

Identification of Oncofetal Antigen/Immature Laminin Receptor Protein Epitopes That Activate BALB/c Mouse OFA/iLRP-Specific Effector and Regulatory T Cell Clones¹

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During tumor development in mice and humans, oncofetal Ag/immature laminin receptor (OFA/iLRP)-specific Th1, CTL, and IL-10-secreting T (Ts) cells are induced. The presence of too many Ts or too few effector T cells appears to predict a poor prognosis. We established clones of OFA/iLRP-specific splenic Th1, CTL, and Ts cells from the OFA/iLRP⁺ MCA1315 fibrosarcoma-bearing BALB/c mice or from BALB/c mice vaccinated with 1 or 10 μ g of rOFA/iLRP. The MCA1315 tumor cell-reactive T cell clones were characterized as to surface Ag phenotype, cytokine secretion profile, and specificity for OFA/iLRP presented by syngeneic splenic APC. OFA/iLRP-specific Th1 and Ts clones were established from all mice. OFA/iLRP-specific CTL could be established from all mice except for mice immunized with 10 μ g of rOFA/iLRP. Analysis of the proliferation profile of the OFA/iLRP-specific clones to overlapping OFA/iLRP 12-mer peptides that spanned the OFA/iLRP protein sequence defined the epitopes to which the T cell clones responded. There was a similar spatial distribution of the epitopes to which the two types of CD8 T cell clones responded. The nonpeptide epitopes of the Ts clones were located between aa 36 and 147 of OFA/iLRP, while the epitopes of the CTL clones were located between aa 52 and 163. Even though the CTL and Ts epitopes shared part of the protein, all of the CD8 CTL epitopes were distinct and separable from those of CD8 Ts cells. *The Journal of Immunology*, 2006, 176: 2844–2856.

There is ample evidence that many tumors express Ags that can be recognized by the adaptive immune system (1) and which can potentially induce therapeutic antitumor immune responses. However, it has been shown in experimental animals that while a tumor will grow and kill the animal if not treated, injection of the same tumor cells at a different site in the animal will lead to rejection of those injected cells (2, 3). This has been termed concomitant immunity (2). A similar phenomenon has been seen in human cancer patients also (3). Boon and Van der Bruggen (4) demonstrated that melanoma-associated Ag (MAGE)³ is a melanoma-specific Ag against which MAGE-associated, MHC-restricted CTL can be induced. Subsequently, human CTL responses specific for human tumor oncogenes (5), mutated p53 (6), and even carcinoembryonic Ag epitopes have been identified (7). Most reported immunotherapy experiments have focused on CD8 CTL responses (8); interest in CD4 Th cells has centered on those interactions which optimize CTL activation (9, 10). CD4 Th1 cells have been shown to be important in tumor rejection (11, 12), but some studies actually have shown that Th1 cell-activated macrophages within the tumor bed promote angiogenesis and, in so doing, promote tumor cell survival (13). One study has shown that tumor Ag-specific Th2 cells can effect CTL-resistant mela-

noma rejection and decrease metastatic melanoma growth by recruiting eosinophils into the tumor bed. The eosinophils degranulate and their granular cytotoxins kill the tumor cells (14). There is much evidence that many tumors have been immunoselected to evade CTL-induced tumor rejection (15). Tumors do this by secreting immunosuppressive cytokines (16), inducing antitumor T cells to secrete immunosuppressive cytokines (17), or down-regulating either the tumor Ags to which responses are being mounted or down-regulating class I MHC expression (14, 18).

We have previously shown that the 32–44 kDa protein oncofetal Ag (OFA) is immature laminin receptor protein (iLRP) (19, 20). Clearly iLRP is an autoimmunogenic tumor rejection Ag when expressed by all tumors, but not by normal cells (21, 22). We have designated the immunogenic, monomeric form of this laminin-binding protein OFA/iLRP. OFA/iLRP is important to the tumor cells for metastasis (23–26). Like most investigators in tumor immunology working with other immunogens, we have found that during tumor development, OFA/iLRP-specific, MHC-restricted effector T cells are induced (CTL and Th1), but, in addition, regulatory T cells (IL-10-secreting, CD8 suppressor cells) are also induced (27, 28). The IL-10 that is secreted inhibits CTL cytotoxic activity and IFN- γ secretion by both CTL and Th1 cells (29). We can increase the ratio of anti-OFA/iLRP CTL:Ts cells by immunizing with a 10-fold lower dose of OFA/iLRP (22) because the Ts cells have always been found to have significantly lower affinity TCRs for OFA/iLRP than the memory CTL (27, 28). However, it would be advantageous to be able to immunize with a mixture of OFA/iLRP peptides that are able to only induce effector T cells and not the Ts cells. The purpose of this report is to identify the OFA/iLRP epitopes recognized by cloned BALB/c mouse OFA/iLRP-specific CTL and Ts cells established from either tumor-bearing mice or mice immunized with different doses of OFA/iLRP. We herein report that while CTL and Ts cell-recognized epitopes are similarly distributed in the OFA/iLRP sequence, they are distinct from one another. This important observation should allow immunization to raise only anti-OFA/iLRP CTL and avoid stimulation of

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³ Abbreviations used in this paper: MAGE, melanoma-associated Ag; OFA, oncofetal Ag; iLRP, immature laminin receptor protein; Ts, IL-10 secreting; NC, nitrocellulose; RT, room temperature; LDH, lactate dehydrogenase; DC, dendritic cell.

Ts cells which can interfere with host T cell antitumor cytotoxicity (29). We also have determined the preferred class I MHC protein for presentation of each OFA/iLRP nonapeptide epitope and demonstrated that each deduced nonapeptide epitope stimulates proliferation of the appropriate clone. We have also shown that immunization of BALB/c mice with syngeneic, bone marrow-derived mature dendritic cells (DC) which were pulsed with either intact OFA/iLRP or mixtures of two CTL clone- or two Ts clone-activating OFA/iLRP peptides inhibits MCA1315 fibrosarcoma cell lung metastasis and growth. However, the activation of Ts cells by OFA/iLRP Ts peptides reduced the inhibition of metastatic growth significantly.

Materials and Methods

Mice

BALB/cAnN female 8- and 10-wk-old mice were used for MCA1315 fibrosarcoma inoculation, for immunization with OFA/iLRP, and as sources of spleen cell APCs and spleen T cells used for MCA1315 fibrosarcoma-reactive T cell cloning. All mice were purchased from Charles Rivers Breeding Laboratories and were maintained in the vivarium of the Department of Comparative Medicine, University of South Alabama.

Tumor cells

MCA1315 fibrosarcoma cells (BALB/c) were grown in RPMI 1640 medium supplemented with essential and nonessential amino acids, sodium pyruvate, sodium bicarbonate, 100 U/ml penicillin G, 100 μ g/ml streptomycin sulfate, 10% heat-inactivated FCS (Sigma-Aldrich) (complete RPMI 1640). The cells were maintained in a 37°C humidified 5% CO₂/95% air atmosphere. The MCA1315 cells were used for restimulation of T cells derived from MCA1315 fibrosarcoma tumor-bearing mice and OFA/iLRP-immune mice for cloning.

Monoclonal Abs

Monoclonal anti-CD8 Ab (hybridoma 53-6.7), monoclonal anti-CD4 Ab (hybridoma GK1.5), monoclonal anti-CD3 Ab (hybridoma 145-2C11), and monoclonal anti- $\alpha\beta$ and anti- $\gamma\delta$ TCR mAbs from hybridomas H57-597 and GL3, respectively, were obtained from BD Biosciences. Monoclonal anti-H2-K^d IgM (hybridoma K3) and control monoclonal mouse IgM (hybridoma MADNP5) were purchased from Serotec.

Ag preparation

A full-length cDNA of OFA/iLRP was cloned into an expression vector under the control of the tac promoter and expressed in *Escherichia coli*. Inclusion bodies were isolated and solubilized in 6 M guanidine hydrochloride in 0.1 M Tris (pH 8.0), 0.1 M NaCl, 2 mM EDTA, and 0.3% dithioerythritol. The solubilized protein was renatured by a rapid 100-fold dilution into the refolding buffer (0.1 M Tris (pH 8.0), 0.5M L-arginine, 8 mM oxidized glutathione, and 2 mM EDTA) as described previously (30) and dialyzed against 20 mM Tris (pH 8.0), 0.1 M NaCl, and 0.04% sodium azide. The purity of rOFA/iLRP was checked by SDS-PAGE. Only one band was seen after staining with Coomassie R250. For the preparation of Ag-bearing nitrocellulose (NC) particles, the method of Abou-Zeid et al. (31) was used as described previously (32). Briefly, solubilized rILRP was subjected to 12% SDS-PAGE according to the method of Laemmli (33) and separated proteins were transferred to NC (34), made visible by staining with Ponceau S (35) and the NC bands carrying the rOFA/iLRP were cut. The NC bands were dissolved and sterilized in DMSO during 1-h incubation at room temperature. Precipitation was subsequently performed by the dropwise addition of 0.05 M carbonate/bicarbonate (pH 9.6) while shaking continuously. Each suspension was washed three times with sterile PBS.

OFA/iLRP peptide preparation

Peptides used in this study were synthesized by Sigma-Genosys using automated solid phase techniques, purified by reversed phase HPLC and their structure was verified by mass spectrometry. All peptides were >95% pure. Peptides were obtained in lyophilized form. Hydrophilic peptides were dissolved in water (pH 7), then the pH was adjusted to improve solubility and diluted with PBS to 2 mg/ml. Hydrophobic peptides were dissolved in DMSO and then diluted with PBS to 2 mg/ml (the final concentration of DMSO in the peptide stock solution is <5%, v/v). Peptide solutions were filtered through a 0.2- μ m pore size membrane and stored at -80°C.

Immunization and challenge of BALB/c mice for T cell clone production

Mice were divided into three groups and two of the groups ($n = 3$) were immunized i.p. twice, separated by 14 days with NC particles to which was bound 1 or 10 μ g of rOFA/iLRP suspended in 1 ml of PBS. The amount of nitrocellulose per mouse was kept constant (50 mg). Two weeks after the second injection, mice were sacrificed by CO₂ asphyxiation, and spleens were excised for cloning of MCA1315-reactive T cells. The third group was injected s.c. with 1×10^5 viable MCA1315 fibrosarcoma cells and 3 wk after injection, all mice had palpable tumors on their backs. These MCA1315 tumor-bearing mice were then sacrificed by CO₂ asphyxiation, and spleens were excised for cloning of MCA1315-reactive T cells.

