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Gene Therapy to Manipulate Effector T Cell Trafficking to Tumors for Immunotherapy

Michael Gough,²*† Marka Crittenden,*† Uma Thanarajasingam,† Luis Sanchez-Perez,*† Jill Thompson,* Dragan Jevremovic,²* ‡ and Richard Vile*†

Strategies that generate tumor Ag-specific effector cells do not necessarily cure established tumors. We hypothesized that the relative efficiency with which tumor-specific effector cells reach the tumor is critical for therapy. We demonstrate in this study that activated T cells respond to the chemokine CCL3, both in vitro and in vivo, and we further demonstrate that expression of CCL3 within tumors increases the effector T cell infiltration in those tumors. Importantly, we show that adenoviral gene transfer to cause expression of CCL3 within B16ova tumors in vivo increases the efficacy of adoptive transfer of tumor-specific effector OT1 T cells. We additionally demonstrate that such therapies result in endogenous immune responses to tumor Ags that are capable of protecting animals against subsequent tumor challenge. Strategies that modify the “visibility” of tumors have the potential to significantly enhance the efficacy of both vaccine and adoptive transfer therapies currently in development. The Journal of Immunology, 2005, 174: 5766–5773.

Tumors can progressively grow in individuals despite the presence of tumor-specific effector cells. A useful concept to explain these observations is that there is an “ignorance” of the developing antigenic tumor. Various models have demonstrated that efficient priming of effector cells does not necessarily mean that Ag-specific T cells will eliminate tumors (1–3). For example, in a model where mice were transgenic for a TCR that recognizes self-MHC presenting an Ag transgenically expressed in normal liver cells, Ag-specific vaccination failed to cause liver pathology (1). In this model, administration of a liver-specific pathogen was required to cause T cell-mediated liver destruction (1). Similar results have been seen in many models in which large numbers of transgenic or in vitro-derived activated CTLs do not consistently cause regression of tumors (2, 3). The ability of T cells to mount an effector response to peripheral Ag is distinct from their specificity for that Ag. Activated effector T cells, rather than naïve cells, are capable of trafficking to peripheral inflammatory sites (4–6). This ability to traffic or home to sites experiencing inflammation is not related to Ag specificity, since effector cells simultaneously generated to Ags not present in an ongoing infection are equally present at an infection site (7). Both naïve and activated effectors have demonstrated that they are unable to traffic to tumor sites expressing their specific Ag; however, delivery of proinflammatory factors that change the tumor microenvironment allows infiltration of activated T cells and tumor destruction (8). In human trials, low numbers of adoptively transferred cells accumulate in tumors despite Ag expression (9–11), strengthening the distinction between Ag specificity and trafficking. However, the overall trafficking of effector cells that do reach a specific site will subsequently be influenced by the presence or absence of specific Ag through retention of Ag-specific cells and release of additional chemoattractants by Ag-specific cells (12).

We hypothesize that increasing the trafficking of effector T cells to tumors will significantly enhance immunotherapy approaches that adoptively transfer or endogenously generate tumor-specific effector T cells. In this study, we demonstrate that T cells express the chemokine receptor CCR5 on activation and that these cells are functionally responsive to its cognate ligand CCL3 both in vitro and in vivo. Expression of CCL3 in tumors enhances trafficking of effector T cells to tumor sites, resulting in enhanced tumor destruction. Finally, we combine intratumoral gene delivery of CCL3 with adoptive transfer of activated effector T cells and demonstrate that enhancing the “visibility” of tumor sites to activated T cells significantly enhances adoptive immunotherapy of tumors.

