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In Vivo Augmentation of Tumor-Specific CTL Responses by Class I/Peptide Antigen Complexes on Microspheres (Large Multivalent Immunogen)Ⅰ

Jodi Goldberg, Protul Shrikant, and Matthew F. Mescher

Tumor membrane Ag immobilized on cell size microspheres (large multivalent immunogen (LMI)) was previously shown to augment tumor-specific CTL activity and reduce tumor growth, and a clinical trial examining this approach is in progress. In the current study, LMI treatment has been examined using adoptive transfer of TCR-transgenic CD8 T cells to visualize Ag-specific cells during the response. OT-I T cells specific for H-2Kb/OVA257–264 were transferred into mice that were then challenged with LMI made by immobilizing H-2Kb/OVA257–264 on microspheres (Kb/OVA257–264-LMI) alone, or along with i.p. challenge with OVA-expressing E.G7 tumor. Kb/OVA257–264-LMI caused significant reduction of tumor growth when administered to E.G7-bearing mice. When administered alone, the Kb/OVA257–264-LMI caused only weak clonal expansion of OT-I cells in the spleen and lymph nodes, although most of the OT-I cells up-regulated expression of CD44 and VLA-4. In contrast, Kb/OVA257–264-LMI administration to E.G7-bearing mice stimulated no detectable expansion of OT-I cells in the spleen and lymph nodes but caused a rapid increase in the number of OT-I cells in the peritoneal cavity, the site of the growing tumor. These results demonstrate the potential for using class I/tumor peptide complexes for immunotherapy. In addition, they suggest a model for the mechanism of CTL augmentation in which recognition of the LMI Ag results in altered trafficking of the tumor-specific CD8 T cells so that they reach the site of a growing tumor more rapidly and in greater numbers, where they may further expand and acquire effector function. The Journal of Immunology, 2003, 170: 228–235.

Numerous experimental models have demonstrated that CD8+ CTL can play a significant role in protecting against challenge with tumor after immunization or reducing growth of established tumors. Aided greatly by advances in the ability to identify class I-restricted epitopes specific to proteins expressed by tumors (1–4) and the development of sensitive means of identifying CD8 T cells specific for peptide Ag using ELISPOT assays (5–7) and staining with tetrameric class I/peptide complex (8–10), it has also become clear that CD8 T cells can be found that are specific for Ags expressed by many human tumors. Thus, there is considerable potential for development of therapeutic vaccine approaches aimed at eliciting clinically effective CD8 mediated antitumor responses. Tumor Ags can be obtained in many forms including synthetic peptides, killed tumor cells or cell lysates, or subcellular fractions derived from the tumor. The challenge remains, however, of delivering these Ags in a manner that elicits effective CTL responses. Many means of accomplishing this are being explored in both animal models and in the clinic, including use of a wide variety of natural and synthetic adjuvants, transfection of tumor cells with surface proteins or cytokines to make them more effective activators of T cells, and loading of dendritic cells with Ag for immunization.

A novel approach for enhancing CTL responses for tumor immunotherapy was suggested by the observation that CD8 T cells are most effectively stimulated by subcellular Ag if it is presented on the surfaces of particles about the same size as cells (11). Plasma membranes or purified class I alloantigen were effective in stimulating in vitro generation of CTL responses or activating cloned CTL effector function when immobilized on 5-μm beads but ineffective if presented on smaller beads or in free form. In contrast, in vivo administration of Ag on cell-sized beads was not sufficient to stimulate detectable CTL responses. However, this form of Ag, termed large multivalent immunogen (LMI),5 was able to very substantially augment CTL responses when administered to mice that had been challenged with cells bearing the same Ag. This was initially demonstrated for responses to allogeneic tumors, using purified class I MHC protein to prepare the LMI (12). Augmentation required that the LMI have the same Ag on the surface as was present on the cells used for the challenge, and the resulting effector CTL retained specificity for the Ag.

The LMI approach was extended to syngeneic tumors using plasma membranes isolated from the tumor cell as the Ag. Administration of LMI at the same time that mice were inoculated with syngeneic tumor resulted in generation of detectable ex vivo tumor-specific cytolytic activity and a concomitant reduction in tumor growth (12, 13). When mice with established tumors were treated, it was found that administering LMI alone did not have a significant effect. However, low dose cyclophosphamide (Cy) and LMI were highly synergistic in reducing growth of established tumors, curing mice inoculated with tumor, and significantly increasing their survival.

