The CC Chemokine CKβ-11/MIP-3β/ELC/Exodus 3 Mediates Tumor Rejection of Murine Breast Cancer Cells Through NK Cells[1]

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CKβ-11 chemoattracts T cells, B cells, dendritic cells, macrophage progenitors, and NK cells and facilitates dendritic cell and T cell interactions in secondary lymphoid tissues. We hypothesized that expression of CKβ-11 in tumor cells may generate antitumor immunity through these interactions. After transduction with the retroviral vector L(CKβ11)SN, the murine breast cancer cell line C3L5 (C3L5-CKβ11) showed expression of retroviral mRNA by Northern analysis and production of functional CKβ-11 by chemotaxis of human NK cells to C3L5-CKβ11 supernatant. Only 10% of mice injected with C3L5-CKβ11 developed tumors, compared with 100% of mice injected with a transduced control C3L5 line (C3L5-G1N). Importantly, the in vitro growth characteristics of the CKβ-11-transduced cell line were unaffected, suggesting the difference in growth in vivo was a result of chemokine production. Vaccination with C3L5-CKβ11 partially protected animals from parental C3L5 challenge. Immunodepletion with anti-asialo-G_{M1} or anti-CD4 during C3L5-CKβ11 vaccination significantly reduced CKβ-11 antitumor activity compared with control and anti-CD8-treated groups. Splenocytes from NK-depleted animals transferred the acquired immunity generated with C3L5-CKβ11 vaccination, while splenocytes from the CD4-depleted animals did not. These results indicate, for the first time, that expression of CKβ-11 in a breast cancer cell line mediates rejection of the transduced tumor through a mechanism involving NK and $CD4^+$ cells. Furthermore, CKβ-11-transduced tumor cells generate long-term antitumor immunity that requires $CD4^+$ cells. These studies demonstrate the potential role of CKβ-11 as an adjuvant in stimulating antitumor responses. ***The Journal of Immunology*****, 2000, 164: 4025–4031.**

Breast cancer is the most common cancer in American women and the second most common cause of cancer death (1). Adjuvant chemotherapy and radiation have improved the survival of women presenting with localized disease, but subsets of women continue to have a poor prognosis (2–4). Metastatic breast cancer has a uniform poor outcome with a median survival rate of ~2 years (3).

Various cytokines and chemokines are known to have antitumor activity, and cytokine-expressing autologous tumor cell vaccines can enhance antitumor immune responses against malignant cells in vivo. Rejection of tumor cells has been noted in various murine tumor models in which tumor cells have been modified with early acting cytokines (GM-CSF, Flt3L, G-CSF) (5–8), immunoregulatory cytokines (IL-2, IL-4, IL-7, IL-10, IL-12) (9–13), inhibitory cytokines (TNF-α, IFN-γ) (14, 15), chemokines (MIP-1α,[3] RANTES, lymphotactin, TCA3, JE/MCP-1/MCAF) (16–23), and costimulatory molecules (CD40L, B7.1) (24, 25).

Chemokines are a large family of cytokines with four subgroups (CC, CXC, CX_3C, and C) based on the N-terminal cysteine motifs (26, 27). The CC chemokine CKβ-11/MIP-3β/ELC/Exodus 3 (CKβ-11) chemoattracts T cells (28–31), B cells (29, 30), dendritic cells (DC) (32), macrophage progenitor cells (33), and NK cells (29), and may be involved in the interactions of DC and T cells in secondary lymphoid tissues (28, 34).

Since CKβ-11, expressed in the secondary lymphoid tissues, has the potential to induce specific homing of most lymphoid cells and of DC, we hypothesized that in vivo expression of CKβ-11 in the murine breast cancer cell line C3L5 may generate antitumor immunity by facilitating localization of lymphocytes and DC at the site of tumorigenesis. After vaccinating animals with CKβ11-transduced C3L5 cells, we found rejection of the CKβ-11-transduced tumor was mediated through NK cells and $CD4^+$ cells (including $CD4^+$ NKT cells), while antitumor immunity in a subsequent challenge with parental C3L5 cells required $CD4^+$ cells at the time of C3L5-CKβ11 vaccination. These results suggest, for the first time, two levels of antitumor activity for CKβ-11; one level is mediated mostly through NK cells, and another is mediated through $CD4^+$ cells.

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[3] Abbreviations used in this paper: MIP, macrophage-inhibitory protein; DC, dendritic cell; Flt3L, Flt3/Flk2 ligand; LTR, long terminal repeat; MCP, monocyte chemoattractant protein; Mo-MLV, Moloney murine leukemia virus; TCA3, T cell activation-specific gene 3; TFA, tumor-free animals.

