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Autoimmune Mediated Regulation of Ovarian Tumor Growth

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Abstract

Objectives—An immune response sufficient to induce organ failure may provide protection and therapy against tumors derived from the targeted organ particularly when removal or ablation of the organ is part of the standard therapy and does not threaten survival. We have previously shown that a targeted immune response directed against the ovarian-specific protein, inhibin- α , causes ovarian failure. Here we determined whether inhibin- α autoimmunity is effective in both prevention and treatment of ovarian tumors.

Methods—A transgene consisting of the SV40 large tumor transformation antigen under the regulation of an anti-Mullerian hormone promoter (AMH-SV40Tag) was transferred by backcrossing for 12 generations to SJL/J mice producing SJL.AMH-SV40Tag (H-2^s) females that develop a high incidence of autochthonous granulosa cell tumors. We determined whether immunization of SJL.AMH-SV40Tag female mice with the IA^s-restricted p215-234 peptide of mouse inhibin- α was capable of preventing and treating these ovarian tumors.

Results—The growth of autochthonous ovarian granulosa cell tumors in SJL.AMH-SV40Tag transgenic mice was significantly inhibited in mice immunized with In α 215-234. In addition, significant inhibition of tumor growth occurred when mice with established ovarian granulosa cell tumors were therapeutically vaccinated with In α 215-234.

Conclusions—Our results indicate that induction of ovarian-specific autoimmunity may serve as an effective way to prevent the emergence of autochthonous ovarian tumors and control the growth of established ovarian malignancies.

Keywords

Autoimmune; Ovary; Cancer Vaccine; Premature Ovarian Failure

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INTRODUCTION

Strategies for cancer therapy have long included targeted vaccination against antigens overexpressed in tumors. Such antigens have included amplified mutated proteins, cancer testis antigens, splice variants, stem cell antigens, and viral proteins (1). In addition, recent studies have indicated that tissue-specific differentiation antigens expressed by both normal tissues and their derived tumors may also serve as effective targets for cancer vaccination. For example, regression of metastatic melanoma is associated with the appearance of autoimmune vitiligo in patients receiving T cells specific for the melanoma antigen recognized by T cells (MART-1)⁴, a normal non-mutated differentiation antigen expressed on both normal melanocytes as well as most melanomas (2,3). In addition, a significant survival advantage occurs in patients with metastatic hormone refractory prostate cancer after transfer of autologous dendritic cells activated and pulsed with prostate acid phosphatase (PAP), a differentiation antigen found in both normal prostate tissues and in the vast majority of prostate tumors (4). Thus, normally expressed autoantigens may be both effective and acceptable targets for cancer vaccination particularly if any resultant autoimmune consequences are tolerable. Such conditions are evident in cancers of the ovary, breast, prostate, and thyroid since the affected organs are often removed or ablated as part of therapy, and their removal, though traumatic, is not life-threatening.

Ovarian function is regulated primarily by a pituitary-ovarian axis comprised of activins and inhibins that are produced primarily in ovarian granulosa cells (5) and serve, respectively, as positive and negative regulators of the pituitary gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH; 6-8). We have previously shown that a targeted autoimmune attack directed against the ovarian-specific protein, inhibin- α , causes a novel form of experimental autoimmune oophoritis (EAO; 9). Immunization of SWXJ (H-2^{q,s}) mice with the p215-234 sequence of inhibin- α (In α 215-234) activates CD4⁺ T cells in an IA^s-restricted manner and induces a profound ovarian failure that mimics primary ovarian insufficiency (POI), a disease that affects 1% of women by age 40 and is responsible for infertility and substantial health risks in women (10).

In the current study we use a transgenic tumor mouse model to show that this same ovarian-specific autoimmunity serves as an effective way to prevent the emergence of ovarian tumors and inhibit the growth of established tumors. Thus, our data establish the efficacy of a full-strength targeted autoimmune attack in both prevention and treatment of ovarian tumors.

