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McNeel et al.

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(54) **COMBINATORIAL ANDROGEN DEPRIVATION WITH AN ANDROGEN RECEPTOR VACCINE**

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(51) **Int. Cl.**

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A61K 39/00	(2006.01)
A61K 38/08	(2019.01)
C07K 16/28	(2006.01)
C07K 16/30	(2006.01)

(52) **U.S. Cl.**

CPC ***A61K 39/001102*** (2018.08); ***A61K 38/08*** (2013.01); ***A61K 38/09*** (2013.01); ***A61K 39/0011*** (2013.01); ***C07K 16/2818*** (2013.01); ***C07K 16/2827*** (2013.01); ***C07K 16/3069*** (2013.01); *A61K 2039/53* (2013.01); *A61K 2039/545* (2013.01); *C07K 2317/76* (2013.01)

(58) **Field of Classification Search**

CPC A61K 39/0011; A61K 38/09; A61K 38/08; C07K 16/3069

USPC 424/174.1

See application file for complete search history.

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(Continued)

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(57) **ABSTRACT**

It is disclosed herein methods of treating prostate cancer comprising administering to the subject the combination of androgen deprivation therapy (ADT) and a vaccine directed against the androgen receptor or a fragment of the androgen receptor. Also disclosed are methods of increasing the efficacy of androgen deprivation therapy in a subject with prostate cancer comprising administering to the subject an effective amount of a vaccine against the androgen receptor or fragments thereof wherein the method inhibits, delays or reduces the growth of the prostate cancer and/or the development of castration-resistant prostate cancer.

19 Claims, 36 Drawing Sheets
(23 of 36 Drawing Sheet(s) Filed in Color)

Specification includes a Sequence Listing.

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FIG. 1

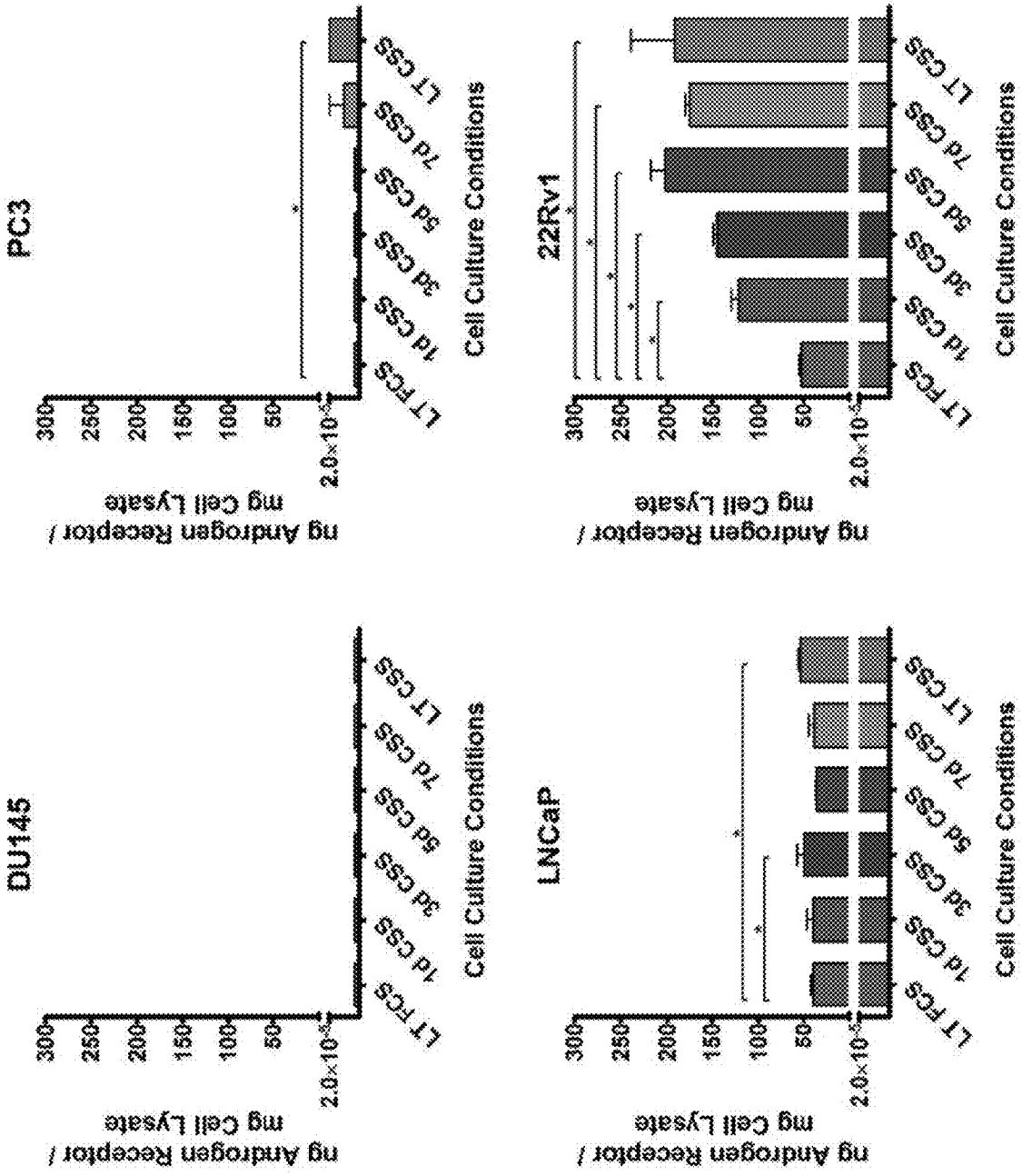
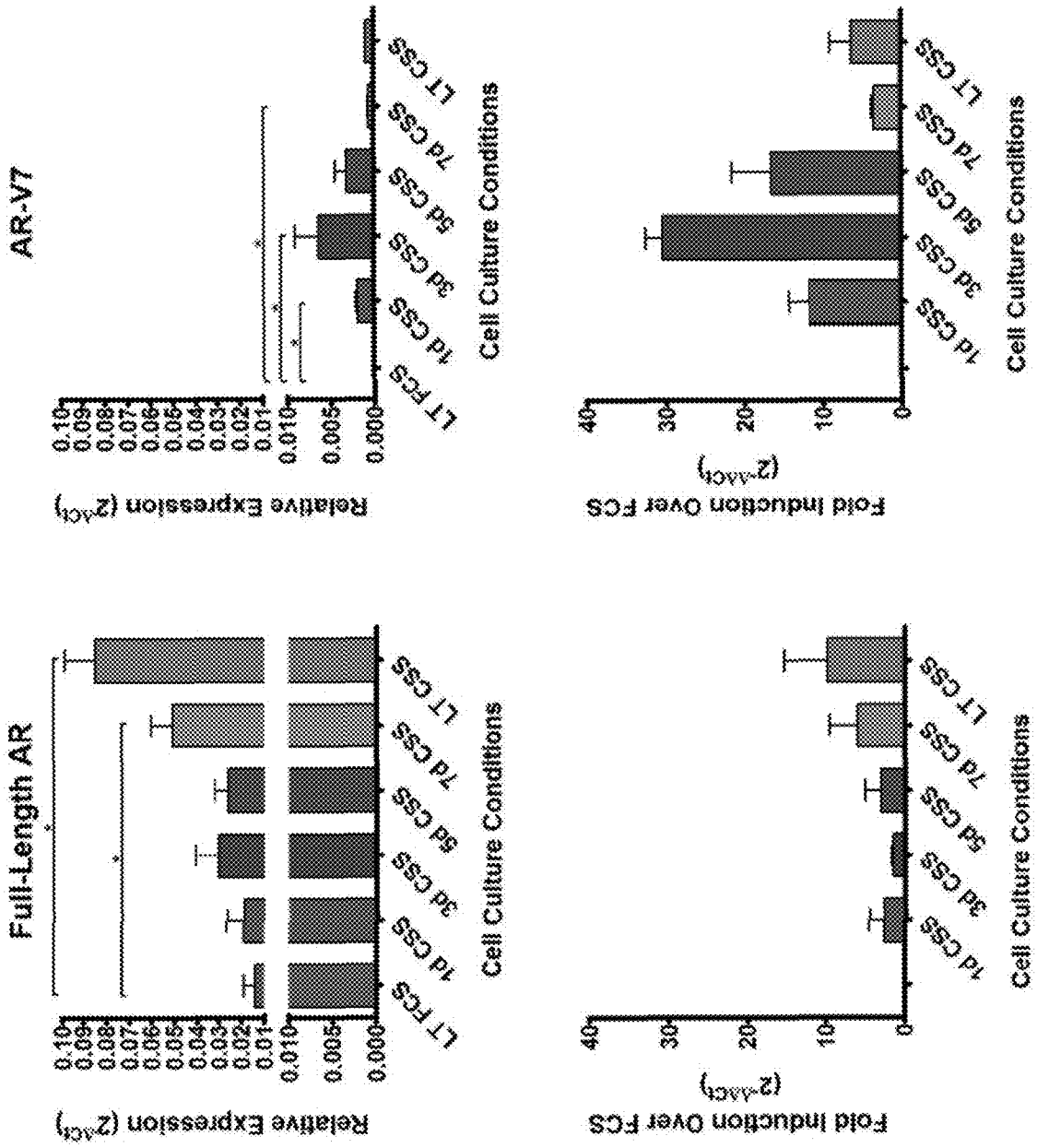
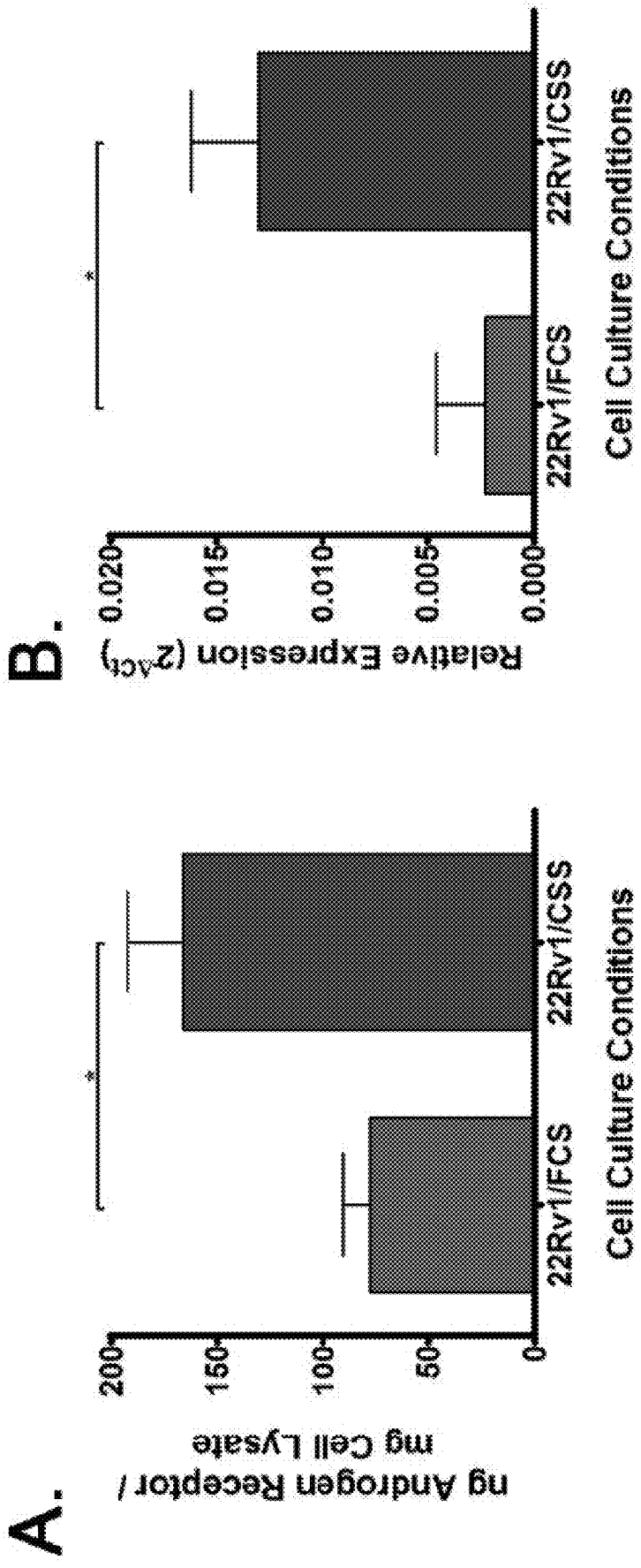

PC3
22Rv1
DU145
LNCaP
ng Androgen Receptor / mg Cell Lysate
Cell Culture Conditions
LT FCS
1d CSS
3d CSS
5d CSS
7d CSS
LT CSS
300
250
200
150
100
50
2.0×10⁻⁴

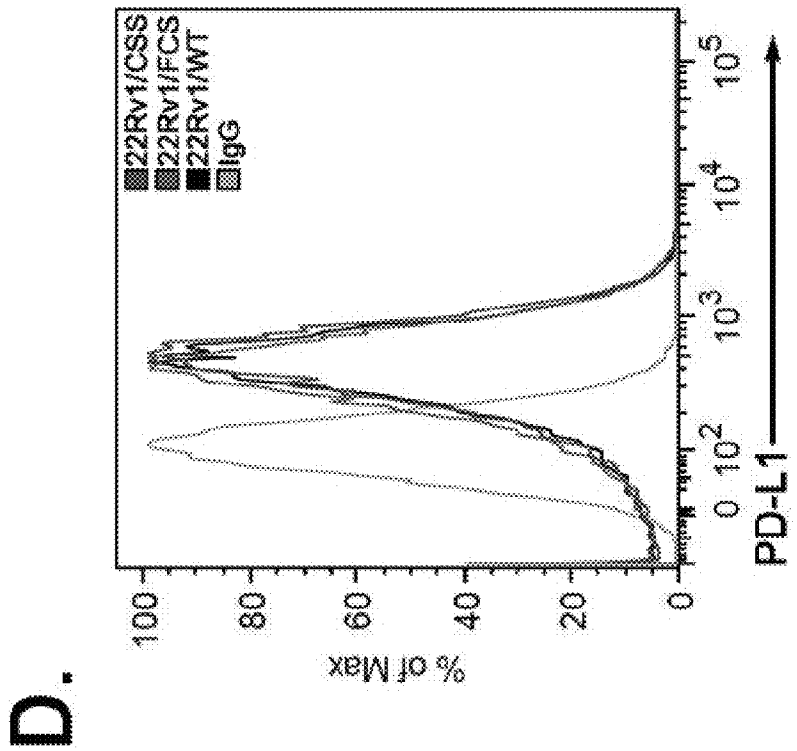
FIG. 2

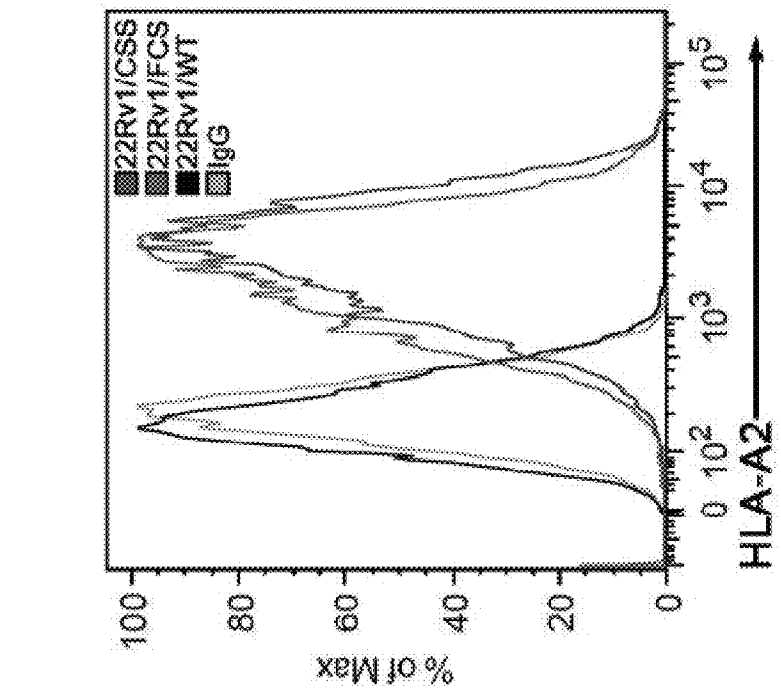

AR-V7
Full-Length AR
Relative Expression ($2^{-\Delta Ct}$)
Fold Induction Over FCS ($2^{-\Delta\Delta Ct}$)
Cell Culture Conditions
LT FCS
1d CSS
3d CSS
5d CSS
7d CSS
LT CSS
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.010
0.005
0.000
40
30
20
10
0