T cell clone production

The spleens excised from BALB/c mice 2 wk after their second i.p. injection of 1 μ g of iLRP:NC or 10 μ g of iLRP:NC particles or 3 wk after s.c. injection of MCA1315 fibrosarcoma cells into normal BALB/c mice were minced, washed in sterile isotonic PBS (pH 7.4), and in complete IMDM. After washing and viability counting, splenic T lymphocytes were stimulated in vitro with irradiated BALB/c MCA1315 fibrosarcoma cells, and the T cells that proliferated (MCA1315 fibrosarcoma-reactive T cells) were cloned by limiting dilution using the method previously published (27). The cells were cultured in this medium supplemented with 100 U/ml recombinant mouse IL-2, 10 U/ml recombinant mouse IFN- γ , and 10 U/ml recombinant mouse IL-6 during initial stimulation, cloning, and expansion of the harvested clones that were established. All of cytokines were obtained from R&D Systems. The IL-2 was used as a growth factor for the T cells, IFN- γ was used to inhibit outgrowth of Th2 Th cells for Ab production (36), and IL-6 was used to promote outgrowth and function of CTL cells (37). After growth of the expanded clones had stabilized, the only cytokine added to the cell cultures was 100 U/ml recombinant murine IL-2. These clones had to be restimulated with irradiated OFA/iLRP⁺ MCA1315 cells every 2 wk in the presence of irradiated syngeneic spleen cells and complete IMDM supplemented with 100 U/ml recombinant murine IL-2 to maintain viability and proliferation.

Determination of T cell clone surface Ag phenotype by mAb and complement depletion

One week after Ag restimulation, part of each T cell clone culture was harvested, washed three times by centrifugation in complete RPMI 1640, and a viability count was done. The cells were diluted to 1×10^6 viable cells/ml and their surface Ag phenotype was determined by cytotoxicity with mAbs plus facilitating anti-Ig antisera plus complement, as previously described (27). The counted cells were pelleted and resuspended in 1 ml of anti-CD4, anti-CD8, anti-CD3, anti- $\alpha\beta$ TCR, or anti- $\gamma\delta$ TCR Ab diluted optimally in complete IMDM. For all Abs used, the optimal dilution was 1/15. Control Ab for the anti-CD4 (a rat IgG2b) and anti-CD8 (a rat IgG2a) was normal rat IgG. Similarly, the control for the hamster IgG monoclonals against mouse CD3 and TCRs was normal hamster IgG. The normal IgGs were obtained from Organon Technika. After Ab and complement treatment, cells were pelleted by centrifugation, washed three times in complete IMDM, and resuspended in 1 ml of complete IMDM. A viability count was done by trypan blue dye exclusion. The percentage of cells specifically killed or lysed by the experimental Ab and complement treatment was calculated by knowing the number of total and viable cells in each tube at the beginning and comparing the nonspecific killing effect of the control Abs plus facilitating antiserum plus complement with the killing by the experimental Abs plus facilitating antiserum plus complement treatment.

Determination of T cell clone specificity by proliferation in response to Ag

At the time of the 2-wk restimulation of the clones to maintain their proliferation, the cloned cells were harvested, washed in complete IMDM, and a viability count was done. A portion of the cells was saved to be used in the proliferation assay. The proliferation assay was done with 10,000 viable cloned cells/well plus irradiated syngeneic spleen cells plus 100 ng/well recombinant murine OFA/iLRP or OVA bound to nitrocellulose particles, an equivalent amount of unconjugated nitrocellulose particles, or 1×10^5 irradiated MCA1315 fibrosarcoma cells in 96-well plates. All wells contained complete IMDM supplemented with 100 U/ml recombinant mouse IL-2 and 1×10^5 irradiated normal BALB/c spleen cells to act as APC. Proliferation was quantitated using the BioTrak BrdU incorporation ELISA (Amersham Biosciences) as described previously (28). Briefly, after 24 h of culture at 37°C in 95% air/5% CO₂ humidified atmosphere, 10 μ l of BrdU was added to each well to a final concentration of 10 μ M

BrdU/well. The cells are then incubated for another 24 h as before. At the end of the second incubation, the plates are centrifuged at $300 \times g$ for 10 min and the labeling medium was removed. The cells were dried for 1 h at 60°C, fixed with an ethanol fixative for 30 min at room temperature (RT) and then the fixative was removed and blocking buffer (1% protein in 50 mM Tris-HCl and 150 mM NaCl (pH 7.4)) was added. The cells were incubated for 30 min at RT, the blocking buffer was removed, and 100 μ l of 1/100 diluted peroxidase-labeled anti-BrdU was added to each well and the plates were incubated for 90 min at RT. The Ab solution was then removed and the wells were washed three times with 300 μ l/well of wash buffer. A total of 200 μ l of 3,3', 5,5'-tetramethylbenzidine (TMB) in 15% (v/v) DMSO was added to each well and the plate was covered and oscillated gently in the dark for 5–30 min at RT. When the required color density was reached, the reaction was stopped by adding 25 μ l of 1 M sulfuric acid to each well and the plate was read on a microELISA reader at 450 nm.

ELISA of IFN- γ , IL-4, and IL-10 production by T cell clones

Quantikine cytokine assay kits for murine IFN- γ , IL-4, and IL-10 from R&D Systems were used. HRP-labeled anti-cytokine Ab were used to detect cytokine captured on the anti-cytokine Ab-coated plates. TMB was the substrate that was added, the color reaction was stopped with 2 N sulfuric acid, and the color was read at 450 nm. The IFN- γ standard curve was linear between 5 and 500 pg/ml, and the minimum amount detectable was 2 pg/ml. The IL-4 standard curve was linear between 8 and 500 pg/ml, and the minimum amount detectable was 5 pg/ml. The IL-10 standard curve was linear between 20 and 1000 pg/ml, and the minimum amount detectable was 4 pg/ml.

Determination of cytotoxic T cell activity of the T cell clones against syngeneic MCA1315 fibrosarcoma cells

Cytotoxicity assays were performed using the CytoTox96 nonradioactive cytotoxicity assay kit produced by Promega (Fisher Scientific). The assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants was measured with a 30-min coupled enzymatic assay resulting in the conversion of a tetrazolium salt to a red formazan product (38). The amount of color formed is proportional to the number of lysed cells. Color was quantitated using a Titertek Multiskan MC ELISA reader (Fisher Scientific) that measured absorbance at 492 nm. The setup of the assay was the same as previously described for testing RFM mouse T cell clone cytotoxicity against RFM thymic lymphoma cells (27), except that the medium used was RPMI 1640 and both the clones and the target cells were from BALB/c mice. All cytotoxicity assays were done with 10,000 irradiated MCA1315 cells/well and an E:T ratio of 50:1 in 96-well plates. Control wells were set up to account for spontaneous LDH release from effectors, spontaneous LDH release from targets, and maximal LDH release from targets as well as the experimental wells. The percent-specific cytotoxicity was calculated using the following formula: percent cytotoxicity = (experimental – effector spontaneous) – target spontaneous/target maximum – target spontaneous.

There is much less of a spontaneous release of LDH in this assay than of ^{51}Cr in a traditional ^{51}Cr release cytotoxicity assay and, therefore, higher specific cytotoxicity percentages are achieved.

Determination of the ability of anti-IL-10 to convert noncytotoxic CD8, iLRP-immune T cell clones to cytotoxic clones

To determine whether the Ts CD8 T cell clones from iLRP-immune mice were inhibited from cytotoxic activity against OFA⁺ syngeneic tumor cells by the IL-10 they were secreting, the cells were harvested 1 day before the normal 2-wk restimulation culture and set up in complete IMDM containing 100 U/ml recombinant murine IL-2 and 10 μ g/ml rat monoclonal anti-mouse IL-10 IgM (clone AB-71-005; BioSource International) or rat monoclonal anti-B220 IgM as a control Ab. The cells were cultured for 24 h as described previously (29) and then harvested, washed three times with complete IMDM, and viability counts were done. The cells were then diluted appropriately and added to a 4-h cytotoxicity assay against syngeneic MCA1315 fibrosarcoma cells as described above except that anti-IL-10 or control IgM was added to a final concentration of 10 μ g/ml in the cytotoxicity assays.

Determination of the OFA/iLRP epitopes recognized by OFA/iLRP-specific CTL, or CD8 Ts clones

Initially, the 13 OFA/iLRP-specific T cell clones established from the spleens of OFA/iLRP-immune or MCA1315 fibrosarcoma tumor-bearing mice were assayed for proliferation to 100 ng/well intact OFA/iLRP or 100

ng/well truncated OFA/iLRP encoded by deletion mutants of the intact OFA/iLRP gene. The truncated OFA/iLRP proteins were of varying lengths, but spanned the entire 295-aa length of the OFA/iLRP. Proliferation assays were performed as described above using the Biotrak BrdU incorporation ELISA except for the stimulant used to induce proliferation. By analysis of the proliferation induced by the various truncated OFA/iLRP proteins and intact OFA/iLRP presented by irradiated, syngeneic spleen cells in the presence of 100 U/ml rIL-2, we could determine the area of the protein which contained the epitope to which each OFA/iLRP-specific T cell clone reacted.

After these initial proliferation assays were analyzed, the OFA/iLRP-specific T cell clones were assayed for proliferation to 100 ng/well 12-mer OFA/iLRP peptides that overlapped by 4 aa and spanned the area of the protein to which a particular clone was stimulated to proliferate. In these assays as in all other proliferation assays, irradiated, syngeneic spleen cells were used as APCs and 100 U/ml rIL-2 was present as a growth factor. As before, proliferation was quantitated by use of the Biotrak BrdU incorporation ELISA as described above and previously (28). Analysis of the proliferation profiles of each clone to the OFA/iLRP peptides used to induce proliferation allowed determination of the amino acid sequence to which a clone was proliferating.

