red Cell Lysis Buffer (Sigma, St. Louis, MO,USA). Pooled DLN, CLLN and splenocyte populations from each group were washed twice with PBS containing 0.5% mouse serum and 1.0% BSA (FACS Buffer). 1.5–3.0×10^5 cells per sample were seeded in wells of round-bottom 96-well plates and prestained with Fc block (0.5 μg/10^6 cells) for 20 min on ice. Cells were stained with FITC-(1.0 μg/10^6 cells), PE-(0.5 μg/10^6 cells) and biotin-conjugated (1.0 μg/10^6 cells) antibodies or isotype controls in 50 μl FACS Buffer for 30 min on ice. Following two washes in 200 μl FACS Buffer, each sample was incubated with Streptavidin-PerCP-Cy5.5 (0.5 μg/10^6 cells) for 20 min on ice to detect biotinylated antibodies. The recombinant CCR7 ligand, ELC-Ig chimera [22], was used to detect CCR7 expression. After prestaining with Fc block, cells were incubated with purified ELC-Ig supernatant (50 μl/sample) for 1 h on ice followed by incubation with PE-conjugated goat antihuman IgG (0.5 μg/10^6 cells) for 30 min on ice. All samples were washed three times with

FACS buffer, fixed in 50 μl of 2% paraformaldehyde (w/v), and analyzed by flow cytometry using a FACScan (BD Biosciences; San Jose, CA, USA) and CellQuest software (BD Biosciences). Amorphous gates were loosely drawn around viable cell populations based on forward and side scatter plots to exclude cellular debris from analysis, and 10,000 gated events were collected per sample. In some experiments, $CD8^+$ T cells were purified by negative selection using the MACS $CD8a^+$ T Cell Isolation Kit (Miltenyi Biotec; Auburn, CA, USA) prior to staining. Flow cytometry showed that >98% of the resulting cell population expressed CD8. NK cells were defined as cells that were $NK1.1^+$, $\alpha\beta TCR^-$; NKT cells as $NK1.1^+$, $\alpha\beta TCR^+$; $\alpha\beta$ T cells as $NK1.1^-$, $\alpha\beta TCR^+$; and CD4 and CD8 T cells as $CD4^+$ $\alpha\beta TCR^+$ and $CD8b^+$ $\alpha\beta TCR^+$, respectively.

In vitro cytokine secretion assay

Draining lymph nodes and splenocytes from each treatment group ($n = 5$ per group) were collected as described above. For each treatment group, 5.0×10^5 of each cell population were plated in triplicate wells of a 96-well plate with either 1.0×10^5 per well mitomycin-C-treated C6VL or MBL-2, 1.0 μg/ml ConA, or with no stimulation (media only) and incubated at 37°C for 72 h (200 μl/well). Wells containing only mitomycin-C-treated C6VL or MBL-2 were included to control for cytokine secretion by stimulator cells. Concentrations of IFN-γ, TNF-α, IL-4 and IL-2 in the culture supernatants were assayed using the BD Murine Th1/Th2 Cytometric Bead Array System (Becton Dickinson, San Jose, CA, USA). A 1x standard solution of mouse Th1/Th2 cytokines was prepared as described in the manufacturer's protocol. The remainder of the assay was performed and analyzed as described by the manufacturer, except all volumes were reduced by one-half. The linear range of detection for each cytokine monitored was 20–2,500 pg/ml. Data are represented as the mean of triplicate samples ± SD. Statistics between groups were measured using the Student's t test, and significance was reached when $P < 0.05$.

In vitro detection of NK- and CTL-mediated lysis

Draining lymph nodes, spleen and peritoneal lavage cells were harvested from mice treated with C6VL-DC, C6VL-DC + IL-15, IL-15 or diluent 18 h following their last IL-15 infusion and processed as described above. Cells were restimulated in vitro for 5 days at a density of 5.0×10^6/ml (1.0 ml per well in 24-well plates) with irradiated C6VL (2000 Rad) in CM containing 20 U/ml rhIL-2. Following restimulation, viable cells were enriched over Lympholyte M density medium (Cedarlane Laboratories Ltd.; Hornby, ON, Canada) per the manufacturer's protocol. Duplicate samples of DLN, splenocytes and lavage cells from each group were plated in round bottom 96-well plates using threefold serial dilutions at a starting con-

centration of 5.0×10^6 cells/ml in CM. C6VL or control MBL-2 cells were labeled for 3 min with Vybrant 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) Cell Labeling Solution (Molecular Probes; Eugene, OR, USA) per manufacturer's protocol for nonadherent cells and 2.0×10^4 cells were added to each well in 100 μl CM, resulting in effector-to-target ratios (E:T) of 50:1, 16.7:1 and 5.6:1 in a total volume of 200 μl/well. To measure NK-mediated lysis, DLN and splenocytes from each group were pooled and plated in duplicate without in vitro stimulation in flat-bottom 48-well plates using fourfold serial dilutions starting at a concentration of 8.0×10^6 cells/ml in 200 μl per well. 2.0×10^4 C6VL or NK-susceptible YAC-1 cells that were labeled as indicated above were added to each well in 200 μl CM, resulting in E:T of 80:1, 20:1 and 5:1 in a total volume of 400 μl/well. Wells containing only DiO-labeled target cells were included as controls for spontaneous death of target cells for both assays. The cells were incubated for 4 h (CTL lysis) or 16 h (NK lysis; based on protocol reported by Kennedy et al. [15]) at 37°C in a 5% CO_2 incubator and then 10 μl of PI cell staining solution (BD Biosciences; San Jose, CA, USA) was added to each sample and incubated an additional 15 min at RT protected from light. To determine percent target cell death in each well, the percent of 7,500 gated DiO^+ C6VL or MBL-2 target cells that were also positive for PI staining was measured by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA). The gate to collect DiO^+ target cells was set on a plot of forward scatter versus DiO intensity to include viable and dead DiO^+ C6VL, MBL-2 or YAC-1 cells and exclude all effector cells. Percent lysis for each sample was calculated as [(% death of target cells plated with effectors)–(% death of target cells without effector cells)]. Data are represented as the mean of duplicate samples ± SD. Statistics between groups were measured using the Student's t test. Statistical significance was reached when $P < 0.05$. This flow cytometric cytotoxicity assay was previously reported to be suitable for measuring both T cell and NK-mediated cytotoxicity with sensitivity comparable to that observed using the standard ^{51}Cr release assay [31], and yields highly reproducible results.