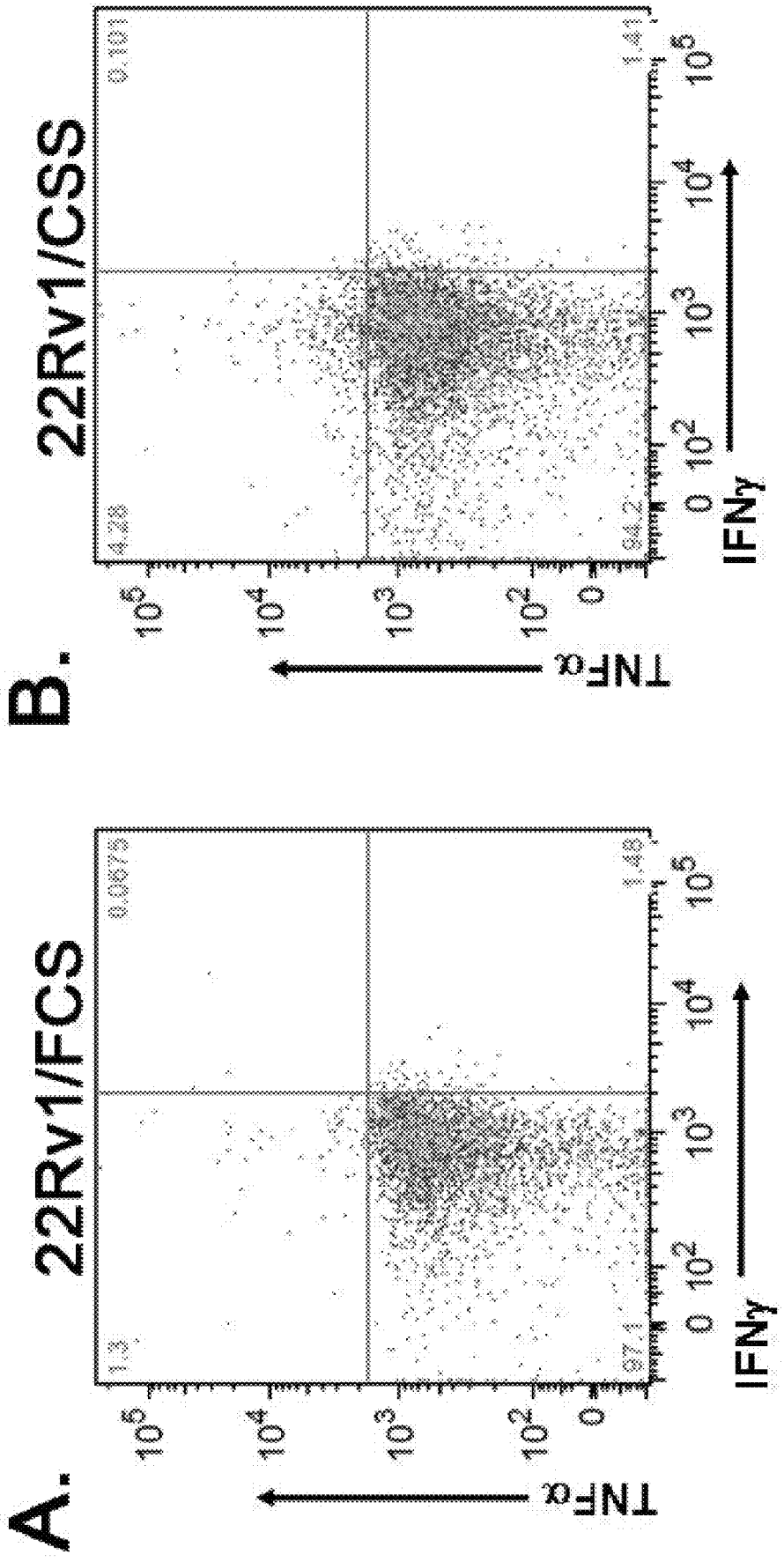

A.
ng Androgen Receptor / mg Cell Lysate
200
150
100
50
0
22Rv1/FCS
22Rv1/CSS
Cell Culture Conditions
*
B.
Relative Expression ($2^{-\Delta Ct}$)
0.020
0.015
0.010
0.005
0.000
22Rv1/FCS
22Rv1/CSS
Cell Culture Conditions
*

FIGS. 3A-3B

FIGS. 3C-3D


D.
22Rv1/CSS
22Rv1/FCS
22Rv1/WT
IgG
% of Max
0
20
40
60
80
100
0
10^2
10^3
10^4
10^5
PD-L1


C.
22Rv1/CSS
22Rv1/FCS
22Rv1/WT
IgG
% of Max
0
20
40
60
80
100
0
10^2
10^3
10^4
10^5
HLA-A2


A.
22Rv1/FCS
0.0675
1.48
1.3
97.1
TNFα
IFNγ
0
10^2
10^3
10^4
10^5
B.
22Rv1/CSS
0.101
1.41
4.28
93.2
TNFα
IFNγ
0
10^2
10^3
10^4
10^5

FIGS. 4A-4B

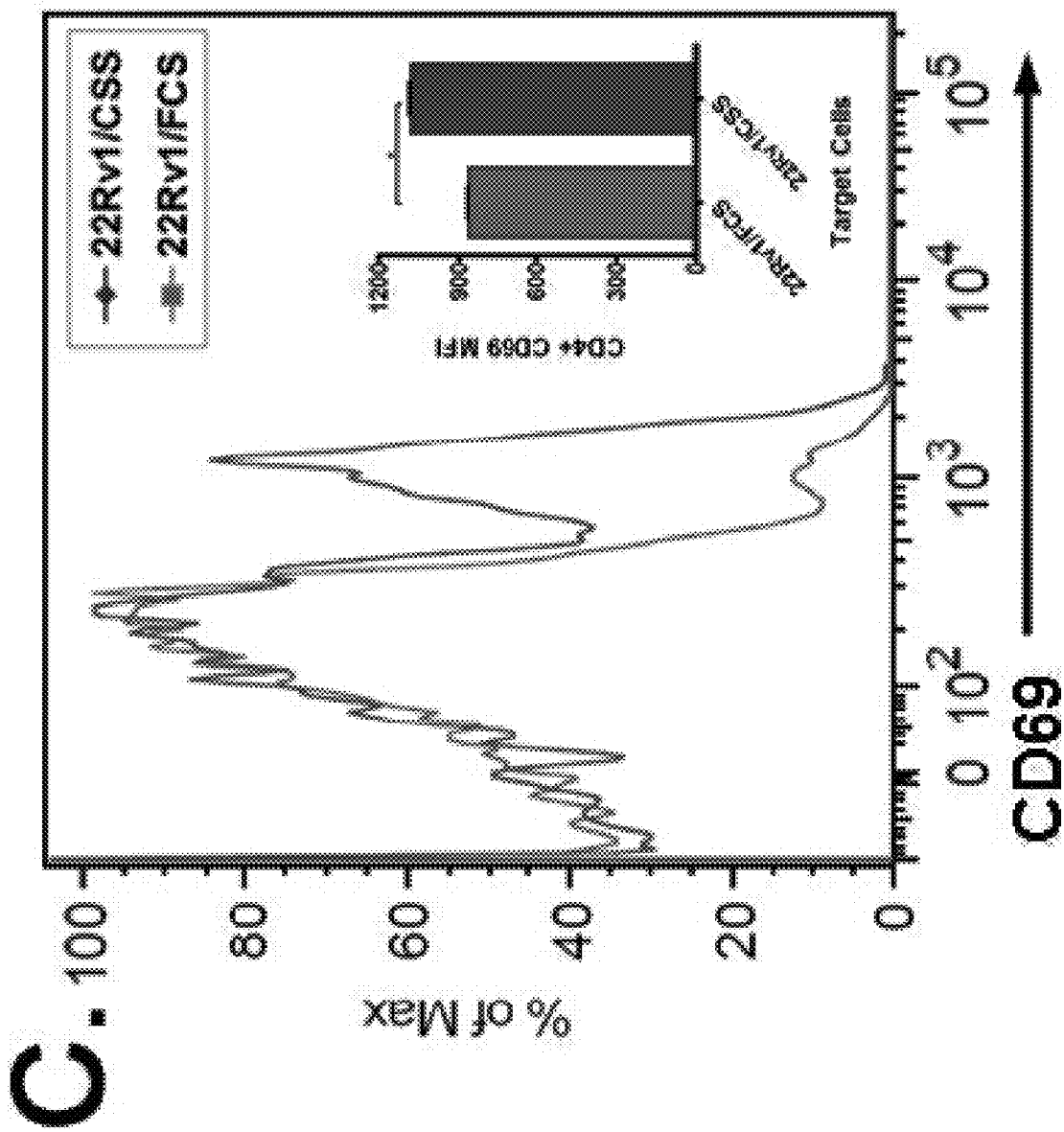

C.
22Rv1/CSS
22Rv1/FCS
% of Max
100
80
60
40
20
0
0 10^2 10^3 10^4 10^5
CD69
Target Cells
CD4+ CD69 MFI
1200
900
600
300
22Rv1/CSS
22Rv1/FCS