Abbreviations used in this paper: LMI, large multivalent immunogen; LN, lymph node; Cy, cyclophosphamide.
tumors and extending survival in several tumor models, including P815 mastocytoma growing as a solid s.c. tumor and fibrosarcoma in a lung metastasis model (14). A substantial fraction of P815-bearing mice treated with Cy and LMI survived long term (>150 days) with no detectable tumor and were immune to rechallenge with the tumor. Based on these studies, a small clinical trial was performed using LMI alone (without Cy) to treat stage III and IV melanoma patients, using plasma membranes derived from two in vitro melanoma lines as the Ag to prepare the LMI. The LMI treatment had no significant adverse effects, and an increased frequency of tumor-specific precursor CTL was found in PBL of the majority of treated patients postvaccination (M. S. Mitchell, J. Kan-Mitchell, P. Morrow, D. Darrah, V. Jones, and M. Mescher, manuscript in preparation). A Phase III trial has now been initiated to examine safety and efficacy of LMI for melanoma and renal carcinoma immunotherapy using autologous tumor as the source of membrane Ag for preparing the LMI, and comparing treatment with LMI alone and together with Cy treatment (15).

The therapeutic effects of LMI in the murine models that were studied were tumor Ag specific and depended on presentation of the Ag on beads; free Ag or Ag with CFA were not effective (12, 14). Based on the effects of LMI on allogeneic responses, where the Ag used was purified class I MHC alloantigen, it appeared likely that the LMI effects seen in the syngeneic tumor therapy models involved tumor-specific Ag being presented by class I MHC proteins on the plasma membranes isolated from the tumors, but this was not directly demonstrated. Furthermore, the mechanism by which Ag presentation on cell-sized beads was able to augment in vivo responses was not defined. The experiments described in this report demonstrate that class I peptide Ag complexes immobilized on cell-sized beads to prepare LMI can mediate augmentation of tumor-specific CD8 T cell responses, results that have implications for the development of better defined LMI for human immunotherapy. Furthermore, the experiments have used adoptive transfer of TCR-transgenic T cells (16, 17), thus allowing identification, enumeration, and phenotypic characterization of the Ag-specific cells during the course of the responses to tumor and LMI. The results have begun to provide some insight into the mechanism by which LMI augment generation of effective tumor-specific CTL responses. An understanding of these mechanisms will be necessary for realizing the full potential of this novel approach for immunotherapy.

Materials and Methods

Animals and cell lines

OT-I (H-2b) mice (18), a gift from Dr. F. Carbone (University of Melbourne, Melbourne, Australia), express a transgenic TCR specific for an OVA-derived peptide (SIINFEKL) presented on H-2Kb (Thy-1.1 congenic and the resulting OT-1PL mice were used as the source of CD8+ OT-I T cells for in vitro experiments and for adoptive transfer into C57BL/6 (Thy-1.2) mice. EL4 murine thymoma derived from the C57BL/6 mouse (H-2b) was maintained in vitro in complete RPMI medium (RPMI 1640, 10% FCS, 0.2% L-glutamine, 0.1% penicillin-streptomycin, 0.1% HEPES, 0.1% non-essential amino acids, 0.01% sodium pyruvate, 0.05% 2-ME. The EL-4 derived E.G7 tumor expressing secreted whole OVA (19) was maintained in vitro in complete RPMI medium containing 400 μg/ml G418. Both EL-4 and E.G7 were washed extensively in PBS before in vivo challenge using 4 × 10⁶ cells/mouse injected i.p. on day 0.

Preparation of Kb/ova complexes and LMI

H-2Kb/ova complexes (SIINFEKL) peptide complexes were generated using a procedure developed by Garboczi et al. (20) but modified by D. Busch and E. Pamer (Yale University, New Haven, CT). Briefly, CD8α encoding a H-2Kb heavy chain-biotinylation site-fusion protein and human β2-microglobulin were expressed in Escherichia coli and purified from inclusion bodies. Denatured Kα and β2-microglobulin proteins were then refolded in the presence of high concentrations of OVA257–264 peptide to form the antigenic complex. Complexes were subsequently biotinylated by BirA ligase (Avidity, Denver, CO).

