A novel treatment strategy for ovarian cancer based on immunization against zona pellucida protein (ZP) 3

Nafis A. Rahman,†,*††,‡‡,§,¹ Herjan J. T. Coelingh Bennink,∥,†,1 Marcin Chrusciel,* Victoria Sharp,¶ Yvette Zimmerman,∥ Roberto Dina,∥ Xiangdong Li,** Antti Ellonen,* Adolfo Rivero-Müller,* Stephen Dilworth,¶ Sadaf Ghaem-Maghami,∥ Olli Vainio,††,‡‡,§,1 and Ilpo Huhtaniemi*∥,¶

*Department of Physiology, University of Turku, Turku, Finland; †Department of Cell Biology, ²Department of Human Molecular Genetics, and ³Department of Obstetrics and Gynecology, Florida International University College of Medicine, Miami, Florida, USA; ⁴Pantarhei Bioscience B.V., Zeist, The Netherlands; ⁵Department of Surgery and Cancer and ⁶Department of Gynecological Oncology, Institute of Reproductive and Developmental Biology, Imperial College London, London, UK; ⁷State Key Laboratory for Agro-Biotechnology, China Agriculture University, Beijing, China; and ⁸Department of Microbiology and Immunology and ⁹Clinical Microbiology Laboratory, Oulu University Hospital, University of Oulu, Oulu, Finland

ABSTRACT We tested the principle of treating malignant ovarian tumors by vaccination against their ectopically expressed protein, zona pellucida glycoprotein (ZP) 3, using as the experimental model the granulosa cell tumors that develop in transgenic mice expressing the simian virus 40 T-antigen under the inhibin-α promoter (inха/Tag). We found high ZP3 expression in granulosa cell tumors of the transgenic mice, in human surface ovarian cancer and granulosa cell lines, and in human granulosa cell tumors and their metastases. Early preventive immunization (between 2 and 5.5 mo of age) of transgenic mice with recombinant human (rh) ZP3 prevented ovarian tumorigenesis, and delayed therapeutic immunization (between 4.5 and 7 mo) reduced weights of existing tumors by 86 and 75%, respectively (P<0.001), compared to vehicle-treated control mice. No objective side effects of the immunizations were observed. Liver metastases were found in nontreated/vehicle-treated controls (n=7/39), but none following active rhZP3 immunizations (n=0/36; P<0.05). Immunization with rhZP3 was highly effective, as demonstrated by the induction of anti-ZP3 antibodies, as well as proliferative responses to the ZP3 antigen. These results signal rhZP3 immunization as a novel strategy to be developed for the immunotherapy of ovarian granulosa cell tumors, as well as for that of other malignancies that may express ZP3.—Rahman N. A., Coelingh Bennink H. J. T., Chrusciel M., Sharp V., Zimmerman Y., Dina R., Li X., Ellonen A., Rivero-Müller A., Dilworth S., Ghaem-Maghami S., Vainio O., Huhtaniemi I. A novel treatment strategy for ovarian cancer based on immunization against zona pellucida protein (ZP) 3. FASEB J. 26, 324–333 (2012). www.fasebj.org

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Ovarian cancers represent one-fourth of all gynecological malignancies and are the leading cause of death in this category, especially in developed countries (1, 2). The largest group (~80%) of ovarian tumors are epithelium-derived, and the less common ones include those of germ cell and sex cord/stromal cell origin (1). Granulosa cell tumors are rare, accounting for 3–7.6% of primary ovarian tumors, but their prognosis is particularly poor (3, 4), with tumor-related mortality rate of 37.3% (5), and ~80% of deaths occurring on recurrence (5). The current therapies for granulosa tumors encompass surgery and/or chemotherapy (6–8), but the effects of various forms of treatment on disease-free or overall survival remain largely unknown (7). Relapse years after the initial diagnosis and treatment is common (7), prompting research into the development of better treatment strategies.

Immunotherapy is a potentially promising therapeutic option for ovarian cancer, but its success has been limited by the lack of suitable and target-defined tumor-associated antigens (TAAs; ref. 9). An ideal TAA for immunotherapy is nonself (i.e., a foreign antigen like human papillomavirus for cervical cancer) or not tolerated, expressed only in the tumor, common in tumors of a given type, required for tumor viability, immunogenic, and preferably cell surface expressed (i.e., can be targeted by antibody; ref. 9). None of the known TAAs expressed in ovarian cancer fulfill all of these criteria. Furthermore, the lack of suitable experimental models has limited the development of ovarian

1 Correspondence: N.A.R., Department of Physiology, University of Turku, 20540 Turku, Finland. E-mail: nafis.rahman@utu.fi; H.J.T.C.B., Pantarhei Bioscience B.V., 3701 Zeist, The Netherlands. E-mail: hcb@pantarheibio.com doi: 10.1096/fj.11-192468 This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.
cancer immunotherapy and the conduction of proof-of-principle experiments in vivo (9). Some transgenic (TG) murine models have proven useful by facilitating the understanding of mechanisms involved in the genesis, growth, and persistence of gonadal tumors (10, 11) and by providing in vivo models for testing novel treatment strategies.