Determination of the OFA/iLRP peptide epitope match with class I H-2^d protein motifs for peptide binding

Two different computer programs were used to identify nonapeptides carrying the motif for H2-D^d, H2-K^d, and H2-L^d which matches the epitope sequences of iLRP deduced experimentally. These programs, available via internet from the National Center for Biotechnology Information (http://bimas.dcr.t.nih.gov/molbio/hla_bind/) and from the University of Tübingen (www.uni-tuebingen.de/uni/kxi/) (39, 40), predict binding affinity and stability based on matrices derived from known epitopes.

Confirmation of the ability of the appropriate H2-K^d-binding nonamer OFA/iLRP peptide to stimulate OFA/iLRP-specific CTL or Ts clone proliferation.

At the time of the 2-wk restimulation of the M3, L4, L6, H2, H4, and H5 CD8 clones to maintain their proliferation, the cloned cells were harvested, washed in complete IMDM, and a viability count was done. A portion of the cells was saved to be used in the proliferation assay. The proliferation assay was done as described above by culturing 10^4 cloned T cells with 1×10^5 irradiated, syngeneic spleen cells and 100 ng/well intact purified, rOFA/iLRP or one of five 9-mer peptides that have the sequence of the OFA/iLRP epitopes deduced to stimulate these T cell clones in complete IMDM supplemented with 100U/ml recombinant mouse IL-2 in 96-well plates. The cultures were incubated at 37°C in a humidified 95% air/5% CO₂ atmosphere for 24 h. At that time BrdU was added to a final concentration of 10 μ M to each well and the cells continued to be incubated at 37°C in a humidified 95% air/5% CO₂ atmosphere another 24 h. As described above, the plates are then harvested and assayed for BrdU incorporation (to monitor proliferation) by ELISA using the BioTrak Cell Proliferation ELISA system (Amersham Biosciences).

Determination of the requirement for MHC recognition by the T cell clones for the proliferative response to the OFA/iLRP nonamer peptide epitopes

The proliferation assay for the clones to the OFA/iLRP nonamer peptides described immediately above were repeated except that a mAb specific for the H2-K^d class I MHC protein or an isotype control Ab was present during the assay. Also, the irradiated syngeneic spleen cells, which served as APCs, were incubated for 30 min at 4°C with the anti-H2-K^d IgM or control IgM before being added to the assay plate wells. In setting up these assays, a standard order of addition of materials was used: first, irradiated syngeneic spleen cells in complete IMDM containing 2 μ g/ml anti-H2-K^d or isotype control mAb plus 100 ng/well intact recombinant OFA/iLRP, the appropriate OFA/iLRP nonamer peptide for the clone being tested, or OVA (as a negative control Ag), and second the OFA/iLRP-specific cloned CTL or Ts cells to be assayed. This was a slight modification of the method of Pawelec et al. (41). As described above, each well of a 96-well plate contained 10,000 cloned T cells to be tested, 100,000 irradiated, syngeneic spleen cells to be the APCs in complete IMDM supplemented with 100 U/ml recombinant murine IL-2. The T cell proliferation was then determined by measuring BrdU incorporation by the cells using the BioTrak ELISA system (28).

Production of BALB/c mouse bone marrow-derived mature DCs

BALB/c mouse DC were established from bone marrow cells using the method of Inaba et al. (42) flushed from the tibias and femurs of these mice. The bone marrow cells were then counted and cultured in 24-well plates at $7-10 \times 10^5$ cells/ml in complete IMDM (supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 μ g/ml streptomycin sulfate, and 10% FCS) which contained 1000 U/ml recombinant murine GM-CSF. Every 2 days, the cells were gently swirled, nonadherent cells were pipetted away, and 1 ml of fresh complete IMDM containing 1000 U/ml GM-CSF was added. After 6 days, the cells were harvested and cultured for 90 min in complete IMDM containing GM-CSF \pm 25 μ g/ml recombinant intact murine OFA/iLRP or mixtures of two CTL- or two Ts-activating OFA/iLRP 9-mer peptides (representing aa 80–88 and 155–163 for CTL clone-recognized epitopes and 36–44 and 139–147 for Ts clone-recognized epitopes). The cells were then washed and resuspended in complete IMDM containing 100 ng/ml recombinant murine IL-1 α , 25 ng/ml TNF- α , and 5 ng/ml recombinant murine IL-6 as described by Holtl et al. (43). The cells were cultured overnight to allow DC maturation. The cells were then harvested, washed, and counted for viability. To monitor maturation, the DCs were assayed for class II MHC and CD80 expression.

Determination of antitumor immunity induced by injection of syngeneic mature DCs pulsed with OFA/iLRP protein or CTL- or Ts-inducing OFA/iLRP peptides

Normal male 8- to 12-wk-old BALB/c mice were injected i.p. with 1×10^6 viable, mature DCs which had been pulsed with either FCS, recombinant, intact murine OFA/iLRP, a mixture of two OFA/iLRP CTL clone-activating peptides (sequences representing OFA/iLRP aa 80–88 and 155–163), or a mixture of two OFA/iLRP Ts clone-activating peptides (sequences representing OFA/iLRP aa 36–44 and 139–147). Two weeks later, the mice received a second identical immunization with Ag-pulsed DCs i.p. Two weeks later, all control FCS:DC-, intact OFA/iLRP: DC-, or OFA/iLRP peptide:DC-immunized mice were challenged i.v. with 5×10^4 , 1×10^5 , or 1×10^6 viable MCA1315 fibrosarcoma cells, which express OFA. After 2 wk, the mice were sacrificed and their lungs, livers, and spleens observed. No tumor foci could be seen in the livers or spleens of the mice, but foci could be seen to various degrees in the lungs. Therefore, the lungs were excised, washed in sterile 0.83% ammonium chloride to lyse erythrocytes in contaminating blood, and then colonies were counted using a dissecting microscope.

Analysis of cytokine secretion by spleen CD8 T cells derived from mice that had been immunized with FCS-, intact OFA/iLRP-, or OFA/iLRP Ts or CTL-activating peptide mixture-pulsed DCs

One-fourth of the mature DC-immunized mice were not challenged i.v. with tumor cells, but were sacrificed at the time the other mice were challenged. Their spleens were excised, minced, washed in complete IMDM medium, and the cells were counted. The spleen cells are separated on monoclonal anti-CD2 Ab-coated petri plates into CD2⁺ and CD2⁻ cells using a modification of the panning technique of Wysocki and Sato (44). The negatively selected CD2⁻ cells (T cell-depleted) were x-irradiated at 3000 rad and used as APCs. The eluted CD2⁺ T cells were incubated with anti-CD8 mAb-coated magnetic beads and positively selected for CD8 T cells using the IMag magnet (BD Biosciences) for magnetic cell sorting. The CD8 T cells from the various DC-immunized mouse groups were mixed with the irradiated, syngeneic, T cell-depleted spleen cells and 100 ng/well intact OFA/iLRP or OVA as a negative control in 96-well plates. These cells are cultured in complete IMDM supplemented with 100 U/ml IL-2 at 37°C in a 95% air/5% CO₂ humidified atmosphere for 48 h. At the end of that incubation, the cells are pelleted by centrifugation at $300 \times g$ for 10 min in the plates and the supernatants were harvested and assayed for IL-4, IL-10, and IFN- γ using the R&D Systems Quantikine ELISAs described above.

Statistical analysis of data

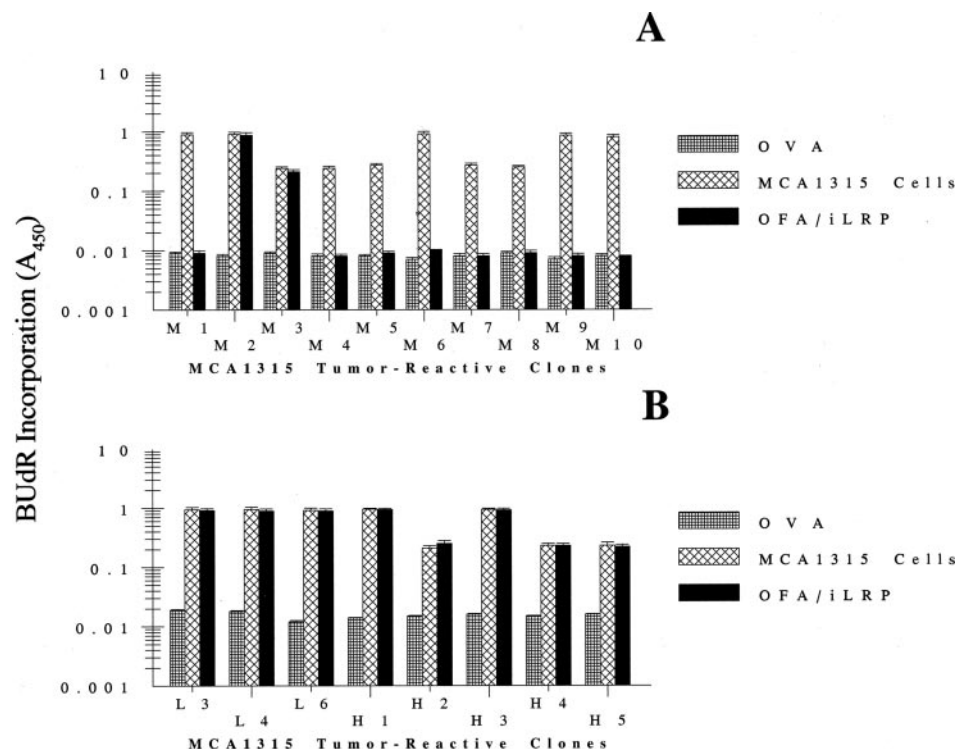
All experiments were repeated three times and, in most cases, the Student *t* test was used to determine the statistical significance of experimental value differences from appropriate control values. We considered a *p* value of <0.05 significant.