Materials and Methods

Cell lines, reagents, and mice

The B16ova cell line was kindly provided by Dr. E. Celis (Mayo Clinic, Rochester, MN). The B16pCR3.1 and B16CCL3 cell lines have been previously described (14). Briefly, B16 cells were transfected with pCR3.1 empty vector or pCR3.1 incorporating murine CCL3 and clones selected based on resistance to 5 mg/ml neomycin. Individual clones were screened for secretion of CCL3 by specific ELISA, and a clone secreting high levels of CCL3 was designated B16CCL3. This clone and a CCL3-negative empty vector-transfected clone designated B16pCR3.1 showed identical in vitro growth rates to parental B16 cells. The directly conjugated Abs CCR5-PE, CD8-PE, CD69 FITC, and CD62 ligand (CD62L)3-allophycocyanin were purchased from BD Biosciences. SIINFEKL peptide was synthesized by the Mayo Clinic Protein Core Facility and oligonucleotide primers were synthesized by the Mayo Clinic Oligonucleotide Core Facility. Six-week-old C57BL/6 mice were age and sex matched for individual

* Abbreviations used in this paper: Indo-1-AM, indomethacin 1 acetyl ester; CD62L, CD62 ligand; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.
experiments. OT1 mice, transgenic for a TCR that recognizes the SIINFEKL peptide of OVA presented on H-2Kb, have been previously described (15).

Preparation of primary murine cells

For preparation of activated OT1 CTLs, spleen and lymph nodes from OT1-transgenic mice were combined and crushed through a 100-μm filter to prepare a single-cell suspension. RBC were removed by a 2-min incubation in ACK buffer (sterile dH2O containing 0.15 M NH4Cl, 1.0 mM KHCO3, and 0.1 mM EDTA adjusted to pH 7.2–7.4). Remaining cells were adjusted to 2.5 × 106 cells/ml in IMDM plus 5% FCS, 10−3 M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin and stimulated with 1 μg/ml SIINFEKL peptide and 50 IU/ml human IL-2. Every 2–3 days one-half of the medium was removed and replaced with fresh medium containing 50 IU/ml IL-2. For use in vivo, nonadherent and loosely adherent cells were harvested following one activation cycle of 3–5 days and viable cells were purified by density gradient centrifugation using Lympholyte-M (Cedarlane Laboratories) according to the manufacturer’s instructions.

Cell labeling and analysis

For analysis of phenotype, 1 × 106 cells were washed in PBS containing 0.05% BSA (wash buffer), resuspended in 50 μl of wash buffer, and exposed to directly conjugated primary Abs for 30 min at 4°C. Cells were then washed and resuspended in 500 μl of PBS containing 4% formaldehyde. Cells were analyzed by flow cytometry and data were analyzed using CellQuest software (BD Biosciences). For in vivo tracking, cells were labeled with the viable cell dye chloromethyl benzamidoxo(1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) according to the manufacturer’s instructions. Briefly, viable cells were resuspended in PBS at 1 × 106 cells/ml and incubated for 30 min with 2.5 μM DiI. Cells were washed, resuspended in IMDM plus 5% FCS, 10−3 M 2-ME.100 U/ml penicillin, and 100 μg/ml streptomycin and incubated for an additional 15 min on ice. Finally, cells were washed another three times in PBS before in vivo injection of 2 × 105 cells. For analysis of labeled cell infiltrate, tumors were harvested and fixed in 10% Formalin in PBS, then paraffin embedded and sectioned. Unstained sections were visualized for accumulation of DiI-labeled cells. H&E-stained sections were prepared for analysis of tissue destruction and gross infiltrate. A pathologist examining H&E sections, while blinded to the experiment design, scored the degree of necrosis.

In vitro response of CD8 T cells to chemokines

For functional responses to chemokines, viable cells were washed and resuspended at 1 × 106 cells/ml in HBSS plus 10 mM HEPES. Cells were mixed with an equal volume of HBSS plus 10 mM HEPES containing 20 μM indomethacin 1 acetyl ester (Indo-1AM; Molecular Probes) and incubated for 30 min at 37°C. Cells were washed twice in HBSS plus 10 mM HEPES plus 0.05% BSA (wash buffer) and resuspended at 5 × 106 cells/ml in HBSS buffer for analysis. Where appropriate, following Indo-1AM labeling, cells were cotained with directly conjugated primary Abs for 15 min on ice. Cells were then washed one time in wash buffer and stored on ice until immediately before analysis. For analysis, samples were individually warmed to 37°C and analyzed on a FACStar plus flow cytometer (BD Biosciences). Baseline readings were performed for 1 min, then agonist was added and the ratio of FL4 (390 nm):FL5 (500 nm) Indo-1AM labeling, cells were costained with directly conjugated primary Abs for 30 min at 4°C. Cells were then collected after 48 h and tested by specific ELISA for IFN-γ; BD OptEIA (BD Biosciences).