Zona pellucida (ZP) glycoproteins 1, 2, and 3 are synthesized exclusively by growing oocytes (12–15) and secreted into the ZP layer surrounding the oocyte. Both native and recombinant ZP3 have been successfully used in animal studies as antigens for active immunization to study contraception through antibody-mediated disruption of sperm-egg interaction (15–17). However, a deleterious effect on ovarian function was found through iatrogenic autoimmune oophoritis (IAO) mediated by ZP3-specific antibodies and autoreactive T cells and consequent destruction of the ovaries (17–19). We now tested whether this deleterious effect of IOA on active immunization against ZP3 could be transformed into a novel treatment strategy for ovarian granulosa cell tumors.

The TG mouse model used in the current study expresses the simian virus 40 (SV40) T antigen under the murine inhibin-α promoter (inh/Tag), and develops ovarian granulosa cell tumors with 100% penetrance at the age of 4–5 mo (20, 21). The tumors are gonadotropin dependent, produce inhibin B, and metastasize to the liver and lungs (22), thus resembling human ovarian granulosa cell tumors. Primary tumors appear in the inh/Tag mouse ovaries with ongoing folliculogenesis (23).

We initially found that primary granulosa cell tumors and cell lines derived from these tumors of the inh/Tag transgenic mice, as well as human granulosa and surface epithelial tumor (KGN) cells (24), expressed abundant ZP3 mRNA and protein, suggesting that ZP3 could be a suitable candidate for the immunotherapy of granulosa cell tumors. Active immunization with recombinant human (rh) ZP3 protein induced antigen-specific humoral and cellular immune responses that led to IAO in all mice treated, delayed the appearance of tumors in animals treated in a preventive setting, and reduced the size of the tumors in animals treated in a curative setting. The present data demonstrate the effectiveness of this immunotherapeutic strategy in a mouse model. The human relevance of this novel treatment is implied by the expression of ZP3 in human ovarian granulosa cell tumors and their metastases.

MATERIALS AND METHODS

Production of rhZP3 protein

Stably transfected Chinese hamster ovary (CHO) cells (Zoagen/Repros, The Woodlands, TX, USA), as described previously by Harris et al. (25), were used for the production of rhZP3. Briefly, CHO cells (dhfr−) were transfected with expression plasmid pBBS35. This plasmid uses SV40 early promoter for recombinant gene expression, carries the mouse DHFR gene (for selection and amplification of transfected cells), and was modified to include a His6 codon cassette in front of the termination codon (thereby allowing recombinant protein purification by metal affinity chromatography). Cloned full-length ZP3 cDNA and CHO shuttle plasmid pZZ298 (hZP3 with native leader, aa 1–383) were used for this work.

Methotrexate (50 μM) was used for selection and amplification of the hZP3 sequence integrated into the genome of the transfected cells. CHO cells were then cultured in Opti-MEM medium (Invitrogen, Paisley, UK) until it was determined that the cell line was stable and producing a suitable quantity of the desired protein. The transfected cells were then transferred to a triple-layer flask in Hyclone HYQ SFM4CHO Utility medium (Fisher Scientific, Loughborough, UK). The medium was changed and stored until purification (−20°C), every 2–3 d over a 3-wk period, so that each flask yielded ~1 L of cell medium supernatant.

To purify the secreted rhZP3, supernatant was thawed but kept cold and diluted 2:1 in dilution buffer (30 mM Na acetate and 23 mM EDTA, pH 4.8) and loaded onto an SP Sephrose Fast Flow column (GE Healthcare, Chalfont, UK). The bed was pre-equilibrated with equilibration buffer (20 mM Na acetate, 40 mM NaCl, and 15 mM EDTA, pH 4.8). The supernatant was bound to the SP Sephrose over 2 h at 4°C; the column was then washed in the wash buffer (20 mM Na acetate and 40 mM NaCl, pH 4.8), and protein was eluted with SP elution buffer (20 mM Tris, 50 mM NaH2PO4, 100 mM NaCl, pH 8.0). The SP column elution fractions were loaded onto a pre-equilibrated Talon Metal Affinity Resin column (Clontech Europe, Saint-Germain-en-Laye, France; binding time 20 min at room temperature), washed with equilibration/wash buffer (50 mM NaH2PO4 and 300 mM NaCl, pH 7.0) and eluted in 1× elution buffer (50 mM NaH2PO4, 300 mM NaCl and 150 mM imidazole, pH 7.0). The fractions containing rhZP3 protein were identified by Western blot analysis, dialyzed at 4°C into PBS at a final concentration of 1 mg/ml and stored at −80°C.