FIG. 4C

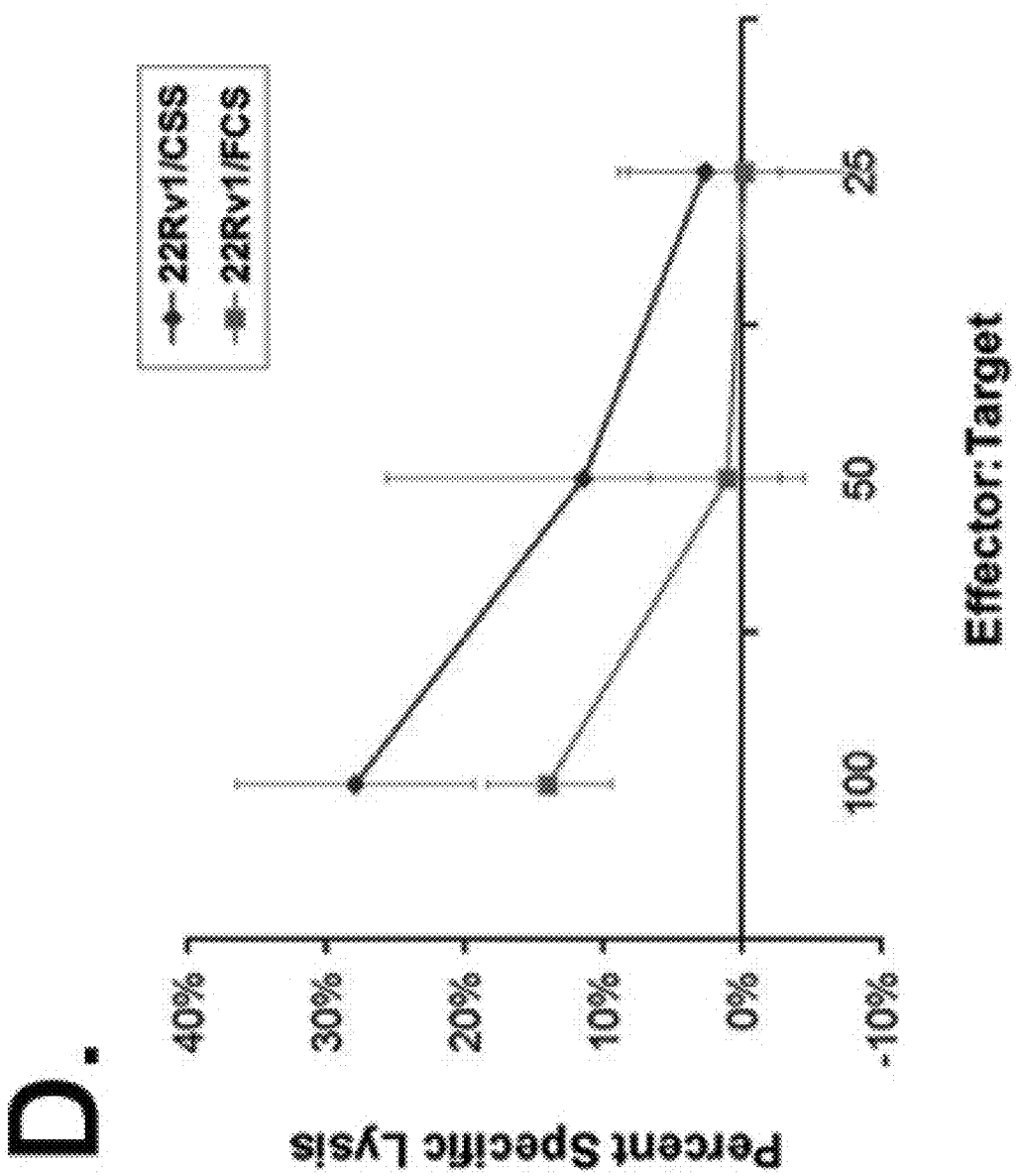

D.
Percent Specific Lysis
40%
30%
20%
10%
0%
-10%
Effector:Target
25
50
100
22Rv1/CSS
22Rv1/FCS

FIG. 4D

FIG. 5A

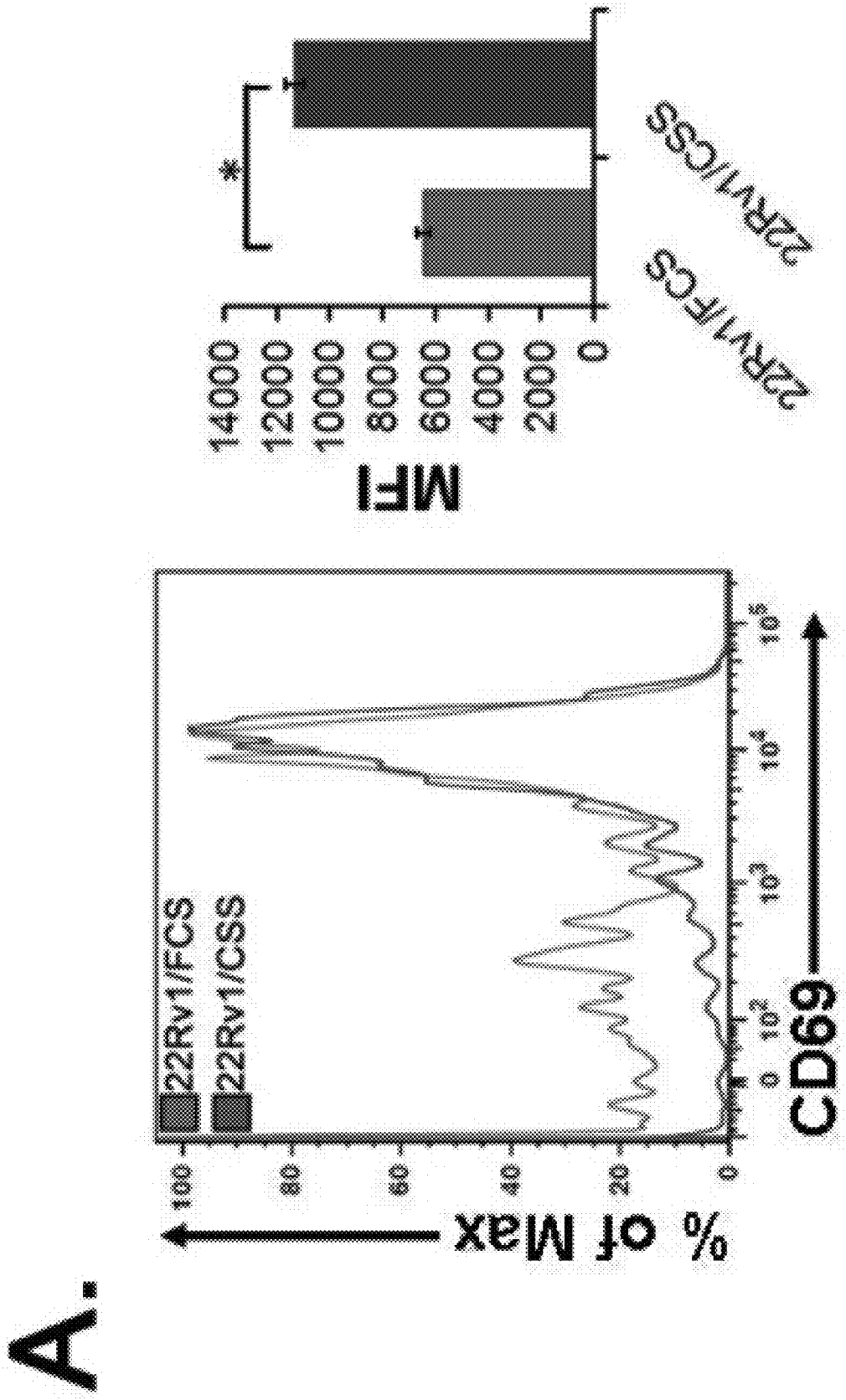
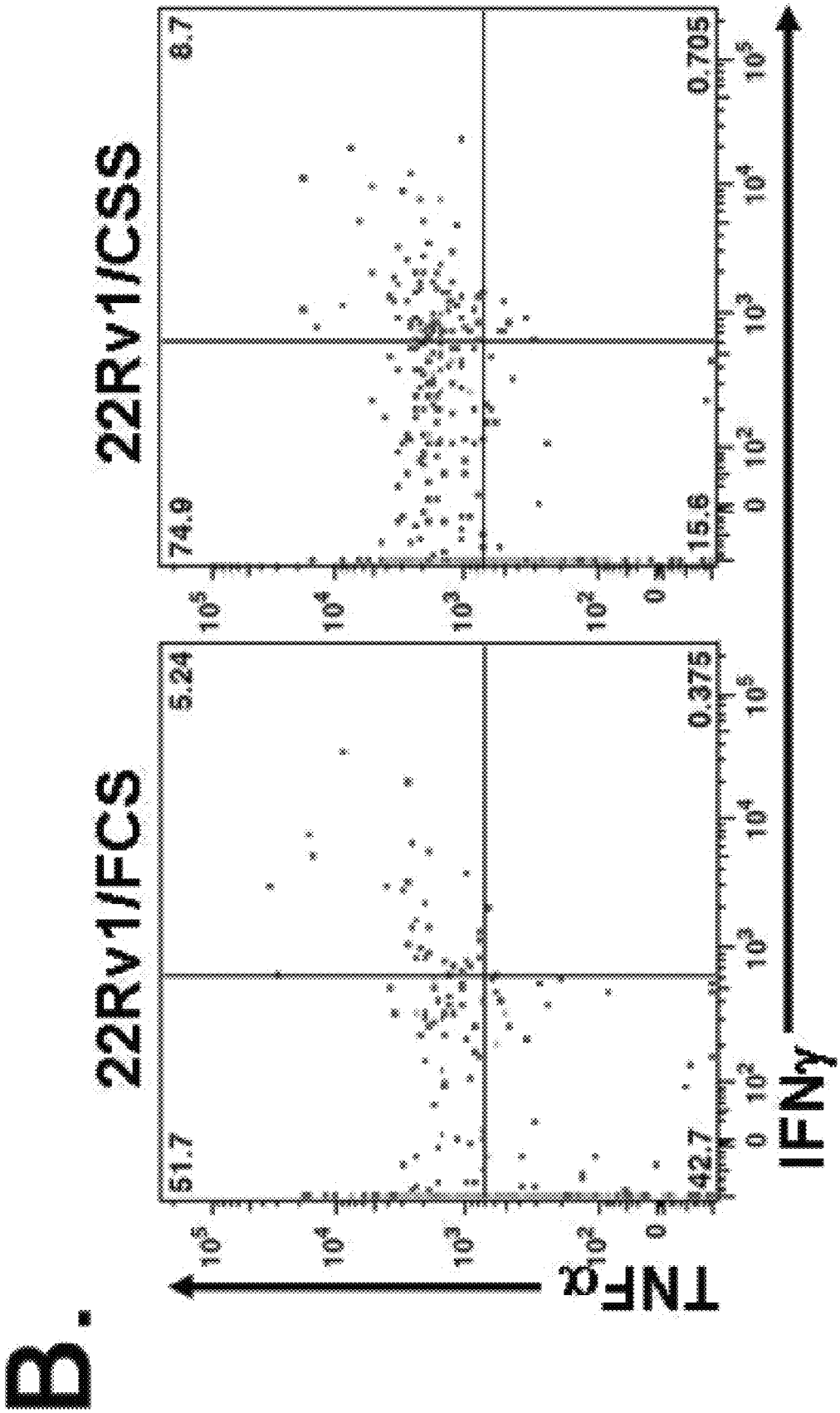

A.
% of Max
100
80
60
40
20
0
22Rv1/FCS
22Rv1/CSS
0
10^2
10^3
10^4
10^5
CD69
MFI
14000
12000
10000
8000
6000
4000
2000
0
*
22Rv1/FCS
22Rv1/CSS

FIG. 5B


B.
22Rv1/FCS
22Rv1/CSS
TNFα
IFNγ
51.7
5.24
42.7
0.375
74.9
8.7
15.6
0.705
0
10^2
10^3
10^4
10^5

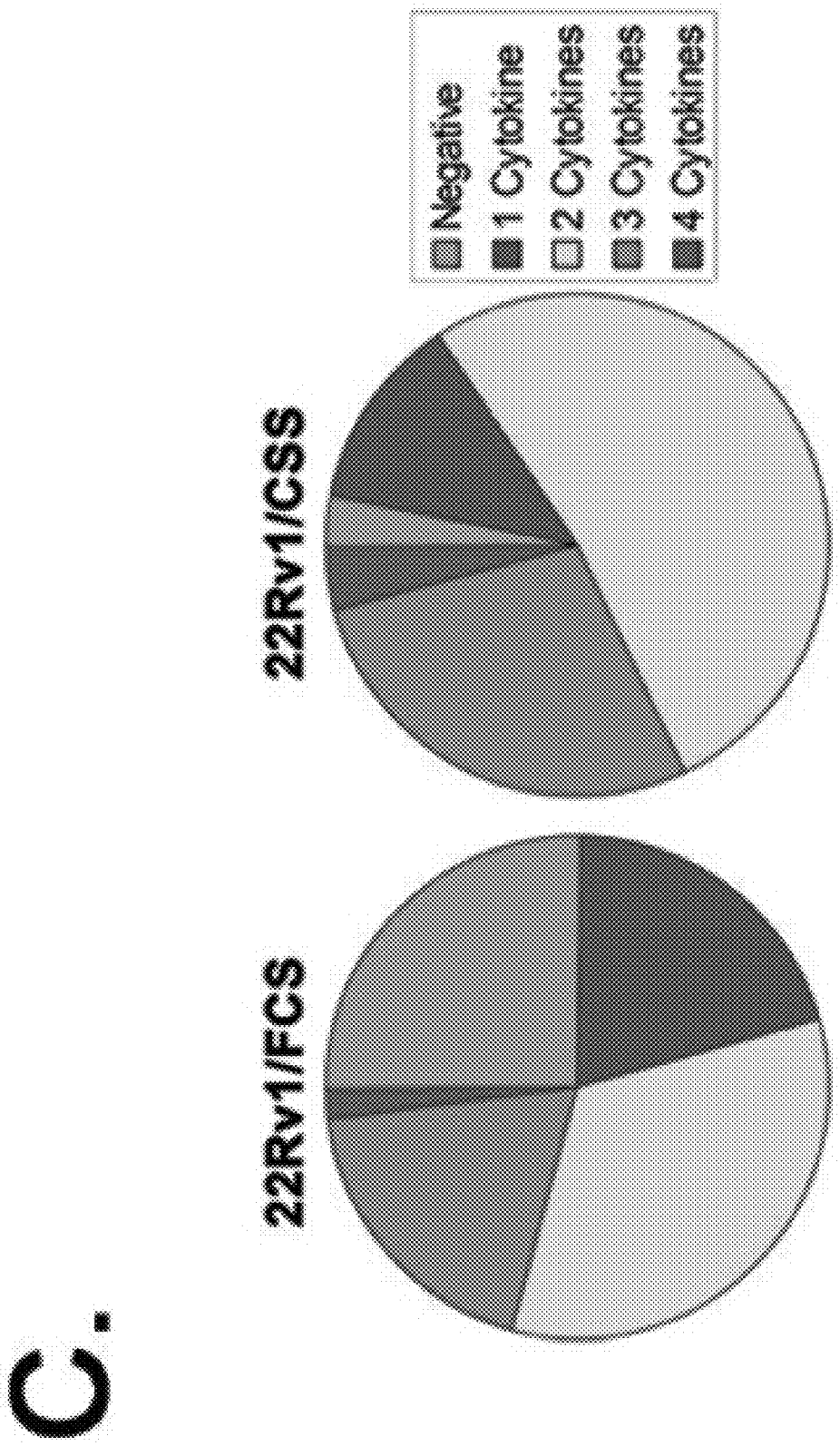

C.
22Rv1/FCS
22Rv1/CSS
Negative
1 Cytokine
2 Cytokines
3 Cytokines
4 Cytokines

FIG. 5C

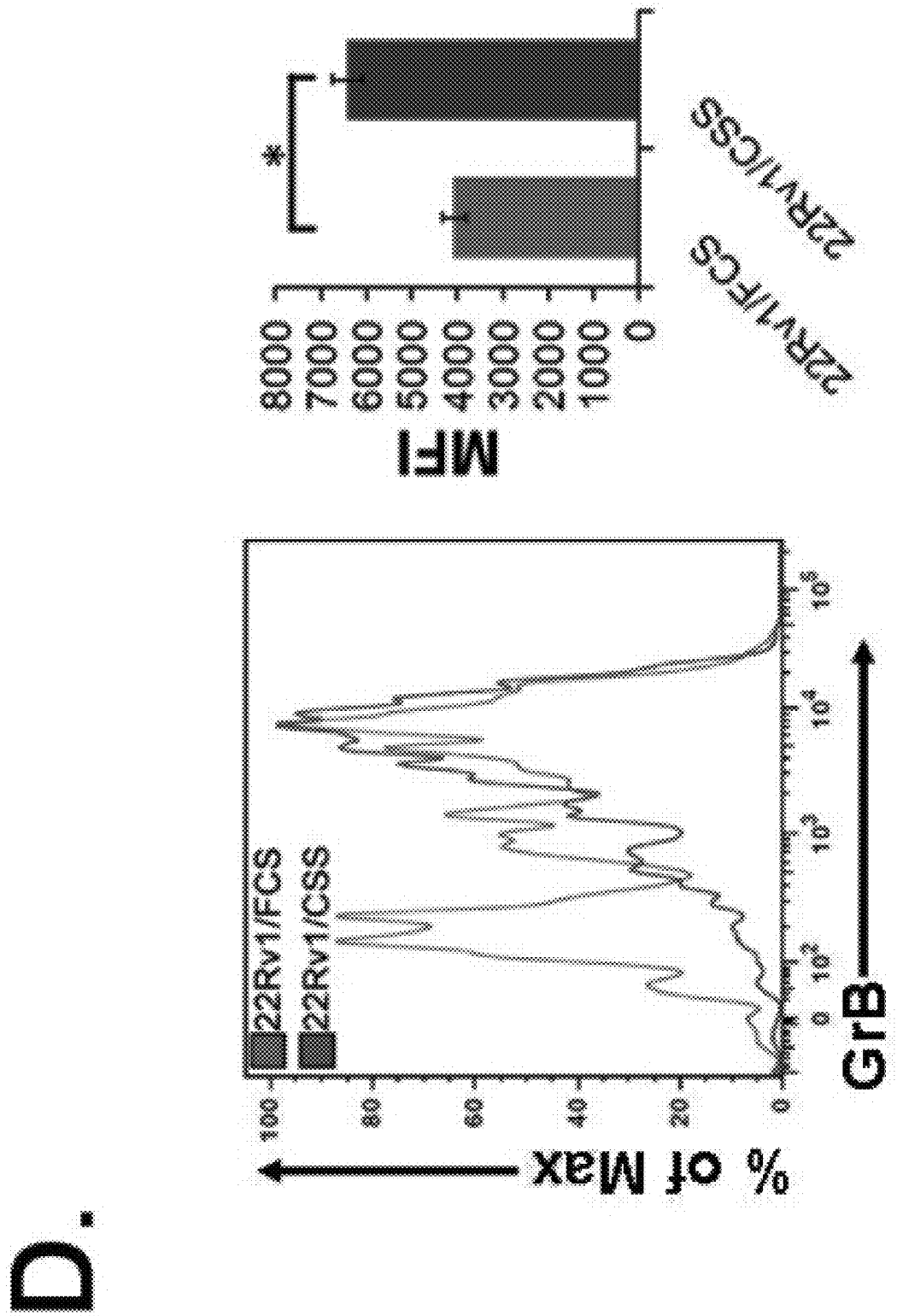
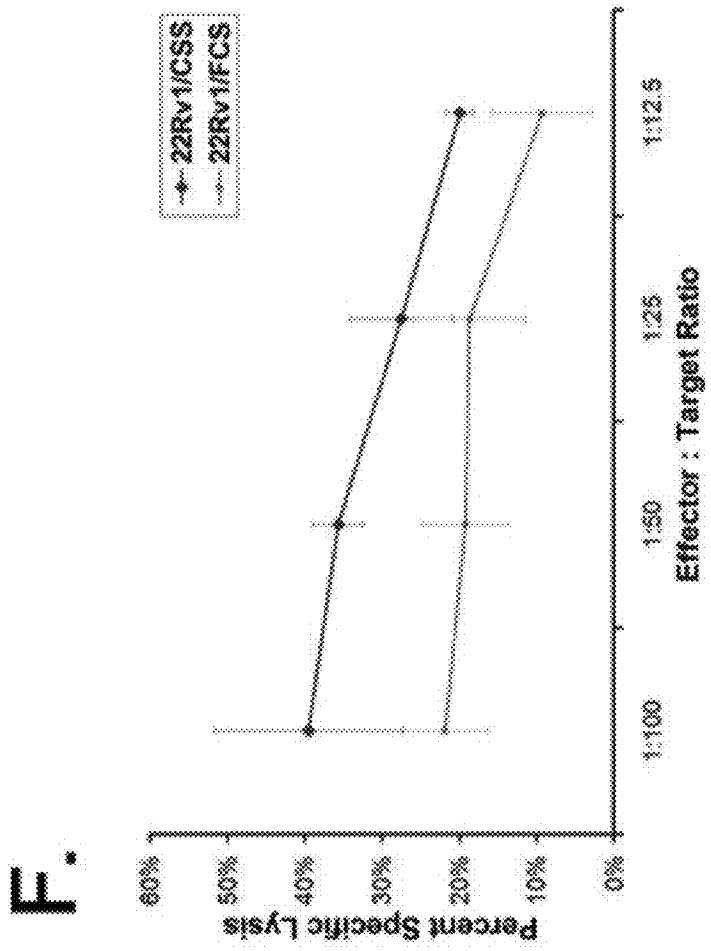
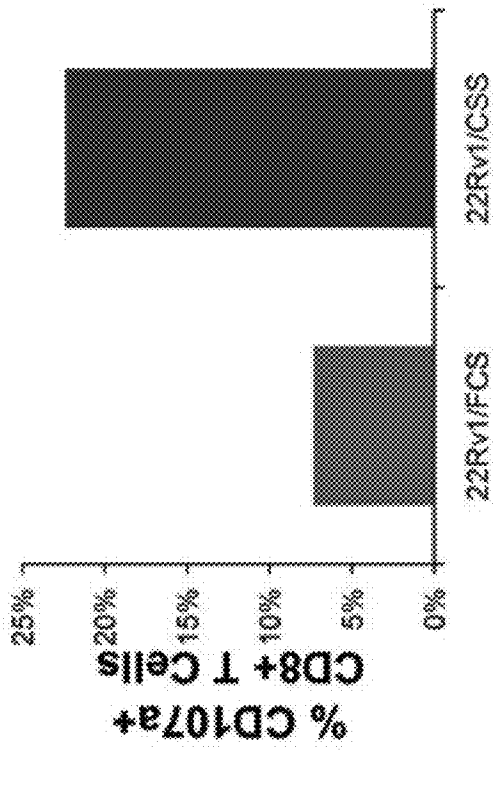