MATERIALS AND METHODS

Mice

The generation of the AT-t94 transgenic mice has been described previously (11,12). Briefly, the AMH-SV40Tag transgene is a fusion construct containing 3.6 kb of the 5' flanking region of the human anti-Mullerian hormone (AMH) gene upstream of the SV40 proto-oncogene encoding the large transforming antigen (SV40-Tag). Fertilized eggs of B6D2F₁ (H-2^{b,d}) mice were microinjected with the fusion genes, and eggs were transferred into oviducts of pseudopregnant B6CBAF₁ (H-2^{b,k}) females. Transgenic offspring were backcrossed for several generations with C57BL/6 mice, and males from the AT-t 94 (H-2^b)

⁴Abbreviations used: 9-fluorenylmethoxy-carbonyl (FMOC); anti-Mullerian hormone (AMH); complete Freund's adjuvant (CFA); epidermal growth factor receptor tyrosine kinase (HER2/neu); experimental autoimmune oophoritis (EAO); follicle stimulating hormone (FSH); luteinizing hormone (LH); Magnetic Resonance Imaging (MRI); melanoma antigen recognized by T cells (MART-1); mouse ovarian surface epithelial cells (MOSEC); p215-234 peptide sequence of inhibin- α (In α 215-234); primary ovarian insufficiency (POI); prostate acid phosphatase (PAP); quantitative real-time RT-PCR (qRT-PCR); rapid acquisition with relaxation enhancement (RARE); simian vacuolating virus 40 large transforming antigen (SV40Tag)

transgenic line were mated at the Cleveland Clinic with female SJL/J (H-2^s) mice purchased from Jackson Laboratories (Bar Harbor, ME). The resultant SJL $\times$ AT-t94 (H-2^{b,s}) transgenic offspring were backcrossed for 12 generations to SJL/J mice producing SJL.AMH-SV40Tag (H-2^s) mice used in the current study. Female SJL.AMH-SV40Tag mice develop a high incidence of autochthonous granulosa cell tumors and are able to respond to the IA^s-restricted In α 215-234. Transgenic mice were identified by RT-PCR amplification of the human AMH promoter from tail DNA.

Peptide synthesis

In α 215-234 (FLVAHTRARAPSAGERARRS) was synthesized by the Molecular Biotechnology Core Facility of the Lerner Research Institute using standard solid phase methodology and FMOC side chain-protected amino acids. The peptide was purified >97% by reverse phase HPLC, and amino acid composition was confirmed by mass spectrometry.

Vaccination

Mice were immunized *s.c.* in the abdominal flank with 200 μ g of In α 215-234 in 200 μ l of an emulsion of equal volumes of water and complete Freund's adjuvant (CFA) containing 400 μ g of *Mycobacteria tuberculosis* H37RA (Difco, Detroit, MI). Control mice were immunized with CFA alone. To determine efficacy of tumor prevention, female SJL.AMH-SV40Tag mice were immunized at 2 months of age and euthanized at 18 months of age for analysis of tumor load. To determine efficacy of tumor treatment, female SJL.AMH-SV40Tag mice with established tumors as determined by Magnetic Resonance Imaging (MRI) were immunized at 15 months of age and euthanized after a second MRI at 19 months of age. All mice were euthanized by asphyxiation with CO₂ followed by cervical dislocation, and each individual ovary was excised and weighed. All protocols received prior approval from the institutional animal care and use committee of the Cleveland Clinic in compliance with the Public Health Service policy on humane care and use of laboratory animals.

Magnetic Resonance Imaging (MRI)

Mice were anesthetized under 2.0 % isoflurane gas in oxygen for 5 minutes and transferred to a Bruker Biospec 9.4T MRI scanner (Bruker BioSpin, Billerica, MA) at the small animal MRI facility of Case Western Reserve University. A nosecone was used to deliver 1-2% isoflurane in oxygen to the animals during the MRI scans. The respiratory rate and core body temperature of each animal was maintained throughout the imaging procedure to ensure the safety of the animal. The sedated animals were placed into a cylindrical mouse volume coil (ID = 35 mm). A rapid acquisition with relaxation enhancement (RARE) pulse sequence was used to obtain high resolution T2-weighted coronal images of mouse ovaries (TR/TE = 2800/44 ms, 1 mm $\times$ 250 μ m $\times$ 250 μ m, RARE-factor = 8, number of averages = 4). Fat suppression was utilized in each acquisition to optimally delineate ovarian tumors. The area of each tumor was measured in each image (slice) by manually tracing along the edge of the tumor. The total tumor volume was calculated by multiplying the slice-by-slice tumor areas by the slice thickness (1 mm) and summing the individual slice volumes.