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Materials and Methods

Construction of the retroviral vector

The cDNA for CKβ-11 was generated by RT-PCR of total mRNA from human PBMC. After reverse transcription with oligo(dT) primer and Superscript II (Life Technologies, Rockville, MD), the forward primer (5′-GTT CGG TAC CTG CCT CTG TTC ACC CTC CAT G-3′) and the reverse primer (5′-AGT GCT CGA GTT ACT TGT CAT CAT CGT CCT TGT AGT CAC TGC TGC GGC GCT TCA TCT T-3′) were used to amplify the complete coding sequence. PCR product was digested with *Xho*I and *Kpn*I and cloned into the expression vector pREP4 (Invitrogen, Carlsbad, CA). Cloning of CKβ-11 cDNA was confirmed by sequencing (35).

pREP4-CKβ-11 was transfected into 293-EBNA cells (Invitrogen) by a standard electoporation method, and hygromycin-resistant cells were selected at 400 μg/ml of hygromycin. Supernatants from 293-EBNA cells and 293-EBNA-CKβ-11 cells were tested for chemotactic activity for NKL cells, as previously described (29). Three-day culture supernatants from 293-EBNA-CKβ-11, but not 293-EBNA cells, showed a chemotactic activity equal to that of 50–100 ng/ml control CKβ-11 protein (30).

The retroviral vector L(CKβ11)SN (see Fig. 1*B*) was generated by cloning the 350-bp cDNA fragment from EBNA-CKβ-11 into the *Eco*RI and *Xho*I sites of pLXSN (36) with CKβ-11 transcriptionally regulated by the Moloney murine leukemia virus (Mo-MLV) long terminal repeat (LTR). Correct orientation was verified by sequence analysis (Biochemistry Biotechnology Facility, Indiana University School of Medicine, Indianapolis, IN).

Cell culture

C3L5 (37, 38), AM12 (39), and GP+E86 (40) cells were cultured in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker) at 37°C with 5% CO_2. The NK cell line NKL was cultured in RPMI 1640 (BioWhittaker) plus 15% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 100 U/ml IL-2 (R&D Systems, Minneapolis, MN) at 37°C with 5% CO_2.

Gene transfer

The retroviral vector L(CKβ11)SN was shuttle packaged through the ecotropic packaging cell line and GP+E86 into the amphotropic packaging cell line AM12. Supernatant (0.45 μm filtered) from these cells plus Polybrene (8 μg/ml; Sigma, St. Louis, MO) was used to transduce the murine breast cancer cell line C3L5 (C3L5-CKβ11), as previously described (6, 7), before selection in media containing G418 (400 μg/ml; Life Technologies/BRL, Grand Island, NY). Fig. 1 represents a schematic of the retroviral vectors used in these studies. G1N (neo control)-transduced murine breast cancer cells (C3L5-G1N) were described in our previous study (6). The vector G1Na (see Fig. 1*A*) was provided by Genetic Therapy (Gaithersburg, MD).

Chemotaxis assay

Cultures of untransduced, C3L5-G1N and C3L5-CKβ-11 cells (1.5×10^6) were plated in 3 ml DMEM without serum or with 10% FCS for 48 or 72 h. Cellular debris was removed from the conditioned media by filtering or centrifugation. NKL cells were washed in PBS and resuspended in DMEM plus 0.5% BSA chemotaxis media. Costar Transwells (6.5 mm diameter, 5 μm pore size, polycarbonate membrane; Cambridge, MA) were used to separate the conditioned media from NKL cells for 3–4 h at 37°C with 5% CO_2. The number of cells that transmigrated through the membrane were counted using the FACScan cell analyzer (Becton Dickinson, San Jose, CA), and chemotaxis was calculated as the percentage of the input cells.

In vivo studies

To determine the protection from tumor growth by CKβ-11, mice were vaccinated with 1×10^4 transduced cells injected s.c. into the anterior chest wall of female C3H/HeN mice (tumor vaccine). Tumor growth was measured weekly and tumor volume was calculated (vol = width^2 × length/2). Animals tumor free at 4 wk were rechallenged with 1×10^4 parental C3L5 cells in the contralateral chest wall (parental tumor challenge). Animal studies were reviewed and approved by the Institutional Review Board.

In adoptive transfer studies, 1×10^7 splenocytes from animals tumor free after 4 wk were harvested and injected i.v. into previously untreated (naive) mice that simultaneously received 1×10^4 untransduced C3L5 cells in the anterior chest wall.

To understand the mechanism and cellular mediators of antitumor activity, mice were injected with PBS, control rabbit serum (100 μl i.p. per mouse on days −3, 0, and +3) (Sigma), rabbit anti-asialo-GM1 to immunodeplete NK cells (20 μl i.p. per mouse every 4 days during the primary tumor challenge or on days −3, 0, and +3) (Wako Pure Chemical Industries, Richmond, VA), anti-CD4 (clone GK1.5; PharMingen, San Diego, CA), and anti-CD8 (clone 53-6.7; PharMingen) Abs (0.1 mg per mouse on days −3, 0, and +3).