To prepare LMI, 5-μm sulfated polystyrene latex beads (Interfacial Dynamics, Portland, OR) were coated with streptavidin-APC (BD Pharmingen, San Diego, CA) by incubating with 1.25 μg/10⁶ beads in PBS for 20 min at 4°C. Beads were then washed, resuspended in PBS at 1 × 10⁷ beads/ml, and incubated with biotinylated Kb/ova for 1–2 h at 4°C. Beads coated with differing amounts of complex were prepared for the experiments shown in Fig. 1. All in vivo experiments were performed using Kb/ova-LMI prepared by incubating 25 ng complex per 10⁶ streptavidin-coated beads. After inoculation with the complex, an aliquot of beads was removed and stained with Y3-FITC (mouse anti-Kb) mAb to determine the level of Ag immobilization. The remaining beads were blocked with 1% normal mouse serum 30 min at 4°C, centrifuged, and resuspended at 2 × 10⁷ LMI/ml in PBS. LMI were sterilized by gamma-irradiation and injected i.p. or i.v. (tail vein) at 10⁶ beads/mouse.

In vitro proliferation assay

Lymph node (LN) cells from OT-1PL mice were isolated and adhered depleted for 1 h at 37°C. Remaining cells were enriched for CD8+ cells using Cellset columns (Cedarlane, Vancouver, Canada). CD8-enriched cells, containing >90% CD8+ cells, were plated in a flat-bottom 96-well plate (Falcon) at 1 × 10⁵ cells/well. LMI beads were added at 2 × 10⁶/well in a final volume of 0.2 ml. Where indicated, recombinant human IL-2 (TEG-329S-NIH) was added at 25 U/well. Cultures were incubated at 37°C for 48 h, 1 μCi [3H]thymidine per well was added for the final 6 h, and incorporation was determined.

Adoptive transfer and analysis by flow cytometry

OT-1/PL lymph node cells were adhered depleted, and the nonadherent cells were washed three times in PBS. CD8 T cells (1–3 × 10⁶/mouse) were adoptively transferred by i.v. injection (tail vein) into Thy-1-congenic C57BL/6 sex-matched recipients, and these mice were challenged 2 or 3 days later with tumor and/or LMI. The adoptively transferred cells equilibrate in the spleen and lymph nodes of the recipients within 1 day. The number of adoptively transferred cells present in the spleen and LN of the recipients varies somewhat between experiments but is very consistent between animals in the same experiment. In some experiments, OT-1 cells were labeled with fluorescent dye before transfer by incubating 10⁶ cells/ml in HBSS at 37°C for 5 min with 3 μM CFSE (Molecular Probes, Eugene, OR). Cells were then washed once in cold complete RPMI medium and three times in PBS before adoptive transfer.

Animals were sacrificed on the indicated days after challenge, and lymph node (LN) cells (pooled inguinal, axillary, brachial, iliac, popliteal, mesenteric, spleen cells, and peritoneal exudate cells were harvested and analyzed by flow cytometry. Peritoneal exudate cells were obtained from two consecutive washes of the peritoneal cavity (PC) using 20 ml HBSS. The number of total live lymphocytes collected from each compartment was determined by flow cytometry using PKH reference beads (Sigma-Aldrich, St. Louis, MO). Cells (2–3 × 10⁵) from transferred recipients were incubated in PBS containing 2% FCS and 0.02% NaN₃, FeC block, anti-CD8-APC mAb, and anti-Thy-1.1-PE mAb were added to each sample. In addition, anti-CD44-FITC or anti-VLA-4-FITC mAbs were added to some samples. FeC block and mAbs were all from BD Pharmingen. Samples were incubated for 1 h at 4°C, washed twice, and fixed in 0.4% paraformaldehyde in PBS for analysis by flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

OT-I cells were identified as CD8+ Thy-1.1+ cells, and this was confirmed by staining for the transgenic TCR α-chain Vα2. No background was present in the OT-I gate if the anti-Thy-1.1 mAb was not included or if cells from normal C57BL/6 mice were stained with the anti-CD8 and anti-Thy-1.1 mAbs. The total number of OT-I cells in a given compartment was determined by multiplying the percent of OT-I cells by the total number of cells recovered. For CFSE experiments, the cells were first gated on the CD8+ Thy-1.1+ population of live lymphocytes and then analyzed for CFSE fluorescence levels.