D.
% of Max
100
80
60
40
20
0
22Rv1/FCS
22Rv1/CSS
0
10^2
10^3
10^4
10^5
GrB
MFI
8000
7000
6000
5000
4000
3000
2000
1000
0
22RV1/FCS
22RV1/CSS
*

FIG. 5D

FIGS. 5E-5F

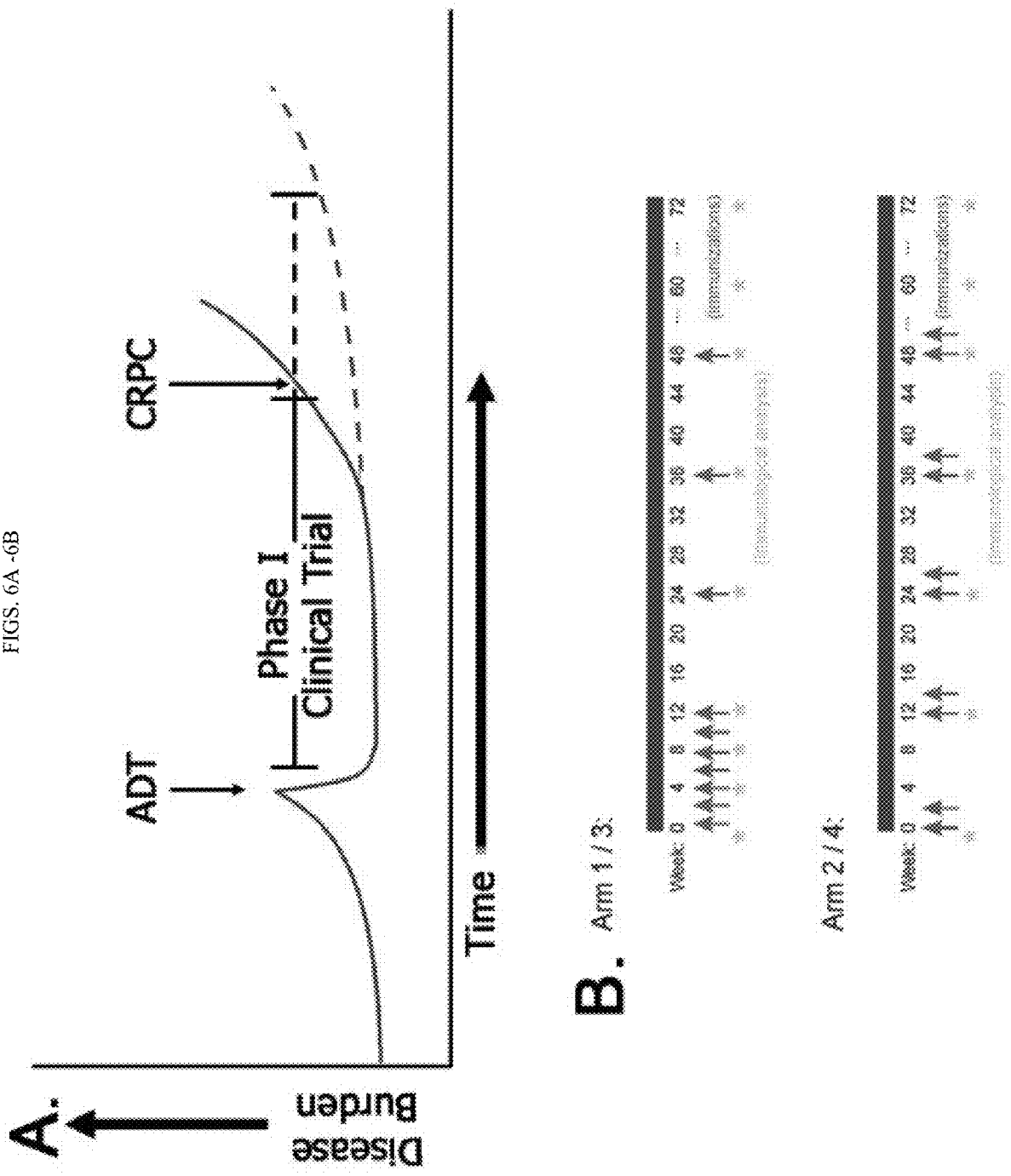

22Rv1/CSS
22Rv1/FCS
Percent Specific Lysis
60%
50%
40%
30%
20%
10%
0%
1:100
1:50
1:25
1:12.5
Effector : Target Ratio

F

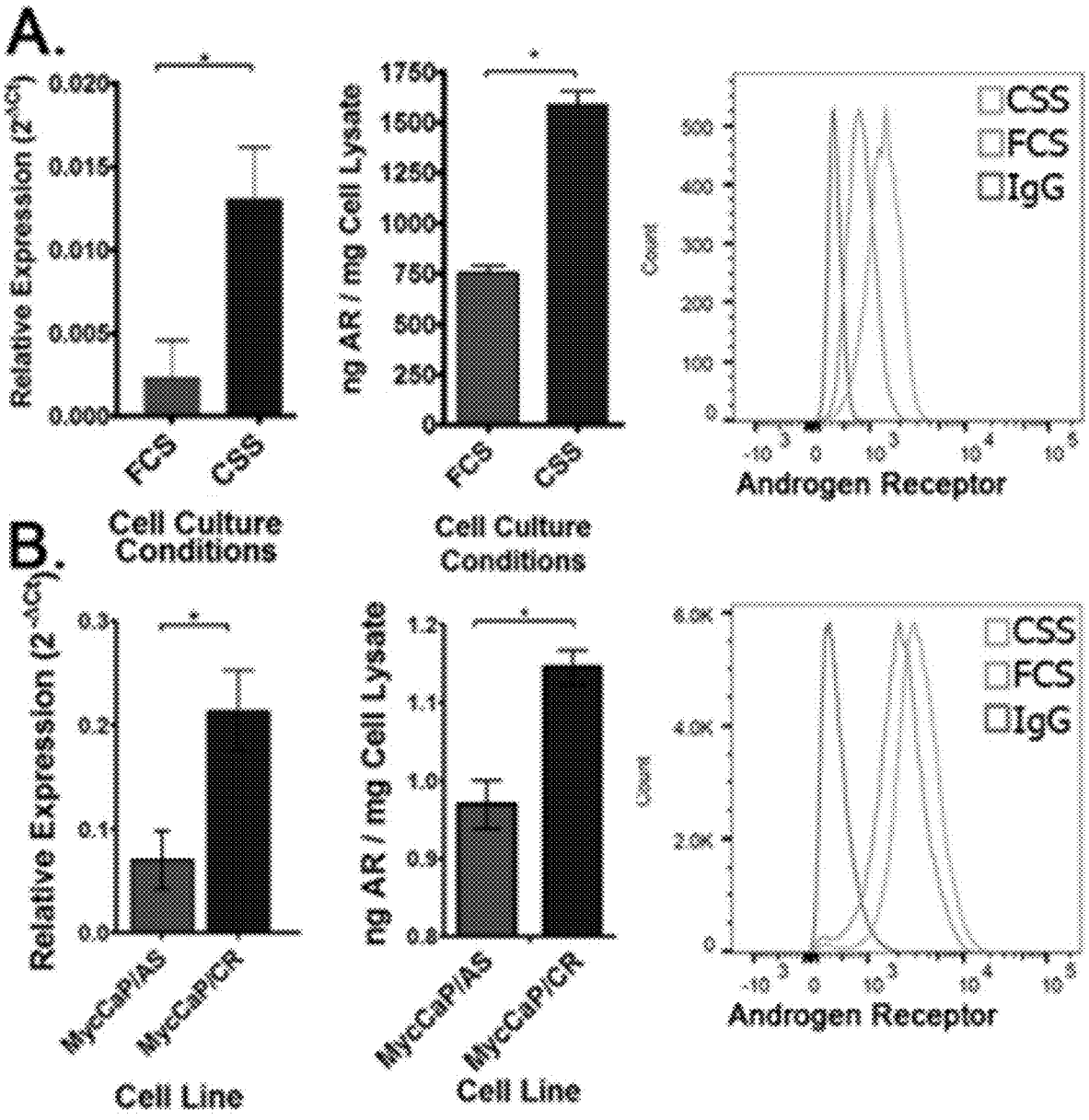

% CD107a+ CD8+ T Cells
25%
20%
15%
10%
5%
0%
22Rv1/FCS
22Rv1/CSS

E

FIGS. 6A -6B


A.
Disease Burden
Time
ADT
Phase I Clinical Trial
CRPC
B.
Arm 1 / 3:
Arm 2 / 4:

FIGS. 7A-7B


A.
Relative Expression ($2^{-\Delta Ct}$)
0.020
0.015
0.010
0.005
0.000
FCS
CSS
Cell Culture Conditions
ng AR / mg Cell Lysate
1750
1500
1250
1000
750
500
250
0
FCS
CSS
Cell Culture Conditions
Count
CSS
FCS
IgG
Androgen Receptor
B.
Relative Expression ($2^{-\Delta Ct}$)
0.3
0.2
0.1
0.0
MycCaPIAS
MycCaPICR
Cell Line
ng AR / mg Cell Lysate
1.2
1.1
1.0
0.9
0.8
MycCaPIAS
MycCaPICR
Cell Line
Count
CSS
FCS
IgG
Androgen Receptor

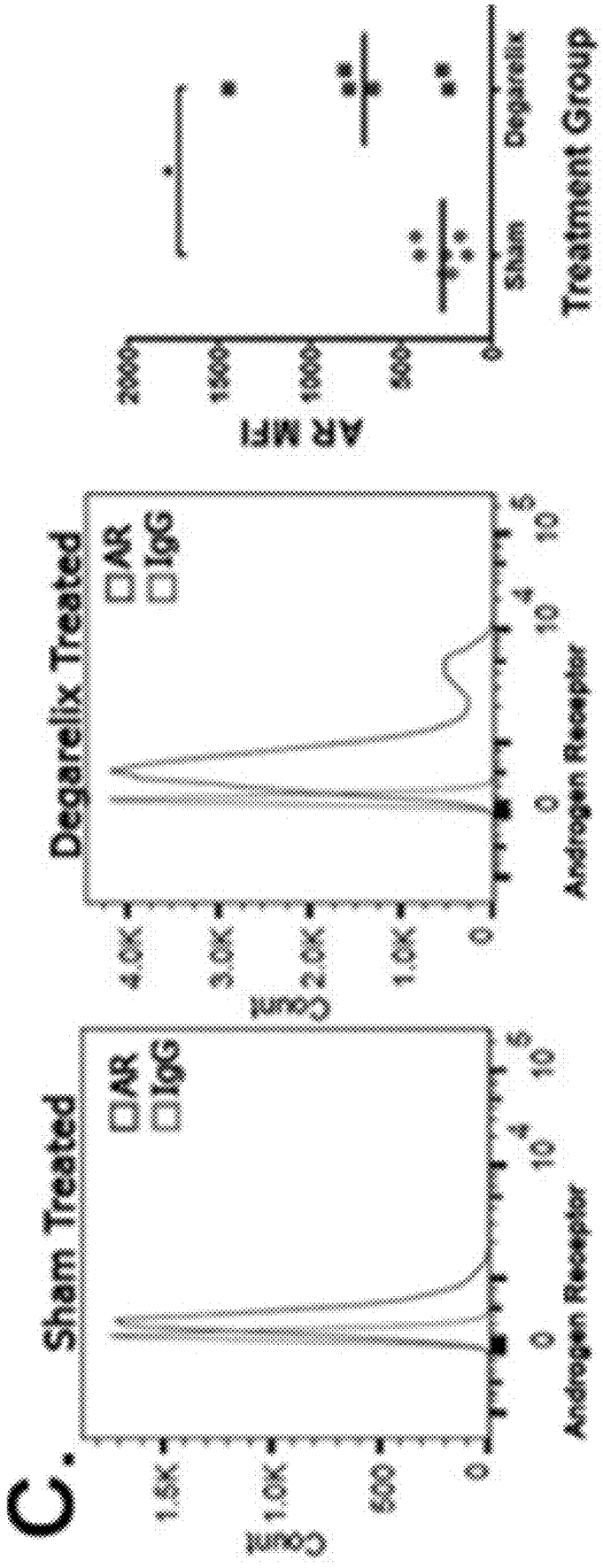
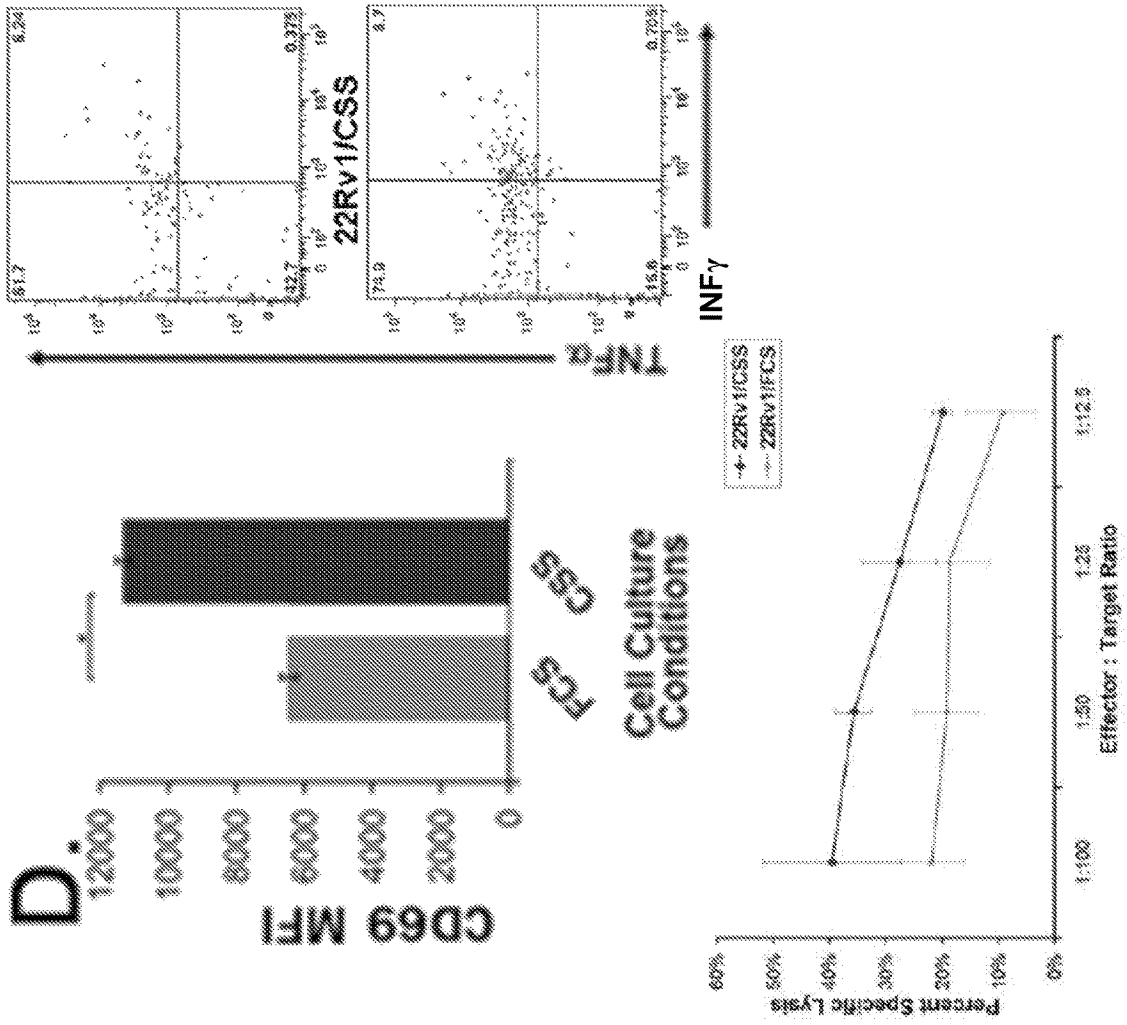
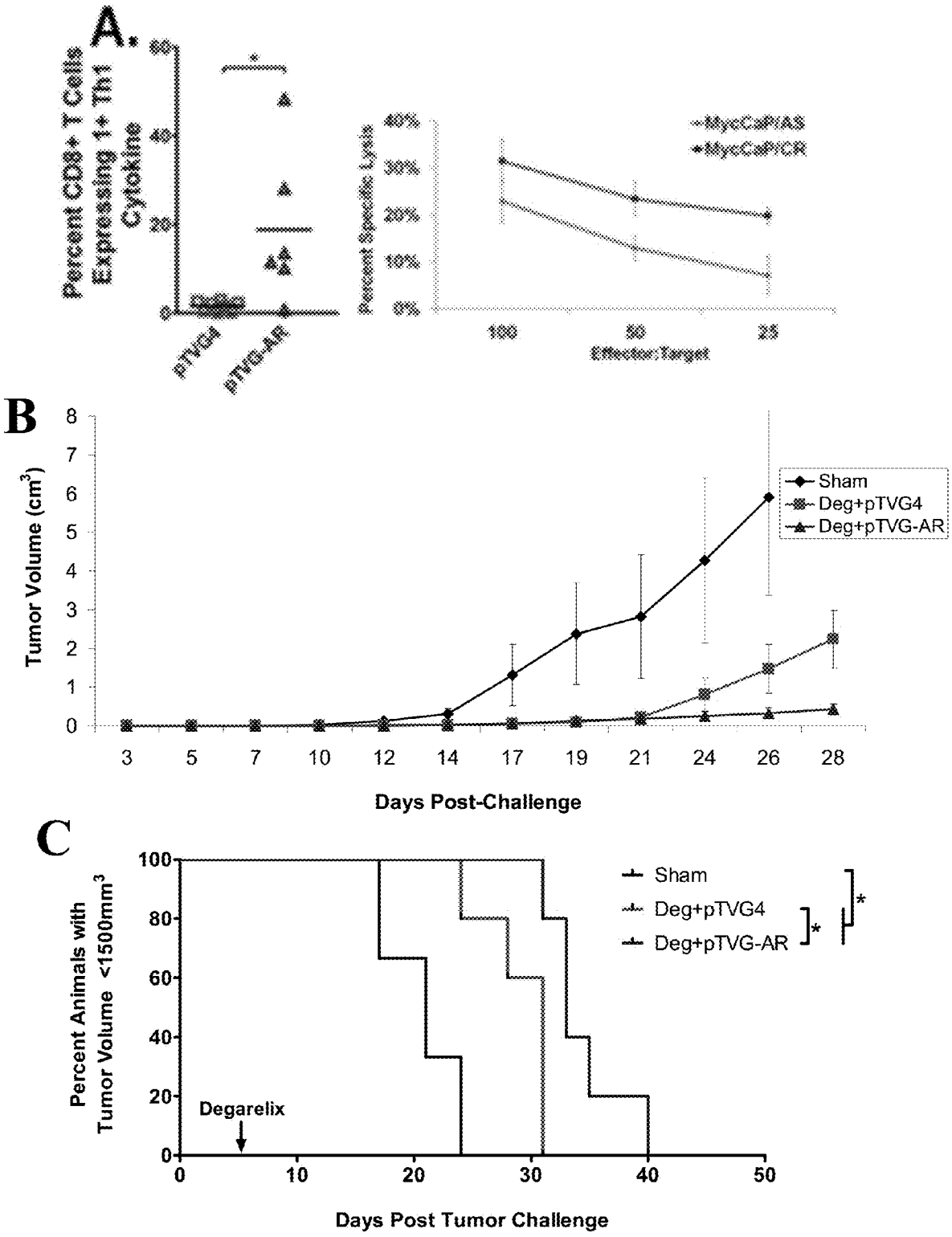