Results

Expression of CCR5 by activated T cells

We have observed that activated OT1 T cells begin to lose the ability to cure B16ova tumors in C57BL/6 mice if tumors are allowed to establish for >7 days before adoptive transfer (data not shown). Increasing the number of T cells transferred into tumor-bearing mice has a limited ability to improve the therapy of established tumors (data not shown). We hypothesized that this inability to eradicate the tumor was attributable to lack of visibility of an established tumor to activated T cells. To test this hypothesis, we examined chemokine receptors on activated T cells. In vitro activation of OT1 splenocyte and lymph node cells with SIINFEKL peptide plus IL-2 leads to an expansion of CD8 T cells,
and an emergence of a CCR5⁺ T cell population (Fig. 1a). Gating on CD8⁺ cells in the spleen and lymph node from naïve OT1 mice confirmed that naïve CD8 T cells have central trafficking properties, with high expression of CD62L and no expression of the early activation marker CD69 (Fig. 1b). There are few CCR5⁺ cells in the naïve lymph node and spleen, and these cells likely represent non-T cell populations. Following activation, CD8⁺ cells up-regulate CD69 and there are both centrally trafficking (CD62L⁺) and peripheral trafficking (CD62L⁻) populations (Fig. 1b). Not all of the CD8⁺ OT1 cells express CCR5 at this stage of culture, with ~5% of CD8 T cells CCR5⁺, and this proportion decreases with continued in vitro culture. We hypothesize that CCR5 expression on CD8 T cells relates to cytokine exposure on activation as has been described for CD4 T cells (18, 19), and that it would be possible to design in vitro activation protocols that generate a much higher proportion of CCR5⁺ CD8 T cells for improved trafficking to inflammatory sites. Gating on the CCR5⁺ cells in this population indicates that they are activated T cells, since they uniformly express CD3, the activation marker CD69, and these cells are exclusively CD62Llow (Fig. 1b). These data suggest that the CCR5⁺ population that appears on activation represent T cells that are capable of peripheral trafficking to sites of inflammation.

**CD8 T cells respond to inflammatory chemokines following activation**

To confirm that the expression of CCR5 on CD8 T cells translated into a functional effect, the ability of CD8 T cells to respond to recombinant chemokine was tested. Activated T cells were labeled with Indo-1, which has distinct emission spectra depending on whether it is calcium bound or unbound, permitting real-time flow cytometric measurements of calcium flux in live cells, an indicator of chemokine receptor signaling. Activated OT1 cells were loaded with Indo1 and counterstained with directly conjugated Abs to confirm the identity of CCL3-responsive cells. During flow cytometry, cells were treated with recombinant chemokines and calcium flux in stained cell populations was determined. Figure 2

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**FIGURE 2.** Functional expression of CCR5 by activated T cells. **a.** In vitro-activated OT1 splenocytes and lymph node cells were labeled with Indo-1, then costained with PE-conjugated CD8 Ab. Calcium flux was measured by flow cytometry, with cells untreated for 60 s to confirm background fluorescence, then 25 ng/ml recombinant chemokine was added and calcium flux over 4 min observed. Plots show the mean ratio of FL4 (calcium bound):FL5 (unbound) Indo-1 fluorescence over time gated on the CD8 (PE) live cell population. Lines represent cells treated with CCL3 (red), CCL21 (green), or CX3CL1 (blue). **b.** In vitro chemotaxis of activated OT1 cells. Activated OT1 T cells were seeded onto a chemotaxis chamber above supernatants of B16 cells stably transfected with CCL3 or transiently infected with adenoviral vectors expressing CCL3 or control genes. Chemotaxis of OT1 cells to the lower chamber was quantitated by MTT assay and compared with a standard curve of MTT of known numbers of OT1 cells to calculate the number of cells responding to chemokine. Data shown represent mean and SE for each sample.
demonstrates that the CD8 population of activated OT1 cells functionally responds to rCCL3. These cells do not respond to the control chemokine CCL21, the receptor for which is primarily expressed on naïve T cells or the unrelated chemokine CX3CL1. Thus, activated T cells both express CCR5 and respond to its cognate ligand CCL3.