Results

CD8+ T cells from OT-I mice respond in vitro to Ag-complexes on LMI

The effects of LMI on tumor-specific CD8 T cell responses have been examined using an adoptive transfer model that allows direct visualization and characterization of the Ag-specific CD8+ T cells during the course of the response. The model uses CD8+ T cells

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from OT-I mice that have a transgenic TCR specific for chicken OVA-derived SIINFEKL peptide (OVA263–276) presented by H-2Kb, and E.G7 tumor, an OVA transfectant of the EL-4 thymoma (19). E.G7 cells express ~100 Kb/SIINFEKL complexes on the cell surface (21) and are specifically lysed by OT-I effector CTL. OVA therefore serves as a pseudo-tumor Ag in this model. This model has been used in several studies of tumor-specific CTL responses (22–25). The OT-I TCR was backcrossed onto the C57BL/6.PL background which expresses the Thy1.1 allele (OT-I.PL). Thus, SIINFEKL-specific CD8+ OT-I cells from OT-I.PL mice can be identified by flow cytometry when adaptively transferred into congenic Thy-1.2+ C57BL/6 recipients using Abs specific for the Thy-1.1 marker and CD8. Greater than 97% of the cells identified as CD8+Thy-1.1+ also displayed the TCR Vα2 receptor encoded by the OT-I transgene (data not shown).

LMI were prepared by adsorbing streptavidin-APC to 5-μm latex microspheres and then incubating with biotinylated-H-2Kb/β2-microglobulin/OVA257–264 peptide complexes to coat the beads with Ag. The amount of Ag on the beads can be controlled by varying the amount of complex incubated with a fixed number of beads, and the extent of Ag immobilization can be determined by staining with anti-class I mAb. LMI prepared using a given amount of Kb/OVA257–264 Ag exhibited a relatively narrow range of Ag density when stained with the Y3 anti-H-2Kb mAb and analyzed by flow cytometry, and varying the amount of Ag used to coat the beads resulted in LMI with varying surface densities of Ag (Fig. 1A). To confirm that Ag was present on the LMI in a form that could be recognized by T cells and to determine the optimal Ag density for stimulation of OT-I cells, CD8 T cells from OT-I.PL mice were incubated with Kb/OVA257–264-LMI or with LMI made using Kb complexed with an irrelevant SIYRYYGL peptide (Kb/SIYRYYGL-LMI) that is recognized by the 2C transgenic TCR (26).

OT-I T cells proliferated in response to Kb/OVA257–264-LMI provided that IL-2 was also added to the cultures, and responses increased with increasing density of Ag on the beads (Fig. 1B). Only weak proliferation occurred in the absence of exogenous IL-2, consistent with the lack of costimulatory ligands on the LMI. The OT-I response to LMI was Ag specific; no response by the OT-I cells was obtained using Kb/SIYRYYGL-LMI in the absence of presence of IL-2 (Fig. 1B). Reciprocal results were obtained when the responding cell population was from a 2C mouse (data not shown). Thus, CD8+ OT-I cells can respond in an Ag-specific manner to the Kb/OVA257–264 presented on LMI. Based on these in vitro results, the in vivo experiments described below were performed with LMI made using 25 ng Kb/OVA257–264 per 106 beads, a level of Ag that stimulates a very weak proliferative response in the absence of IL-2 and a plateau response in the presence of IL-2 (Fig. 1).

**FIGURE 1.** OT-I respond to Kb/OVA257–264-LMI in vitro. A, Kb/OVA257–264-LMI were prepared as described in Materials and Methods using varying amounts of Kb/OVA257–264 complex to prepare the beads, as indicated. The beads were then stained with Y3 (mouse anti-Kb) mAb and goat anti-mouse FITC Ab. Control beads coated with streptavidin but no Ag had only background levels of staining (filled histogram). B, OT-I T cells (105/well) were placed in culture with Kb/OVA257–264-LMI or control beads (2 × 105/well) made using the irrelevant Kb-binding peptide SIYRYYGL in the absence or presence of IL-2 at 2.5 IU/ml. Cultures were incubated for 48 h, and 1 μCi/well [3H]thymidine was added during the final 6 h. [3H]Thymidine incorporation is plotted as a function of the mean fluorescence intensity of the beads prepared using varying amounts of Kb/OVA257–264 as in A. Results are shown as mean ± SD of triplicate samples, and the experiment is representative of three independent experiments.

**LMI-mediated reduction of E.G7 tumor growth**

Having established the efficacy of Kb/OVA257–264-LMI in vitro, experiments were conducted to determine whether these LMI could mediate tumor growth reduction in vivo in the manner previously seen using LMI coated with tumor cell plasma membranes (12). Mice received adoptively transferred OT-I cells i.v. on day −2 and were then injected with tumor (i.p.) and LMI (i.v.) on day 0. Fourteen days later, peritoneal exudate cells were collected, and the number of tumor cells was determined by flow cytometry. Mice that received just tumor had ~250 × 106 tumor cells in the PC at this time, and the tumor burden was not significantly different in mice that had received OT-I by adoptive transfer (Fig. 2). In contrast, mice that received OT-I and Kb/OVA257–264-LMI had a substantially reduced tumor load. Previous studies examining LMI effects on syngeneic tumor growth used LMI made using tumor cell plasma membranes, and thus displaying numerous proteins in addition to class I Ag, and did not address whether class I/peptide Ag was necessary or sufficient to mediate growth reduction. The results in Fig. 2, and additional experiments (data not shown), demonstrate that LMI bearing the appropriate class I Ag in the absence of other cell surface proteins is sufficient to mediate growth reduction of a syngeneic tumor that expresses the same class I Ag.