C.
Sham Treated
AR
IgG
Count
1.5K
1.0K
500
0
0
10^4
10^5
Antigen Receptor
Degarelix Treated
AR
IgG
Count
4.0K
3.0K
2.0K
1.0K
0
0
10^4
10^5
Antigen Receptor
AR MFI
2000
1500
1000
500
0
Sham
Degarelix
Treatment Group

FIG. 7C

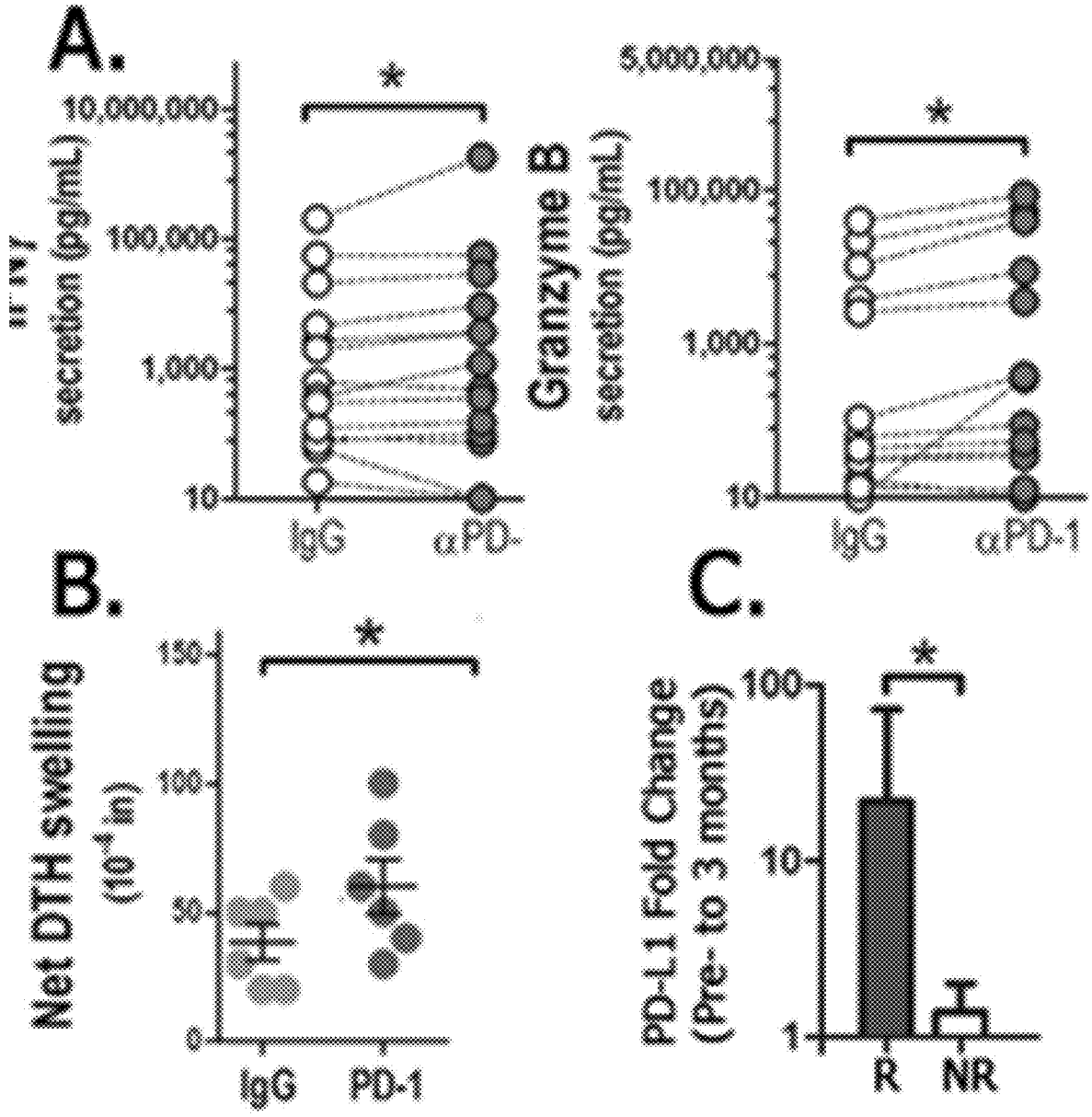
FIG. 7D


22Rv1/FCS
22Rv1/CSS
TNFα
INFγ
D.
CD69 MFI
12000
10000
8000
6000
4000
2000
0
FCS
CSS
Cell Culture Conditions
22Rv1/CSS
22Rv1/FCS
Percent Specific Lysis
60%
50%
40%
30%
20%
10%
0%
1:100
1:50
1:25
1:12.5
Effector : Target Ratio

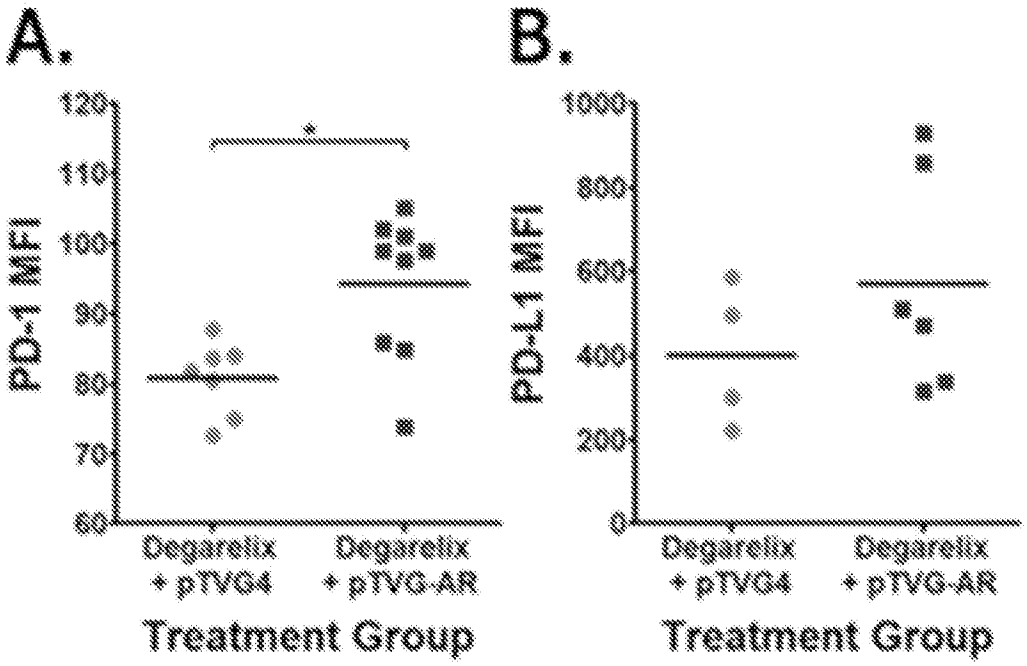
FIGS. 8A-8C


A.
Percent CD8+ T Cells Expressing 1+ Th1 Cytokine
pTVG4
pTVG-AR
Percent Specific Lysis
MycCaPTAS
MycCaPTCR
Effector:Target
B
Tumor Volume (cm3)
Sham
Deg+pTVG4
Deg+pTVG-AR
Days Post-Challenge
C
Percent Animals with Tumor Volume <1500mm3
Degarelix
Sham
Deg+pTVG4
Deg+pTVG-AR
Days Post Tumor Challenge

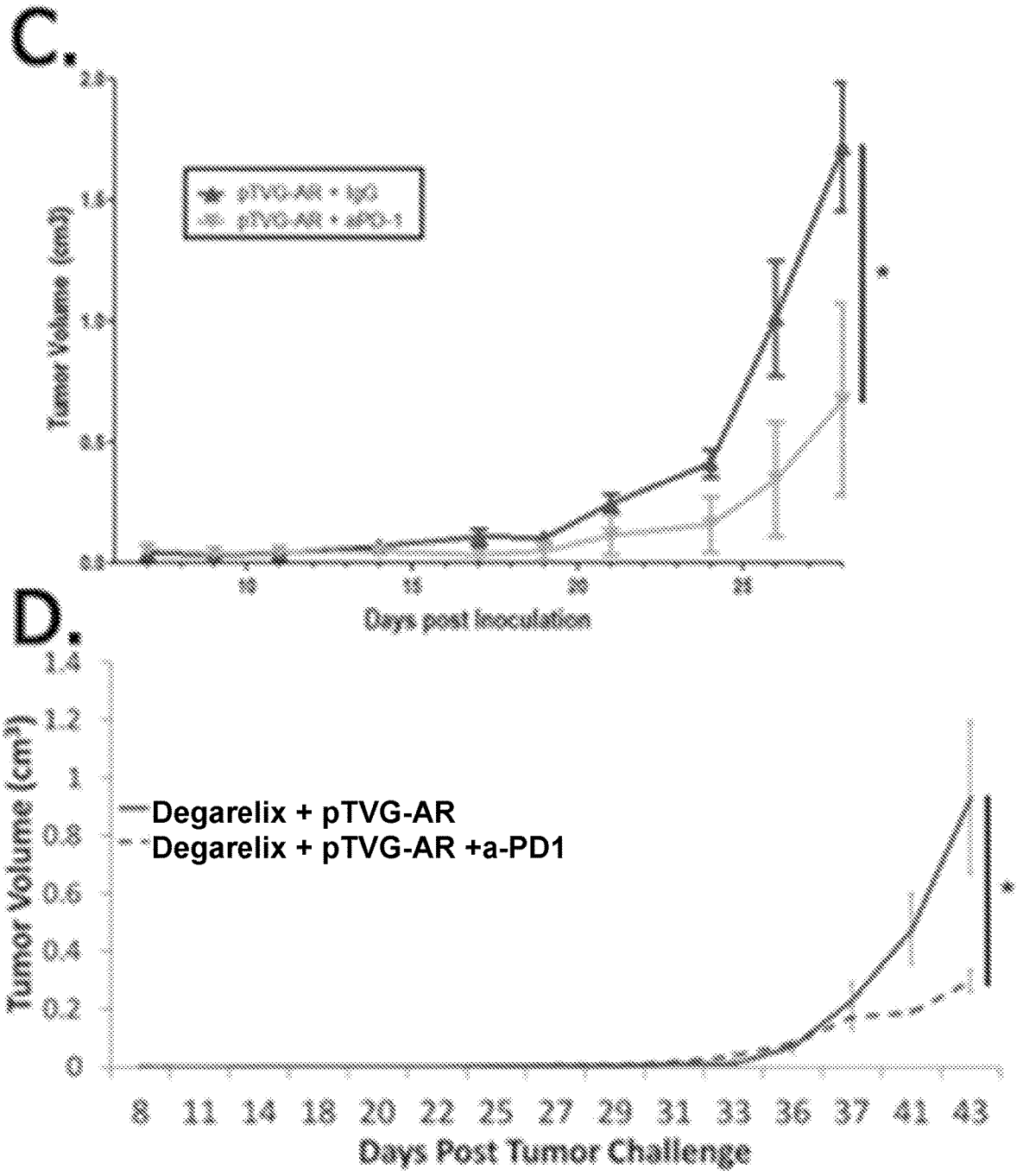
FIGS. 9A-9C


A.
IFNγ secretion (pg/mL)
10,000,000
100,000
1,000
10
IgG
αPD-
Granzyme B secretion (pg/mL)
5,000,000
100,000
1,000
10
IgG
αPD-1
B.
Net DTH swelling (10^{-4} in)
150
100
50
0
IgG
PD-1
C.
PD-L1 Fold Change (Pre- to 3 months)
100
10
1
R
NR

FIGS. 10A-10B


A.
PD-1 MFI
120
110
100
90
80
70
60
Degarelix + pTVG4
Degarelix + pTVG-AR
Treatment Group
B.
PD-L1 MFI
1000
800
600
400
200
0
Degarelix + pTVG4
Degarelix + pTVG-AR
Treatment Group

FIGS. 10C-10D


C.
Tumor Volume (cm³)
Days post Inoculation
D.
Degarelix + pTVG-AR
Degarelix + pTVG-AR +a-PD1
Tumor Volume (cm³)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
8 11 14 18 20 22 25 27 29 31 33 36 37 41 43
Days Post Tumor Challenge
*