Transfer of draining lymphocytes from vaccine- and IL-15-treated mice to naïve recipients

Groups of 13 tumor-challenged donor mice were vaccinated twice with C6VL-DC or the control vaccine, MBL-2 lysate-pulsed DC (MBL-2-DC). IL-15 was administered following each vaccine as described above. Groups of 11 mice vaccinated with C6VL-DC or MBL-2-DC without IL-15 infusion were included as positive and negative controls, respectively. Eighteen hours after the last IL-15 infusion, DLN from each group were harvested, pooled and prepared as described above. DLN were mixed with 5000 C6VL cells (five times the lethal dose, thawed for tumor challenge as above) and injected into the peritoneum of ten naïve recipient mice

per group. Each naïve recipient received either a number of cells equivalent to one donor's DLN (3.2×10^6, C6VL-DC and MBL-2-DC DLN; or 6.7×10^6, C6VL-DC + IL-15 and MBL-2-DC + IL-15 DLN) or a number of cells from C6VL-DC + IL-15 or MBL-2-DC + IL-15 recipient DLN that resulted in the transfer of a number of CD8$^+$ T cells that matched those transferred in one DLN from C6VL-DC and MBL-2-DC donors (1.7×10^6 total cells or 5.9×10^5 CD8$^+$, per recipient). Recipient mice were monitored for survival for 60 days following tumor challenge.

Results

IL-15 increases DLN, CLLN and spleen cellularity

Tumor bearing mice were immunized twice with C6VL lysate pulsed DC with each immunization followed by IL-15 injections. IL-15 was delivered subcutaneously to avoid administering IL-15 to the same site as the tumor (i.p.) and to potentially augment the expansion and survival of vaccine-primed CD8$^+$ T cells in the DLN. Tumor-bearing mice injected with C6VL-DC, IL-15 or diluent alone were included as controls. Eighteen hours following the last IL-15 infusion, the draining lymph nodes (DLN), contralateral lymph nodes (CLLN) and spleens were harvested and analyzed. The lymphocyte viability did not differ among treatment groups (data not shown). C6VL-DC vaccinations alone did not significantly increase DLN cellularity compared to untreated (naïve) tumor-bearing mice ($P = 0.057$) (Fig. 1). However, the combination of C6VL-DC vaccinations and IL-15 infusion (C6VL-DC + IL-15) significantly increased the number of DLN cells, raising it nearly 3.5-fold compared to C6VL-DC vaccine recipients ($P = 0.010$). Similarly, there was an increase in the number of cells isolated from CLLN and spleens of mice treated with C6VL-DC + IL-15 compared to C6VL-DC ($P = 0.05$ and 0.012, respectively), indicating that the IL-15 had a moderate systemic, as well as a local, effect. IL-15 injections alone increased cell numbers in the DLN and CLLN ($P = 0.015$ and 0.045, respectively, compared to naïve mice), but not in the spleen ($P = 0.24$).

C6VL-DC vaccines plus IL-15 synergistically increases the absolute numbers of NK, NKT and CD8$^+$ T cells in DLN

The cellular composition of DLN, CLLN and spleens from mice vaccinated and infused with IL-15 was analyzed by flow cytometry. Subcutaneous IL-15, alone or in combination with C6VL-DC vaccines, increased the percentages of NK and CD8$^+$ T cells in the DLN, CLLN and spleens of mice (Table 1 and Fig. 2a). In addition, increased percentages of NKT and total $\alpha\beta$T cells were evident in the DLN, but not the CLLN or the spleen (Table 1). The increase in
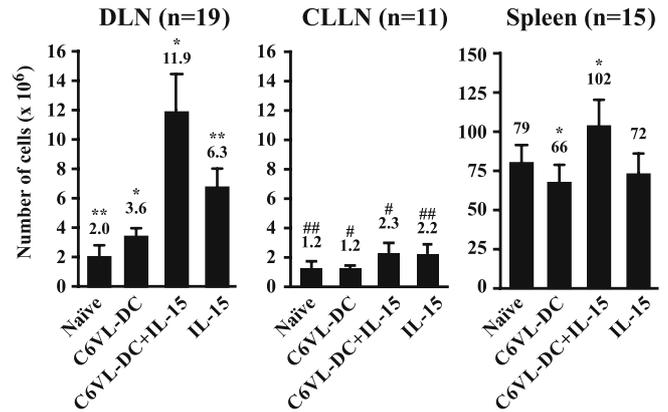

Fig. 1 Interleukin-15 infusion results in increased cell numbers in the DLN, CLLN and spleen. Mice bearing a lethal number of C6VL cells (i.p.) were vaccinated twice s.c. with 5.0×10^5 C6VL-DC or HBSS (*control*). 0.5 µg rhIL-15 was administered s.c. at the vaccine site every 12 h for 5 days following each vaccination. Mice not receiving IL-15 were infused with only the diluent. Eighteen hours following the last infusion, DLN, CLLN and spleens were harvested from mice and viable cells enumerated. *Bars* represent the average number of cells ($\times10^6$) isolated per DLN, CLLN or spleen ($n = 3$–10 mice/group) in 2 (CLLN) or 4 (DLN and spleen) independent experiments $\pm$ SD. *P*-values were calculated using a 2-sample Student's *t*-test (*$P < 0.012$; **$P = 0.015$, $^{\#,\#\#}P < 0.05$)

CD8$^+$ T cells observed in IL-15 recipients was accompanied by a compensatory decrease in the percentage of CD4$^+$ T cells at all three sites. The effect of IL-15 infusion on the cellular composition of the DLN was more evident when the absolute numbers of each population was compared. In the DLN, C6VL-

Table 1 S.c. IL-15 has local and systemic effects on the cellular compostion of the DLN, CLLN and spleens of recipient mice[a]

Treatment	NK	NKT[b]	$\alpha\beta$T[b]	CD4^{+b}	CD8^{+b}
DLN:					
Naïve	0.60	1.03	54.5	27.0	28.9
C6VL-DC	0.57	0.99	50.5	24.0	23.8
C6VL-DC + IL-15	1.37	2.42	60.9	18.7	38.6
IL-15 only	1.21	2.17	60.1	19.8	38.3
CLLN:					
Naïve	0.60	1.03	60.0	27.0	28.9
C6VL-DC	0.67	1.28	66.3	35.0	31.6
C6VL-DC + IL-15	1.01	1.12	69.5	25.8	41.8
IL-15 only	0.91	0.73	66.6	26.2	42.7
Spleen:					
Naïve	3.12	1.46	52.2	25.6	23.0
C6VL-DC	3.37	2.02	48.7	23.7	21.5
C6VL-DC + IL-15	5.60	2.02	50.3	15.4	30.1
IL-15 only	3.66	1.84	48.5	16.2	27.4

[a] Cells were obtained from DLN, CLLN and spleens of mice in each treatment group 1d following the last IL-15 infusion, and analyzed by flow cytometry as described in Materials and methods. Numbers indicate the average percent of total cells in replicate samples. Data shown is representative of 4 independent experiments of $n = 5$–6/group