**Effects of LMI on Ag-specific CD8 T cells in the absence of tumor challenge**

In previous work examining the effects of LMI bearing purified class I alloantigen, it was found that administration of LMI could substantially augment ex vivo cytolytic activity generated in response to allogeneic tumor but elicited no detectable response when administered to mice that were not also challenged with the tumor (12). The ability to identify and characterize adoptively transferred OT-I cells provides a much more sensitive and direct means of determining the effects of LMI on the Ag-specific T cells in vivo. When adoptive transfer recipients of OT-I cells were challenged by i.v. injection of Kb/OVA257–264-LMI, there was a small...
beads (prepared using 25 ng complex/10^6 beads) were administered i.v. (tail vein) on day 0. Peritoneal exudate was collected on day 14, and the number of E.G7 cells was determined by forward/side scatter analysis. Mice that were not challenged with tumor had fewer than 2 × 10^6 cells in the E.G7 gate (left bar).

Values are the mean ± SD for three mice per group, and the experiment is representative of three independent experiments.

but significant increase in the number of OT-I cells in the spleen and LN on days 2 through 4 in comparison with the numbers in mice that did not receive LMI (Fig. 3, A and B).

FIGURE 3. OT-I cell numbers increase in spleen, LN, and PC in response to K^b/OVA_257-264-LMI administration in mice without tumor. OT-I PL LN cells (1.2 × 10^6) were adoptively transferred by i.v. injection of 4 × 10^6 live E.G7 tumor cells (day 0), with the exception of one group that did not receive tumor. Where indicated, 10^7 K^b/OVA_257-264-LMI beads (prepared using 25 ng complex/10^6 beads) were administered i.v. (tail vein) on day 0. Peritoneal exudate was collected on day 14, and the number of E.G7 cells was determined by forward/side scatter analysis. Mice that were not challenged with tumor had fewer than 2 × 10^6 cells in the E.G7 gate (left bar).

Values are the mean ± SD for three mice per group, and the experiment is representative of three independent experiments.

Many of the properties of LMI effects on tumor growth would be consistent with recognition of Ag on the LMI resulting in more rapid trafficking of Ag-specific CD8 T cells to the peripheral site of tumor growth (12, 14). We therefore also examined OT-I cell numbers in the PC after administration of K^b/OVA_257-264-LMI. In the absence of LMI very few OT-I cells could be detected in the PC, but the numbers increased significantly within 3 days of LMI administration (Fig. 3C). This effect was specific to the OT-I cells, because no significant differences were found in total CD8 T cell numbers in the PC in response to LMI (Fig. 3D).

The increase in OT-I cell numbers in the spleen and LN in response to LMI could result from altered migration of the cells or from proliferation and clonal expansion. OT-I cells with light-scattering properties consistent with blastogenesis were present at these sites in LMI-treated mice beginning on day 1 (data not shown), suggesting that some proliferation was occurring. This possibility was directly assessed by labeling the OT-I cells with the fluorescent dye CFSE before adoptive transfer (27). CFSE enters cells and stably resides in the cytosol. When cells divide, the dye is diluted equally among the daughter cells, thereby resulting in a 2-fold decrease in mean fluorescence intensity at each cell division. By day 3 after administration of K^b/OVA_257-264-LMI, a fraction of the OT-I cells in the LN had diluted their CFSE, with some cells having undergone up to eight rounds of division (Fig. 4A). The CFSE profile (Fig. 4A) shows the progeny cells and therefore does not directly reveal the fraction of the starting cells that have divided. Thus, for example, a single original cell that has undergone 4 divisions will be represented by 16 events in the peak with 4-fold diluted CFSE. Analysis of the number of events in each peak shown in Fig. 4A, and calculation of the number of starting cells.
cells from which these events derived showed that 75% of OT-I cells remained undivided. Of the cells that did divide, more than one-half underwent a single cell division, and ~40% underwent two to four cell divisions. In contrast, administration of K\textsuperscript{b}/SIYRYYGL-LMI that are not recognized by OT-I cells in vitro (Fig. 1) resulted in no proliferation, as evidenced by a single bright peak of CFSE fluorescence (Fig. 4B). Similar results were obtained when OT-I cells in the spleen were examined (data not shown).