To confirm expression of functional CCR5 in ligand-directed chemotaxis, OT1 cells were tested in a chemotaxis assay against rCCL3 or supernatants of B16 melanoma cells engineered to express CCL3. Figure 2b demonstrates that compared with supernatants from unmodified B16 cells, activated OT1 cells show significant chemotaxis toward supernatants from B16 cells stably expressing CCL3 ($p < 0.05$), B16 cells infected with AdCCL3 ($p < 0.05$), or rCCL3 alone ($p < 0.01$), but not toward control supernatants from B16 infected with AdGFP ($p = 0.25$). These results demonstrate a specific chemotaxis of activated OT1 cells toward CCL3 that is not influenced by the adenoviral vector and supports the hypothesis that CCL3 is a suitable chemokine to render target sites more “visible” to activated OT1 cells.

**CCL3 attracts CCR5-expressing cells in vivo**

To confirm that CCL3 functions to attract CCR5+ cells in vivo, we injected s.c. sites with three daily doses of CCL3 or PBS and characterized expression of chemokine receptors in the injection site 24 h after the last injection. RT-PCR of RNA prepared from the injection site demonstrated the presence of CCR5 only in sites injected with CCL3 (Fig. 3). Expression of CCR1, CCR6 and CCR7 were not seen in PBS or CCL3 injection sites. The lack of CCR1 expression is interesting since CCL3 has been described to signal via both CCR1 and CCR5 (20). These data suggest that CCL3 displays an in vivo selectivity for cells expressing CCR5; however, we cannot exclude the possibility that expression of CCR1 is below detection in this system.

RT-PCR identifies changes in receptor RNA transcripts and is thus not affected by ligand-mediated receptor modulation that may occur in the injection site. However, to exclude the possibility that chemokine injection increased receptor expression on resident cells rather than caused influx of new cells, we tested the effect of chemokine treatment on activated splenocytes in vitro. High doses (25 ng/ml) of CCL3, CCL20, or CCL21 did not alter surface expression of CCR5 on activated splenocytes (data not shown), supporting the hypothesis that injection of recombinant chemokine causes influx of new CCR5-expressing cells to the injection site.

To confirm that CCL3 injection can cause chemotaxis in vivo, we used a Gelfoam sponge model to measure cell influx to a defined environment. Sponges were established s.c. in mice and injected with rCCL3 or PBS vehicle. Sponges were harvested over a time course, and dissociated and infiltrating cells were counted. Figure 3b shows that the addition of rCCL3 caused a transient influx of cells into the sponge. This influx is over and above the continuing background population of the sponge environment that is seen with the PBS control. These data demonstrate that rCCL3 injection causes an increase in CCR5-expressing cells in the injection site and a transient in vivo chemotaxis.

**Activated effector cells are attracted to sites of Ag and chemokine in vivo**

To test our hypothesis that tumor control by specific effector cells is limited by the restricted access of effector cells to the tumor site, we studied in vivo tracking of effector T cells to the tumor site. In vitro-activated OT1 T cells were labeled with DiI and adoptively transferred into mice bearing tumors on opposite flanks. In mice bearing 10-day B16ova tumors on one flank and control B16pCR3.1 tumors on the opposite flank, more DiI-labeled cells were visible in the Ag-expressing tumor (Fig. 4, a and b). To study
the effect of chemokine expression, tumors were established where 90% of the cells were B16ova and 10% of the cells on one flank were B16CCL3 and on the other flank were B16pCR3.1. Similar numbers of DiI-labeled cells were visible in the B16ova/ B16pCR3.1 to those seen in 100% B16ova tumors (Fig. 4, a and d). However, expression of CCL3 in the tumor enhanced the infiltration of activated T cells (Fig. 4c). These data are summarized in Table I. That expression of CCL3 enhances the infiltration of adoptively transferred activated T cells supports the hypothesis that the infiltrate is limited in tumors that do not express CCL3.