[b] Cell populations categorized based on marker staining as indicated in Materials and methods
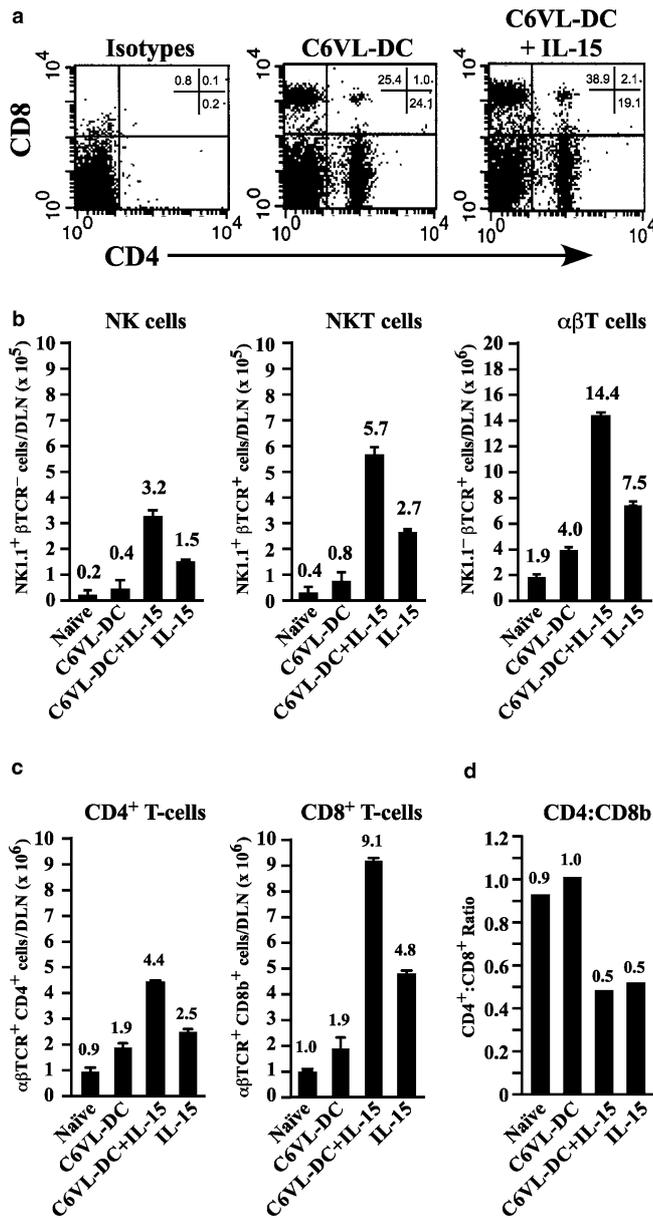
Fig. 2 Recipients of s.c. IL-15 have increased numbers of NK, NKT and T cells in their DLN. Tumor-bearing mice were vaccinated twice s.c. with 5.0×10^5 C6VL-DC or HBSS only. Mice were infused s.c. with IL-15 or diluent only every 12 h for 5 days after each vaccine. Eighteen hours following the last infusion, DLN were harvested and analyzed by flow cytometry for cell surface expression of markers indicative of (**b**) NK, NKT, and $\alpha\beta$ T cells; or (**a**) and (**c**) CD4$^+$ and CD8$^+$ T cell subsets as described in Materials and methods. Average absolute numbers of each cell type per DLN were calculated as [(percentage of total cells staining positive × number of viable cells isolated)/ *n* of mice per group]. **a** Representative primary flow cytometry data monitoring the percents of CD4 and CD8 T cells in the DLN of C6VL-DC recipients +/- IL-15. **b** and **c** *Bars* represent the average absolute numbers of cell types in the DLN of each group (*n* = 5–10 mice/group) in four independent experiments ± SD. **d** *Bars* represent the ratio of CD4$^+$ to CD8$^+$ T cells in the DLN of each group

DC + IL-15 treatment resulted in a 8.0-fold increase in NK, 7.1-fold increase in NKT, and a 3.6-fold increase in $\alpha\beta$T cells per DLN compared to recipients of the C6VL-DC vaccine alone (Fig. 2b). In addition, CD4$^+$ and CD8$^+$ T cell numbers were 2.3- and 4.8-fold higher, respectively, in recipients of C6VL-DC + IL-15 compared to recipients of the C6VL-DC vaccine alone (Fig. 2c). C6VL-DC + IL-15 treatment stimulated less dramatic increases in NK, NKT, $\alpha\beta$T cell, and CD8$^+$ T cell numbers in the CLLN (2.7-, 1.8-, 2.1-, and 2.7-fold increases, respectively) and spleens (1.9-, 1.2-, 1.2-, and 1.6-fold increases) compared to the DLN (data not shown). IL-15 infusions without concomitant C6VL-DC vaccination resulted in intermediate increases in each cell population compared to recipients of both treatments. Despite varying magnitudes of CD8$^+$ T cell expansion among the DLN, CLLN and spleen, however, IL-15 infusion with or without concomitant C6VL-DC vaccination resulted in a decreased CD4$^+$:CD8$^+$ T cell ratio at each site, with ratios ranging from 0.48–0.61 compared to 1.0–1.1 in naïve mice or mice treated with C6VL-DC vaccines alone (Fig. 2d and data not shown).

IL-15 infusion increases numbers of activated- or memory-phenotype CD8$^+$ T cells in DLN