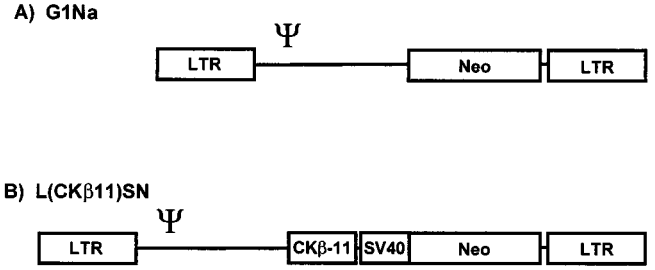

FIGURE 1. Schematic diagrams of the retroviral vectors. *A*, G1Na vector containing the neomycin phosphotransferase (neo) gene transcriptionally regulated by the Mo-MLV LTR. *B*, L(CKβ11)SN vector containing the human CKβ11 cDNA transcriptionally regulated by the Mo-MLV LTR and the neo gene regulated by the SV40 early promoter. Ψ, The RNA packaging signal.

Statistical analysis

Differences in tumor growth were evaluated over 4 wk by the number of animals with tumor and by the tumor volume in these animals. Tumor growth was determined using the Mann-Whitney statistic for nonparametric analysis and the one-tailed critical values of U_s, as described (41). Differences in chemotactic responses and splenocyte percentages were evaluated by Student's *t* test.

Flow-cytometric analysis

Single-cell suspensions of splenocytes were resuspended in PBS with 0.5% BSA and dual stained by incubation with FITC-conjugated anti-CD3ε (clone 145-2c11; PharMingen) or the appropriate isotype control (PharMingen), and with PE-conjugated anti-CD4 (clone RM4-5; PharMingen), PE-conjugated anti-CD8 (clone 53-6.7; PharMingen), PE-conjugated anti-pan NK cells (clone DX5; PharMingen), or the appropriate isotype control (PharMingen) for 1 h at 4°C. C3L5 cells were stained with FITC-conjugated anti-CD1δ (clone 1B1; PharMingen) and the appropriate isotype control (PharMingen). The cells were washed and analyzed for the percentage of cells in each population by flow cytometry using the FACScan cell analyzer (Becton Dickinson).

Results

Transduction and expression of the chemokine CKβ-11 in the murine breast cancer cell line C3L5

To assess the effectiveness of CKβ-11 as a tumor vaccine, we generated the full-length cDNA by RT-PCR and constructed the retroviral vector L(CKβ11)SN with CKβ-11 transcriptionally regulated by the Mo-MLV LTR and the neo gene transcriptionally regulated by the SV40 early promoter (Fig. 1*B*). After shuttle packaging this retroviral vector was used to transduced the murine breast cancer cell line C3L5 before selection in medium containing G418 (C3L5-CKβ11). The control vector G1Na, containing the neo gene transcriptionally regulated by the Mo-MLV LTR (Fig. 1*A*), was also used to generate a control cell line (C3L5-G1N), as previously described (6, 7).

To measure the level of mRNA expression from the integrated provirus, Northern analysis was performed on total cellular RNA isolated from C3L5, C3L5-G1N, and C3L5-CKβ11 cell populations (Fig. 2). Because the vectors contain the neomycin phosphotransferase gene as a common sequence, a neo-specific probe was used for hybridization. The parental C3L5 cells do not hybridize to the neo probe (Fig. 2, *lane 1*). In contrast, the C3L5-G1N- and C3L5-CKβ11-transduced populations hybridized to the neo-specific probe with higher levels of neo expression in C3L5-G1N than

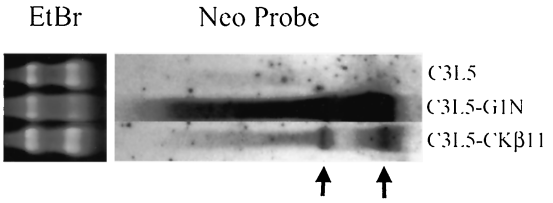

FIGURE 2. Molecular analysis of retroviral mRNA expression. Total RNA from C3L5, C3L5-G1N, and C3L5-CKβ11 cells was isolated, separated by gel electrophoreses, and blotted to nylon membranes. Membranes were probed with a radiolabeled neomycin phosphotransferase (Neo)-specific probe. After washing, the membrane was exposed to film for 6 h. *Bottom frame*, Ethidium bromide (EtBr) staining of RNA gel shows equal loading of samples. *Top frame*, Neo-specific hybridization to C3L5-G1N and C3L5-CKβ11. The arrows show multiple transcript lengths in C3L5-CKβ11 cells resulting from the two promoters.

in C3L5-CKβ11 (Fig. 2, *lanes 2* and *3*). With the C3L5-CKβ11-transduced cells, two transcript lengths are evident (Fig. 2, *lane 3*), consistent with expression from the LTR and the SV40 promoters. This molecular analysis demonstrates expression of the retroviral messenger RNA from the integrated provirus in transduced cells.