Results

Characterization of the OFA/iLRP-specific, MCA1315 fibrosarcoma-bearer and OFA/iLRP-immune mouse-derived T cell clones

Although all of the MCA1315-reactive clones were established from the spleens of BALB/c mice which had been immunized with either 1 or 10 μ g of OFA/iLRP-conjugated nitrocellulose particles proliferated specifically to OFA/iLRP (Fig. 1B), only 20% (2 of

FIGURE 1. Determination of OFA/iLRP-specific, MCA1315 fibrosarcoma-reactive T cell clones by proliferation stimulation (BrdU incorporation). *A*, Proliferation to MCA1315 fibrosarcoma cells, OFA/iLRP, or OVA by MCA1315 fibrosarcoma-reactive T cell clones established from spleens of MCA1315 fibrosarcoma-bearing BALB/c mice in the presence of irradiated syngeneic splenic APCs and IL-2. *B*, Proliferation to MCA1315 fibrosarcoma cells, OFA/iLRP, or OVA by MCA1315 fibrosarcoma-reactive T cell clones established from spleens of 1 or 10 μ g of recombinant murine OFA/iLRP:NC particle-immune BALB/c mice in the presence of irradiated syngeneic splenic APCs and IL-2. These data represent the means of three separate proliferation assays \pm SEM.



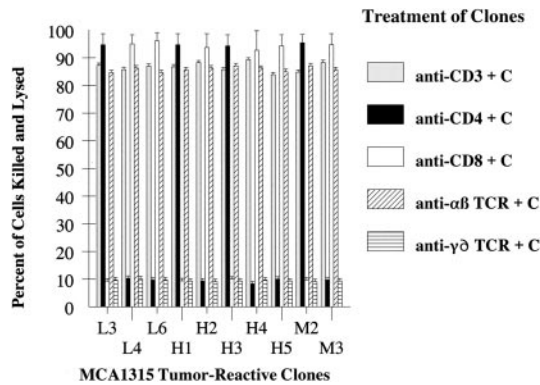


FIGURE 2. Surface Ag phenotype of OFA/iLRP-specific, MCA1315-reactive T cell clones by mAb + facilitating anti-IgG + complement cytolytic assay. These data represent the means of three separate complement-mediated cytolytic assays ± SEM.

10) of the MCA1315 fibrosarcoma-reactive clones (clones M2 and M3) established from MCA1315 fibrosarcoma-bearing BALB/c mice proliferated specifically to OFA/iLRP (Fig. 1A). Thus, the majority of the antitumor cell T cell response is targeted at other MCA1315 tumor Ags such as the MCA1315 individual tumor-specific transplantation Ag (TSTA). Of the two OFA/iLRP-specific splenic T cell clones derived from MCA1315 fibrosarcoma-bearing BALB/c mice, one was a CD8 T cell clone and the other was a CD4 T cell clone (Fig. 2). They both expressed CD3 and αβ T cell Ag receptors. These two clones appear to represent one CD4 Th1 clone (clone M2), and one CD8 CTL clone (clone M3) in that the CD4 clone (M2) secretes IFN-γ, but neither IL-4 nor IL-10 upon stimulation with OFA/iLRP presented by irradiated, syngeneic spleen cells in the presence of IL-2.(Fig. 3B). The CD8 CTL clone M3 shows the same OFA/iLRP-stimulated cytokine profile as the Th1 clone (secretes only IFN-γ) (Fig. 3A) and causes 54% cytotoxicity of MCA1315 target cells, but only ~5% cytotoxicity

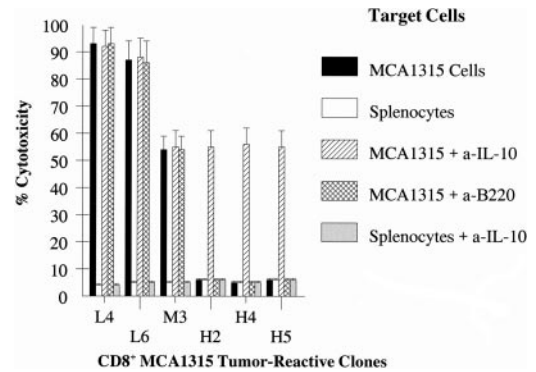


FIGURE 4. Determination of the anti-MCA1315 fibrosarcoma cytotoxic activity of CD8 OFA/iLRP-specific T cell clones in the presence and absence of neutralizing anti-IL-10 Ab. These data represent the means of three separate cytotoxicity assays ± SEM.

on syngeneic spleen cells. Also, addition of neutralizing anti-IL-10 Ab has no effect on its cytotoxicity levels (Fig. 4). The lower than usual cytotoxicity is probably due to that clone expressing a low to moderate affinity receptor for the OFA/iLRP peptide: class I MHC complex considering the lower amount of proliferation induced in this clone by 100 ng/well iLRP or MCA1315 irradiated tumor cells (Fig. 1A).

The MCA-1315-reactive T cell clones established from the spleens of 1 or 10 μg OFA/iLRP:NC particle-immune mice all proliferated specifically to OFA/iLRP in the presence of irradiated, syngeneic spleen cells and IL-2 (Fig. 1B). From the 1-μg OFA/iLRP:NC particle-immune spleens, we were able to establish one MCA1315 tumor cell-reactive CD4 Th1 clone (clone L3) (Figs. 2 and 3), two CD8 CTL clones (clones L4 and L6), and no CD8, T cell clones (Figs. 2-4). All of these clones express CD3 and have αβ TCRs (Fig. 2).

From the spleens of 10-μg OFA/iLRP:NC particle-immune BALB/c mice, we established no CD8 CTL clones (Figs. 2-4), but

FIGURE 3. Determination of the cytokine secretion profile of OFA/iLRP-specific, MCA1315-reactive T cell clones by anti-cytokine ELISA. A, IL-4, IL-10, and IFN-γ secretion profiles of CD8 OFA/iLRP-specific T cell clones. B, IL-4, IL-10, and IFN-γ secretion profiles of CD4 OFA/iLRP-specific T cell clones. These data represent the means of three separate cytokine secretion assays ± SEM.

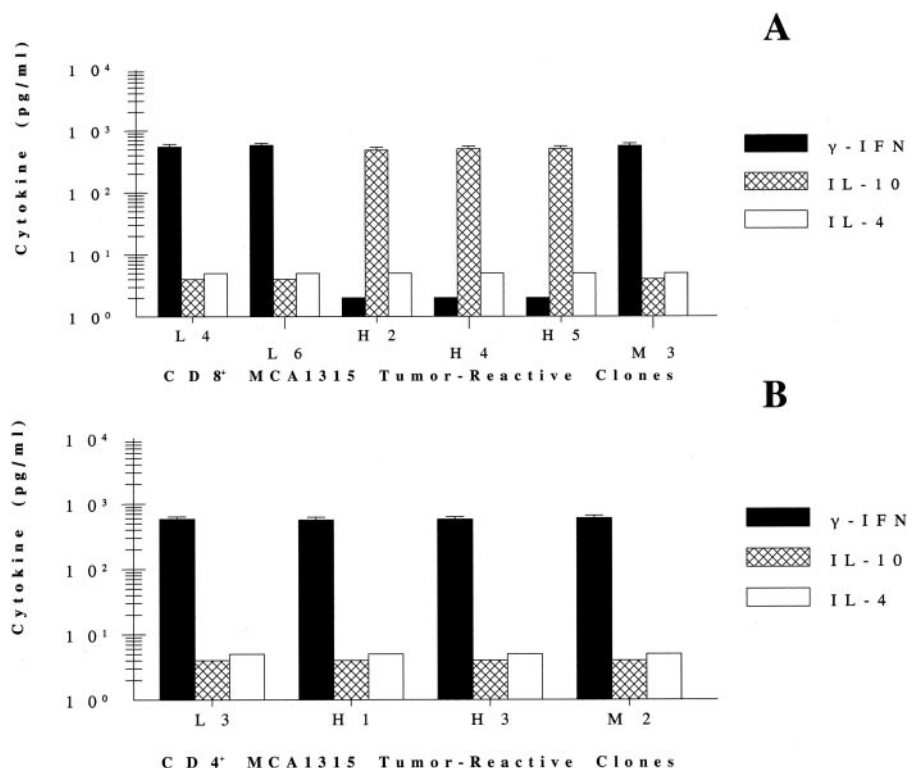
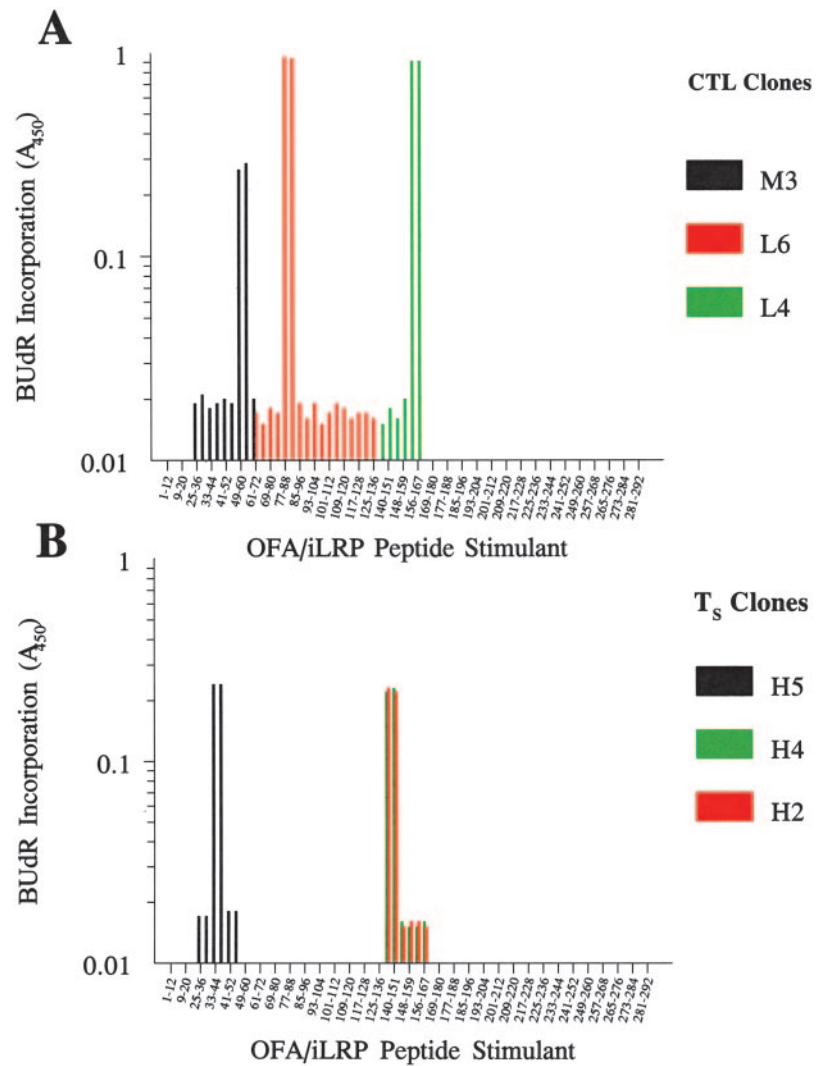