Expression of CCL3 in tumors enhances antitumor efficacy of adoptively transferred effector T cells

Serial sections from tumors shown in Fig. 4 were H&E stained, and tissue destruction and cellular infiltrate were studied. B16pCR3.1 tumors were essentially undisturbed, with limited necrosis and limited infiltration (Fig. 5a). Tumors expressing OVA demonstrated infiltration and small areas of necrosis (Fig. 5, a and d). In contrast, tumors expressing OVA plus CCL3 demonstrated significantly larger infiltrates and larger areas of necrosis than tumors expressing OVA alone (Fig. 5, c and d). Pathology scores of necrosis are shown in Table II.

To test whether this enhanced tumor destruction had a significant impact on tumor growth, we established B16ova tumors in mice and allowed the tumors to grow to 0.3 cm in diameter. Tumors were then treated with three daily injections of recombinant adenoviral vector expressing CCL3 or control adenovirus expressing GFP. On the second day of adenovirus injection, one-half were treated with adoptive transfer of in vitro-activated OT1. AdCCL3 significantly delayed tumor growth (median survival 30 days) compared with control AdGFP injection (median survival 23 days, p < 0.001; Table III). Adoptive transfer of OT1 cells to AdGFP-treated mice significantly delayed tumor growth (median survival 39 days, p < 0.001); however, all mice eventually succumbed to tumor. Adoptive transfer of these numbers of effectors alone, though causing significant delays in tumor growth, consistently fails to cure mice of tumors once established for >1 wk. The combination of AdCCL3 with adoptive transfer of OT1 significantly delayed tumor growth (median survival 42 days) compared with AdCCL3 alone (p < 0.05; Table IV). Expression of CCL3 combined with adoptive transfer of OT1 did not significantly enhance survival of mice over AdGFP in combination with OT1 (p = 0.06), although 40% of mice survived with AdCCL3 plus OT1 compared with 0% with AdGFP plus OT1 (Table III).

To exclude the potential effect of adenoviral infection on the trafficking and cytotoxicity of adoptive T cell therapy, the effect of chemokine expression by tumors on adoptive T cell therapy was tested in a constitutive rather than gene therapy model. Mice were injected s.c. with B16 tumor cells consisting of 90% B16ova and 10% of either B16CCL3 or control B16pCR3.1 cells. On the same day, mice were treated i.v. with adoptive transfer of activated OT1 T cells and tumor development was monitored. Only a combination of adoptive therapy and chemokine expression led to a significant survival benefit (p < 0.05) in tumor-bearing animals (summarized in Table IV). Although adenoviral vector components could be playing a role in the gene therapy model described above, these data suggest that chemokine expression in the absence of adenoviral vector delivery can significantly increase the efficacy of adoptive T cell therapy.

Mice rejecting tumors following intratumoral chemokine expression demonstrate both adoptively transferred and endogenous T cell responses

We were surprised to observe that adoptive transfer of SIINFEKL-specific OT1 cells cleared tumors in four of five mice in which 10% of the tumor cells did not express the ova Ag (Table IV). These data suggest that an endogenous immune response may play some role in the tumor clearance initiated through adoptive transfer and chemokine expression. To confirm this result and identify

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**Table I. Score of DiI CD8 T cell infiltrate into tumors**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16pCR3.1</td>
<td>−</td>
</tr>
<tr>
<td>B16ova</td>
<td>+</td>
</tr>
<tr>
<td>B16ova/B16pCR3.1</td>
<td>+ +</td>
</tr>
<tr>
<td>B16ova/B16CCL3</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

*a Mice were injected with tumor cells on opposite flanks, and once established to 0.3 cm in diameter, mice were injected i.v. with 2 × 10⁶ in vitro-activated OT1 T cells that had been labeled with the viable cell dye DiI. Four days after injection, tumors were harvested, Formalin fixed, and paraffin sections taken for analysis of infiltrate of fluorescent cells. Multiple fields were assessed and scored on a scale from − (no infiltrating cells stain with DiI) to ++++++ (100% of infiltrating cells stain with DiI).