CD8$^+$ T cells were purified from the DLN of each treatment group by negative selection and were stained for the activation marker, CD44, expression in combination with several other activation markers CD62L, CD122, CCR7, CD43, and CD69, to further characterize the influence of IL-15 infusion on the CD8$^+$ T cell population in the DLN. Mice that were infused with IL-15 alone or in conjunction with C6VL-DC vaccines had significantly higher percents of CD8$^+$ CD44^{hi} cells in their DLN compared to tumor-bearing mice that received HBSS or C6VL-DC vaccines only (Fig. 3b left panel), resulting in 5.8- and 7.6-fold increases in the absolute numbers of CD8$^+$ CD44^{hi} T cells, respectively (Fig. 3b, right panel). Among the CD8$^+$ CD44^{hi} population, both CD62L$^+$ and CD62L$^-$ cells were expanded (Fig. 3a, c), resulting in 2.1- and 3.2-fold increases in the percents of CD44^{hi} CD62L$^+$ and CD44^{hi} CD62L$^-$ CD8$^+$ T cells, respectively, when mice were treated with C6VL-DC + IL-15 compared to C6VL-DC alone. While the CD44^{hi} CD62L$^-$ phenotype has been used to indicate populations of effector CD8$^+$ T cells, we could not detect a concomitant decrease in CCR7 or increase in CD43 expression to further indicate that these were indeed effector CD8$^+$ T cells. In contrast, increases in CD44^{hi} CD122$^+$, CD44^{hi} CCR7$^+$, CD44^{hi} CD69^{int}, CD44^{hi} CD25^{int} and CD44^{hi} CD43$^-$ CD8$^+$ T cells were observed upon IL-15 infusion (Fig. 3d and data not shown), consistent with the phenotype of CD8$^+$ central memory T cells [22]. Expansion of these cells was greater in recipients of both C6VL-DC vaccines and IL-15 infusion compared to recipients of either treatment alone and occurred irrespective of the presence of tumor in the peritoneum of recipient mice (data not shown). The effects of IL-15 infusion on the percents of CD8$^+$
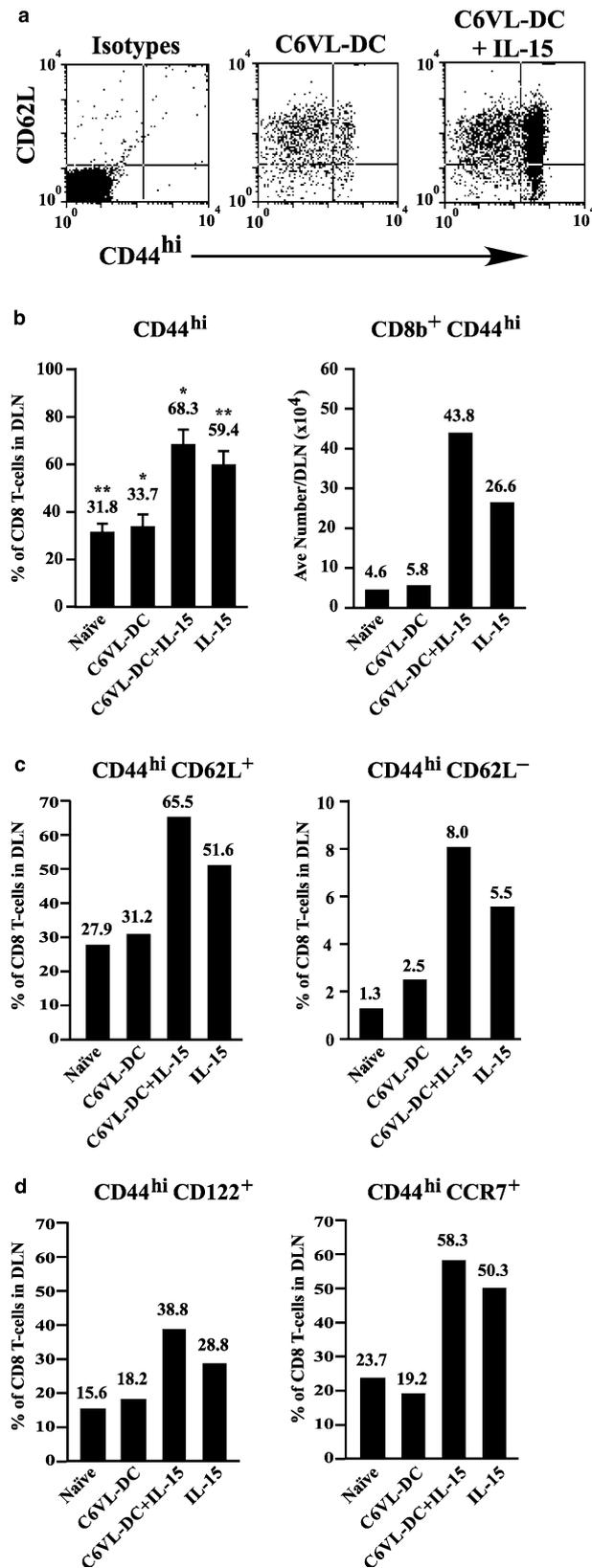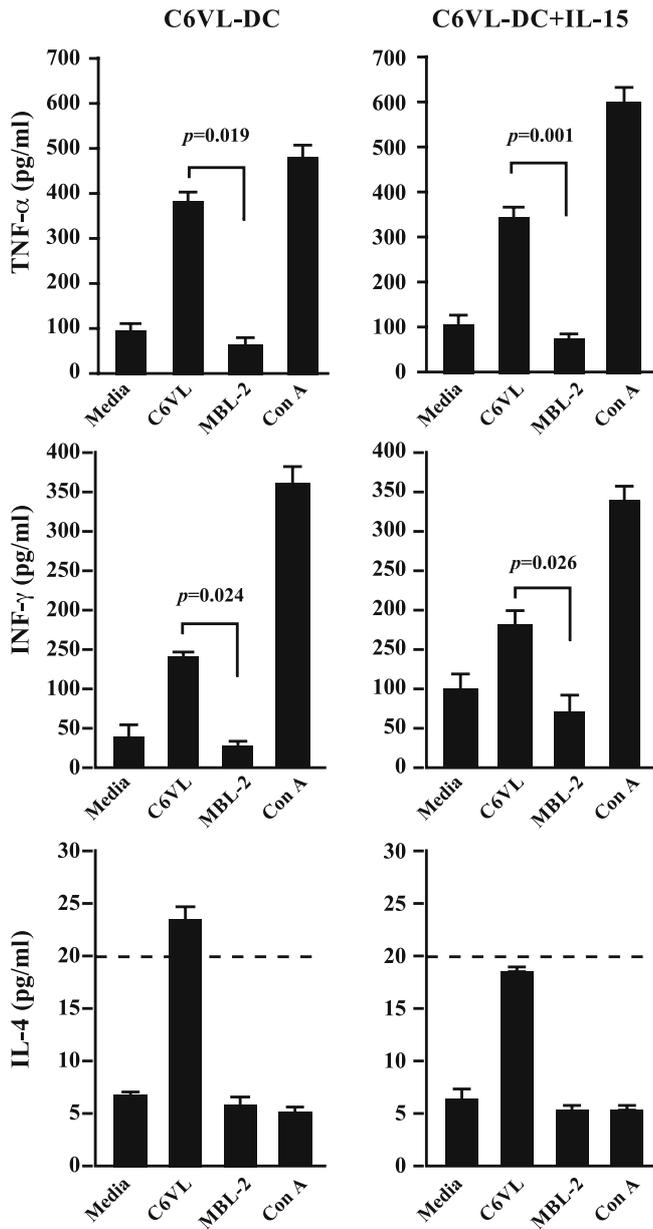
Fig. 3 Antigen-activated or memory-phenotype CD8$^+$ T cells are expanded by IL-15 infusion. Groups of 10 (+IL-15) or 20 (no IL-15) tumor-bearing mice were vaccinated and infused with IL-15 as indicated in the Materials and methods. Eighteen hours following the last IL-15 injection, DLN were harvested and CD8$^+$ T cells purified by negative selection. The CD8$^+$ T cells were stained for cell surface expression of CD8b (PerCP-Cy5.5) and CD44 (FITC) alone (**b**), or in combination with PE-conjugated CD62L, CD122, or CCR7 (**a–c**) and analyzed by flow cytometry. *Bars* indicate (**b**) the average (%) (*left*) and absolute number (*right*) of CD8b$^+$ CD44^{hi} cells per DLN in each treatment group, or **c, d** the percentage of CD8b$^+$ cells in the DLN that are both CD44hi and CD62L$^{+/-}$, CD122$^+$ or CCR7$^+$. *P* values were calculated using a two sample Student's *t* test (* and **$P < 0.0001$)