FIGURE 5. Proliferation profile of OFA/iLRP-specific CD8 clones to overlapping 12-mer OFA/iLRP peptides which in toto span the OFA/iLRP protein. *A*, Proliferation of OFA/iLRP-specific CTL clones to 100 ng/well 12-mer OFA/iLRP peptides. *B*, Proliferation of OFA/iLRP-specific Ts clones to 100 ng/well 12-mer OFA/iLRP peptides. These data represent the means of three separate peptide-induced proliferation assays.



established three CD8 Ts clones (clones H2, H4, and H5). These mice also provided two CD4 Th1 clones (clones H1 and H3) (Figs. 2 and 3). The OFA/iLRP-immune CTL clones (clones L4 and L6) killed from 87 to 95% of MCA1315 fibrosarcoma cells, but killed normal spleen cells at background levels ($\sim 5\%$ cytotoxicity). Also, neutralizing anti-IL-10 Ab or control IgM (anti-B220) had no effect on the cytotoxicity levels exhibited by the OFA/iLRP-immune CTL clones (clones L4 and L6) (Fig. 4). The superior killing ability of clones L4 and L6 compared with that of CTL clone M3 correlates with the increased proliferation of the OFA/iLRP-immune CTL clones L4 and L6 to 100 ng/well OFA/iLRP presented by irradiated, syngeneic splenic APCs in the presence of IL-2 (Fig. 1*B*). All of the CD8⁺, OFA/iLRP-specific Ts clones (clones H2, H4, and H5) exhibited cytotoxicity against OFA/iLRP-expressing MCA1315 fibrosarcoma cells only in the presence of neutralizing anti-IL-10 Ab. However, substituting an irrelevant IgM Ab (anti-B220) eliminated the ability of the Ts clones to kill the OFA/iLRP-expressing tumor cells. The Ts cells killed only $\sim 55\%$ of the MCA1315 tumor cells in the presence of anti-IL-10. This is about half as much killing of MCA1315 tumor cells as was seen with OFA/iLRP-immune CTL clones L4 and L6 (Fig. 4). This is about as much cytotoxicity as OFA/iLRP-specific CTL clone M3 displayed and like clone M3, all Ts clones proliferated significantly less than the OFA/iLRP-immune CTL clone cells in response to 100 ng/well OFA/iLRP presented by syngeneic APCs in the presence of IL-2 (Fig. 1). This is probably due to the Ts clones and CTL clone M3 having lower affinity TCRs for OFA/iLRP than

do the other OFA/iLRP-immune CTL clone cells (clones L4 and L6).

OFA/iLRP epitopes for CTL, and Ts T cell clones are scattered throughout the OFA/iLRP protein sequence

The proliferation profile of OFA/iLRP-specific CTL clones to overlapping 12-mer OFA/iLRP peptides shows that each clone responds optimally to a pair of OFA/iLRP peptides (Fig. 5*A*). The two peptides that make up each pair to which the clones respond share an 8-aa sequence (Table I). We have denoted those sequences as the deduced OFA/iLRP epitopes recognized by these CTL clones. Two of the three clones appear to have high affinity TCRs specific for the OFA/iLRP epitopes to which they are specific because they respond to 100 ng/well of the peptides presented by syngeneic APCs by incorporating 55.4- to 59.7-fold more BrdU into their DNA than the baseline BrdU incorporation. These two clones (clones L4 and L6) are probably the progeny of memory OFA/iLRP-specific CTL. The other OFA/iLRP CTL (clone M3) probably was cloned from a naive OFA/iLRP-specific splenic CTL present in the spleen of that tumor-bearing mouse because it incorporates only 14.5-fold more BrdU than baseline when exposed to 100 ng/well of the OFA/iLRP peptides containing the epitope to which it is specific in the presence of irradiated, syngeneic APCs and IL-2. The strength of the proliferation response by each of these OFA/iLRP CTL clones to 100 ng/well

Table I. Summary of iLRP epitopes recognized by CD8⁺ iLRP-specific CTL and Ts clones

iLRP-Specific T Cell Clone	T Cell Type	Proliferation-Inducing iLRP Peptides	Proliferation-Inducing iLRP Peptide Amino Acid Sequences	Deduced iLRP Epitope Sequences
M3	CTL	49-60	INLKRTWEKLLL	RTWEKLLL
		53-64	RTWEKLLLAARA	(aa 53-60)
L6	CTL	77-88	ISSRNTGQRAVL	NTGQRAVL
		81-92	NTGQRAVLKFAA	(aa 81-88)
L4	CTL	152-163	SPLRYVDIAIPC	YVDIAIPC
		156-167	YVDIAIPCNNKG	(aa 156-163)
H5	Ts	33-44	QMEQYIYKRKSD	YIYKRKSD
		37-48	YIYKRKSDGIYI	(aa 37-44)
H2	Ts	136-147	EASYVNLPTIAL	VNLPTIAL
		140-151	VNLPTIALCNTD	(aa 140-147)
H4	Ts	136-147	EASYVNLPTIAL	VNLPTIAL
		140-151	VNLPTIALCNTD	(aa 140-147)

intact OFA/iLRP or to 10⁵ irradiated MCA-1315 fibrosarcoma cells (which express OFA/iLRP) is the same as for their proliferation response to 100 ng/well of the OFA/iLRP peptides which contain the epitopes to which they are specific (Fig. 1). The OFA/iLRP epitopes to which these CTL clones respond by proliferation appear to be spatially distributed somewhat more in the middle of the OFA/iLRP protein (between aa 52 and 160 (Fig. 5A and Table I).

The proliferation profile of OFA/iLRP-specific Ts clones to overlapping 12-mer OFA/iLRP peptides shows that each clone responds optimally to a pair of OFA/iLRP peptides (Fig. 5B). The two peptides that make up each pair to which the clones respond contain a shared 8-aa sequence (Table I). We have denoted those sequences as the deduced OFA/iLRP epitopes recognized by these OFA/iLRP-specific Ts clones. All three clones appear to have low to moderate affinity TCRs specific for the OFA/iLRP epitopes to which they are specific because they respond to 100 ng/well of the appropriate peptides presented by syngeneic APCs by incorporating 11.3- to 15.1-fold more BrdU into their DNA than the baseline BrdU incorporation. Thus, these Ts cell clones are probably not memory T cell clones. The strength of the proliferation response by each of these OFA/iLRP-specific Ts clones to 100 ng/well intact OFA/iLRP or to 10⁵ irradiated MCA-1315 fibrosarcoma cells (which express OFA/iLRP) is the same as for their proliferation response to 100 ng/well of the OFA/iLRP peptides which contain the epitopes to which they are specific (Fig. 1). The OFA/iLRP epitopes to which these Ts clones respond by proliferation appear to be spatially distributed similarly to those recognized by the OFA/iLRP-specific CTL clones (between aa 32 and 147 (Fig. 5B and Table I)). Interestingly, clones H2 and H4 both appear to recognize the

Table II. OFA/iLRP CTL and Ts clone epitope fit with H-2K^d motif

iLRP-Specific T Cell Clone	T Cell Type	Deduced iLRP Epitope Sequence	H-2K ^d Motif ⁺ Nonapeptide	Parker Score	Tubingen Score
M3	CTL	RTWEKLLL	K RTWEKLLL ^a	5.760	15
L6	CTL	NTGQRAVL	R NTGQRAVL	40	13
L4	CTL	YVDIAIPC	R YVDIAIPC	144	16
H2	Ts	VNLPTIAL	Y VNLPTIAL	40	9
H4	Ts	VNLPTIAL	Y VNLPTIAL	40	9
H5	Ts	YIYKRKSD	Q YIYKRKSD	12	15

^a Bold letters represent anchor residues for peptide in MHC protein.

Table III. OFA/iLRP CTL and Ts clone epitope fit with H-2D^d motif

iLRP-Specific T Cell Clone	T Cell Type	Deduced iLRP Epitope Sequence	H-2K ^d motif ⁺ Nonapeptide	Parker Score	Tubingen Score
M3	CTL	RTWEKLLL	K RTWEKLLL ^a	0.360	NA ^b
L6	CTL	NTGQRAVL	R NTGQRAVL	0.300	NA
L4	CTL	YVDIAIPC	R YVDIAIPC	0.120	NA
H2	Ts	VNLPTIAL	Y VNLPTIAL	1.000	NA
H4	Ts	VNLPTIAL	Y VNLPTIAL	1.000	NA
H5	Ts	YIYKRKSD	Q YIYKRKSD	0.015	NA

^a Bold letters represent anchor residues for peptide in MHC protein.

^b Not available.

same epitope. Either they both were derived from the same clone or the epitope recognized by them is one which is immunodominant in H-2^d mice. Even though the OFA/iLRP peptides containing the epitopes to which the CTL and Ts clones partially are spatially overlapped, the epitopes themselves are distinct and separable for these two types of OFA/iLRP T cells. This is even though the CTL and the Ts cells are both CD8⁺ class I MHC-restricted T cell clones.