To further assess the effects of LMI, the phenotypes of the OT-I cells in LN, spleen, and the PC during the course of the response to K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI were determined using a panel of CD8 T cell activation markers. No changes in CD25 or L-selectin expression were observed between days 1 and 4 post-LMI treatment (data not shown). However, increased expression of the CD44 and VLA-4 surface receptors were seen in the spleen by day 2 and in the LN by day 3 post-LMI treatment, and expression of these markers remained high through day 4 (Fig. 5). In addition, almost all OT-I cells found in the PC of LMI-treated mice displayed an activated CD44\textsuperscript{high} VLA-4\textsuperscript{high} phenotype (data not shown). The LMI-mediated changes in CD44 and VLA-4 expression levels were specific to the OT-I cells; no changes in expression of these markers were found when tumor was present (Fig. 6). Thus, although LMI administration in the absence of Ag contributed to more rapid and extensive increase in the number of OT-I cells at the site of tumor growth, thereby contributing to reduced tumor load in the PC. We therefore examined the responses of adoptively transferred OT-I cells in mice challenged by i.p. injection of E.G7 tumor and either left untreated or treated with K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI or control LMI (beads coated with streptavidin but no Ag) at the time of tumor challenge by either i.p. or i.v. injection. In marked contrast to the clonal expansion of OT-I in the spleen and LN caused by LMI in the absence of tumor (Fig. 3), no significant differences in the numbers of OT-I cells at these sites were found when tumor was present (Fig. 6A). The numbers of OT-I cells in spleens and LN of the K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI-treated mice on day 4 were the same as those in the tumor-bearing mice.

**FIGURE 5.** OT-I cells up-regulate surface expression of CD44 and VLA-4 in response to K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI. CD8 OT-1.PL LN cells (1.2 × 10\textsuperscript{5}) were adoptively transferred into C57BL/6 recipients (day 0). On day 0, one-half of the mice received 10\textsuperscript{7} K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI by i.v. (tail vein) injection (○), and the remaining mice were left untreated (□). Pooled LN (A and C) and spleen (B and D) cells were harvested on days 1–4 and analyzed by flow cytometry. CD44 (A and B) and VLA-4 (C and D) expression were determined for OT-I cells (gating on CD8\textsuperscript{+} Thy-1.1\textsuperscript{+}). Also shown are expression levels on the host CD8 T cells in mice that had received K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI (▲). Values are means ± SD of two to four mice per group. Results are representative of three independent separate experiments.

**FIGURE 6.** Treatment with K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI results in increased numbers of OT-I cells in the PC of mice challenged with E.G7. CD8 OT-1.PL cells (2.2 × 10\textsuperscript{5}) were adoptively transferred into C57BL/6 recipients (day 0). On day 0 mice were challenged by i.p. injection of 4 × 10\textsuperscript{7} E.G7 cells. On the same day mice received either K\textsuperscript{b}/OVA\textsubscript{257-264}-LMI or control LMI (coated with streptavidin) by i.v. (tail vein) or i.p. injection, as indicated. On day 4, animals were sacrificed, and the number of OT-I cells in various compartments was assessed by flow cytometry. A, OT-I cells in pooled LN (■) and spleen (□); B, OT-I cells in the PC. Values are the mean ± SD for two to four mice per group. Results are representative of three independent experiments.
that received no LMI or control LMI, and in mice that were adoptively transferred but received neither tumor nor LMI (Fig. 6A). The fact the the numbers of OT-I cells do not increase at these sites is not due to a limitation of the maximum number of OT-I cells that can be present, because challenge of adoptively transferred mice with peptide Ag and adjuvant results in $>2 \times 10^6$ OT-I cells at these sites (data not shown).

Although increases in OT-I numbers were not found in the spleen and LN, significant effects of Kb/OVA257–264-LMI administration were found when OT-I cells in the PC, the site of tumor growth, were examined on day 4. OT-I cell numbers were increased in untreated tumor-bearing mice in comparison with mice that received OT-I cells but were not challenged with tumor (Fig. 6B). These numbers were significantly further increased in mice that had received Kb/OVA257–264-LMI either i.p. or i.v. but were unaffected by treatment with control LMI. Again, the effect of the Kb/OVA257–264-LMI was specific to the OT-I cells; the number of CD8+ Thy-1.1+ host T cells showed some increase in the PC when tumor was present, but this was unaffected by LMI treatment (data not shown).