Immunocytochemistry

Ovarian tumors were fixed in 10% phosphate-buffered formalin (Fisher Scientific, Fair Lawn, NJ) and embedded in paraffin. Ovaries were sectioned at 5 μ m thickness, mounted on superfrost glass slides, and unmasked in 1 mM EDTA (pH 8.0) at 94°C for 6 minutes followed by a 30 minute cool-down period at room temperature. For identification of T cells, sections were blocked with 5% normal goat serum (Abcam, Cambridge, MA) and incubated with a 1:10 dilution of rat anti-mouse CD3 (NCL-CD3-12; Novocastra

Laboratories, Newcastle Upon Tyne, UK). For intracellular detection of IFN γ , sections were treated with 10% Triton (Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature, blocked with 5% normal goat serum (Abcam), and incubated with a 1:200 dilution of rat anti-mouse IFN γ (clone XMG1.2; BD Biosciences, San Diego, CA). Sections treated with primary antibodies were subsequently incubated with a 1:50 dilution of mouse-adsorbed biotinylated goat anti-rat IgG (BD Biosciences) in the presence of 5% BSA. Endogenous peroxidase activity was blocked by incubating mounted tissues with 1.5% H₂O₂ in methanol for 10 minutes, and detection of CD3+ and IFN γ + immunostaining was determined following incubation with streptavidin-horseradish peroxidase complex (ABC kit; Vector, Burlingame, CA) and subsequent treatment with diaminobenzidine and H₂O₂ substrate (BioGenex, San Ramon, CA). Between all steps, sections were washed thoroughly with PBS at room temperature. Slides were counterstained with hematoxylin 2 (Richard-Allan Scientific, Kalamazoo, MI), dehydrated in an ascending gradient of ethanol followed by xylene, and mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ) for examination by light microscopy.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from formalin fixed paraffin embedded ovarian tumors and formalin fixed paraffin embedded naive ovarian tissue with RNeasy FFPE kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cDNA was synthesized with random hexamers (Applied Biosystems, Foster City, CA) using M-MLV reverse transcriptase (Promega, Madison, WI). Expression of the genes encoding IFN γ , TNF α , IL-1 β , IL-17a, and IL-10 were quantified with the SYBER Green PCR Master Mix kit (Applied Biosystems) using primer pairs selected for amplification of each individual cytokine including: IFN γ sense 5'-TGATGGCCTGATTGTCTTTCAA-3', antisense 5'-GGATATCTGGAGGAACTGGCAA-3'; TNF α sense 5'-CAAAGGGAGAGTGGTCAGGT-3', antisense 5'-ATTGCACCTCAGGGAAGAGT-3'; IL-1 β sense 5'-GAGTGTGGATCCCAAGCAAT-3', antisense 5'-AGACAGGCTTGTGCTCTGCT-3'; IL-17a sense 5'-CTCCACCGCAATGAAGAC-3', antisense 5'-CTTCCCTCCGCATTGAC-3'; and β -actin sense 5'-GGTCATCACTATTGGCAACG-3', antisense 5'-ACGGATGTCAACGTCACACT-3'. Relative gene-expression was determined as the ratio of cytokine to β -actin gene expression levels for each tissue.

Biostatistical analysis

The unpaired Student *t*-test was used to analyze differences in mean ovarian tumor weights, mean change in tumor volumes, and mean ratios of cytokine expression levels in ovarian tumors between inhibin- α and control immunized mice. The paired Student *t*-test was used to analyze differences between pre- and post-treatment tumor volumes.