Immunoization of inh/Tag TG female mice

Either 8- or 17-wk-old inh/Tag TG female mice of the C57BL/6J strain, genotyped by PCR (20), were used for the experiments. The mice were housed 2–4/cage under controlled light (12 h light:12 h cycle) and temperature (21 ±1°C), and fed with mouse chow SDS RM-3 (Whitham, Essex, UK) and tap water ad libitum. The ethics committees for animal experimentation of the University of Turku and the State Provincial Office of Southern Finland approved the experiments.

The inh/Tag TG females were immunized at 2 time points, at the age of 8 (first immunization, preventive treatment group) or 17 wk (first immunization, curative treatment group), and thereafter followed by boosters every 3 wk for 3–4 times and killed 14 d after the last booster. Granulosa cell tumors appear in the Inh/Tag mice around the age of 3–4 mo (20, 26). In the preventive setting, immunization was started at 8 wk of age, and the mice were killed at the age of 22 wk. In the curative setting, immunization was started at 17 wk, and the mice were terminated at the age of 28 wk. Each treatment setting consisted of 3 groups of TG females: nontreated (n=9–10), treated with the immunization vehicle (n=9–10), and rhZP3-immunized (n=11 in preventive, n=16 in curative treatment). Age-matched wild-type (WT) littermates (n=6) were used as controls for normal ovarian weights and hormone levels.
**Immunizations**

The mice first received first immunization of 10 µg of rhZP3 subcutaneously, divided over 2 injection sites, in a volume of 100 µL, where the antigen was emulsified (1:1) in complete Freund’s adjuvant (CFA; Difco Laboratories, Detroit, MI, USA; 100 µg ZP3/ml final concentration). After the first immunization, mice received boosters every 3 wk, where incomplete Freund’s adjuvant (IFA; Difco Laboratories) was replaced by CFA. Control mice were immunized with a mixture of PBS/CFA on the first immunization, and boosted further every 3 wk with PBS/IFA. The mice were killed 2 wk after the last boost by cervical dislocation. During the treatments, we closely followed the body weights of the mice as a sign of their wellbeing. On autopsy, tumor weight and spread were monitored, and the ovaries, adrenals, lungs, and liver were snap-frozen, or fixed in 4% paraformaldehyde (PFA) or Bouin’s solution, for mRNA, protein, and histopathological analyses. Serum was collected for hormone measurements. Mesenteric lymph nodes and spleen were collected for humoral and cellular immune response experiments.

**Blood sampling and anti-ZP3 antibody measurement**

Anti-ZP3 antibody titers were measured in blood samples (20–30 µL) collected from femoral veins of the immunized, nontreated, and control-treated mice using thin glass capillaries. Blood samples (n=6–8/time point) were collected 3 times from each treatment group, 1 d prior to the beginning of the treatment, 1 d prior to the second booster, and prior to autopsy. Antibody titers were analyzed by Anti-Zona Pellucida Antibody ELISA Ig-Classifier Kit (Bioserv Diagnostics, Rostock, Germany), according to the manufacturer’s manual with minor modifications, i.e., substituting the secondary kit antibodies with sheep anti-mouse HRP-linked secondary antibodies (GE Healthcare).

**T-cell proliferation response**

To determine T-cell proliferation responses, we collected samples of spleen and mesenteric lymph nodes, and we harvested and isolated from them a viable mononuclear cell population for *in vitro* studies. Red blood cells from the spleen cell suspension were removed (not necessary for lymph node suspensions). A 1-step gradient method (27), and the cell suspensions were frozen in 20% FCS and 10% DMSO in liquid nitrogen for subsequent assessment of immune responses by [3H]-thymidine incorporation (27). Cell suspensions of splenocytes or lymph nodes were plated at a concentration of 0.5 × 10^6 or 0.2 × 10^6/well, respectively, on 24-well plates and grown in quadruplicates for 72 h in DMEM/F12 medium, containing 10% heat-inactivated FCS and 50 µg/ml gentamicin (37°C, 5% CO₂ atmosphere). As a positive control for proliferation, cells were stimulated with mitogens concanavalin A (ConA; 6.25 µg/ml; Sigma, St. Louis, MO, USA) and phytohemagglutinin (PHA; 20 µg/ml; Sigma) for 72 h. After incubation, the cultures were pulsed for 48 h with [3H]-thymidine (0.5µCi/well; Amersham Biosciences, Little Chalfont, UK; ref. 28). The data were expressed as mean ± se counts per minute (cpm) of quadruplicate sample cultures.

**Human granulosa cancer samples**

Human granulosa cell tumor paraffin blocks for immunohistochemistry analyses, both in primary (n=7), as well as recurrent (n=3) and metastases (n=5) were obtained from the Department of Gynecological Oncology, Imperial College London. Local ethics committee approval was in place for the use of these samples for research purposes.