Tumor-specific secretion of cytokines is not enhanced by s.c. IL-15 infusion

To evaluate whether IL-15 infusions augmented tumor-specific vaccine-primed cellular immunity or innate clearance of C6VL, groups of 5–6 mice were immunized twice with C6VL-DC alone or with s.c. IL-15 infusion. Mice treated with only IL-15 or its diluent were included as controls. Eighteen hours following the last IL-15 infusion, DLN and spleens were harvested from each group and analyzed in vitro for C6VL-specific cytokine release and CTL- and NK-mediated killing of C6VL.

Consistent with our previous findings [10], DLN cells from C6VL-DC vaccinated mice secreted significant amounts of Th1-type cytokines, TNF-α and IFN-γ compared to IL-4 (Fig. 4, left panel). The secretion of the cytokines was tumor-specific, as their amounts were significantly lower upon in vitro stimulation control MBL-2 tumor. DLN lymphocytes from recipients that were treated with IL-15 in conjunction with C6VL-DC vaccines also secreted TNF-α and IFN-γ at high levels (Fig. 4, right panel). Secretion of TNF-α and IFN-γ by C6VL-DC + IL-15 DLN cells were also significantly increased upon stimulation with C6VL compared to MBL-2. However, addition of IL-15 to C6VL-DC vaccine therapy did not significantly increase the secretion of tumor-specific TNF-α and IFN-γ by bulk draining lymph node cells compared to treatment of the mice with C6VL-DC alone (*P* values of 0.30 and 0.062 for TNF-α and IFN-γ secretion, respectively). Similarly, while high levels of tumor-specific TNF-α and IFN-γ were secreted by splenocytes from C6VL-DC + IL-15 recipients upon stimulation with C6VL compared to MBL-2, the levels were not significantly higher than those secreted by splenocytes from recipients of C6VL-DC alone (data not shown). Secretion of IFN-γ, TNF-α and IL-4 by DLN or splenocytes from tumor-bearing mice treated with only IL-15 or diluent and secretion of IL-2 by any of the groups were below the limits of detection of the assay (≤ 20.0 pg/ml, data not shown). Similarly, secretion of the cytokines by mitomycin C-treated C6VL or MBL-2 stimulator cells was not detected (data not shown).
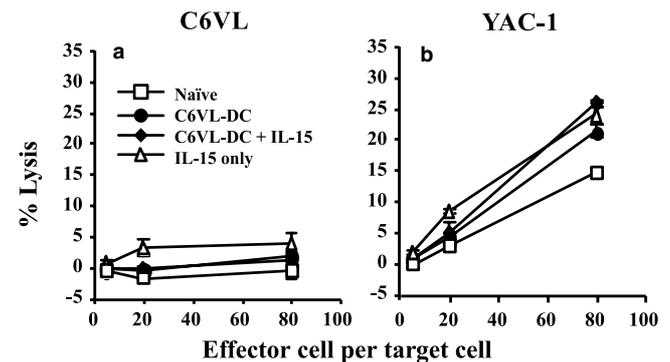
CD44^{hi} cells in the CLLN and spleen were also evident (data not shown), although the change in absolute number of the cells was of lesser magnitude than in the DLN.

C6VL-DC   **C6VL-DC+IL-15**

◄

Fig. 4 Interleukin-15 infusion does not increase vaccine-primed, tumor-specific cytokine release by DLN cells. Groups of five mice were vaccinated with C6VL-DC or HBSS and infused with rhIL-15 as described in Materials and methods. Eighteen hours following the last IL-15 infusion, DLN were harvested and stimulated for 72 h in vitro with mitomycin-C-treated C6VL or control tumor cells at a ratio of one tumor cell per five responder cells. Cytokine concentrations in culture supernatants were measured as described in the Materials and methods. *Bars* represent the mean of triplicate samples $\pm$ SD. The *dotted line* represents the assays lower limit of detection (20 pg/ml). *P* values were calculated using a two sample Student's *t* test. Cytokine secretion by mitomycin-C-treated tumor cells and DLN from recipients of only IL-15 or its diluent were below the limits of detection in all cases. Representative data from one of three independent experiments is shown

5 days was sufficient to generate LAK activity capable of killing C6VL in vitro, DLN and spleens from mice treated as described above were harvested, their cells pooled, and their ability to lyse C6VL and YAC-1 tumor cells was immediately assessed in a 16 h cytotoxicity assay. Pooled effector populations from tumor-bearing naïve mice demonstrated low but significant lysis of NK-susceptible YAC-1 targets (Fig. 5b), but were unable to lyse C6VL (Fig. 5a) ($P < 0.0015$, lysis of C6VL compared to YAC-1). The level of YAC-1 lysis mediated by the naïve effectors was very similar to data previously reported by Kennedy et al. [15]. As expected based on our previous results [10], vaccination of mice with C6VL-DC did not increase NK lysis of C6VL compared to naïve effectors. Also, IL-15 treatment alone or in combination with C6VL-DC vaccines did not increase

IL-15 adjuvant does not stimulate NK-mediated clearance of C6VL or augment the tumor-specific CTL activity stimulated by C6VL-DC vaccination of tumor-bearing mice

Overexpression of IL-15 in tumor-bearing hosts, expression of secretable IL-15 in tumor cells and injection of high doses of recombinant IL-15 protein have all been reported to stimulate NK-mediated clearance of tumors in vivo [24, 26, 44]. Culturing naïve splenocytes with 150 ng/ml rhIL-15 for 48 h resulted in LAK activity that efficiently lysed both C6VL and MBL-2 cells in vitro, while fresh splenocytes could efficiently lyse the NK-susceptible line, YAC-1, but not C6VL or MBL-2 target cells (data not shown). To determine whether the dose of IL-15 that we administered in vivo over