Determination of the H-2^d class I protein restriction of the deduced OFA/iLRP peptide epitopes

Comparison of the deduced OFA/iLRP epitopes recognized by CTL and Ts clones with the K^d, L^d, and D^d peptide-binding motifs showed that all CTL and Ts clone nonapeptide epitopes should be moderately bound by K^d as shown by both the Parker score and the Tubingen score (Table II). In contrast, none of the OFA/iLRP nonapeptide epitopes which induces proliferation of the CTL and Ts clones appear to be able to be presented by D^d class I proteins because the Parker scores are near 0 for the D^d motif (Table III). The OFA/iLRP nonapeptide epitopes recognized by all clones except CTL clone L4 and Ts clone H5 probably can weakly be presented by L^d proteins because their Tubingen scores are very similar to those for K^d protein motif binding. The Parker score, however, is considerably lower for nonapeptide epitopes of all clones when calculated for L^d compared with K^d (Table IV). The nonapeptide epitopes recognized by CTL clone L4 and Ts clone H5 appear to probably only be able to be presented by K^d considering that Parker and Tubingen scores for the L4 epitope are 1.000 and 0, respectively for L^d and 144.000 and 16, respectively, for K^d. Similarly, the Parker and Tubingen scores for the H5 epitope are 0.100 and 1, respectively for L^d and 12 and 15 for K^d (Tables II and IV). Table V summarizes this and shows that probably all of the CTL and nonapeptide epitopes of the Ts

Table IV. OFA/iLRP CTL and Ts clone epitope fit with H-2L^d motif

iLRP-Specific T Cell Clone	T Cell Type	Deduced iLRP Epitope Sequence	H-2L ^d Motif ⁺ Nonapeptide	Parker Score	Tubingen Score
M3	CTL	RTWEKLLL	K RTWEKLLL ^a	0.500	11
L6	CTL	NTGQRAVL	R NTGQRAVL	5.000	12
L4	CTL	YVDIAIPC	R YVDIAIPC	1.000	0
H2	Ts	VNLPTIAL	Y VNLPTIAL	5.000	12
H4	Ts	VNLPTIAL	Y VNLPTIAL	5.000	12
H5	Ts	YIYKRKSD	Q YIYKRKSD	0.100	1

^a Bold letters represent anchor residues for peptide in MHC protein.

Table V. Summary of OFA/iLRP CTL and Ts clone epitope MHC restriction

iLRP-Specific T Cell Clone	T Cell Type	Nonapeptide Epitope Sequence	Epitope Position	Class I H-2 ^d restriction
M3	CTL	KRTWEKLLL	52–60	K ^d >>>L ^d
L6	CTL	RNTGQRAVL	80–88	K ^d >>>L ^d
L4	CTL	RYVDIAIPC	155–163	K ^d
H2	Ts	YVNLPITAL	139–147	K ^d >>>L ^d
H4	Ts	YVNLPITAL	139–147	K ^d >>>L ^d
H5	Ts	QYIYKRKSD	36–44	K ^d

clones are mostly presented on K^d class I proteins, but all except that for L4 and H5 could probably weakly be presented on L^d class I proteins also.

Demonstration that the deduced nonamer OFA/iLRP peptides do induce proliferation by the appropriate OFA/iLRP-specific T cell clone

Fig. 6A shows that the OFA/iLRP CTL clones M3, L4, and L6 specifically are induced to proliferate by either intact OFA/iLRP or the nonamer peptide deduced from their proliferative profile (Fig. 5 and Table II). That is, M3, L4, and L6 cloned cells proliferate to the nonamer peptides with the sequence of OFA/iLRP amino acids 52–60, 155–163, and 80–88, respectively. Each clone responds equally to the intact OFA/iLRP and to the appropriate OFA/iLRP peptide presented by irradiated, syngeneic spleen cells (Fig. 6A). Similarly, the OFA/iLRP-specific CD8, Ts clones H2, H4, and H5 proliferate to intact OFA/iLRP or to nonamer peptides with the sequence of OFA/iLRP aa 139–147 and 36–44, respectively (Fig. 6B). As with the CTL clones, the Ts clones responded equally to the intact OFA/iLRP and to the peptide which contained the sequence of their epitope. As was seen earlier, (Fig. 5 and Table II), the same nonamer peptide (peptide H2-aa 139–147) induces proliferation of clone H2 and H4 to equivalent amounts. That this is a specific response is shown by the lack of response to any of the

FIGURE 6. Proliferation of OFA/iLRP-specific CD8 clones to intact OFA/iLRP or 9-mer OFA/iLRP peptides which appear to be the epitopes to which the clones should respond. A, Proliferation of OFA/iLRP-specific CTL clones to 100 ng/well intact OFA/iLRP or OFA/iLRP peptides representing aa 36–44 (pep H5), 52–60 (pep M3), 80–88 (pep L6), 139–147 (pep H2), or 155–163 (pep L4). B, Proliferation of OFA/iLRP-specific Ts clones to 100 ng/well intact OFA/iLRP or the same OFA/iLRP 9-mer peptides. These data represent the mean of three separate peptide-induced proliferation assays.

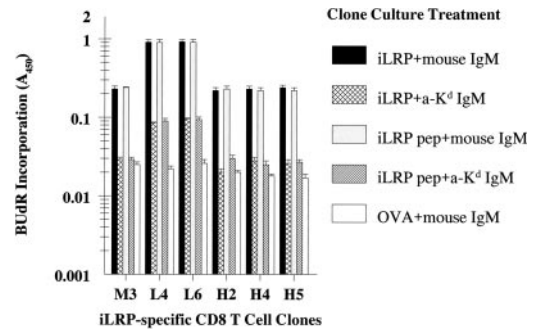
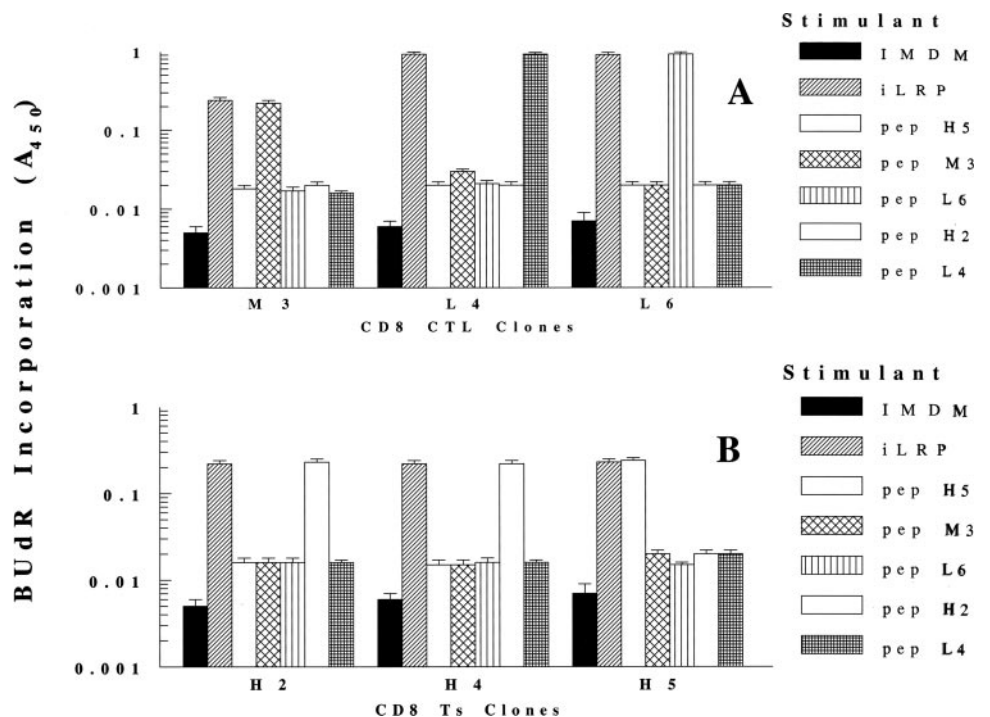


FIGURE 7. Inhibition of proliferation of OFA/iLRP-specific CD8 CTL and Ts clones to intact OFA/iLRP or 9-mer OFA/iLRP peptides to which the clones respond by anti-H2-K^d Ab. These data represent the mean of three separate inhibition of proliferation assays.

other peptides. As in the other proliferation assays, the nonamer peptides induced the same amounts of proliferation as the intact protein or 12-mer peptides. Thus, CTL clones L4 and L6 proliferated strongly to all, while CTL clone M3 and all Ts clones proliferated significantly less well. This may be due to clones L4 and L6 having higher affinity TCRs for their epitopes than do CTL clone M3 or any of the Ts clones.

The proliferation of OFA/iLRP CD8 clones to intact rOFA/iLRP or to the appropriate OFA/iLRP nonamer peptide epitope is inhibited by the presence of monoclonal anti-H2-K^d Ab

As was predicted by motif analysis of the OFA/iLRP peptides that appeared to induce proliferation of the CD8 clones (Tables II–IV), anti-H2-K^d Ab inhibits the ability of OFA/iLRP-presenting APCs to induce proliferation of the CTL and Ts clones (Fig. 7). Anti-K^d Ab inhibited the proliferation of the OFA/iLRP-specific CTL clones to intact OFA/iLRP 87.4–90.7% (mean ± SEM, 89.2 ± 0.9) and inhibited the proliferation of Ts clones to intact OFA/iLRP 87.7–90.9% (mean ± SEM, 89.3 ± 0.9). Similarly, in response to the appropriate peptide epitope sequence for each clone,

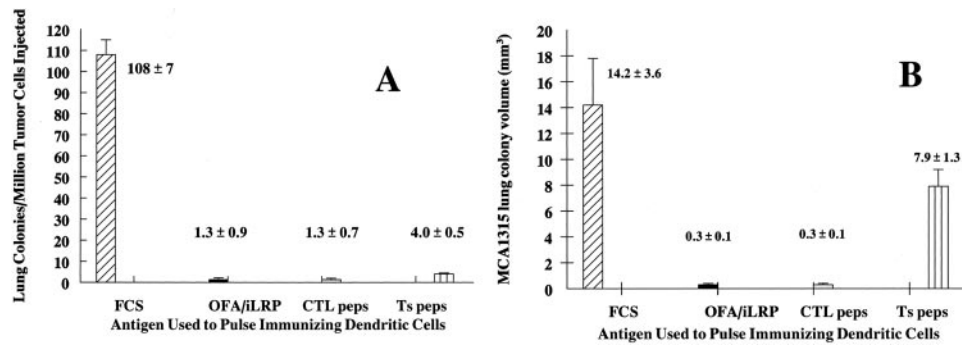


FIGURE 8. Inhibition of in vivo MCA1315 fibrosarcoma lung metastasis and lung colony growth in BALB/c mice immunized with syngeneic mature DCs which were pulsed with intact OFA/iLRP, mixtures of two OFA/iLRP CTL clone-activating peptides (pep L4 and pep L6) or two OFA/iLRP Ts clone-activating peptides (pep H2 and pep H5). *A*, Lung colonies/ 10^6 injected MCA1315 tumor cells in FCS-, intact OFA/iLRP-, or OFA/iLRP peptide-pulsed mature DC-immunized mice 2 wk after i.v. tumor cell injection. *B*, MCA1315 tumor lung colony volume in FCS-, intact OFA/iLRP-, or OFA/iLRP peptide-pulsed mature DC-immunized mice 2 wk after i.v. tumor cell injection.

the CTL clones were inhibited 87.9–90% in the presence of monoclonal anti-H2-K^d Ab (Fig. 7). The OFA/iLRP-specific proliferative responses of the Ts clones to the appropriate peptide epitope sequences were inhibited 87–88.6% by the presence of anti-H2-K^d mAb. That the proliferative responses of the clones are specific is shown by the fact that they proliferate in response to either intact OFA/iLRP or the appropriate nonamer peptide sequence presented by irradiated syngeneic spleen cells 9.2- to 41.3-fold more than to OVA presented by the same APCs. Thus, the nonamer sequences listed in Table II which were deduced to be the epitopes recognized by these T cell clones and the clones apparent MHC restriction to K^d are borne out in Figs. 6 and 7 in that the deduced nonamer peptide epitopes do specifically induce proliferation by the appropriate clone and all are inhibitable by inclusion of anti-K^d Ab in the proliferative assays.