To demonstrate production of the chemokine CKβ-11, we measured the functional activity of CKβ-11 in conditioned media, as Abs against murine CKβ-11 are not commercially available. Supernatant from parental C3L5 and C3L5-CKβ11-transduced populations and a soluble rCKβ-11 positive control (100 ng/ml) were studied for chemoattractant potential in chemotaxis assay with the human NK cell line NKL. As shown in Table I, a previously determined optimal concentration of CKβ-11 (100 ng/ml) attracted 42 $\pm$ 11% of input NKL cells through the membrane (mean for three separate experiments). From previous studies, the maximum sensitivity of this assay is ~1 ng/ml and the responsiveness of NKL cells to CKβ-11 is bell shaped, with concentrations higher than 100 ng/ml desensitizing chemotaxis (29). Supernatant from the C3L5-CKβ11 cells attracted 24 $\pm$ 3.4% of the NKL cells through the membrane, while only 5 $\pm$ 2.2% of cells transversed the membrane when supernatant from the parental C3L5 cells was tested (Table I). Although we cannot determine the precise concentration, from these results, we concluded that C3L5-CKβ11 supernatants contain between 1 and 10 ng/ml of CKβ-11 (0.5–10 ng/10^6 cells/24 h) and that these cells produce biologically relevant levels of CKβ-11 protein.

To determine the effect of CKβ-11 expression on the growth of C3L5 cells, the growth rate of transduced cells was studied. As shown in Fig. 3, expression of the chemokines in C3L5 cells did not significantly ($p > 0.05$) alter the in vitro growth rate of these cells compared with parental C3L5 and with G1N-transduced C3L5 cells.

Table I. *Chemotaxis of NKL cells with conditioned medium from transduced cells or recombinant CKβ-11*[a]

	Expt. 1[b]	Expt. 2	Expt. 3[c]	Mean $\pm$ SD
CKβ-11 (100 ng/ml)	44	52	30	42 $\pm$ 11[e]
C3L5-control	2[d]	7	6	5 $\pm$ 2.2
C3L5-CKβ11	20	24	27	24 $\pm$ 3.4[e]

[a] Results presented are the percent of input cells that migrate through the membrane with each treatment.
[b] Dilution of supernatant (1:2).
[c] Medium without serum for 48 h.
[d] C3L5-LXSN transduced cells.
[e] Significantly different ($p < 0.05$) than C3L5-control.

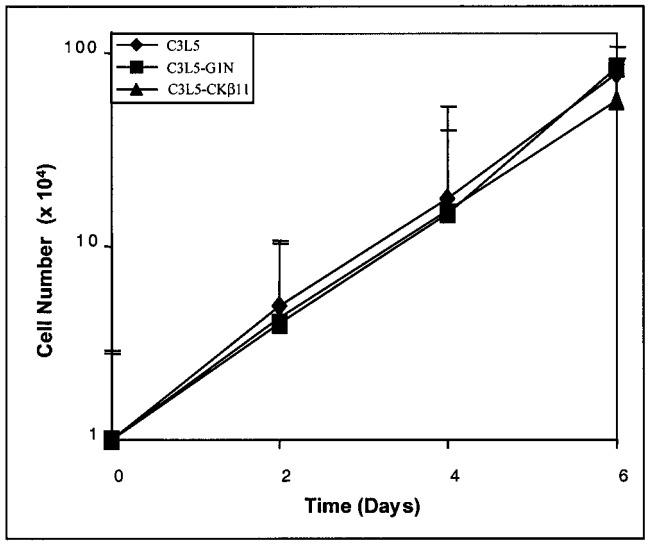

FIGURE 3. In vitro growth curve of C3L5, C3L5-G1N, and C3L5-CKβ11 cells. Cells were plated at 1×10^4 cells/well and cultured for 6 days. Viable cell number was determined using a hemocytometer every 2 days. Results are given as means $\pm$ SD from triplicate cultures.