**Table II. Pathology scores of necrosis in tumors**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Area of Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16pCR3.1</td>
<td>+</td>
</tr>
<tr>
<td>B16ova</td>
<td>+ +</td>
</tr>
<tr>
<td>B16ova/B16pCR3.1</td>
<td>+ +</td>
</tr>
<tr>
<td>B16ova/B16CCL3</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

*a Mice were injected with tumor cells on opposite flanks, and once established to 0.3 cm in diameter, mice were injected i.v. with 2 × 10⁶ in vitro-activated OT1 T cells. Four days after injection, tumors were harvested, Formalin fixed, and paraffin sections taken and H&E stained. Stained sections were assessed for degree of necrosis by pathologists blinded to the experiment design. The degree of necrosis was scored on a scale from − (no necrosis) to ++++++ (total necrosis).

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**FIGURE 5.** Expression of CCL3 in tumors enhances tumor destruction. Mice were injected with tumor cells on opposite flanks, and once established to 0.3 cm in diameter, mice were injected i.v. with 2 × 10⁶ in vitro-activated OT1 T cells. Four days following injection, tumors were harvested, Formalin fixed, and paraffin sections taken and H&E stained. a and b, Mouse injected with B16ova (a) on one flank and B16pCR3.1 (b) on the opposite flank. c and d, Mouse injected with 90% B16ova/10% B16CCL3 (c) on one flank and 90% B16ova/10% B16pCR3.1 (d) on the opposite flank. Images show representative areas in tumor sections. Original magnification, ×10.
whether these endogenous effectors could sustain a functional anti-tumor response, we rechallenged the survivor mice indicated in Table III on the opposite flank with parental B16 tumors. These tumors will present melanoma epitopes recognized by endogenous T cells, for example gp100 and Trp2, but not recognized by the adoptively transferred OT1 T cells that are restricted to the SIINFEKL epitope of OVA. The last two columns of Table III summarize these experiments, showing that mice rejecting 0.3-cm B16ova tumors, via intratumoral AdCCL3 alone or combined with adoptive transfer of OT1 cells, subsequently rejected parental B16 tumors. To monitor the specificity of the response to the secondary tumor challenge, splenocytes from mice rejecting both B16ova and subsequent B16 tumors were stimulated in vitro with a panel of peptides and IFN-γ ELISA was performed on cell supernatants. Figure 6 demonstrates that these mice retain a specific response to SIINFEKL, with IFN-γ secretion significantly greater than in un-stimulated cells ($p < 0.05$) or in the presence of the irrelevant 2C peptide ($p < 0.05$). Importantly, in one-half of the survivor mice there is a significant IFN-γ response to Trp2 peptide compared with untreated or 2C peptide-treated cells ($p < 0.0001$); however, when considered as a group there is no significant difference between Trp2-treated and untreated or 2C-treated cells. Similarly, one-half of the mice secreted significantly more IFN-γ in response to gp100 peptide than untreated cells or to irrelevant 2C peptide, although there is no significant difference when analyzed as a group. As expected, naive age-matched control mice did not secrete IFN-γ in response to SIINFEKL or melanoma differentiation Ags. Thus, these intratumoral therapies both enhance adoptive immunotherapy and stimulate functional endogenous tumor-specific immune responses that in some cases can be identified as targeting known melanoma-specific differentiation Ags.

**Discussion**

We demonstrate that a subpopulation of T cells expresses the inflammatory chemokine receptor CCR5 on activation and that these T cells represent a peripheral trafficking effector population. These chemokine receptors are functional, and injection of the cognate ligand CCL3 causes attraction of receptor-expressing cells to the injection site. Expression of CCL3 in tumors in vivo enhances the trafficking of adoptively transferred effector T cells to the tumor site and results in enhanced tumor destruction. We further demonstrate that these data can be translated into therapy via adenovirus-mediated gene transfer of CCL3 into tumors, which enhances the survival of tumor-bearing mice following adoptive transfer. Importantly, the intratumoral immunotherapy also resulted in endogenous tumor-specific T cell responses that were capable of rejecting subsequent tumor challenge.