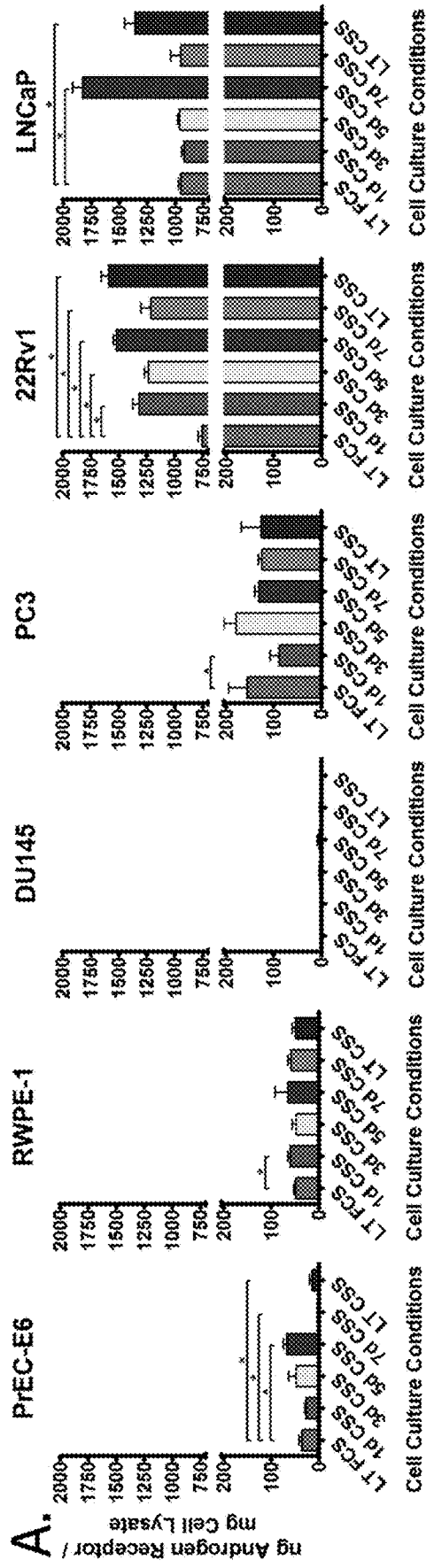
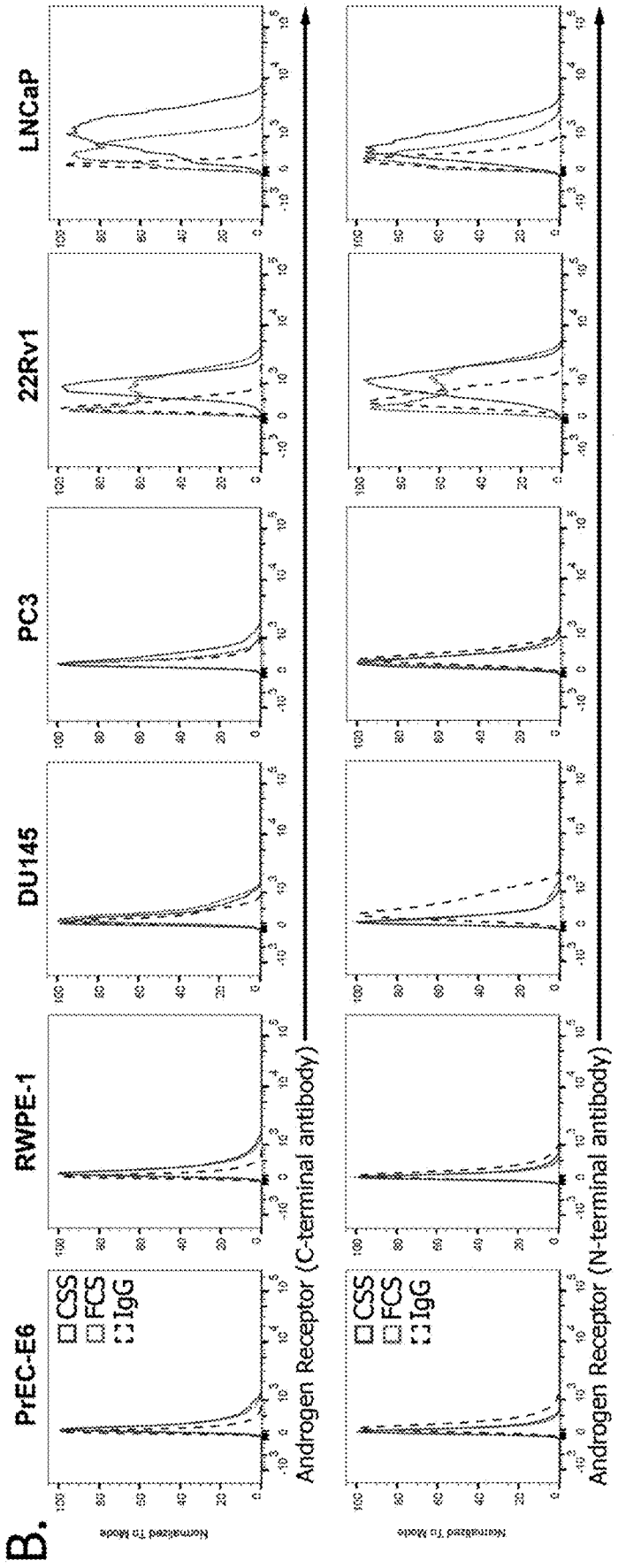
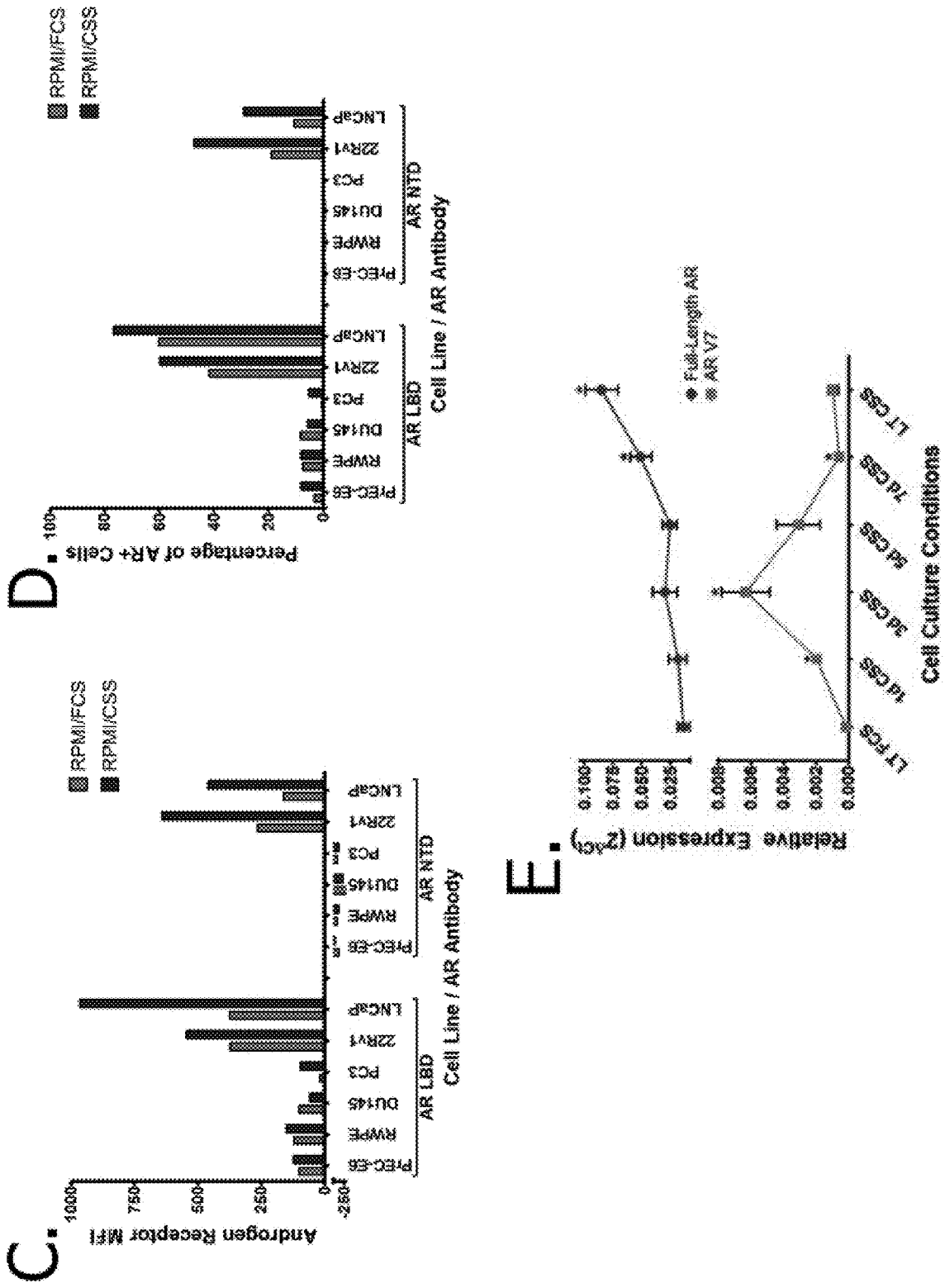

A.
ng Androgen Receptor / mg Cell Lysate
PrEC-E6
RWPE-1
DU145
PC3
22Rv1
LNCaP
Cell Culture Conditions
LT FCS
1d CSS
3d CSS
5d CSS
7d CSS
LT CSS

FIG. 11A

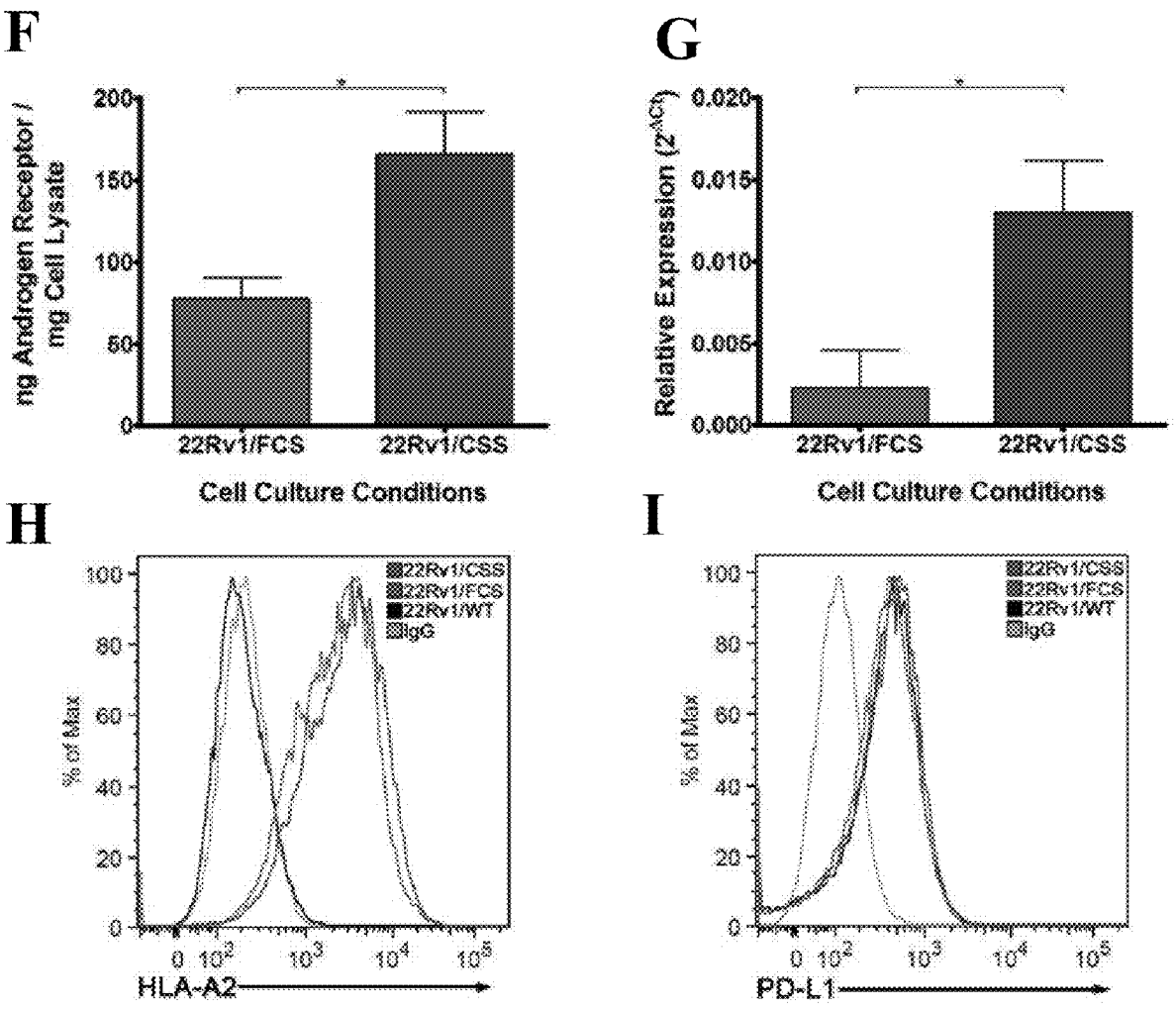
FIG. 11B


B.
PrEC-E6
RWPE-1
DU145
PC3
22Rv1
LNCaP
CSS
FCS
IgG
Normalized To Mode
Androgen Receptor (C-terminal antibody)
Androgen Receptor (N-terminal antibody)

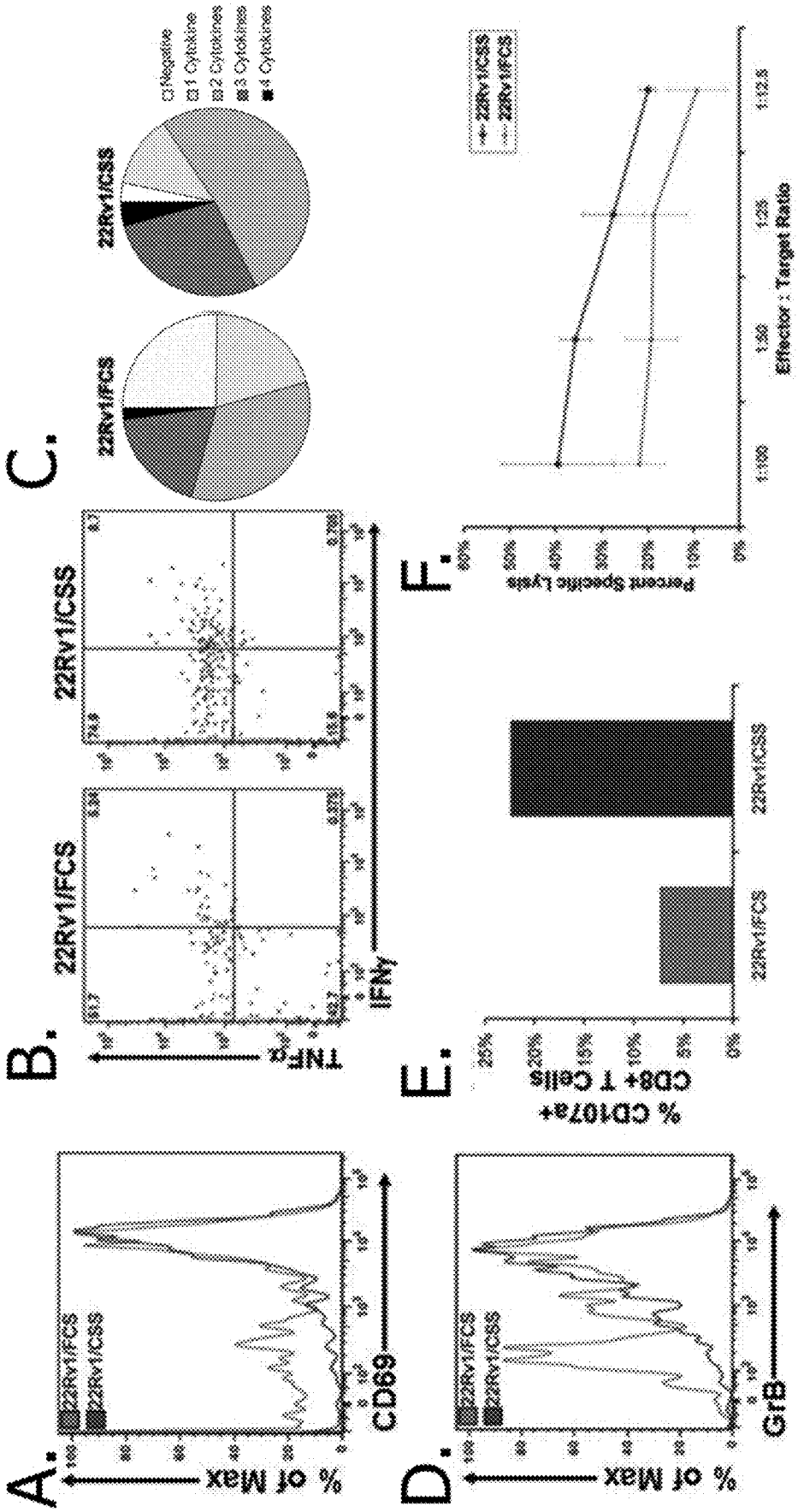
FIGS. 11C-11E


C.
Androgen Receptor MFI
RPMI/FCS
RPMI/CSS
PrEC-EB
RWPE
DU145
PC3
22Rv1
LNCaP
AR LBD
AR NTD
Cell Line / AR Antibody
D.
Percentage of AR+ Cells
E.
Relative Expression (2^-Ct)
Full-Length AR
AR V7
LT FCS
1d CSS
3d CSS
5d CSS
7d CSS
LT CSS
Cell Culture Conditions