Tumor vaccine and parental tumor challenge

To determine whether chemokine expression prevented tumor formation, female C3H/HeN mice were vaccinated with 1×10^4 C3L5-G1N or C3L5-CKβ11 cells s.c. in the anterior chest wall (tumor vaccine). Animals were followed for 4 wk, and tumor volume and the percentage of tumor-free animals (TFA) were determined. After inoculation with the tumor vaccine (Fig. 4), C3L5-CKβ11-vaccinated mice (90% TFA, vol = 1.9 cm^3) had

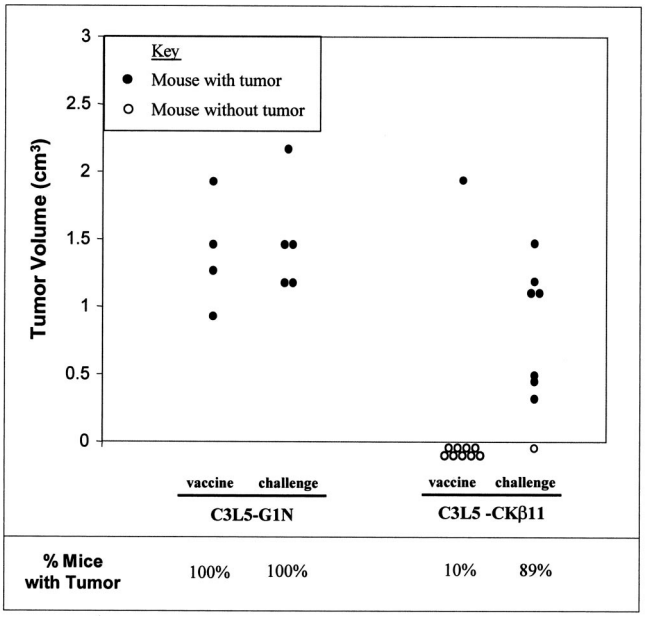

FIGURE 4. In vivo tumor size of C3L5-G1N and C3L5-CKβ11 vaccine. From the results of two independent experiments, the percentage of animals that developed tumor 4 wk after vaccination (1×10^4 transduced cells per animal) or after parental C3L5 challenge (1×10^4 cells per animal) is represented under the figure. The animals without tumor (○) are represented below the ordinate, and the animals with tumor (●) are represented by the tumor volume.

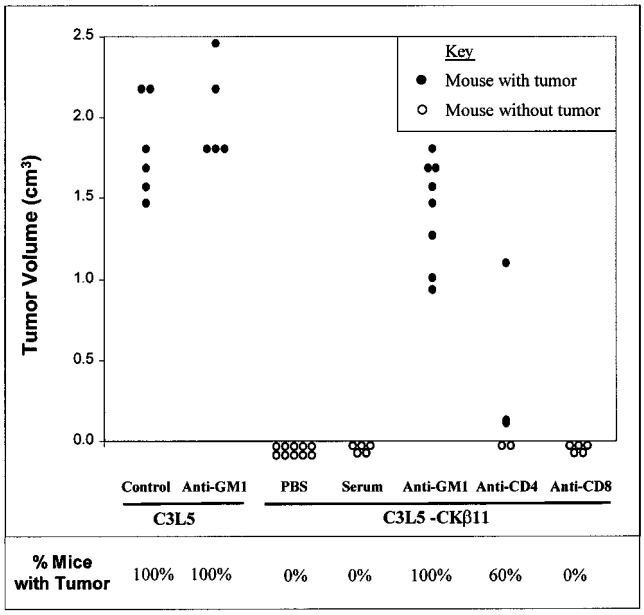

FIGURE 5. In vivo tumor size of C3L5 and C3L5-CKβ11 vaccine. From the results of two independent experiments, the percentage of animals that developed tumor 4 wk after vaccination (1×10^4 transduced cells per animal) and after immunodepletion of specific lymphocyte subsets with Abs (anti-asialo-GM1 for NK cells, anti-CD4 for Th cells, anti-CD8 for CTL) is represented under the figure. The animals without tumor (○) are represented below the ordinate, and the animals with tumor (●) are represented by the tumor volume.

significantly decreased tumor formation ($p < 0.05$) when compared with control C3L5-G1N-vaccinated animals (0% TFA, vol = 1.6 ± 0.29 cm^3).

To determine whether C3L5-CKβ11-vaccinated animals had generated tumor immunity, TFA at 4 wk were rechallenged with 1×10^4 parental C3L5 cells in the contralateral chest wall (parental tumor challenge). During this secondary challenge with parental C3L5 cells (Fig. 4), vaccination with C3L5-CKβ11 significantly reduced ($p < 0.05$) the tumor growth, but did not prevent tumor formation (11% TFA, vol = 0.87 ± 0.45 cm^3). These results indicate that CKβ-11-transduced cells can mediate tumor rejection and generate a low level of antitumor immunity.