Although the number of OT-I cells did not increase in the spleens of tumor-bearing mice in response to Kb/OVA257–264-LMI, the OT-I cells at this site had responded to the LMI as evidenced by the finding that the majority of the cells had up-regulated CD44 expression on day 4 (Fig. 7A). In contrast, the majority of the OT-I cells in the spleens of tumor-bearing mice treated with control LMI had not up-regulated CD44 (Fig. 7B). Similar results were obtained when OT-I cells in the LN of these mice were examined (data not shown). Spleen and LN OT-I cells from Kb/OVA257–264-LMI-treated mice also showed increased VLA-4 expression on day 4, whereas those from mice treated with control LMI did not (data not shown). As expected, almost all of the OT-I cells in the PC of tumor-bearing mice express high CD44 levels whether treated with Kb/OVA257–264-LMI or control LMI (Fig. 7, C and D).

The above results show that recognition of Ag delivered on LMI results in altered expression of surface receptors and rapid trafficking to the site of tumor growth. To determine whether the increased numbers of OT-I in the PC required that the tumor express specific Ag or whether it might simply reflect trafficking and increased numbers at an inflammatory site, experiments were conducted to compare LMI effects in mice challenged with E.G7 vs mice challenged with EL-4, the parental tumor that does not express OVA. Again, OT-I cell numbers in spleens and LN did not differ significantly as a result of LMI treatment and were the same in mice challenged with either E.G7 or EL-4 (Fig. 8A). In contrast, OT-I cell numbers were significantly increased in the PC on Kb/OVA257–264-LMI administration to mice challenged with E.G7, but not in the PC of mice challenged with EL-4, whereas control LMI had no effect in either case (Fig. 8B). Thus, LMI-mediated expansion of the Ag-specific OT-I cells at the site of tumor growth occurs only when the tumor bears that specific Ag.

**Discussion**

LMI made by coating tumor membrane Ag onto cell-sized microspheres have been shown to mediate tumor growth reduction in several murine tumor models (12, 14). In the clinic, LMI administration has had no significant side effects (M. S. Mitchell et al., manuscript in preparation), and efficacy is being evaluated for melanoma and renal carcinoma in trials using autologous tumor as the source of membrane Ag (15). In the murine models, LMI were shown to act, at least in part, by augmenting tumor-specific CTL responses, and LMI made by coating microspheres with purified alloantigens caused substantial augmentation of CTL responses to
allogeneic tumor (12). As demonstrated here, LMI made by coating microspheres with the appropriate class I/peptide complexes can specifically and effectively reduce syngeneic tumor growth (Fig. 2). Given the large number of class I-restricted tumor-specific peptide epitopes that have been identified for human tumors (1–4), and the ability to prepare HLA-class I/peptide complexes in relatively large quantities (8), this raises the possibility of preparing well-defined LMI for use in clinical trials. Potential advantages of this approach include the ability to prepare LMI having higher Ag densities than can be achieved using tumor membranes, in which the amount of tumor-specific Ag is not known and cannot be controlled, and the ability to treat patients from whom sufficient autologous tumor material cannot be obtained to prepare LMI.

In previous studies, the effects of LMI treatment on CD8 T cell responses could only be indirectly assessed by measuring ex vivo cytolytic activity (12). In the experiments described here, the use of adoptively transferred TCR-transgenic CD8 T cells specific for the Ag on the LMI made it possible to more directly examine the mechanism by which LMI augment responses. OT-I mice express a transgenic TCR specific for OVA257–264 peptide bound to H-2Kb class I protein (18) and can specifically lyse E.G7 tumor, the EL-4 thymoma transfected with OVA. Thus, OVA serves as a pseudotumor Ag in this model, allowing the OT-I T cell response to the tumor to be monitored during the course of the response. By adoptively transferring OT-I CD8 T cells into normal recipients in small numbers and then challenging with tumor, the entire course of the response can be followed with respect to the locations, activation status, and clonal expansion of the tumor-specific CD8 T cells, and several studies have used this model (22–25).