Fig. 5 Interleukin-15 infusions do not stimulate NK-mediated killing of C6VL. Six tumor-bearing mice per group were vaccinated with C6VL-DC or HBSS and infused with IL-15 as indicated in the Materials and methods. Mice were infused s.c. with 0.5 µg rhIL-15 every 12 h for five consecutive days following each vaccine. Eighteen hours following the last IL-15 infusion, DLN and spleens were harvested, pooled and used immediately in an overnight CTL assay. Specific lysis of DiO-labeled C6VL (**a**) and NK-susceptible YAC-1 (**b**) tumor cells at E:T of 80:1, 20:1 and 5:1 were assessed by flow cytometry following a 16-h incubation of targets with effectors. Percent lysis was calculated as described in the Materials and methods. Data points represent the mean of duplicate samples $\pm$ SD. Representative data from one of three independent experiments is shown
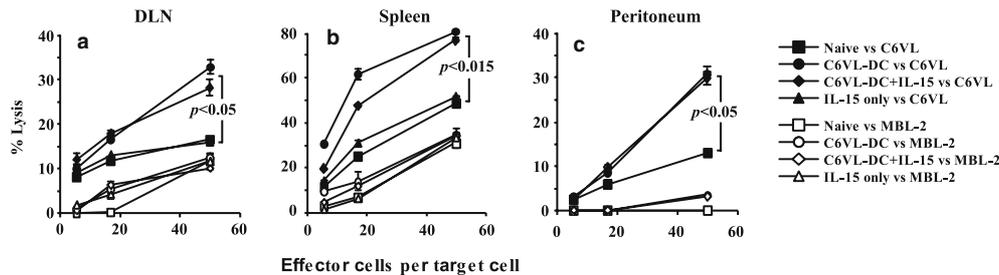
Fig. 6 Interleukin-15 does not augment the tumor-specific CTL activity stimulated by C6VL-DC vaccines in tumor-bearing mice. Six mice per group were vaccinated with C6VL-DC or HBSS and were given IL-15 or diluent only as detailed in the Materials and methods. Eighteen hours following the last IL-15 infusion, DLN, splenocytes and peritoneal lavage samples were harvested and restimulated in vitro for 5 days with one irradiated C6VL cell per five responder cells. Specific lysis of DiO-labeled C6VL and MBL-2 (*control*) tumor cells by (**a**), DLN; (**b**), splenocytes; or **c**, peritoneal lavage cells at E:T of 50:1, 17:1 and 5.6:1 was assessed by flow cytometry following a 4-h incubation of targets with restimulated effectors. Percent lysis was calculated as described in the Materials and methods. Data points represent the mean of duplicate samples ± SD. *P* values were calculated using a two sample Student's *t* test at each data point. Representative data from one of two independent experiments is shown

the ability of the effectors to lyse C6VL cells (Fig. 5a, all $P > 0.05$), indicating that the dose of IL-15 that we administered was insufficient to generate significant LAK activity in the DLN or spleen. Surprisingly, lysis of YAC-1 cells was not augmented by IL-15 treatment alone or in conjunction with C6VL-DC vaccines, despite increased numbers of NK cells in IL-15 recipients.

C6VL-specific CTL activities in the DLN, spleens and peritoneal cavity of treated mice was also measured. Incubation of target cells with restimulated DLN, splenocytes and peritoneal cells from C6VL-DC recipients resulted in high levels of killing of C6VL at all E:T ratios (Fig. 6a, b, c). The CTL activity was specific as MBL-2 target cells were killed at significantly lower levels than C6VL at all ratios ($P < 0.0047$, $P < 0.0047$ and $P < 0.001$ for DLN, spleen and peritoneum, respectively). Likewise, cells harvested from recipients of C6VL-DC + IL-15 lysed C6VL efficiently compared to MBL-2 ($P < 0.0024$, < 0.0032 and < 0.001 for DLN, splenocytes and peritoneal cells, respectively). However, despite increased numbers of activated or memory-phenotype $CD8^+$ T cells in mice treated with C6VL-DC + IL-15 compared to C6VL-DC alone, killing of C6VL tumor cells was not enhanced in mice infused with IL-15 (Fig. 6a, b, c). In vitro stimulation with C6VL likely gave rise to some specific CTL activity against C6VL in the cells isolated from the control treated mice. However, killing of C6VL by cells harvested from the DLN, spleen and peritoneum of C6VL-DC and C6VL-DC + IL-15 recipients remained significant compared to the background killing of C6VL by cells in the control groups, with *P* values of < 0.044, < 0.015 and < 0.05 for all ratios in the DLN, spleen and peritoneum, respectively.

IL-15 infusion does not enhance the survival of tumor-bearing C6VL lysate-pulsed DC vaccine recipients

Prior experiments have shown that C6VL lysate-pulsed DC vaccines are able to stimulate a protective immune response in mice bearing a lethal number of C6VL cells [10]. We looked to see if IL-15 infusions would enhance the efficacy of our C6VL-DC vaccine. Mice were tumor challenged and immunized three times with IL-15 given between each immunization. Mice receiving MBL-2-DC vaccines or only IL-15 were included as control groups. As expected, C6VL-DC vaccines significantly enhanced the survival of tumor-bearing mice compared to mice vaccinated with MBL-2-DC ($P = 0.039$), with 30% of the tumor-challenged mice surviving (Fig. 7). C6VL-DC vaccines coupled with IL-15 also significantly enhanced the survival of tumor-bearing mice compared to MBL-2-DC + IL-15 recipients ($P = 0.039$). However, IL-15 infusion in addition to C6VL-DC vaccines did not augment the survival of mice compared to C6VL-DC vaccines alone ($P = 0.72$). None of the control treated groups survived the tumor challenge confirming our in vitro evidence that IL-15 infusion does not result in background clearance of the C6VL tumor. Modifying our dosing schedule to give the same 0.5 μg/mouse IL-15 every 12 h, but for five consecutive days prior to the second and third vaccines, or for more prolonged peri-
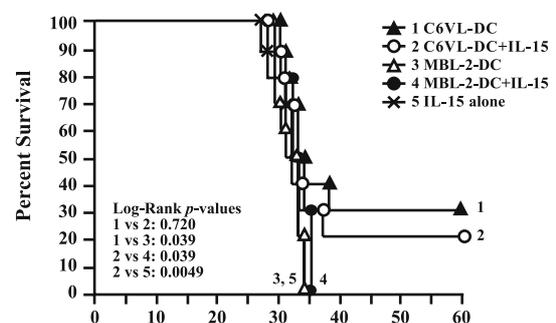

Fig. 7 Interleukin-15 infusion does not enhance the survival of tumor-bearing C6VL lysate-pulsed DC vaccine recipients. Naïve mice were challenged with a lethal dose of C6VL. Twenty-four hours following tumor challenge, mice received their first vaccine of 5.0×10^5 C6VL or MBL-2 lysate-pulsed DC. The vaccines continued every 10 days for three vaccines. Beginning 1d following each vaccine and continuing every 12 h for 5 days, 0.5 μg rhIL-15 was administered s.c. at the vaccine site. Control mice receiving DC vaccines but not IL-15 were injected with diluent alone on the same schedule. The mice were monitored for survival for 60 days following tumor challenge

ods (14 consecutive days, beginning 1 day following the first C6VL-DC vaccine) or giving the IL-15 i.p. at lower doses also failed to improve the efficacy of the C6VL-DC vaccine (data not shown).