Immunodepletion of lymphoid subsets with Ab injections

Several mechanisms may be involved in generating antitumor activity by CKβ-11-transduced C3L5 cells. Because CKβ-11 is known to chemoattract effector cells, such as naive and memory T cells and NK cells, we studied the role of $CD4^+$, $CD8^+$, and NK cells in CKβ-11 antitumor activity by immunodepleting these cells in vivo with Abs. As shown in Fig. 5, immunodepletion of NK cells with anti-asialo-GM1 and of $CD4^+$ cells with anti-CD4 during the C3L5-CKβ11 vaccination period, resulted in reduction of CKβ-11 antitumor activity ($p < 0.05$) (0% TFA, vol = 1.3 ± 0.25 cm^3; 40% TFA, vol = 1.3 ± 0.25 cm^3, respectively) compared with vaccinated animals treated with control serum (100% TFA) and of $CD8^+$ cells immunodepleted with anti-CD8 (100% TFA).

To confirm the immunodepletion of each cellular subset by in vivo Ab treatments, splenocytes were isolated 1 or 4 days after the final Ab treatment and stained for $CD3^+$, $CD4^+$, $CD8^+$, and $DX5^+$ cells (Table II). The $CD3^+$ T cells are divided into $CD4^+$ and $CD8^+$ fractions. NK cells are divided into $CD3^-$ NK cells and the recently described $CD3^+$ NKT subset, which also coexpress $CD4^+$ (42). As shown in Table II, normal spleen cells are 19% $DX5^+$ cells and 43–45% $CD3^+$ T cells. This $CD3^+$ population contains 31 ± 3.7% $CD4^+$ and 15 ± 2.4% $CD8^+$ T cells.

After immunodepletion of the NK cells with anti-asialo-GM1, the percentage of pan $CD3^-$ NK splenocytes (3.6%) was significantly reduced (72%; $p < 0.05$) compared with control animals receiving no treatment (PBS) or rabbit serum (13 ± 3.7%). After immunodepletion of the $CD8^+$ cells with anti-CD8, the percentage of $CD3^+$ $CD8^+$ splenocytes (1.4%) was significantly reduced (90%; $p < 0.05$) compared with the normal controls (15 ± 2.4%). Additionally, the percentage of $CD3^+CD4^-$ splenocytes (3.6%) was also significantly reduced (71%; $p < 0.05$) compared with the normal controls (12 ± 1.5%), which independently confirms the reduction of $CD8^+$ T cells. After immunodepletion of the $CD4^+$ cells with anti-CD4, the percentage of $CD3^+CD4^+$ splenocytes (3.2%) was significantly reduced (90%; $p < 0.05$) compared with the normal controls (31 ± 3.7%). Additionally, the $CD3^+CD8^-$ splenocytes (5.8%) were significantly decreased (81%; $p < 0.05$), and the $CD3^+CD4^-$ splenocytes (21%) were significantly increased (69%; $p < 0.05$) compared with normal controls (30 ± 3.1%; 12 ± 1.5%, respectively), again independently confirming the immunodepletion of $CD4^+$ cells and the compensatory increase in $CD3^+CD8^+$ splenocytes. Interestingly, because the NKT cells are $CD3^+CD4^+$ NK cells, during immunodepletion with anti-CD4, the percentage of $DX5^+$ $CD3^+$ splenocytes (2.5%) was also significantly reduced (58%; $p < 0.05$) compared with normal controls (6 ± 1.3%). These results indicate that the target subpopulations were substantially reduced by the Ab treatments.

Transfer of splenocytes from vaccinated and immunodepleted animals

To determine the role of the various subpopulations in the generation of acquired immunity, splenocytes from animals immunodepleted of lymphocyte subsets during C3L5-CKβ11 vaccination were transferred to naive animals during challenge with parental C3L5 cells. Transfer of splenocytes from C3L5-CKβ11-vaccinated animals significantly reduced ($p < 0.05$) the tumor volume of the parental tumor challenge compared with PBS-treated or naive splenocyte-treated animals (Table III). Treatment with anti-asialo-GM1 and anti-CD8 did not inhibit the acquired immunity

Table II. *Analysis of splenic CD3, CD4, CD8, and NK subsets after immunodepletion with specific Abs*[a]

	$CD3^+$ (%)		$CD3^+$ (%)		NK Cells (%)	
	$CD4^+$	$CD4^-$	$CD8^+$	$CD8^-$	$CD3^-$	$CD3^+$
Normal	31 ± 3.7	12 ± 1.5	15 ± 2.4	30 ± 3.1	13 ± 3.7	6.0 ± 1.3
Anti-CD4	3.2 (−90)	21 (+69)	—[b]	5.8 (−81)	—	2.5 (−58)
Anti-CD8	—	3.6 (−71)	1.4 (−90)	—	—	—
Anti-G_{M1}	—	—	—	—	3.6 (−72)	—

[a] Results presented are the percent of the total splenocytes that represent these phenotypes and the change in these populations as the result of the treatments.
[b] Not significantly different ($p > 0.05$) from normal untreated or rabbit serum-treated animals.