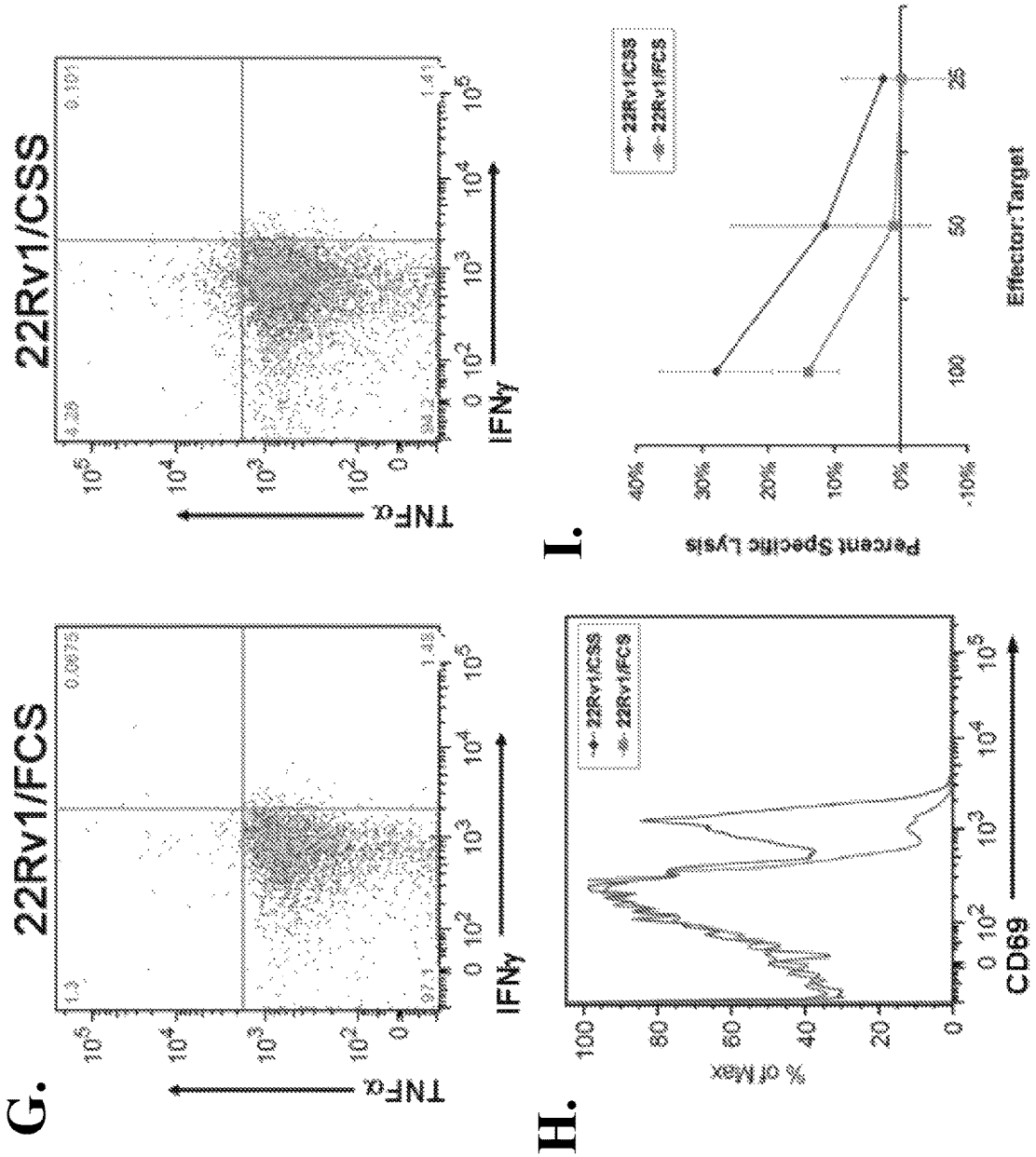
FIGS. 11F-11I


F
ng Androgen Receptor / mg Cell Lysate
200
150
100
50
0
*
22Rv1/FCS
22Rv1/CSS
Cell Culture Conditions
G
Relative Expression ($2^{-\Delta Ct}$)
0.020
0.015
0.010
0.005
0.000
*
22Rv1/FCS
22Rv1/CSS
Cell Culture Conditions
H
% of Max
100
80
60
40
20
0
22Rv1/CSS
22Rv1/FCS
22Rv1/WT
IgG
0 10^2 10^3 10^4 10^5
HLA-A2
I
% of Max
100
80
60
40
20
0
22Rv1/CSS
22Rv1/FCS
22Rv1/WT
IgG
0 10^2 10^3 10^4 10^5
PD-L1

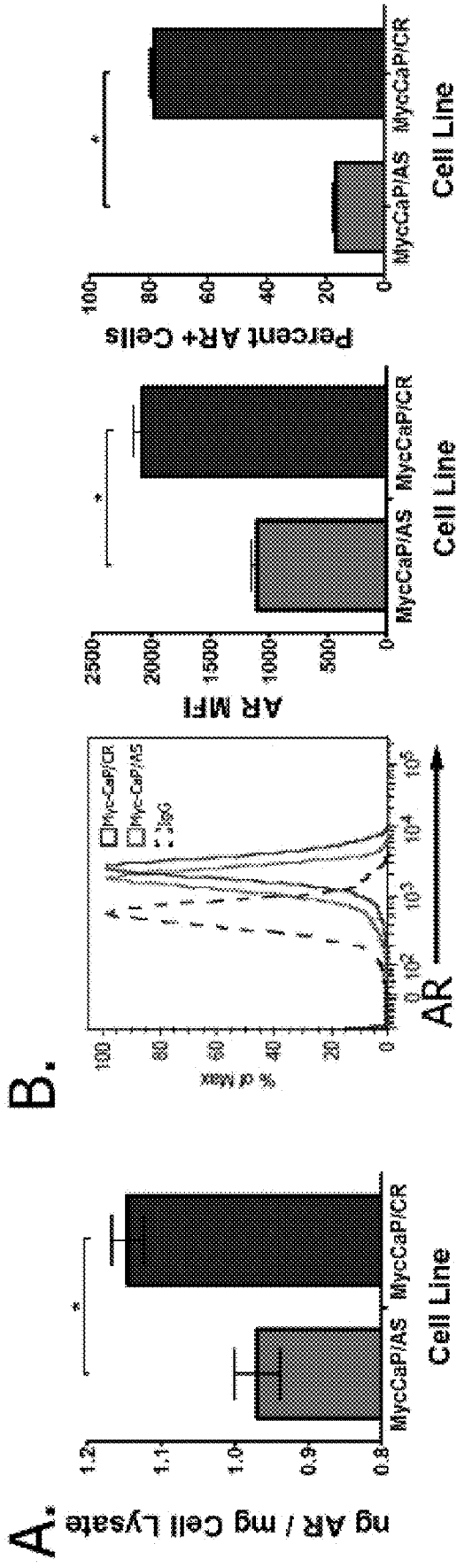
FIGS. 12A-12F


A.
% of Max
CD69
22Rv1/FCS
22Rv1/CSS
B.
22Rv1/FCS
22Rv1/CSS
TNFα
IFNγ
C.
22Rv1/CSS
22Rv1/FCS
Negative
1 Cytokine
2 Cytokines
3 Cytokines
4 Cytokines
D.
% of Max
GrB
22Rv1/FCS
22Rv1/CSS
E.
% CD107a+ CD8+ T Cells
22Rv1/FCS
22Rv1/CSS
F.
Percent Specific Lysis
Effector : Target Ratio
22Rv1/CSS
22Rv1/FCS

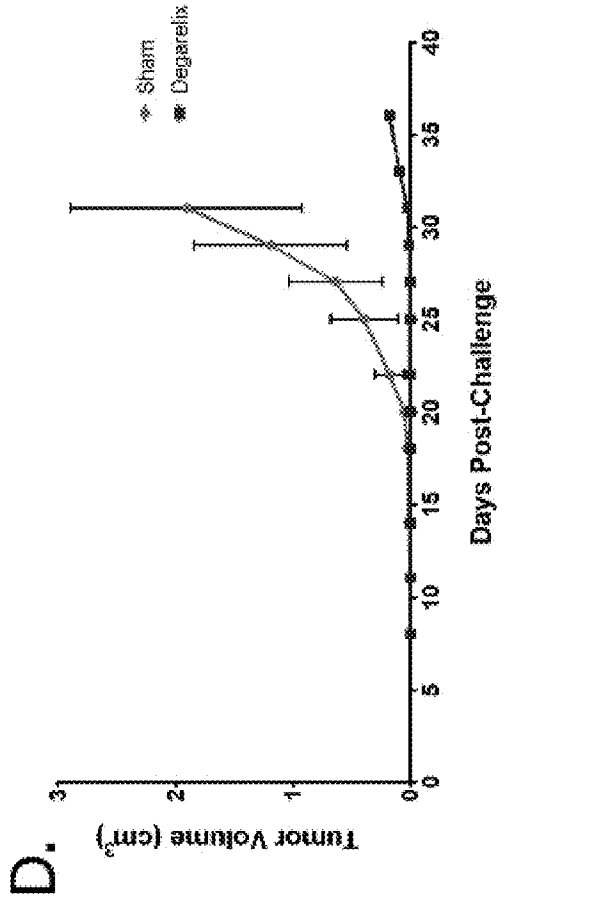
FIGS. 12G-12I

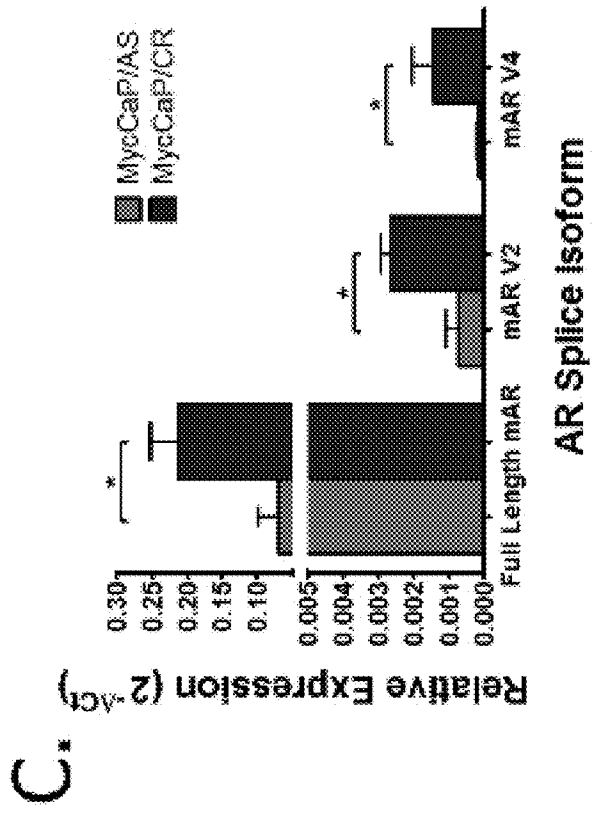

G.
22Rv1/CSS
22Rv1/FCS
TNFα
IFNγ
H.
22Rv1/CSS
22Rv1/FCS
% of Max
CD69
I.
22Rv1/CSS
22Rv1/FCS
Percent Specific Lysis
Effector:Target

FIGS. 13A-13B


A.
ng AR / mg Cell Lysate
1.2
1.1
1.0
0.9
0.8
MycCaP/AS
MycCaP/CR
Cell Line
B.
MycCaP/CR
MycCaP/AS
AR
AR MFI
2500
2000
1500
1000
500
0
MycCaP/AS
MycCaP/CR
Cell Line
Percent AR+ Cells
100
80
60
40
20
0
MycCaP/AS
MycCaP/CR
Cell Line

FIGS. 13C-13D


D.
Tumor Volume (cm³)
Days Post-Challenge
Sham
Degarelix
0
1
2
3
0
5
10
15
20
25
30
35
40


C.
Relative Expression (2-ΔCt)
AR Splice isoform
MycCaP/AS
MycCaP/CR
Full Length mAR
mAR V2
mAR V4
0.30
0.25
0.20
0.15
0.10
0.005
0.004
0.003
0.002
0.001
0.000
*
*
*