Ag-specific vaccination strategies generally result in accumulation of specific cells at the vaccination site (12, 21, 22). Local

### Table III. Survival following gene therapy and adoptive transfer to mice bearing established tumors and rechallenge of survivors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Adenovirus Vector</th>
<th>Adoptive Transfer</th>
<th>Median Survival (days)</th>
<th>Survivors</th>
<th>Rechallenge</th>
<th>Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16ova</td>
<td>AdGFP</td>
<td>—</td>
<td>23</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16ova</td>
<td>AdGFP</td>
<td>OT1</td>
<td>39</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16ova</td>
<td>AdCCL3</td>
<td>—</td>
<td>30</td>
<td>0/10</td>
<td>B16</td>
<td>1/1</td>
</tr>
<tr>
<td>B16ova</td>
<td>AdCCL3</td>
<td>OT1</td>
<td>42</td>
<td>4/10</td>
<td>B16</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Mice were injected s.c. with B16ova cells, and once established to 0.3 cm in diameter, three daily intratumoral injections of $1 \times 10^8$ PFU of AdGFP or AdCCL3 were performed. On the second day, mice were injected i.v. with $2 \times 10^8$ in vitro activated OT1 cells. Tumor growth was monitored and mice were sacrificed if tumors exceeded 1.2 cm in diameter. Mice surviving for $>80$ days were rechallenged on the opposite flank with $2 \times 10^6$ parental B16 cells. Tumor growth was monitored and mice were sacrificed if tumors exceeded 1.2 cm in diameter.

### Table IV. Survival following adoptive transfer of T cells to mixed tumors expressing Ag and CCL3 or control tumors with Ag alone

<table>
<thead>
<tr>
<th>90% Tumor</th>
<th>10% Tumor</th>
<th>Adoptive Transfer</th>
<th>Median Survival (days)</th>
<th>Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16ova</td>
<td>B16pCR3.1</td>
<td>—</td>
<td>21</td>
<td>0/5</td>
</tr>
<tr>
<td>B16ova</td>
<td>B16pCR3.1</td>
<td>OT1</td>
<td>18</td>
<td>1/5</td>
</tr>
<tr>
<td>B16ova</td>
<td>B16CCL3</td>
<td>—</td>
<td>49</td>
<td>1/5</td>
</tr>
<tr>
<td>B16ova</td>
<td>B16CCL3</td>
<td>OT1</td>
<td>&gt;60</td>
<td>4/5</td>
</tr>
</tbody>
</table>

* Mice were injected s.c. with $2 \times 10^6$ B16 cells, consisting of 90% B16ova cells and $2 \times 10^5$ of either B16CCL3 or control B16pCR3.1 cells. On the same day, mice were injected i.v. with $2 \times 10^6$ in vitro-activated OT1 cells. Tumor growth was monitored and mice were sacrificed if tumors exceeded 1.2 cm in diameter.

![FIGURE 6. IFN-γ secretion by draining lymph node cells following peptide stimulation. Splenocytes were harvested from survivor mice (a) following AdCCL3 plus OT1 therapy of B16ova tumors initiated at 0.3 cm in diameter, which subsequently rejected B16 tumors, or naive age-matched control mice (b). Splenocytes were stimulated for 48 h with 5 μM peptide matching the H2-Kb-binding epitopes for OVA (SIINFEKL), 2C (SIVRYYGL), gp100 (EGSRNQDWL), or Trp2 (SVYDFFVWL), and 48-h supernatants were ELISA tested in triplicate for the presence of IFN-γ.](http://www.jimmunol.org/Downloadedfrom/5771)
inflammation is a critical component of vaccination, such that vacci-
ination in the absence of inflammation induces like adjuvant,
limit Ag-specific immune responses (23). However, the presence of
adjuvant also influences the ability of effector cells to reach the
Ag site (12). Inflammation in peripheral tissue sites has multiple
roles in initiating local immune responses, including maturation of
APCs (24–27), influencing the cytokine milieu in draining lymphoid organs (28–30), and activation of local endothelia (31–33).