RESULTS

Development of an ovarian autoimmune/tumor model system

We hypothesized that a full strength autoimmune disease capable of causing organ-specific failure would also provide protection and therapy against tumors derived from the autoimmune targeted organ. To test this hypothesis, we developed a complex model system consisting of both a characterized autoimmune-induced ovarian failure and a derived ovarian tumor, all within the same mouse. We generated the former autoimmune component when we found that SWXJ (H-2^{q,s}) mice develop POI following immunization with the IA^s-restricted In α 215-234 (9). Incorporation of the tumor component was facilitated by mating AT-t94 (H-2^b) transgenic mice (obtained from Dr. Jean-Yves Picard, INSERM U782 and Univ Paris-Sud, Clamart 92140, France) with SJL/J (H-2^s) mice to produce SJLxAT-t94

(H-2^{b,s}) transgenic offspring. AT-t94 mice express the SV40Tag proto-oncogene under regulation of an anti-Mullerian hormone promoter (AMH-SV40Tag), and thereby develop a high incidence of autochthonous granulosa cell tumors (11,12). Backcrossing of (SJLxAT-t94)F₁ transgenic mice to the SJL/J (H-2^s) mouse strain for over 12 generations resulted in the production of SJL.AMH-SV40Tag (H-2^s) mice used in the present study. Similar to the original AT-t94 transgenic line, our SJL.AMH-SV40Tag mice develop granulosa cell tumors starting at 8-10 months of age and show an incidence of affected ovaries that exceeds 90% by 18 months of age.

Vaccination with Inα 215-234 prevents the emergence of autochthonous ovarian tumors

To determine whether autoimmune vaccination prevents the emergence of autochthonous ovarian tumors, we immunized 2 month old female SJL.AMH-SV40Tag mice with Inα 215-234 in CFA or with CFA alone and euthanized the mice at age 18 months. We found that mice immunized at 2 months of age with Inα 215-234 showed a decreased tumor incidence per mouse and per ovary with 31% of the treated mice being tumor free compared to none of the CFA immunized controls (Table 1). Median ovarian tumor load was 33 times lower in Inα 215-234 immunized mice and the difference in mean ovarian tumor weights between both groups was highly significant ($P<0.0006$; Figure 1a,b). Our results indicate the effectiveness of using a full strength autoimmune induced organ failure to prevent tumors derived from the targeted organ.

Vaccination with Inα 215-234 inhibits growth of pre-existing established ovarian tumors

We next determined whether vaccination with Inα 215-234 was effective in inhibiting tumor growth in mice with established ovarian granulosa cell tumors. To this end, we measured ovarian tumor volumes by MRI analysis in 15 month old SJL.AMH-SV40Tag female mice and subsequently immunized tumor bearing mice with Inα 215-234 in CFA and with CFA alone. Four months later, tumor volumes were measured again by MRI and the mice were euthanized.

Our results showed that all five individual control mice immunized with CFA had marked increased ovarian tumor growth over the four month post-vaccination period whereas 5/6 individual mice vaccinated with Inα 215-234 had only modest ovarian tumor growth and the remaining mouse vaccinated with Inα 215-234 showed an actual decrease in ovarian tumor volume by the end of the four month testing interval (Figure 2a; Figure 3). Ovarian tumor size was significantly increased in control mice immunized with CFA ($P=0.007$) but was not significantly increased in mice vaccinated with Inα 215-234 ($P=0.083$; Figure 2b). Moreover, the difference in total ovarian tumor growth (post-treatment minus pre-treatment tumor volumes) between CFA immunized mice and Inα 215-234 vaccinated mice was highly significant ($P=0.009$; Figure 2c, 3). A similar inhibitory trend in the growth of metastases was observed in Inα 215-234 vaccinated mice. However, the relatively low metastatic frequency precluded observing statistically significant differences in the development of extragonadal tumors (data not shown).