Immunization of BALB/c mice with syngeneic mature DCs pulsed with intact OFA/iLRP or mixtures of OFA/iLRP peptides inhibit tumor metastasis and growth in vivo

Immunization with intact OFA/iLRP-pulsed mature DCs reduced MCA1315 lung colony counts by 98.8% (Fig. 8A). Not only were there significantly fewer lung tumor colonies in the intact OFA/iLRP-pulsed mature DC-immunized mice, but the tumor colony volume was also reduced by 97.9% compared with the FCS/medium-pulsed DC-injected control mice (Fig. 8B). When BALB/c mice were immunized with mature DCs that had been pulsed with the mixture of CTL (clones L4 and L6) activating OFA/iLRP peptides and subsequently injected i.v. with MCA1315 fibrosarcoma cells, there was the same reduction in tumor lung colony numbers and tumor lung colony volume as occurs after intact OFA/iLRP-pulsed DC immunization (Fig. 8).

Surprisingly, immunization with mature DCs pulsed with the OFA/iLRP peptides that activate the H2/H4 and H5 Ts clones, resulted in 96% inhibition of MCA1315 lung colony numbers after i.v. tumor cell injection compared with that in mice immunized with FCS-pulsed DCs (Fig. 8A). However, the tumor colonies which did form in the lungs of Ts clone-activating OFA/iLRP peptide-pulsed DC-immunized mice were significantly larger than those of mice immunized with intact OFA/iLRP- or CTL clone-activating OFA/iLRP peptide-pulsed DCs (Fig. 8B). Indeed, the mice immunized with Ts clone-activating OFA/iLRP peptide-pulsed DCs, had MCA1315 tumor colonies that had only 56.7% less volume than did the FCS-pulsed DC-immunized (normal control) mice. Intact OFA/iLRP- or CTL clone-activating OFA/iLRP peptide-pulsed DC immunization resulted in 97.9% reduction in

colony volume on top of 98.8% reduction in the number of colonies. Thus, the OFA/iLRP peptides identified as activating proliferation of OFA/iLRP-specific T cell clones can be used to effectively immunize against tumor cell metastasis and growth in vivo.

Spleen CD8 T cells from mature DC-immunized mice differ in the cytokines induced by intact OFA/iLRP depending on what the DCs had been pulsed

Fig. 9 shows that while none of the spleen CD8 T cells secrete IL-4 after stimulation with 100 ng/well intact OFA/iLRP presented by x-irradiated, T cell-depleted, syngeneic spleen cells, distinct patterns of IL-10 and IFN- γ secretion was seen depending on the Ag presented in the mice by the mature DCs. Because the stimulation culture was done in complete IMDM which was supplemented with 10% FCS, a 6-fold-increase over baseline detectable levels of both γ -IFN and IL-10 was seen with splenic CD8 T cells derived from mice immunized with FCS-pulsed mature DCs. However, the splenic CD8 T cells harvested from BALB/c mice immunized with mature DCs which had been pulsed with intact OFA/iLRP or a mixture of two OFA/iLRP-specific CTL clone-activating OFA/iLRP nonamer peptides responded to 100 ng/well intact OFA/iLRP by secreting 48.9- or 34.3-fold more IFN- γ , respectively, than did the FCS-pulsed DC-immune spleen CD8 T cells when cultured with OFA/iLRP in vitro. Those same CD8 T cells secreted only 1.6- and 1.2-fold more IL-10, respectively, than the FCS-pulsed DC-immune CD8 T cells when cultured with intact OFA/iLRP in vitro. Thus, the intact OFA/iLRP and OFA/iLRP peptides

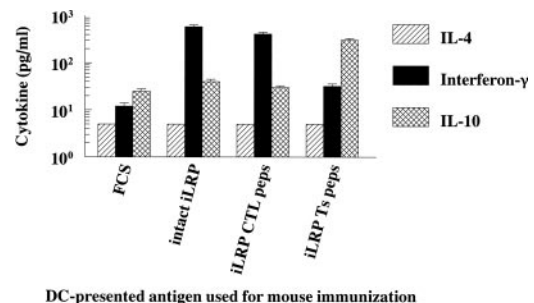


FIGURE 9. OFA/iLRP induces different cytokine profiles in vitro from CD8 T cells from spleens immunized in vivo with OFA/iLRP-specific Ts clone-activating peptides (pep H2 and H5), OFA/iLRP-specific CTL clone-activating peptides (pep L4 and L6), or intact OFA/iLRP presented by mature syngeneic DCs.

that activate CTL clones, not only induce potent inhibition of tumor lung colony frequency and volume when presented by mature DCs, but also induce spleen CD8 T cells that predominantly secrete IFN- γ with only some IL-10 when stimulated in vitro by intact OFA/iLRP.

In contrast, the splenic CD8 T cells from mice immunized with mature DCs pulsed with a mixture of two OFA/iLRP-specific Ts clone-activating OFA/iLRP peptides, secreted 12.3-fold more IL-10 than the FCS-pulsed mature DC-immunized mouse CD8 splenic T cells, but only 2.7-fold more IFN- γ when stimulated with 100 ng/well intact OFA/iLRP in vitro. Thus, OFA/iLRP-specific Ts clone-activating OFA/iLRP peptides presented by mature, syngeneic DCs induce splenic CD8 T cells in vivo that secrete 10 times more IL-10 than IFN- γ in response to intact OFA/iLRP presented by irradiated, syngeneic, T cell-depleted spleen cells in vitro. Therefore, even though the Ts peptide-pulsed DC-immune mice had a potent inhibition of tumor lung colony frequency and some inhibition of tumor lung colony volume, the CD8 T cells induced were predominantly secreting IL-10.

No detectable IL-10, IL-4, or IFN- γ was found in any of the CD8 T cell cultures when the T cells are cultured with 100 ng/well OVA presented by irradiated, syngeneic, T cell-depleted spleen cells (data not shown). Thus, the cytokine responses shown in Fig. 9 were specific because they did not occur if the stimulating Ag used in vitro was irrelevant to the in vivo immunization procedure that induced those CD8 T cells.

Discussion

A problem with trying to use the immune system to defeat cancer is that most of the proteins expressed by tumors are also expressed by normal cells and so only a small number of proteins are available to target. Also, some of those tumor proteins can be recognized by T lymphocytes only because of mutations that have induced neopeptides (6). Thus, a second mutation in the tumor cells and amplified by selection to escape immunity may end the effectiveness of that T cell response. A number of tumor type-specific Ags recognized by CTL are being used for immunotherapy targeting of those tumors. But, even these clinically effective treatments are limited because only a certain type of tumor, such as melanoma, may express that immunogen. Because of immune surveillance, a selection of tumor cells which have developed a method of evading immune effectors constantly occurs. This may be through tumor cell down-regulation of the protein against which the T lymphocytes are responding (45), down-regulation of class I MHC expression to effectively block CTL recognition of the tumor cells (18), or through initiation of immunosuppressive cytokine secretion by the tumor cells (46). To overcome all of these potential impediments to use effectively an antitumor T lymphocyte response therapeutically, one needs an immunogenic tumor protein which the tumor cell cannot easily alter because of multiple immunogenic epitopes which activate both Th and CTL cells. Also, if it is a protein which is important for the tumor cell to use for its growth or metastasis, it may be difficult for the tumor cell to survive or thrive if mutations or down-regulation occur.

We have previously shown that rodent tumor bearers and human breast cancer patients produce a vigorous CTL and Th1 response to OFA/iLRP during tumor development (27, 28). We have also shown that OFA/iLRP begins to be expressed in tumor cells early in their transformation (47). Others have reported that iLRP expression is important for tumor cell metastasis and that the most metastatic human tumors express increased amounts of this protein (23, 24). Immature laminin receptor protein is up-regulated upon laminin binding by some of the surface iLRP and integrin laminin receptors (25). The iLRP is coregulated with certain of those lami-

nin-binding integrins (48). Not only are iLRP and the laminin-binding integrins up-regulated by laminin binding, but that also induces metalloproteinase secretion and activation which promotes metastatic ability (26). Also, iLRP interaction with laminin induces breakdown of laminin into peptides that promote tumor cell motility, thus also promoting metastatic ability (49). Thus, OFA/iLRP is important to the metastatic potential of the tumor cells. One would think, therefore, that tumor cells would not thrive if they down-regulated OFA/iLRP to escape an anti-OFA/iLRP effector T cell response. On top of this, the sequence of OFA/iLRP is evolutionarily conserved to a very high degree (19, 50). Murine and human OFA/iLRP differ by only a few amino acids of 295 total in the protein. That suggests that very little mutation of the *OFA/iLRP* gene is allowable for a functional protein to be made. If that is the reason for the evolutionary conservation of the OFA/iLRP sequence, then there should be little selective pressure for the tumor cells to try and escape anti-OFA/iLRP attack through mutation of the OFA/iLRP itself. Doing so might adversely affect the survival of the tumor cells in the cancer patient or tumor-bearing animal. Indeed, immunization with DCs pulsed with renal cell carcinoma lysate (which contains OFA/iLRP) has prolonged the life of terminal renal cell carcinoma patients by 4- to 6-fold longer than their typical lifespan with metastatic renal cell carcinoma. Also, the disease process in those patients is either stable or cured (43). That this was due to OFA/iLRP immunity induced by the DCs was suggested by the fact that those patients with a Th1 response to OFA/iLRP were the only ones in which remission or stabilization of the disease occurred.