Failure of IL-15 infusion to enhance vaccine-primed clearance of C6VL in vivo is likely not due to vaccine-primed and IL-15 expanded cells being sequestered in the secondary lymphoid organs

Phenotypic characterization of the $CD8^+$ T cells in the DLN of IL-15 recipients indicated that activated T cells with a phenotype characteristic of central memory T cells (T_{CM}) comprised a significant proportion of all cells in the draining lymph nodes of mice receiving IL-15 in addition to the C6VL-DC vaccine (Fig. 3b). T_{CM} home to secondary lymphoid organs due to their high levels of CCR7 and CD62L expression [37, 41] and lack immediate inflammatory and cytotoxic function. However, they can mediate rapid recall responses with greater potency against viral infections and established tumors than effector-phenotype $CD8^+$ T cells [19, 42] when used for adoptive immunotherapy. Further, murine antigen-primed $CD8^+$ T cells cultured in IL-15 resemble T_{CM} in phenotype and function, and thus require restimulation with antigen to acquire cytolytic function [22]. Based on this information, we were concerned that possibly C6VL-specific T_{CM} in the DLN and peripheral lymphoid organs were not trafficking to tumor sites where they would be restimulated and acquire effector function [22, 41]. It was also possible that our in vitro CTL assay was not sensitive enough to detect IL-15 induced differences in tumor-specific CTL activity, as the assay uses small numbers of cells per sample and the percent of $CD8^+$ expansion was not as dramatic as the change in absolute numbers of cells. To address these concerns, we performed an in vivo CTL assay to compare both the C6VL-clearing capacity of an entire lymph node-equivalent of cells and the clearing capacity of a defined number of $CD8^+$ T cells from C6VL-DC and C6VL-DC+IL-15 recipient mice. DLN cells from each group were injected i.p into mice bearing five times the lethal dose of C6VL cells in their peritoneum. Transfer of 3.2×10^6 DLN cells (equivalent to one DLN) pooled from ten C6VL-DC vaccinated mice resulted in significant protection of naïve tumor-bearing recipient mice compared to transfer of MBL-2-DC-primed DLN ($P=0.0022$) (Fig. 8a, b). While the transfer of 1 DLN equivalent of cells from C6VL-DC+IL-15-treated mice (6.7×10^6 cells pooled from 13 mice) also resulted in significant protection of naïve recipient mice compared to the transfer of an equal number of cells from mice treated with MBL-2-DC+IL-15 ($P<0.001$), the protection was not increased compared to transfer of cells primed with C6VL-DC alone ($P>0.92$) (Fig. 8a). Further, the transfer of a $CD8^+$ T cell-matched number of DLN (1.7×10^6 total cells or 5.9×10^5 $CD8^+$) primed with C6VL-DC+IL-15 did not confer significant protection
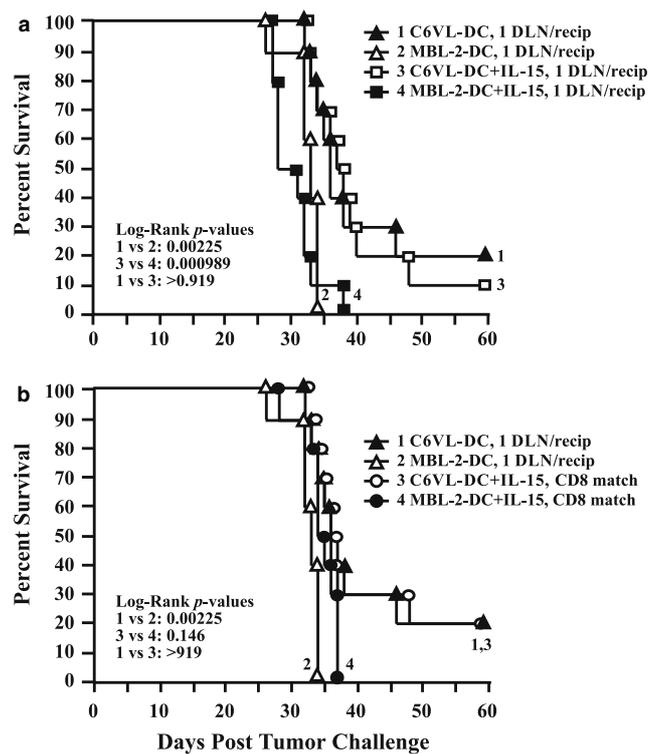

Fig. 8 C6VL-DC+IL-15 recipients do not have enhanced C6VL-specific CTL activity per-DLN or per-$CD8^+$ T cell compared to recipients of C6VL-DC vaccines alone. Tumor-bearing donor mice ($n=13$ per group) were vaccinated twice with 5.0×10^5 C6VL- or MBL-2-lysate-pulsed DC and infused s.c. with 0.5 µg rhIL-15 twice daily for 5 days following each vaccine. Mice vaccinated with C6VL-DC or MBL-2-DC without IL-15 infusion ($n=11$ per group) were control donors. Eighteen hours following the last IL-15 infusion, DLN from each group were harvested, pooled, mixed with C6VL cells and injected into the peritoneum of ten naïve recipient mice per group. Each naïve recipient received 5000 C6VL cells and either **a** a number of cells equivalent to one donor's DLN (3.2×10^6, C6VL-DC and MBL-2-DC DLN; or 6.7×10^6, C6VL-DC+IL-15 and MBL-2-DC+IL-15 DLN) or **b** a number of cells from IL-15 recipient DLN that resulted in the transfer of a number of $CD8^+$ T cells that matched those transferred in 1 DLN from C6VL-DC and MBL-2-DC donors (1.7×10^6 total cells or 5.9×10^5 $CD8^+$ per recipient)

compared to transfer of the same number of MBL-2-DC+IL-15-primed DLN cells ($P=0.146$) (Fig. 8b).