Table III. *Protection of animals with splenocytes transferred from C3L5-CKβ11-vaccinated animals*[a]

Splenocytes from Animals Vaccinated with	Immunodepletion (in vivo)	Size of the Tumor (cm^3)
No splenocytes		1.08 ± 0.15
Unvaccinated		1.07 ± 0.27
C3L5-CKβ11	Control serum	0.55 ± 0.33[b]
C3L5-CKβ11	Anti-CD4	1.09 ± 0.24
C3L5-CKβ11	Anti-CD8	0.59 ± 0.35[b]
C3L5-CKβ11	Anti-asialo GM1 (NK cells)	0.78 ± 0.28[b,c]

[a] Animals were immunodepleted of CD4, CD8, and NK cells during vaccination period.
[b] Significantly different ($p < 0.05$) from the unvaccinated group.
[c] Significantly different ($p < 0.05$) from control serum treatment.

generated during the C3L5-CKβ11 vaccination, as the adoptive transfer of splenocytes from these animals significantly reduced ($p < 0.05$) tumor volume compared with control animals (Table III). In contrast, adoptive transfer of splenocytes from the anti-CD4-depleted animals lost this acquired immunity, as tumor volume was not significantly different ($p > 0.05$) than controls (Table III). Although adoptive immunity was apparent with the transfer of splenocytes, no in vitro antitumor activity was detectable (data not shown). These results indicate that C3L5-CKβ11 vaccination generates some level of transferable immunity and that this response is not generated through NK^+ or $CD8^+$ cells, but through $CD4^+$ cells.

Discussion

Chemokines were originally identified and described by their involvement in the inflammatory response and their capacity to chemoattract leukocytes. However, it has recently been observed that some chemokines influence the trafficking of lymphocytes to primary (thymus), secondary (lymph nodes), and tertiary (periphery) lymphoid tissues (34). CKβ-11, a ligand for CCR7 (35), is expressed at high level in the thymus and in the lymph nodes, constitutively in DC within the T cell zone of the lymph node, and is also known to strongly chemoattract $CD8^+$ T cells, naive $CD4^+$ T cells, mature DC, and NK cells. CCR7 was found recently to coordinate the primary immune response in receptor knockout mice, as naive T cells and activated skin DC are unable to migrate into the lymph nodes (43). We hypothesized that expression of CKβ-11 in tumor cells would facilitate the formation of antitumor immunity, an effect possibly mediated by NK cells and lymphocytes.

These results indicate, for the first time, that expression of CKβ-11 in a tumor model inhibited formation of the transduced tumor (even though in vitro growth of the transduced tumor cell was not effected) and that CKβ-11-transduced tumor vaccine generated antitumor immunity in vivo. These results also suggest that NK cells and $CD4^+$ cells are involved in the antitumor activity elicited by CKβ-11 and that $CD4^+$ cells are necessary for establishing antitumor immunity.

Many immunoregulatory molecules have been shown to mediate antitumor activity in a variety of tumor models. The murine breast cancer cell line C3L5 used in these studies is poorly or nonimmunogenic, as 10^6 irradiated C3L5 cells offer no protection against future tumor challenges (data not shown). We have previously shown that both Flt3L- and GM-CSF-transduced C3L5 cells generate antitumor immunity (6, 7). Flt3L and GM-CSF are both known to stimulate proliferation and differentiation of DC (44–46), although the immunity generated with Flt3L-transduced tumor vaccine was stronger than GM-CSF- and CKβ-11-transduced tumor vaccines ((7) and these data). Flt3L is also known to stimulate proliferation of NK cells in vivo (47, 48), and we showed that NK cells are involved in mediating antitumor activity of Flt3L-transduced tumor cells (7). CKβ-11 does not activate NK cells (data not shown), but does chemoattract both DC and NK cells (29, 32).

Several groups have shown antitumor activity with a variety of chemokines. With nine known CC chemokine receptors and five known CXC chemokine receptors, expression of receptors in the lymphoid cells varies widely. Additionally, the specificity of chemokine receptors is promiscuous, allowing for redundant activity of several chemokines on leukocytes that express the receptor. Therefore, the type of infiltrating leukocytes is determined by the expression profile of chemokines (34).