**RT-PCR analysis**

Total RNA was extracted using the NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany). Prior to reverse transcription, using the DiNamo cDNA Synthesis Kit (Finzymes, Espoo, Finland), total RNA was treated with deoxyribonuclease I (Invitrogen). First-strand cDNA (5 µl) was used as template in PCR (initial denaturation of 96°C for 3 min, then 35 cycles of 94°C for 1 min, 57°C for 45 s, and 72°C for 45 s, with a final extension period of 5 min at 72°C). Primer sequences for each gene were as follows: mouse ZP3 (mZP3; 150 bp), gene forward primer 5'-GAGCTTTTGCGGATTCAGACG-3' and reverse primer 5'-AGCTTTCATGACGATTGC-3'; human ZP3 (hZP3; 185 bp), gene forward primer 5'-GGTGGATATAGGGGAGCAT-3' and reverse primer 5'-TTCTCTCTGTGGTATGG-3'; mouse cyclophilin A (mPPIA; 165 bp), gene forward primer 5'-CATCCTAAAGCATAACGAGCGATC-3' and reverse primer 5'-TCCATGGCTTCCAATGTT-3'; human cyclophilin A (hPPIA, 165 bp), gene forward primer 5'-CATCCTAAAGCATAACGAGCGATC-3' and reverse primer 5'-TCCATGGCTTCCAATGTT-3'. All primers were designed using Primer3 (http://frodo.wi.edu/cgi-bin/primer3), and sequences of murine and human ZP3 were obtained from GenBank (accession no. NM_001110354 and NM_007155.5, respectively).

**Immunohistochemistry**

Paraformaldehyde-fixed paraffin serial sections (5 µm) of the murine and human ovarian granulosa cell tumors, and WT murine or normal human ovaries as positive controls, were deparaffinized and rehydrated. To perform antigen retrieval, all sections were boiled for 15 min in 10 mM citric acid (pH 6.0). In the next step, after blocking endogenous peroxidase activity in 3% H₂O₂ in methanol (10 min), the sections were incubated with blocking solution for 1 h at room temperature [15% normal goat serum (NGS) and 5% BSA in 0.1% Tween PBS; Sigma] to reduce nonspecific background staining. Sections were then incubated overnight at 4°C with rabbit polyclonal antibody anti-ZP3, diluted 1:250, (Zonagen/Repro). Primary antibodies of ZP-3 were linked with the goat anti-rabbit IgG biotin conjugate secondary antibody (dilution 1:1000; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The avidin-biotin immunoperoxidase system was used to visualize bound antibody (Vectorstain Elite ABC Kit; Vector Laboratories) with 3,3'-diaminobenzidine (Sigma) as a substrate. Between each stage of the staining, the sections were washed 3 times with PBS. As a control for the antibodies, adjacent sections were incubated with either 1% NGS in PBS or anti-rabbit IgG as a primary antibody to differentiate unspecific from specific staining.

**Immunofluorescence staining**

KK-1, KGN, OVCAR-3, and MSC-1 cells were grown on surface of sterile coverglass slides in appropriate medium supplemented with 10% FCS (Promocell, Heidelberg, Germany) and penicillin-streptomycin solution (Sigma). After washing with PBS, the cells were fixed in 4% paraformaldehyde (5 min at room temperature). To block autofluorescence, the cells were incubated with 100 mM NH₄Cl for 3 min at room temperature (Sigma). Before overnight incubation (4°C) with primary rabbit polyclonal antibody anti-ZP3 (diluted 1:200), the cells were washed with 0.1% Triton X-100 PBS (3x5 min) and incubated with blocking solution for 1 h at room temperature [15% NGS and 5% BSA in 0.1% Triton
We first studied whether ZP3 murine and human granulosa cell tumors express were carried out for the analysis of groups with nongaussian (SAS Institute, Cary, NC, USA). Logarithmic transformations were performed with the SAS Enterprise Guide 3.0 program (Bonferroni test, using the SAS Enterprise Guide 3.0 program post hoc Statistical analyses were carried out by ANOVA with manufacturer’s instructions. Inhibin B was measured by DSL-10-84100i ELISA Inhibin B ng/ml, for FSH 0.03 ng/ml, for progesterone 0.5 nM, and for estradiol. The approximate assay sensitivity for LH is 0.1 extraction. Delfia Progesterone and Estrogen Kits (Perkin-Vernon Hills, IL, USA), as described previously. Serum progesterone and estradiol were measured after diethyl ether extraction. Delfia Progesterone and Estrogen Kits (Perkin-Elmer-Wallac, Madison, WI, USA) were used as standards. After electrophoresis, proteins were transferred to Amersham Hybond-P PVDF membrane (GE Healthcare) using a Trans-Blot SD cell (Bio-Rad, Hercules, CA, USA), 20 V for 60 min. After blocking the membrane with TBS containing 5% fat-free milk powder and 0.05% Tween, incubation with the primary antibody (anti-ZP3 rabbit polyclonal antibody, 1:1000 dilution; Zonagen/Repros) was performed overnight at 4°C. As a secondary antibody, ECL goat anti-rabbit IgG HRP linked (GE Healthcare) was used at 1:10,000 dilution. Signals were visualized using the Amersham ECL Plus Western blotting detection system (GE Healthcare), and the images were recorded with Fujifilm LAS-4000 chemiluminescent film (Fujifilm, Japan). The intensity of specific bands was quantified using the ImageJ 1.42q visual system (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij). Hormone measurements Serum levels of LH (29) and FSH (30) were measured by immunofluorometric assays (Delfia; Perkin-Elmer-Wallac, Vernon Hills, IL, USA), as described previously. Serum progesterone and estradiol were measured after diethyl ether extraction. Delfia Progesterone and Estrogen Kits (Perkin-Elmer-Wallac) were used for measuring serum progesterone and estradiol. The approximate assay sensitivity for LH is 0.1 ng/mL, for FSH 0.03 ng/mL, for progesterone 0.5 nM, and for estradiol 50 pM, respectively. The intra-assay and interassay coefficients of variation for these assays were below 10%. Inhibin B was measured by DSL-10-84100i ELISA Inhibin B kit (Beckman Coulter, Carlsbad, CA, USA), according to the manufacturer’s instructions. Statistical analyses Statistical analyses were carried out by ANOVA with post hoc Bonferroni test, using the SAS Enterprise Guide 3.0 program (SAS Institute, Cary, NC, USA). Logarithmic transformations were carried out for the analysis of groups with nongaussian distribution. Values of P < 0.05 were regarded as statistically significant. All values are presented as means ± se.