It is important to note that two mice with the largest tumors were included in the Inα 215-234 vaccination treatment group. This somewhat non-randomized selection process was done simply to prevent premature death of the two mice with large tumor loads. This anticipated protection was based on our observation that immunization with Inα 215-234 served as an effective preventive vaccine (Table 1; Figure 1). Our concerns were justified when one of the CFA vaccinated mice died soon after the first MRI analysis and was unable to be incorporated into our analysis of tumor volume changes. None of the Inα 215-234 vaccinated mice died prior to the final MRI analysis. It is worth noting that despite the higher pre-treatment tumor load in Inα 215-234 vaccinated mice (Figure 2a,b), their post-

treatment tumor volumes when considered independently from the pre-treatment volumes were significantly lower ($P=0.025$) than the post-treatment tumor volumes of CFA immunized control mice. Although it is possible that the large initial tumor load in In α 215-234 vaccinated mice may have induced greater tumor immunogenicity, analysis of tumor growth excluding the two In α 215-234 vaccinated mice with the largest tumor loads from the statistical analysis still indicated a significant inhibition of tumor growth in In α 215-234 vaccinated mice compared to CFA immunized mice ($P=0.03$). Thus, our results indicate that induction of an autoimmune response sufficient to induce ovarian failure by vaccination with In α 215-234 regulates growth of established ovarian tumors.

IFN γ producing T cells infiltrate ovarian tumors of mice vaccinated with In α 215-234

Since immunoregulation of tumor growth is mediated predominantly by T cells, we next addressed the role of T cells in regulating the growth of ovarian tumors in mice immunized with In α 215-234. We originally planned to evaluate tumor growth in recipients of defined populations of primed T cells and B cells. However, the SJL.AMH-SV40Tag females underwent a lengthy period of breeding deficiency similar to those observed in all of the originally described transgenic lines expressing the AMH-SV40Tag transgene (11,12). Our alternative approach involved immunocytochemical and molecular analysis of ovarian tumors taken 16 months after immunization with either In α 215-234 or CFA alone at 2 months of age. We observed an extensive infiltration of CD3+ T cells in ovarian tumors from In α 215-234 vaccinated mice but not in tumors from CFA immunized control mice (Figure 4a). Intracellular staining of the tumor infiltrating CD3+ T cells showed substantial production of IFN γ consistent with type-1 proinflammatory T cells (Figure 4b). Molecular analysis of formalin fixed ovarian tumor tissue by qRT-PCR confirmed the highly active proinflammatory nature of the T cells infiltrating the regulated tumors of In α 215-234 vaccinated mice by showing highly significant overexpression ($P<0.0005$ in all cases) of IFN γ , TNF α , IL-1 β , IL-17a, but not IL-10 when compared to tumors from mice immunized with CFA alone or when compared to formalin fixed ovaries from normal untreated mice (Figure 4c). Taken together, our data show that regulation of ovarian tumor growth in In α 215-234 vaccinated mice is accompanied by a very prominent and active infiltration of the tumors with type-1 proinflammatory T cells producing IFN γ .

DISCUSSION

Our study indicates that an immune response to an ovarian-specific differentiation protein sufficient to mediate ovarian failure is capable of providing both protection against the emergence of autochthonous ovarian tumors as well as therapy against established ovarian malignancies.

It is readily apparent that a strategy based on targeting tumor-specific antigens would theoretically provide precise destruction of tumors with minimal collateral damage to surrounding normal tissue and avoidance of the side-effects associated with non-specific targeting. However, such targeted strategies require the availability of a T cell repertoire capable of responding and expanding in an immunodominant manner to the tumor-specific antigen. Since many tumor-specific antigens are derived from defective, ectopic, or dysregulated proto-oncogenes, their ubiquitous overexpression during development may predispose to efficient thymic negative selection and the availability of a T cell repertoire comprising low affinity clones and an overall ineffective low avidity immune response (13). Indeed, such non-dominant and perhaps cryptic responses are a dynamic component of self-recognition plasticity in autoimmunity and may collectively provide clinically significant inflammation (14). However, individual non-dominant autoimmune responses often have limited clinical consequences and would likely provide sub-optimal tumoricidal activity. On the other hand, selection of immunodominant tissue-specific differentiation antigens capable

of causing targeted clinical organ failure may provide vigorous anti-tumor responsiveness albeit at the expense of incorporating substantial collateral damage to surrounding normal tissue. Thus, the trade-off for a vigorous immune response capable of inducing organ failure may be best suited for tumors derived from organs that are ablated or removed as part of therapy and whose removal is not life-threatening such as ovary, breast, prostate, and thyroid as previously suggested (3).