Chemokines play a critical role in the distribution of effector
cells following Ag-specific priming. A range of chemokines in-
duced during inflammation enhances lymphocyte binding to en-
dothelia (31), and experiments applying pertussis toxin to block
chemokine-receptor signal transduction inhibits this tight adhesion
(34, 35), correlating with deficient in vivo trafficking and function
(36). Thus, chemokines are a valid therapeutic target to manipulate
the distribution of selected cell populations in vivo. The data pre-
mitted in this article studying CCR5 and CCL3 are consistent with
similar experiments performed with the chemokines CXCL10 and
XCL1, which also enhance effector T cell trafficking to tumors and
synergistically enhance adoptive T cell therapy (37, 38). Together
these data emphasize that immunotherapies that can generate large
numbers of effector T cells in vivo and that simultaneously recruit
these effector T cells to the tumor site will significantly enhance
immune control of tumors.

CCL3 represents an attractive molecule for intratumoral expres-
sion, since it is commonly associated with IFN-γ expression and
acts in concert with the cytokine IFN-γ and other chemokines to
drive Th1 responses in vivo (39). CCR5 is expressed on the ef-
fector-effector/memory population (6, 40, 41) and this interaction
is known to be critical for control of T cell responses in a number
of models (4–6, 31). Interestingly, the presence of IL-12 during T
cell activation has been described to enhance the differentiation of
T cells into a phenotype expressing CCR5 (42). The expression of
IL-12 is closely tied to expression of IFN-γ (43) and CCR5 is
responsible for T cell migration to tumors in IL-12-treated
mice (44).

That CCL3 expression alone significantly extends survival, but
does not itself abrogate growth of s.c. B16 tumors is in agreement
with published studies (14, 45). It has previously been demon-
strated that constitutive expression of CCL3 in tumors causes in-
flux of CD8 T cells (14, 45); however, this influx was transient
(45). This could be a result of a changed cytokine environment
caused by the new cells, adaptation of the response to a stable
chemokine gradient, or reflect a stable balance between new cell
influx and existing cell exit or death. We demonstrate that the
chemotactic response to recombinant chemokine is, as expected,
transient and limits the usefulness of recombinant protein for tu-
mor modification. For these reasons we developed a gene therapy
model for treatment of established tumors, where adenoviral vec-
tors were used to deliver CCL3 or control genes into the tumor site.
In our hands, adenoviral vector delivery of CCL3 is more effective
in delaying tumor growth than constitutive expression of CCL3 in
selected B16 clones (data not shown). Similarly adoptive transfer
of OT1 T cells to an AdGFP-treated tumor enhanced survival
more than adoptive transfer to mice bearing untreated tumors (data
not shown). Replication-defective adenoviral vectors have intrinsic
immunogenicity (46, 47) and this may explain the enhanced im-
munogenicity of adenovirus-infected tumors. In this regard, tu-
mos excised from mice within 2–3 wk of adenoviral vector de-

delivery still contained small areas of GFP expression where treated
with AdGFP, and culture of tumor cells followed by specific
ELISA demonstrated continued expression of CCL3 in tumors

treated with AdCCL3 (data not shown). However, we also show that
adoptive transfer of OT1 T cells is also enhanced by consti-
tutive expression of chemokines in the absence of adenoviral de-

divery. Thus, although vector components may play a role in the
genere therapy model, chemokine expression significantly enhances
the efficacy of Ag-specific adoptive therapy.

In these experiments, animals developed endogenous T cell re-
sponses not encoded by the adoptively transferred T cells. The
mechanism by which this occurs requires further study. Although
the data suggest that epitope spreading has occurred, we have pre-
viously demonstrated that expression of CCL3 in tumors, when
combined with Ag release, can generate tumor-specific T cell re-
sponses that are capable of clearing a subsequent tumor challenge
(14). Alternatively the involvement of additional T cell epitopes
could be required for the success of the therapy (48), particularly
since in our hands Ag-directed therapies for B16 melanoma com-
monly result in the emergence of Ag escape variants (T. Kotke
and R. Vile, manuscript in preparation).

In summary, these data suggest that strategies that aim to gen-
erate immunotherapies to treat tumors must take into account the
accessibility of the tumor site to immune cells. Vaccination distant
from the site of tumor growth may efficiently activate Ag-specific
effector cells that may not adequately traffic to the tumor site.
Therefore, it may be necessary to incorporate strategies to render
effectors better able to traffic to tumors (49) or to modify the tumor site to increase its accessibility to effectors.

Disclosures
The authors have no financial conflict of interest.

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