RESULTS

Murine and human granulosa cell tumors express ZP3

We first studied whether ZP3 expression could be detected in the KK-1 murine granulosa tumor cell line, derived from an inhα/Tag ovarian tumor (20), and in the KGN human granulosa cancer cells (24). Abundant ZP3 expression was found in these cell lines both at mRNA (by RT-PCR) and protein (by immunofluorescence) levels (Fig. 1). mRNAs from mouse WT and human ovaries were used as positive controls and those from a murine Sertoli tumor cell line (MSG-1; ref. 31) and human total blood cells as negative controls for mZP3 and hZP3 expression, respectively. The 150-bp mZP3 and 183-bp hZP3 DNA amplicons detected in the KK-1 and KGN granulosa cell lines were identical to those of the mouse and human ovaries, respectively, but absent in MSC-1 and human blood cells (Fig. 1A). On immunofluorescence, clear cytoplasmic localization of the ZP3 protein was detected in KK-1 and KGN but not in MSC-1 cells (Fig. 1B).

To elucidate the human relevance of the planned immunization strategy, we investigated the ZP3 protein expression in human granulosa cell tumors (Fig. 1C). We checked primary granulosa cell tumors (n=7); metastatic deposits to liver, spleen, and peritoneum (n=5); and recurrent granulosa cell tumors (n=3). In total, 11 of 15 tumor samples stained positive for the ZP3 protein by immunohistochemistry. Primary tumors were not always positive (4 of 7), but most important, all recurrences and metastases expressed the ZP3 protein.

rhZP3 immunization reduces ovarian tumor size

We observed a clear reduction of ovarian tumor weights with rhZP3 immunization of the inhα/Tag mice both in the preventive (treatment initiated before apparent tumorigenesis) and curative (treatment initiated with existing tumors) settings (Fig. 2A, B). When rhZP3 immunization was initiated at 8 wk of age (n=11) and continued for ~3.5 mo, total ovarian weights were reduced by 86% (P<0.001) in comparison to untreated (n=9) and vehicle-treated controls (n=9) (Fig. 2A). Where TG females already possessed discernible ovarian tumors, the curative treatment that involved vaccination was started at 17 wk of age and continued for 3.5 mo. The total ovarian weights of these mice decreased by 75% after rhZP3 immunization (n=16) in comparison to age-matched untreated (n=10) and vehicle-treated controls (n=10) (P<0.001; Fig. 2B). The tumor burden (tumor weight/body weight) responded very similarly to the total ovarian weights (Supplemental Fig. S1) The spleen weights, as a sign of immunization efficacy, increased significantly after rhZP3 treatments in both treatment groups compared to untreated and control-treated mice (Fig. 2C, D). There were no statistically significant differences in total body, adrenal, or uterine weights between the treated and nontreated groups in either immunization experiment (Supplemental Fig. S2).

rhZP3 treatment specifically affects tumorous granulosa cells

To assess whether rhZP3 immunization reduced the ZP3 expression levels in comparison to nontreated and
control groups, we carried out a semiquantitative analysis of ZP3 protein in extracts of the TG ovaries. Western hybridization revealed a decrease in ZP3 protein in the rhZP3-treated ovaries in both preventive and curative groups, compared to nontreated and vehicle-treated control groups (P<0.05; Fig. 3). As the murine granulosa tumor cells express ZP3 protein abundantly, this provided further evidence for destruction of the ZP3-expressing tumor cells during the immunization (Fig. 3), in line with the reduced tumor weights (Fig. 2).