It is important to note that ovarian stromal cancers derived from granulosa cells represent only a small portion of ovarian tumors and that more than 80% of malignant ovarian cancers are of epithelial origin (15). Thus, it may be useful to determine whether autoimmune ovarian failure targeted to inhibin- α would be therapeutic in treating the more common epithelial derived forms of ovarian cancer. To this end TgMISIIR-TAg transgenic mice that spontaneously develop ovarian epithelial carcinomas (16) and transformed C57BL/6 mouse ovarian surface epithelial cells (MOSEC; 17) may serve as valuable transgenic reagents.

It is worth noting that the failure of the ovary *per se* does not account for the regulation of ovarian tumor growth. We have previously shown that ovarian failure is due to antibody mediated dysregulation of pituitary FSH release and occurs by 43-45 weeks after immunization of normal 2 month old mice (9). However, IFN γ producing T cells and overexpression of proinflammatory cytokines are prominent within ovarian tumors 64 weeks after immunization of 2 month old SJL.AMH-SV40Tag mice. Thus, long after the ovaries fail and atrophy, ovarian tumors are markedly inflamed and show extensive T cell infiltration and overexpression of proinflammatory cytokines. The lingering persistence of prominent intra-tumor T cell infiltrates long after the period when the ovaries show complete failure suggests that regulation of tumor growth may be mediated by T cells rather than by antibody mediated dysregulation of FSH. This view is supported by an extensive literature that has failed to implicate dysregulation of FSH or its receptor in the pathogenesis of ovarian cancer despite elevated serum levels in patients with ovarian malignancies (18-20). Experiments designed to distinguish the relative contributions of lymphocyte subsets and defined endocrine irregularities in tumor progression in our ovarian cancer model are currently underway.

Based on our prior report on ovarian autoimmunity (9), the therapeutic outcome in our current study is associated exclusively with CD4 $^{+}$ T cells responding in an IA S -restricted manner to the In α 215-234 peptide. Although CD4 $^{+}$ T cells often may be more efficient at tumor rejection than CD8 $^{+}$ cells (21), optimal tumoricidal activity typically occurs when tumor responses involve both CD4 $^{+}$ and CD8 $^{+}$ T cells (22). Thus, it is possible that incorporation of a CD8 $^{+}$ response in the induction of autoimmune ovarian failure may provide an even more profound therapeutic outcome in the treatment of ovarian malignancy. In addition, incorporation of a dendritic cell vaccine approach (23) offers the potential for *in vitro* shaping of the anti-tumor T cell repertoire perhaps by enhancement of the autoaggressive Th17 lineage (24) and/or by selective costimulatory/co-inhibitory manipulation to produce optimized autoimmunity (25,26). A complementary and perhaps synergistic strategy may also involve inhibition or elimination of FoxP3 $^{+}$ regulatory T cells that inhibit autoimmunity (27,28). In any event, our data provide direct experimental support for the view that the ability of tissue-specific differentiation antigens to induce targeted autoimmune organ failure predicts their potency in regulating tumor growth and providing therapeutic tumor vaccination.