We have previously shown that in human and rodent cancer, not only are OFA/iLRP-specific effector Th1 and CTL induced, but also regulatory Ts cells which are potentially CTL, but which secrete IL-10 that inhibits both Th1 activation and cytokine synthesis and also inhibits CTL activity (27–29). These Ts cells are OFA/iLRP-specific. We have shown both in this paper and previously that these Ts cells appear to have a low affinity TCR for OFA/iLRP: class I MHC complex and so they are induced and respond only when at least 10-fold higher doses of OFA/iLRP is present than is required to induce the effector T cells (22, 27, 28). Thus, one could potentially get around the IL-10-secreting Ts cells induced by OFA/iLRP if lower amounts of OFA/iLRP are used for immunization. In the renal cell carcinoma study (43), immunization of the patients with more than three doses of tumor lysate-pulsed DCs produced no amelioration of disease and may have been due to induction of those Ts cells. Some data has suggested that breast cancer patients which express levels of Ts cells above some threshold are more likely to have recurrences than patients with either a higher T effector:Ts ratio or more T effector cells specific for OFA/iLRP (28). The data presented herein shows that besides dose, one should be able to immunize with selected OFA/iLRP peptides and not have to worry about the Ts cells. We find that even though they and the OFA/iLRP-specific CTL are both K^d-restricted CD8 T cells, none of the epitopes which are recognized by the CTL clones are recognized by the Ts cells (Fig. 5, Tables I and V). This also suggests that just picking out a peptide or mixture of peptides from the OFA/iLRP sequence that will be bound and presented by an appropriate class I MHC protein may not produce the effects desired if the dose is too high because it may be the Ts cell epitope which is selected (51).

By combining T cell cloning or polyclonal T cell ELISPOT analysis with MHC motif database searching, an optimal set of OFA/iLRP peptides containing only CTL or Th1 and CTL epitopes may be selected so optimal immunotherapy is possible. That this protein expresses epitopes able to activate Th1 cells (21, 28) combined with our finding that immunization with higher

doses of OFA/iLRP induces high titers of anti-OFA/iLRP IgG (22) suggests that probably Th2 cells also recognize OFA/iLRP. This is important because it has been reported that tumors which have down-regulated their class I MHC molecules can still be killed by Th2-induced eosinophil activation and degranulation in the tumor bed (14). It is, therefore, important to have epitopes recognized by CD4 T cells. We herein show that OFA/iLRP is immunogenic for CD4 and CD8 T cells. We have identified epitopes recognized by CD8 CTL and IL-10-secreting Ts cells that are distinct. Also, those epitopes are scattered between aa 32 and 160 (of 295 total) so tumor cell mutation of a small section of the protein, even if it does not effect the protein's function, should not block immune recognition and attack of the tumor cells.

Usually a protein has a few immunodominant epitopes for a given MHC haplotype (52). That four of the six CD8 T cell clones recognized distinct OFA/iLRP epitopes which appear to be presented by K^d class I proteins may be due to the small number of clones tested. Even if these epitopes are not the immunodominant OFA/iLRP epitopes presented by K^d, it is possible for subdominant epitopes to be immunogenic and even induce protective immunity (53, 54).

In analysis of the binding ability for our deduced nonapeptide OFA/iLRP epitopes, the Tubingen and Parker scores are determined by an extended anchor-based motif algorithm (40) and an affinity matrix algorithm (39, 55), respectively, do not return exactly the same results due to variations in matrix development and scoring methods. Anchor-based motif algorithms are somewhat insensitive, in that they miss all ligands that do not express the dominant anchors (56).

That these algorithms show that our nonapeptide epitopes are at best able to moderately bind the K^d or L^d class I proteins, may be offset by the affinity of the T cell clones' TCRs. This is especially true because of the degeneracy of TCR recognition of peptide:MHC protein complexes (57, 58). The data presented herein suggest that different functional types of OFA/iLRP-specific T cell clones are restimulated to proliferate and secrete cytokines by distinct OFA/iLRP epitopes.

We have shown that immunizing BALB/c mice with syngeneic, bone marrow-derived DCs which have been pulsed with different mixtures of OFA/iLRP peptides containing two CTL or two Ts epitopes or with intact OFA/iLRP inhibits metastasis and growth of subsequently i.v. injected MCA1315 fibrosarcoma cells. Surprisingly, immunization with Ts clone-activating OFA/iLRP peptide-pulsed DCs induced potent inhibition of lung colony establishment by the tumor cells, though not quite as much as did intact OFA/iLRP or OFA/iLRP-specific CTL clone-activating OFA/iLRP peptide-pulsed DC immunization (96 vs 98.8%, respectively). That the Ts cells can inhibit CTL activity and Th1 and CTL secretion of γ -IFN (29) would have suggested that tumor promotion would occur after such immunization. That it did not may reflect the potent type I immunity induced by mature DC immunization (59). However, when one considers the biology of the CD8, IL-10-secreting OFA/iLRP-specific T cell clones, the data do make sense. We have denoted these IL-10-secreting CD8 OFA/iLRP-specific T cells as Ts cells, but their suppression is totally mediated by the IL-10 they secrete (29). Indeed, if anti-IL-10 mAbs are included in the cytotoxicity assays, even the "Ts" cells are cytotoxic for OFA/iLRP-expressing, syngeneic tumor cells (Fig. 4 and Ref. 29). This is true for Ts clones derived from tumor-bearing mice, OFA/iLRP-immunized mice, and human breast cancer patients (28, 29). It is also true for noncloned splenic T cells derived from OFA/iLRP-immunized mice (22). Although the IL-10 inhibits CTL killing of OFA/iLRP-expressing tumor cells, it has been reported by others that IL-10 can inhibit metastasis by

activating tissue inhibitor of metalloproteinases (60) and thus block enzymatic digestion of extracellular matrix collagen which is needed to allow extravasation from the blood stream into tissue or for migration from a tissue into the blood stream. Indeed, we have found that IL-10 can inhibit MCA1315 fibrosarcoma cell invasion of extracellular matrix protein-coated filters in vitro in a dose-dependent manner (our unpublished results). Thus, the reduction in tumor lung foci in the OFA/iLRP-specific Ts clone OFA/iLRP peptides-pulsed DC-immunized mice is probably due to the effects of IL-10 on TIMP activation in the immunized mice resulting in reduced intravasation into the lung because of reduced tumor cell metalloproteinase activity (60). That the tumor immunity inhibiting effects of IL-10 were probably present in the Ts clone-activating peptide-immunized mice is suggested by the fact that although the tumor colony volume was reduced from the control level in such mice, there was a 26.3-fold increase in tumor colony volume in those mice compared with mice immunized with intact OFA/iLRP- or OFA/iLRP-specific CTL clone-activating peptide-pulsed DCs. The cytokine profiles of spleen cells taken from the four groups of immunized mice at the time other members of those groups were being injected i.v. with tumor cells show that the OFA/iLRP Ts clone-activating peptides-pulsed DC-immunized mice would have had significantly increased levels of IL-10 and decreased levels of IFN- γ compared with the OFA/iLRP-specific CTL clone-activating peptide- or intact OFA/iLRP-pulsed DC-immunized mice (Fig. 9).

Although the tumor lung colony frequency reductions by all OFA/iLRP-pulsed DC immunizations were very similar numerically, the biological mechanisms resulting in those reduced tumor lung colony counts were probably distinct. That is, the mice immunized with OFA/iLRP-specific CTL clone-activating OFA/iLRP peptides- or intact OFA/iLRP-pulsed DCs, developed, one would expect, high numbers of OFA/iLRP peptide:K^d protein-specific CTL which reduced tumor lung colony frequency and volume by killing the invading tumor cells. Also, upon activation, those cells secreted large amounts of IFN- γ , which further activates immune effectors and inhibits type II immunity. However, as suggested above, the OFA/iLRP-specific Ts clone-activating OFA/iLRP peptides-pulsed DC-immunized mice developed high numbers of OFA/iLRP peptide:K^d protein-specific Ts cells which upon activation by the appropriate K^d protein-presented OFA/iLRP peptide began secreting large amounts of IL-10 which would have activated TIMP (60) and thus have inhibited tumor cell metalloproteinase digestion of extracellular matrix and so have limited the frequency of the tumor lung colonies. Unfortunately, the IL-10 would also have interacted with any tumor-reactive CTL induced by the influx of tumor cells and the IL-10 would have inhibited the ability of those CTL (as well as, themselves) from killing the tumor cells that did get into the lung (29). So, in the Ts peptide-immunized mice, the tumor colonies that did occur were significantly larger than in the CTL peptide-immunized mice.

An alternative explanation of the differences between OFA/iLRP-specific CTL clone-activating peptides-pulsed DC- and OFA/iLRP-specific Ts clone-activating peptides-pulsed DC-immunization effects on tumor lung colony frequency and volume is that the Ts cell-inducing OFA/iLRP peptides are less immunogenic than are the CTL-inducing OFA/iLRP peptides and thus less killing of tumor cells and larger tumor colony volumes occurred in the former. However, the fact that Fig. 9 shows a significant difference in cytokine secretion profiles of spleen cells taken from the mice so immunized when the CD8 spleen cells are cultured with APCs and intact OFA/iLRP suggests that the immunizations induced qualitatively different immune states, at least by cytokine profile measurements. Indeed, as much as a 10-fold increase in

IL-10 is produced by the CD8 spleen cells derived from Ts OFA/iLRP peptides-DC in vivo immunization compared with the CD8 spleen cells derived from CTL OFA/iLRP peptides-DC in vivo immunization when exposed in vitro to identically presented doses of intact OFA/iLRP.

The experiments shown herein identify the epitopes recognized by CD8 T cell clones specific for OFA/iLRP and directly show the ability of immunization with DCs pulsed with OFA/iLRP or OFA/iLRP peptides to limit tumor growth and metastasis in vivo. They also demonstrate differences in cytokine production and thus, effector function, induced by different OFA/iLRP peptides presented in vivo by DCs.

Disclosures

The authors have no financial conflict of interest.

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