Figure 1. ZP3 mRNA and protein expression in the murine KK-1 granulosa tumor cell line derived from the ovarian tumor of an Inhα/Tag mouse, human KGN granulosa, and human serous epithelial (OVCAR-3) cancer cell line; and ZP3 protein expression in human granulosa cancer cells. A) RT-PCR analysis of murine ZP3 (mZP3), human ZP3 (hZP3), and control murine and human cyclophilin A (mPPIA, hPPIA) mRNA. Murine WT and human ovaries were used as positive controls; murine Sertoli cell line MSC-1 and human blood cells as negative controls. Amplicon sizes are presented at right and left. B) Immunofluorescence visualization of cytoplasmic localization of the ZP3 protein in KK-1 (a–c), OVCAR-3 (d–f) and KGN (g–i) cells. a, d, g Detection with goat anti-rabbit IgG-Alexa Fluor 594 (red fluorescence). b, e, h, k Nuclei stained with DAPI (blue). c, f, i Merged images. j, k) MSC-1 cells, used as negative control. C) Immunohistochemical (ZP3 stained) images of human ovarian granulosa cell tumors and oocytes. Immunohistochemical cytoplasmic immunostaining of ZP3 protein in primary ovarian granulosa cell tumor (a), recurrent granulosa cell tumor vaginal vault (b), and granulosa cell tumor metastatic deposit (c); positive control from a normal human ovary (d) shows highly specific protein expression of the ZP3 protein only in the ZP-layer of the oocyte. Arrows indicate ZP3-immunostained tumor cells and ZP layer. Scale bars = 25 μm (Ba–f, j–k); 20 μm (Bg–i); 50 μm (C).

Endocrine consequences of rhZP3 immunization

In intact inhα/Tag mice, serum progesterone and inhibin B levels rise and those of gonadotropins decrease, along with the ovarian tumor progression (22, 26). After the 3.5-mo rhZP3 immunizations, serum progesterone was significantly decreased in both treatment groups, compared to nontreated and vehicle-treated controls (P<0.05; Fig. 4A, B). Serum LH levels were increased in the rhZP3-treated mice (P<0.05, rhZP3 vs. controls; Fig. 4C, D). Serum inhibin B levels

Figure 2. Ovarian (A, B) and spleen (C, D) weights of the inhα/Tag TG females and WT control nontreated littermate mice. A, C) Data from the preventive experiment (early immunization at 2 mo of age before the appearance of tumors; n=9–11/group). B, D) Data from the curative treatment (later immunization at 4.5 mo of age with existing tumors; n=10–16/group). Ovarian data are means ± SE of both (left and right) ovaries. *P < 0.001 vs. nontreated and vehicle-treated control TG groups.
were significantly decreased after rhZP3 treatment in both treatment groups (P<0.05; Fig. 4G, H). A reciprocal increase in FSH reached statistical significance in the curative group (P<0.05, ZP3 vs. the two control groups; Fig. 4F). The hormone levels were thus in agreement with the positive treatment response to rhZP3 immunization. Neither serum estradiol levels nor uterine weights (reflecting the integrated level of estrogen exposure) responded significantly to the treatments (Supplemental Fig. S2).

Histological and immunohistochemical analyses demonstrate antitumoral effect of rhZP3 immunization

Histological analysis of the ovaries confirmed that their weight reduction (Fig. 2) on rhZP3 immunization was due to loss of the tumor mass (Fig. 5F, H, N, P). The nontreated and vehicle-treated control sections showed predominantly tumor cells that expressed the ZP3 protein, along with the few existing follicles with ZP layer (Fig. 5A–D, I–L). Almost all follicles, even the primordial ones, were lost in the rhZP3-treated ovaries (Fig. 5E–H, M–P), whereas normal follicles were present in abundance in all nontreated and vehicle-treated ovaries (Fig. 5A–D, I–L). Immunohistochemical analysis showed abundant ZP3 staining of the follicles and tumor cells of nontreated and vehicle-treated control ovaries in both the preventive and curative treatment groups (Fig. 5B, D, J, L). In contrast, in the rhZP3-treated group, ZP3 expression was scant and displaced to the periphery of the ovary (Fig. 4F, H, N, P). The positive control from a WT ovary showed highly specific protein expression of the ZP3 protein only in the ZP layer of the oocyte (Fig. 5Q, R).