Studies have shown antitumor activity for MIP-1α, RANTES, lymphotactin, and TCA3 (16–19). MIP-1α expression in adenocarcinoma cells, but not IL-8 expression, led to reduced tumor formation and increased infiltration of macrophages and neutrophils (18). After subsequent challenges with MIP-1α-expressing and parental tumor cells, protective immunity was also observed (18). With TCA3 expression, tumor formation was inhibited and tumor-specific immunity was generated; however, histological analysis revealed mostly neutrophils around the TCA3-expressing tumor (17). RANTES also inhibited tumor formation and generated tumor immunity (16). Additionally, immunodepleting $CD8^+$ T cells, blocking macrophage migration with Abs to adhesion receptors, and, to a lesser extent, immunodepleting $CD4^+$ T cells all restored RANTES-transduced tumor formation (16). Lymphotactin, by itself, reduced tumor growth; however, in combination with IL-2, it enhanced antitumor immunity and inhibited an established tumor (19). These interactions were mediated through $CD4^+$ and $CD8^+$ T cells (19).

JE/MCP-1/MCAF (MCP-1) was shown to reduce in vivo growth of tumor cells and increase infiltration of macrophages/monocytes to the tumor site (20–23). Localization of fibroblasts engineered to express MCP-1 recruits migration of macrophages to the lungs and suppresses lung metastasis of renal adenocarcinoma (21). This may occur because MCP-1 and LPS are synergistic in activating cytotoxicity of macrophages against tumor cells (49). Additionally, the combined expression of MCP-1 and the addition of Abs against P-glycoprotein inhibited tumor formation of multidrug-resistant lung cancer cells in vivo (22).

In the normal host immune response, DC acquire Ag and present them to $CD4^+$ and $CD8^+$ T cells through MHC class I and II molecules. NK cells will kill a target cell unless a signal from the target cell MHC class I is received through the NK cell inhibitory receptor Ly-49A (50). Expression of cytokines or chemokines in the tumor cells stimulates the normal immune response to mediate the antitumor response. Host immune cells may infiltrate the tumor site, proliferate, differentiate, secrete other secondary cytokines or chemokines, recruit other effector cells, or become activated, eventually targeting tumor cells for destruction.

Because DC are chemoattracted by CKβ-11, these cells may be involved in presenting tumor-specific Ag to the $CD4^+$ cells that are mediating the antitumor immunity. Studies have demonstrated that Ag-activated $CD4^+$ NKT cells express CD40 ligand (CD154) and engage the CD40 receptor on APC, which then produce IL-12 (51) for the activation of NKT cells (52). The transduced tumor cells are probably not presenting the tumor-specific Ag to the NKT cells, as we found that C3L5-CKβ11 cells had equivalent levels of CD1δ expression as C3L5-G1N cells (data not shown). As noted previously, IFN-γ expression in C3L5, although it raises the level of MHC class I and II expression, does not mediate antitumor responses as strong as that of Flt3L expression (7). Although Flt3L

is known to stimulate DC and NK cells, we found a predominate role for NK cells in antitumor activity in this tumor model.

It has been suggested that effector cells in innate immunity play a pivotal role in shaping initial T cell activation (51), possibly by conditioning DCs for subsequent immune responses (52). NKT cells are $CD3^+$ T cells that also express NK cell markers and usually the invariant Vα14-Jα281 chain of the TCR (53) to interact with CD1δ (MHC-like) molecules on APC (54). NKT cells are important in IL-12-mediated antitumor activities, as mice with a deletion of CD1 gene or the Jα281 gene segment, and the subsequent loss of NKT cells could no longer mediate the IL-12-induced rejection of tumors (13). These studies have not separated the effects of the NK^+ $CD3^-$ cells or NK^+ $CD3^+$ NKT cells from the $CD4^+CD3^+$ T cells in generating antitumor activity or priming immunity against the transduced tumor cells.

Our results with CKβ-11 suggest two levels of responsiveness: antitumor activity against the transduced cells mediated mostly by NK^+ cells, and antitumor immunity mediated through $CD4^+$ cells. More recently, several groups showed that MCP-1, MIP-1α, and RANTES chemoattract immature DC migration (32, 55), and that MCP-1 also activate CTL and NK cytolytic responses (56). Our results with CKβ-11 and Flt3L support the notion that NK cells activate adaptive immunity and may suggest a common mechanism for antitumor activity with other stimulatory molecules. Whether these same interactions are responsible for the increased antitumor response we observed with Flt3L compared with GM-CSF or CKβ-11 needs further studies.

Because chemokines have important functions in host defenses, they may be used to modulate other activities, such as antitumor immunotherapy. Local expression of chemokines by transduced tumor cells may augment other therapeutic modalities, particularly those that stimulate DC, NK cells, or T cells, such as GM-CSF, Flt3L, IL-2, IFN-γ, or IL-12. Future studies are necessary to support its role in clinical immunotherapeutic trials.

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