Ag-bearing LMI are uniquely effective in mediating reduction of syngeneic tumor growth; the same tumor membrane Ag administered alone or in CFA has little or no effect (14). It was therefore somewhat surprising to find that administration of Kb′/OVA257–264-LMI caused relatively little clonal expansion of OT-I T cells when tumor was not present (Fig. 3). Only ~3- to 5-fold expansion occurred in the spleen or LN on days 2 through 4, and ~25% of the OT-I cells divided as measured by CFSE dye dilution (Fig. 4). This is in marked contrast to immunization of adoptive transfer recipients with peptide in CFA or along with LPS, where the Ag-specific cells expand 30- to 60-fold by day 3 and all of the cells undergo multiple rounds of division (Ref. 28 and J. Goldberg and M. F. Mescher, unpublished results). Although division and clonal expansion in response to LMI were limited, the majority of the OT-I cells up-regulated CD44 expression, and about one-half also had increased VLA-4 expression (Fig. 5). Thus, it appears that most of the OT-I cells had recognized Ag and responded by altering expression of some surface receptors but that this did not lead to proliferation of most of the cells.

It is generally thought that initial T cell clonal expansion in response to Ag occurs in lymphoid organs, with the activated cells then migrating to peripheral sites. However, when OT-I adoptive transfer recipients are challenged by i.p. injection of E.G7 cells, increased numbers of OT-I cells were observed in the PC beginning about day 4 postchallenge, and no increases could be detected in draining LN or spleen (Ref. 24 and Fig. 6). When OT-I adoptive transfer recipients were challenged by i.p. injection of E.G7, administration of Kb′/OVA257–264-LMI resulted in substantially increased numbers of OT-I cells in the PC by day 4, 3- to 10-fold more than in untreated mice (Figs. 6 and 8). A significant fraction (>20%) of the OT-I cells in the PC at this time exhibit high forward and side scatter characteristic of blasts, indicating that some of the OT-I cells are proliferating at this site (data not shown). This increase was specific in that it required that the LMI bear the Kb′/OVA257–264 Ag (Fig. 6) and occurred only if the tumor growing in the PC expressed OVA (Fig. 8).

The effects of Kb′/OVA257–264-LMI administration on OT-I cells in the spleen and LN differed markedly depending on whether or not tumor was growing in the PC. The clonal expansion induced by LMI in LN and spleen in the absence of tumor (Fig. 3) was absent when tumor was present (Fig. 6A). Although expanded numbers of OT-I cells were not detected in LN and spleen of tumor-bearing mice, the cells at these sites had recognized and made some response to the Kb′/OVA257–264-LMI as indicated by the fact that they had up-regulated CD44 (Fig. 7) and VLA-4 surface expression (data not shown).

Together these results suggest that administration of Ag-bearing LMI may augment tumor-specific CD8 T cell responses to syngeneic tumor by altering trafficking of the T cells so that they reach the site of growing tumor more rapidly and in greater numbers, where they may further expand and acquire effector function. It is not surprising that the majority of Ag-specific cells do not proliferate in response to the LMI (Figs. 3 and 4), because costimulatory ligands are not present and the LMI may not induce production of inflammatory cytokines needed to support an effective CD8 T cell response (28–30). The majority of cells do recognize the LMI Ag, however, leading to altered expression of surface receptors (Figs. 5 and 7). VLA-4 is particularly interesting in this regard, as this integrin has been shown to be up-regulated in response to priming with Ag (31, 32) and has been implicated in having a role in directing migration of T lymphocytes to peripheral sites (31, 33). Thus, cells in the spleen and LN that have recognized LMI Ag and altered their expression of VLA-4, and perhaps other receptors that regulate trafficking, may migrate rapidly to peripheral sites and be further stimulated to undergo proliferation and development of effector function if they re-encounter Ag in an inflammatory environment. More efficient migration to the PC after recognition of LMI Ag is demonstrated by the fact that very few OT-I cells can be recovered from the PC of untreated mice, whereas they are present at this site in readily detectable numbers in mice treated with Kb′/OVA257–264-LMI (Fig. 3C).

Ag-specific CD8 T cells may directly recognize and respond to Ag displayed on the surface of the LMI in vivo. This can clearly occur in vitro (Fig. 1), and the ability of alloantigen-bearing LMI to augment CTL responses to allogeneic tumor (12) suggests that it can also occur in vivo, because it is unlikely that the native class I alloantigen on the LMI would be taken up and represented in intact form by host APC. However, in the case of LMI-bearing Ag to syngeneic tumor, cross-priming may also occur (34–37), with the host APC taking up the LMI and presenting tumor-specific peptides (or OVA). Further work will be needed to determine the in vivo fate of LMI and whether direct recognition, cross-priming, or both are involved in the LMI-mediated augmentation.

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References