We then studied the sections of several other organs (lung, pancreas, liver, spleen, adrenal, and uterus) for metastases and side effects of the treatment. In line with our earlier reports of a 15–20% rate of metastasis to the liver at the age of 6 mo (20, 26), we found at 7 mo in

Figure 3. Western blot analysis for ZP3 protein in the rhZP3 immunized, nontreated, and vehicle-treated control mice. Protein extracts (30 μg) from female inhox/Tag mice with nontreated, control-treated, and ZP3-treated ovarian tumor samples from the preventive and curative treatments were analyzed. WT ovary and KK-1 cells were used as positive controls; WT murine testes as negative controls. Top panel: representative data of Western blot analysis of the ZP3 protein expression from 1 of 3 similar experiments. Molecular mass (kDa) of protein is presented at right. Bottom panel: densitometric quantification of the ZP3 80-kDa band, corrected for intensity of the 42-kDa β-actin band. Bars represent percentages of ZP3 (mean±se of 3 independent experiments) in the control, preventive, and curative treatment groups, calculated in relation to the amount of ZP3 (100%) measured in the nontreated mice of the corresponding group. *P < 0.05 vs. nontreated and control groups.

Figure 4. Effects of rhZP3 immunization on serum hormone levels. Concentrations of progesterone (A, B), LH (C, D), FSH (E, F) and inhibin B (G, H) in the nontreated, control-treated, and ZP3-treated inhox/Tag TG female mice, as well as in WT control littermate females. A, C, E, G) Data from the preventive treatment groups. B, D, F, H) Data from the curative treatment groups. Values are means ± se (n=9–16/group). n.d., not determined. *P < 0.05 vs. nontreated and vehicle-treated control TG groups.
the curative, nontreated, and control-treatment groups 2 cases of liver metastases in each (4/19 cases), but none in the active rhZP3 treatment groups (0/16 cases). Because of the low frequency of metastases, we pooled the observations from our earlier pilot experiments with similar immunization schedule with the current definitive experiments. In this way, the increased number of observations revealed a statistically significant reduction ($P<0.01$) in the occurrence of liver metastases between control (7/39) and ZP3-treated (0/36) TG mice.

**Immunization boosting with rhZP3 was effective, proven by anti-ZP3 titers and cellular responses**

We checked the rise of antibody titers by ELISA in each treatment group from serum samples collected before the treatments, 1 d prior to the second boost, and at the time of termination of the mice. There was up to 5-fold increase from the pretreatment levels in IgG and IgM before the second booster (Fig. 6A). IgG titers further increased up to 7-fold at the time of autopsy, whereas a sharp concomitant decline was found in the IgM titers (Fig. 6A). There were no antibody responses in the nontreated and vehicle-treated control groups (Fig. 6A).

We then performed a cell proliferation study in order to evaluate the cell-mediated immune response against rhZP3. Isolated mononuclear cell populations drained from mesenteric lymph nodes and splenocytes were cultured *in vitro*. The assessment of the cellular immune response was determined by cell proliferation with $[^3H]$-thymidine incorporation analysis. As positive control, we stimulated the cells with two strong mitogens, ConA or PHA. rhZP3 immunization induced a 3–4-fold higher proliferation of both lymph nodes and splenocytes compared to that of the nontreated cells for both treatment groups (Fig. 6B). The results, expressed as stimulation index, are shown in Supplemental Fig. S3.

**DISCUSSION**

Active immunization against ZP3 as a suitable TAA provides a novel treatment strategy for granulosa cell tumors. Inflammatory reaction has been suggested as the primary cause of tissue destruction, in particular, in cases when highly overexpressed TAAs (in our case, the highly glycosylated rhZP3 protein) are used with strong adjuvant (i.e., complete Freund’s adjuvant). After the use of ZP vaccine, strong inflammatory reaction (oophoritis) and subsequent ovarian destruction were observed earlier in a mouse model (32). The feasibility of the ZP3 approach was hypothesized on the basis of two additional observations. First, immunotherapy of cancer does not necessarily require direct targeting of the immune responses to tumor antigens themselves. A good example is the killed Bacille Calmette-Guérin (BCG) bacteria that, when injected into bladder tissue, generate broad host defense mechanisms; among others, T cells reactive to the active tumor (33). This type of indirect immunotherapy is now established as the most effective treatment for superficial bladder cancer (34–36). Second, inflammatory oophoritis and subsequent ovarian destruction were observed in mice, rab-
bits, and monkeys immunized against ZP antigens (32). We anticipated that, similar to the BCG immunization, ZP vaccination would activate autoimmune responses to ovarian tissue. Furthermore, earlier studies with a ZP3 peptide vaccine carried out in an homologous murine system showed that immunization with ZP3 can lead to epitope spreading, with the induction of amplified antibodies to ZP3 that did not react with the immunizing peptide, thereby augmenting and extending the immune response (37). In turn, activation of tumor-specific T cells may ensure genuine effectors to control further tumor spread, whereas a circulating pool would reach and eliminate the metastases. We also found that the murine granulosa tumor cells expressed high levels of ZP3, which made the rhZP3 antigen an ideal TAA for an immunization trial.