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Figure 1. Ovarian-specific autoimmunity prevents the growth of autochthonous ovarian tumors SJL.AMH-SV40Tag mice were vaccinated at 2 months of age with either the IA^s-restricted In α 215-234 peptide or with CFA alone. At 18 months of age, ovaries were weighed and examined for the presence of ovarian tumors. **(A)** The weight of each ovary in each group was plotted and normalized by expression as percentile. Median ovarian tumor load was 33 times higher in CFA-immunized control mice compared to In α 215-234 vaccinated mice. The difference in mean ovarian tumor weights between vaccinated and control immunized mice was highly significant ($P < 0.0006$). **(B)** Representative left and right ovaries showing extensive growth of ovarian tumors in CFA immunized SJL.AMH-SV40Tag mice (upper panels) compared to In α 215-234 vaccinated mice (middle panels) and ovaries from an age-matched normal untreated non-transgenic littermates (lower panels). Solid black bar represents 1.0 cm.

Figure 2. Ovarian-specific autoimmunity inhibits the growth of established ovarian tumors

Coronal MRI images were obtained from SJL.AMH-SV40Tag mice at 15 months of age, and tumor-bearing mice were vaccinated with either Inα 215-234 peptide or with CFA alone. Four months later, a second MRI was taken and ovarian tumor volumes were determined. **(A)** All five individual control mice immunized with CFA showed marked increased ovarian tumor growth over the four month post-vaccination period whereas 5/6 individual mice vaccinated with Inα 215-234 had only modest ovarian tumor growth and the remaining individual mouse showed an actual decrease in ovarian tumor volume by the end of the four month testing interval. **(B)** Ovarian tumor size was significantly increased in control mice immunized with CFA ($P=0.007$) but was not significantly increased in mice vaccinated with Inα 215-234 ($P=0.083$). **(C)** The difference in total ovarian tumor growth (post-treatment minus pre-treatment tumor volumes) between CFA immunized mice and Inα 215-234 vaccinated mice was highly significant ($P=0.009$). All error bars show $\pm$ SD.

Figure 3. Representative MRI showing inhibited growth of established ovarian tumors in Inα 215-234 vaccinated mice

Outlined dashed lines in coronal section in left upper panel shows small normal appearing right ovary (RO) and large left ovary (LO) in 15 month old control SJL.AMH-SV40Tag mouse prior to immunization with CFA. Right upper panel shows substantially larger growth of both right ovary (RO) and left ovary four months later representing an overall 7-fold increase in total ovarian tumor load four months after CFA immunization. Lower panels show substantial shrinkage of right ovary and increased size of left ovary representing an overall 32% decrease in total ovarian tumor load in an SJL.AMH-SV40Tag mouse four months after vaccination at 15 months of age with Inα 215-234.

Figure 4. Inhibited growth of ovarian tumors in In α 215-234 vaccinated mice is accompanied by tumor infiltration of type-1 proinflammatory T cells

SJL.AMH-SV40Tag mice were vaccinated at 2 months of age with either In α 215-234 or CFA alone, and ovarian tumors were immunostained and evaluated for gene expression 16 months later. **(A)** Extensive infiltration of CD3+ T cells (arrows) occurred in ovarian tumors from In α 215-234 vaccinated mice (left panel) but not in tumors from CFA immunized control mice (right panel). **(B)** Intracellular staining showed numerous IFN γ producing T cells (arrows) in tumors from In α 215-234 vaccinated mice (left panel) but not in tumors from CFA immunized control mice (right panel). **(C)** qRT-PCR analysis showed highly significant overexpression of IFN γ , TNF α , IL-1 β , IL-17a, but not IL-10 in formalin fixed tumors from mice immunized with In α 215-234 compared to mice immunized with CFA

alone or compared to formalin fixed ovaries from normal untreated mice. All scale bars shows 50 μm . All error bars show $\pm\text{SD}$. * indicates $P<0.0005$.

Table 1

Autoimmune-induced ovarian failure prevents the development of ovarian tumors in SJL.AMH-SV40Tag mice

Treatment	Tumor Incidence in Mice	Tumor Incidence in Ovaries	Median Ovarian Tumor Weight (gm)	Mean Ovarian Tumor Weight (gm)
CFA	19/19 (100%)	35/38 (92%)	1.305	2.58 ±0.53 SE
Ina 215-234	20/29 (69%)	33/58 (57%)	0.0398	1.40 ±0.36 SE

$P<0.0006$