Discussion

Our laboratory previously demonstrated that vaccination of tumor bearing mice with lysate-pulsed DC stimulated potent $CD8^+$ T cell-mediated clearance of C6VL, and resulted in an average of 40% survival [10]. While the use of IL-15 as a vaccine adjuvant has been reported to enhance the antitumor activity of adoptively transferred $CD8^+$ T cells transgenic for tumor antigens [19, 35], the ability of IL-15 to augment vaccine-primed responses by endogenous wild-type $CD8^+$ T cells has not been assessed in a therapeutic tumor model. We hypothesized that the administration

of IL-15 in conjunction with C6VL-DC vaccines would enhance vaccine-primed CD8$^+$ T cell immunity against C6VL and thus result in higher levels of survival in our therapeutic model. Instead, we found that IL-15 administration neither helped nor hindered C6VL-DC-primed T cell responses against C6VL or the survival of tumor-bearing mice. Our collective data indicates that while IL-15 infusion following C6VL-DC vaccines resulted in dramatic expansion of NK, NKT and activated CD8$^+$ T cells at both local (DLN, Figs. 2 and 3a) and distal sites (CLLN and spleen; data not shown), T cells with specificity for C6VL were not significantly or preferentially expanded by the treatment (Figs. 4, 6, 7, 8).

Recent studies of systemic IL-15 administration in adoptive transfer models demonstrated that systemic administration of IL-15 augmented the primary antigen-specific CD8$^+$ T cell response following a peptide-pulsed DC vaccine [35] and potentiated the in vivo tumor activity of antigen-specific CD8$^+$ T cells [18]. However, these studies monitored antigen-specific responses of transgenic CD8$^+$ T cells following the transfer of a relatively large number (1.0–5.0×10^6) of splenocytes or purified CD8$^+$ T cells that were activated in vivo by vaccination [19, 35]. Thus, the inability of IL-15 to enhance survival in our tumor model may be due to naïve mice having comparably few resident tumor specific CD8$^+$ T cells that are available for stimulation by our DC vaccines and expansion by IL-15. Similarly, Oh et al. [28] recently reported that IL-15 only slightly increased the frequency of antigen specific CD8$^+$ T cells primed by HIV vaccine vectors in wild-type mice.

It is possible that using a different IL-15 administration schedule, route or timing relative to the DC vaccine may be more efficacious. Our administration of IL-15 is similar, but not exactly the same as that reported by others. Rubinstein et al. [35] reported that one round of IL-15 infusion (0.5 μg/mouse every 12 h,×7 days) following a single vaccination with OVA peptide-pulsed DC enhanced the primary antigen-specific response of adoptively transferred OT-1 CD8$^+$ T cells. We also administered 0.5 μg IL-15 every 12 h, but for 5 days following each of two vaccines. While many groups have reported biological activity with 0.5 μg IL-15 twice daily, doses as high as 108 μg twice daily for 3 days have been used in other murine models [19], with the strong caveat that many manuscripts published on the topic have not included the specific activity of the recombinant IL-15 used in the studies. We chose to deliver the IL-15 s.c. at the site of DC vaccines instead of i.p., as has been traditionally done. This avoids administering the IL-15 to the same site as the tumor (i.p.) and potentially biases expansion and survival of vaccine-primed CD8$^+$ T cells in the DLN. The rational for administering the IL-15 at the vaccine site is further supported by recently published studies by Dubois et al. [6] showing that the IL-15Rα chain functions to cross-present IL-15 from antigen-presenting cells to T cells. Importantly, injecting IL-15

s.c. still resulted in expansion of T cells at distal sites in vivo, indicating that our dose was sufficient to induce systemic effects. Several other schedules and amount of IL-15 were tried in our model without any significant improvement in vaccine efficacy.

We used recombinant human IL-15 in our studies, as many groups studying the in vivo and in vitro effects of IL-15 have done. While doing so risks raising antihuman IL-15 antibodies in vivo, we were unable to detect IL-15-specific antibodies by ELISA in any of the sera from 36 individual IL-15-treated mice used in three independent experiments (assayed 10 days following the last IL-15 infusion; data not shown). Also, while IL-15 supports the growth and survival of some human malignant T cell lines in vitro [33, 45], culturing C6VL for 96 h in the presence of up to 1 μg/ml IL-15 did not influence the proliferation rate or viability of the C6VL cells (data not shown) nor did it hasten the demise of tumor challenged mice (Fig. 7). Finally, while it was recently noted that IL-15 can expand de novo-induced human antigen-specific regulatory CD4$^+$ T cells in the presence of cognate antigen, CD25$^+$ CD4$^+$ T cells were not expanded by IL-15 infusion in our studies (data not shown), nor do we have evidence that regulatory T cells specific for C6VL antigens are induced by vaccination against, or tumor challenge with, C6VL (data not shown). These results are in line with previously published data showing that IL-15 stimulates little, if any, proliferation of murine CD4$^+$ T cells [46].

We did not find that IL-15 enhanced tumor specific cytokine secretion or CTL activity in DLN, CLLN or spleen cells harvested 6 days following the second DC vaccine. It is possible that due to the timing of our analysis or the in vitro assays that we used, some CD8$^+$ T cell responses were augmented by IL-15 and went undetected. Others have detected augmented in vitro CTL activity among transgenic CD8$^+$ T cells as early as 6 days following a single peptide-pulsed DC vaccine when rhIL-15 was administered using the same dose and time schedule that we used. However, they did not assess the in vivo tumor clearing capacity of the cells [35]. In another study using the same model, IL-15 without concomitant DC vaccination increased the number of antigen-reactive T cells without improving the overall survival of the treated mice [25]. While these studies showed that IL-15 expanded early responses of antigen-specific CD8$^+$ T cells, Oh et al. [28] showed that the utility of IL-15 as adjuvant for vaccination against HIV antigens was the induction of longer-lived CD8$^+$ T cell immunity in vaccine recipients, suggesting that IL-15 during priming may alter the quality rather than just the quantity of CD8$^+$ T cells during the immune-induction phase. As our analysis did not correlate the immunophenotype of a cell to its response upon restimulation by C6VL, we cannot say what proportion of the CTL responses that we measured were mediated by primary effector versus early memory CD8$^+$ T cells primed by the C6VL-DC vaccines, or whether IL-15 infusion changed the

amount of cytokine a single cell type secretes upon tumor recognition. We were consistently unable to demonstrate any augmented antitumor immune response among mice receiving IL-15 in addition to C6VL-DC vaccine. If any augmented responses went undetected by our in vitro analysis, they were not of sufficient magnitude to positively affect the survival of C6VL-bearing mice.

Interleukin-15, indeed, has many attributes that make it an attractive choice for use as a vaccine adjuvant to augment $CD8^+$ T cell responses. While our results do not support its use to enhance a primary antitumor immune response, it might be very effective in enhancing recall responses or to bolster $CD8^+$ T cell responses following adoptive transfer of tumor-specific T cells. We believe that further elucidation of the kinetics of $CD8^+$ T cell primary responses and memory cell development following vaccination against tumor antigen, and how they are affected by the presence or absence of tumor in vivo, will help investigators use IL-15 to its fullest adjuvant potential in models of active immunotherapy.

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