ZP3 immunization significantly reduced the ovarian tumor mass in the inh/H9251/Tag mice. We initially hypothesized that the autoimmune vaccination responses occur through activation of host defense mechanisms. The tumor-related antigens within the ovary would be targeted as part of the ongoing autoimmune response and would therefore eliminate the tumors. However, we now assume that two distinct mechanisms of tumor ablation took place. First, the hypothesized indirect autoimmune vaccination response eliminated the tumors by disrupting normal folliculogenesis and ovarian function. Second, a direct mechanism is possible, involving a specific immune response toward the granulosa tumor cell-expressed ZP3.

The functional response of the ovaries to rhZP3 immunization was reflected by changes in serum hormone levels. Progesterone and inhibin B levels decreased, and those of gonadotropins increased, after rhZP3 immunization, as signs of reduction of endocrinologically active tumor tissue and lower negative feedback to the hypothalamus-pituitary level. Human granulosa cell tumors generally produce estradiol, but at least 30% of them are steroidogenically inactive (38, 39). The granulosa tumor cells in the inh/H9251/Tag ovaries represent functionally the late preovulatory stage, thus producing mainly progesterone (20); therefore, estradiol is not an optimal endocrine marker for them (38, 40). It is postulated that inhibin B acts in the normal ovary as a defense mechanism against proliferative effects of elevated gonadotropins (41, 42). This is supported by the fact that ovarian granulosa cell tumors are often associated with elevated levels of inhibin B and reduced levels of serum FSH (38, 40, 43). Inhibin B is produced by human granulosa cell tumors, and it has been proposed as a marker of primary and recurrent granulosa cell tumors (38, 40, 44, 45). Accordingly, we found suppression of circulating progesterone and inhibin B, and increase of gonadotropins, in response to the treatments.

Histological and immunohistochemical analyses after rhZP3 immunization confirmed the reduction of tumor mass, and residual tumor tissue could be observed by ZP3 immunolocalization only in limited areas of the ovarian periphery. Moreover, the relatively rare occurrence of metastases (20%) in these TG females (20, 26), was only observed in control animals (n=4/19), but none in those receiving active rhZP3 treatment (0/16). Combining these findings with our pilot data with similar immunization made the reduction of metastasis rate statistically significant (0/36 vs. 7/39; P<0.05). Since the ZP antigens can only be found around the oocytes, and in granulosa cell tumors, as demonstrated here, active immunization with rhZP3 was not expected to damage any other organs, thus being specific and safe. This is supported by the lack of

Figure 6. Humoral and cellular immune responses to rhZP3 immunization. A) Anti-ZP3 antibody titers (IgM and IgG) from serum of rhZP3 immunized, nontreated, and vehicle-treated control TG mice at 3 time points: before treatment, after the second booster, and at autopsy (sacrifice time). Antibody response is expressed as net optical density value at 450 nm. Values are averages ± se of 4–8 samples repeated twice. Antibody response of mice immunized with rhZP3 was 6-fold higher than that of mice immunized with vehicle only (IFA) or of nontreated controls (P<0.05). There was no difference between the nontreated and control groups. B) Cellular immune response of splenocytes and mononuclear cells from draining mesenteric lymph nodes as measured by [3H]-thymidine incorporation (cpm) in T-cell proliferation assay. Results are means ± se. *P < 0.05 vs. nontreated and vehicle-treated control TG groups.
any detectable changes in other organs as checked by histopathological analysis.

The highly positive humoral and cellular response, namely, the sharply increased blood antibody titer by ELISA compared to preimmunization serum, and the significantly higher proliferation of mononuclear cells from splenocytes and mesenteric lymph nodes of the rhZP3 immunized mice, further supported the efficacy of the rhZP3 immunization. The rhZP3 immunization had both preventive and curative effect in the presence of the ZP antigens. Still naïve T cells were expected to acquire the property to recognize tumor-specific antigens and mature into antigen-specific T-effector cells due to the optimal environment for T-cell stimulation following vaccination.

Taken together, the present data provide strong evidence for a rapid, effective, and specific effects of rhZP3 immunization against granulosa cell tumors. This strategy is likely to be effective in the treatment of primary, metastatic, and recurrent granulosa cell tumors. Recurrence of disease may also be prevented if immunization is given as prophylaxis following treatment of primary tumors. The relevance of our findings to human cancer therapy is emphasized by the presence of ZP3 in primary human granulosa cell tumors, their metastases, and recurrences. These promising experimental data prove the principle that ZP3 immunization provides a novel lead into the immunotherapy of human ovarian cancer and other malignancies eventually expressing ZP3.

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