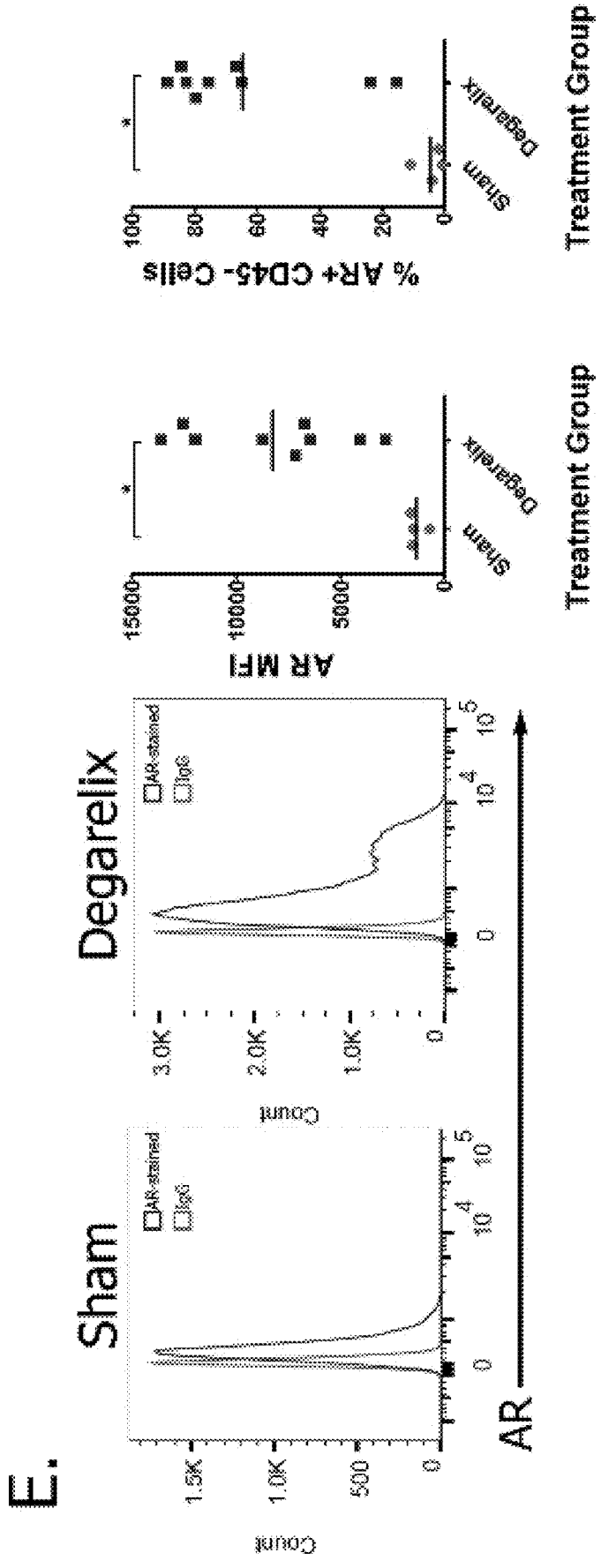
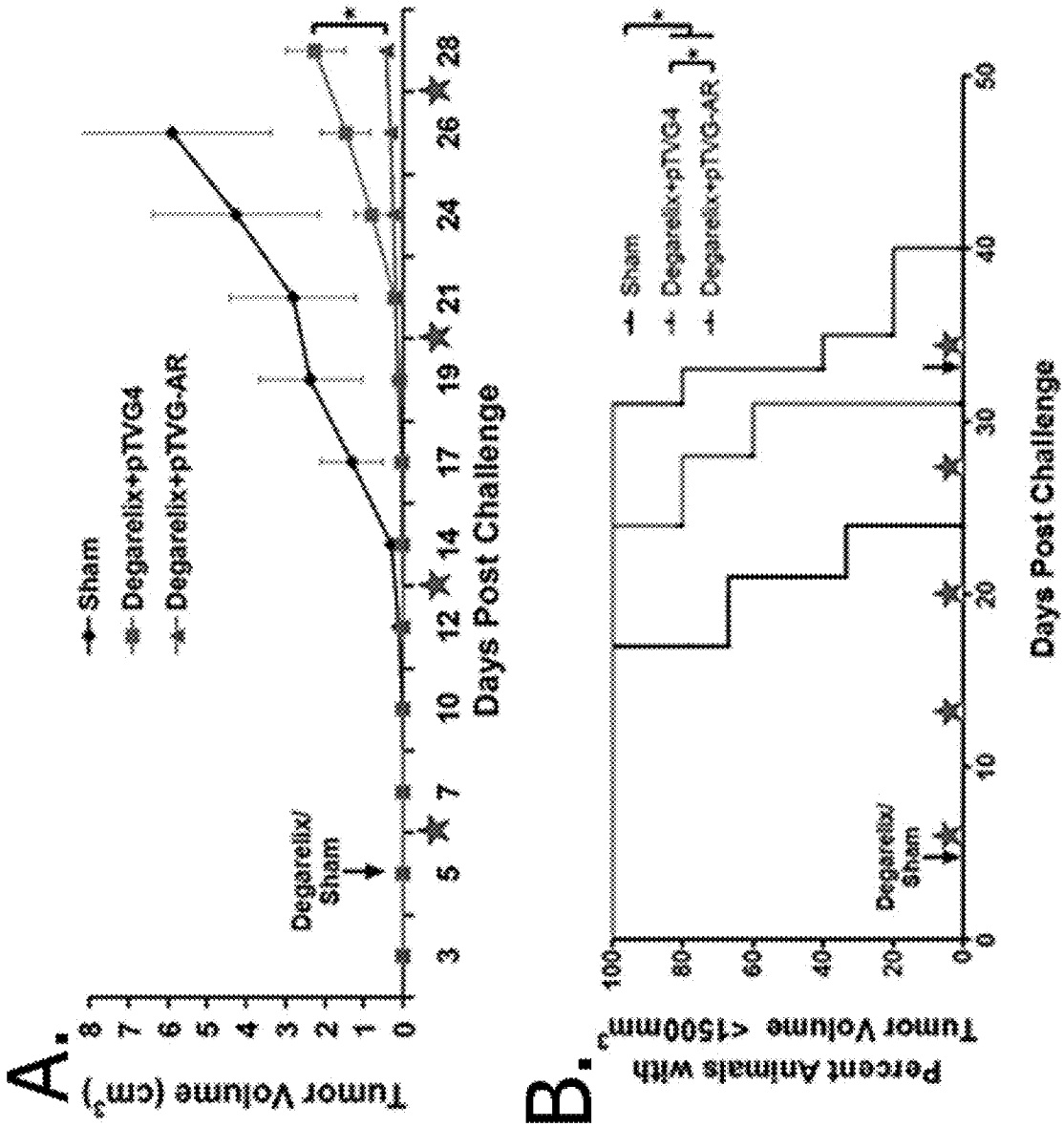
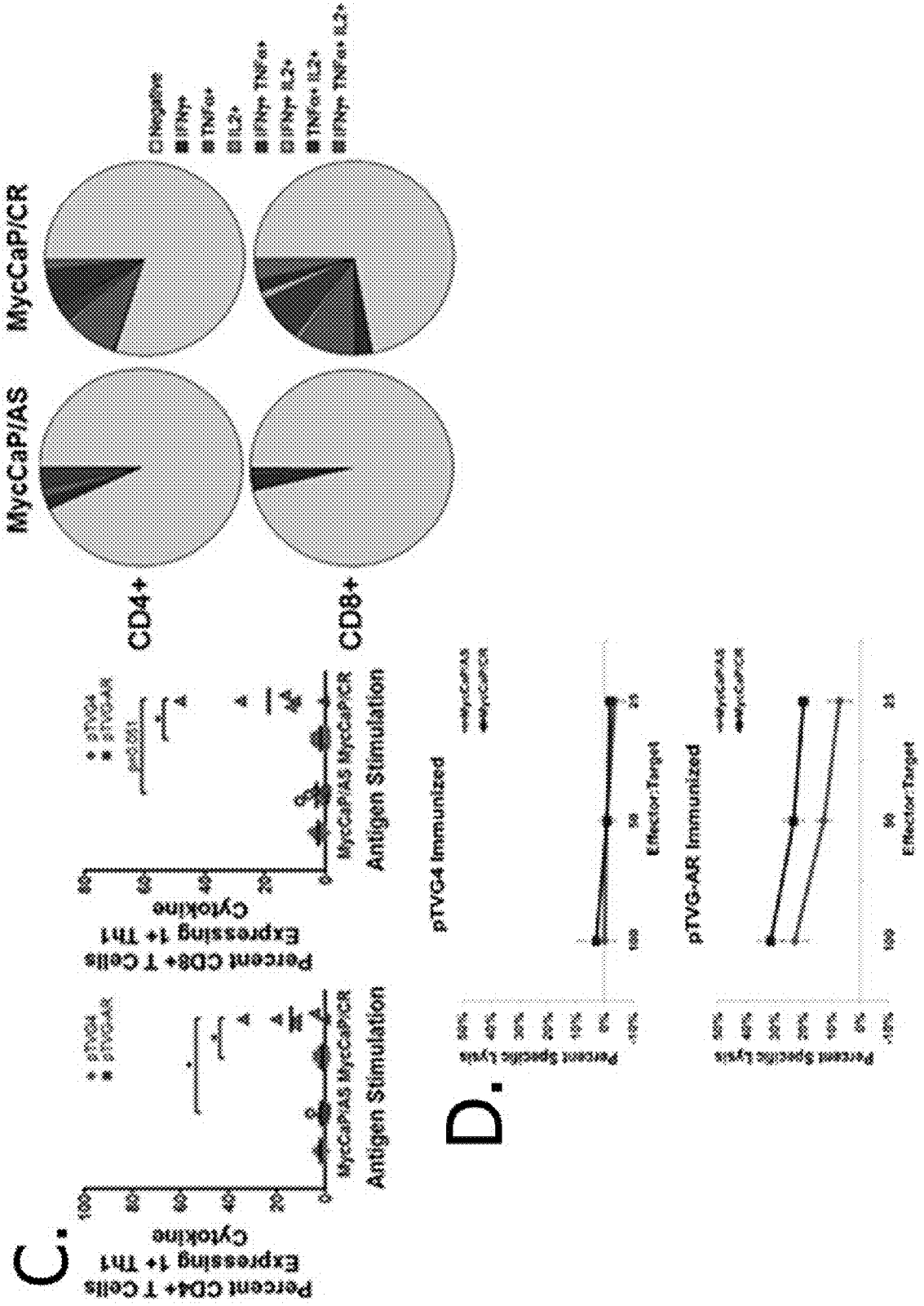

E.
Sham
Degarelix
AR-stained
Ig6
Count
AR
AR MFI
% AR+ CD45- Cells
Treatment Group
Sham
Degarelix

FIG. 13E

FIGS. 14A-14B

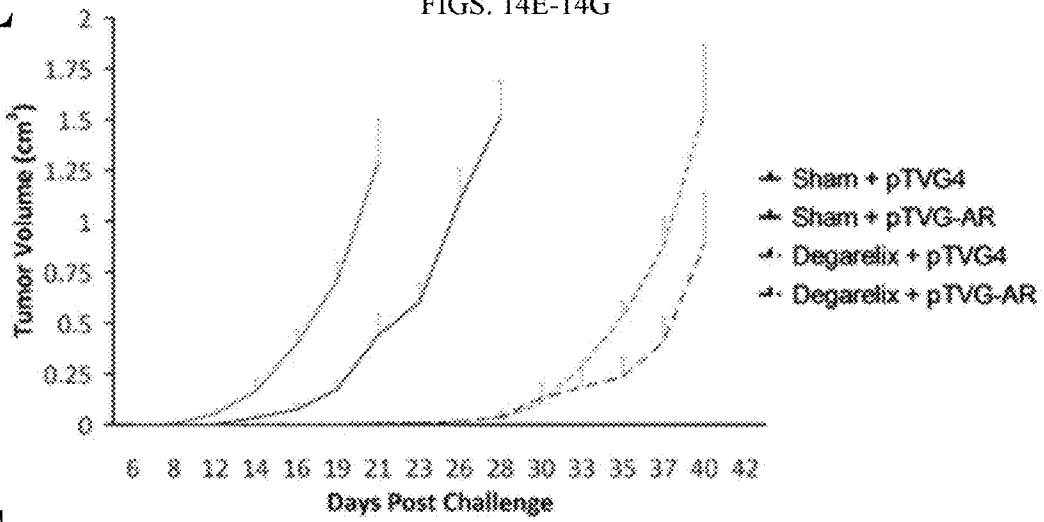

A.
Tumor Volume (cm³)
Sham
Degarelix+pTVG4
Degarelix+pTVG-AR
Degarelix/ Sham
Days Post Challenge
B.
Percent Animals with Tumor Volume <1500mm³
Sham
Degarelix+pTVG4
Degarelix+pTVG-AR
Degarelix/ Sham
Days Post Challenge

FIGS. 14C-14D

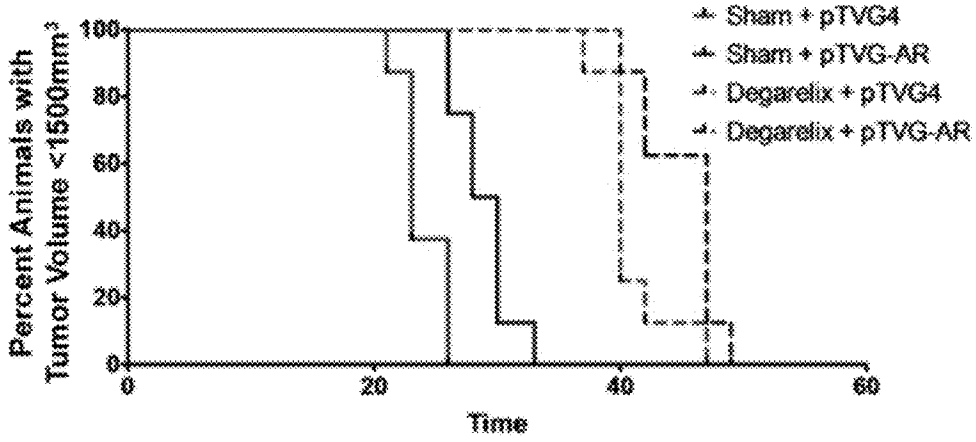

C.
Percent CD4+ T cells Expressing 1+ Th1 Cytokine
Percent CD8+ T cells Expressing 1+ Th1 Cytokine
Antigen Stimulation
MycCaP/AS MycCaP/CR
* pTVG4
* pTVG-AR
p=0.001
CD4+
CD8+
MycCaP/AS
MycCaP/CR
Negative
IFNγ+
TNFα+
IL2+
IFNγ+ TNFα+
IFNγ+ IL2+
TNFα+ IL2+
IFNγ+ TNFα+ IL2+
D.
pTVG4 Immunized
pTVG-AR Immunized
Percent Specific Lysis
Effector:Target

FIGS. 14E-14G

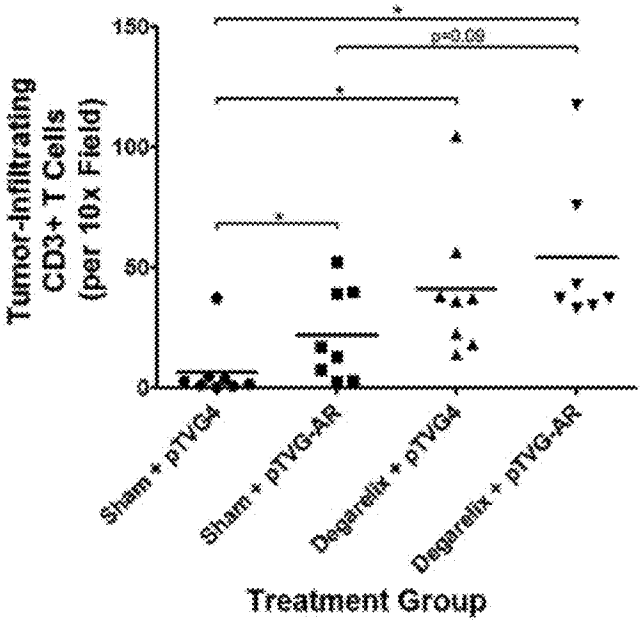
E

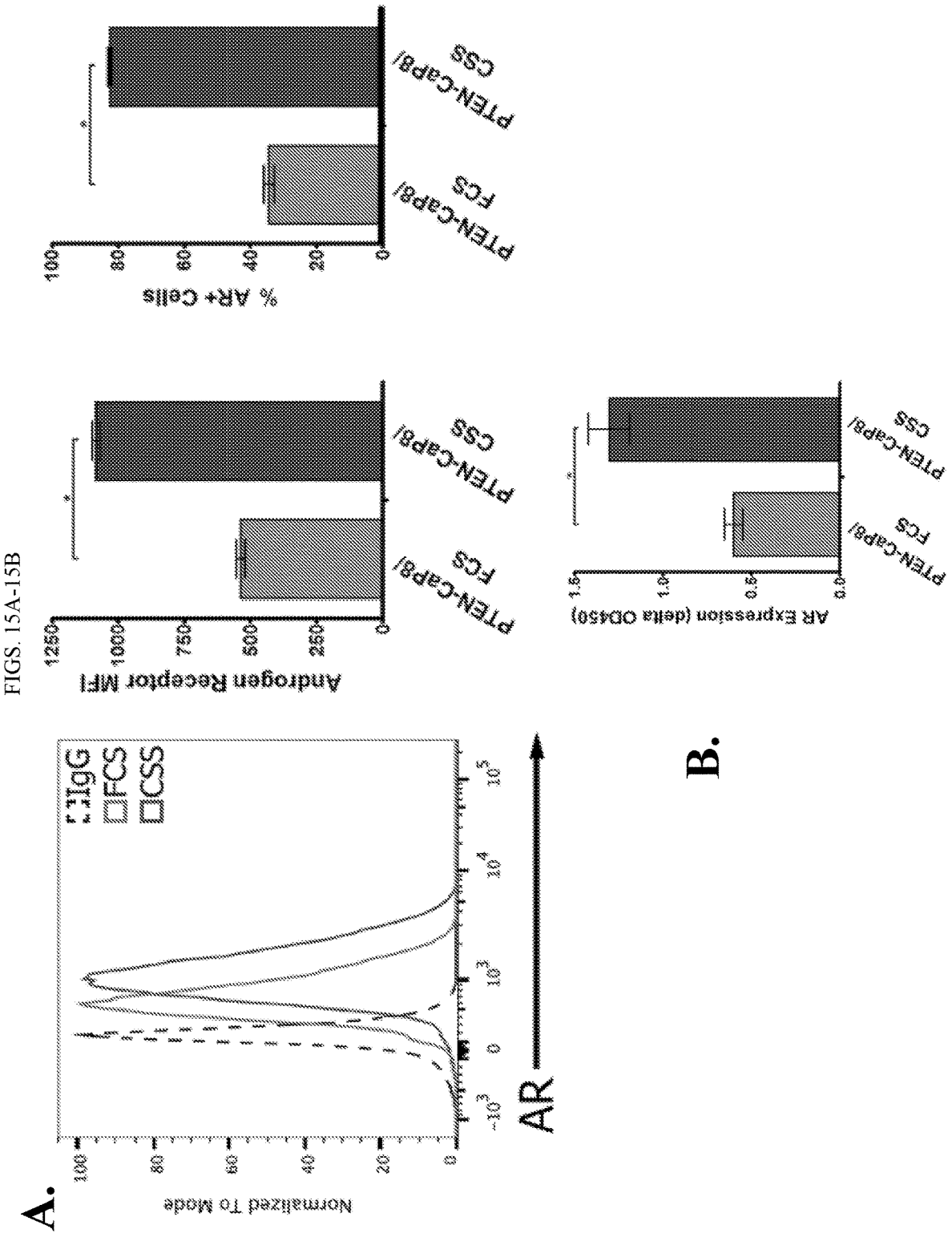


F



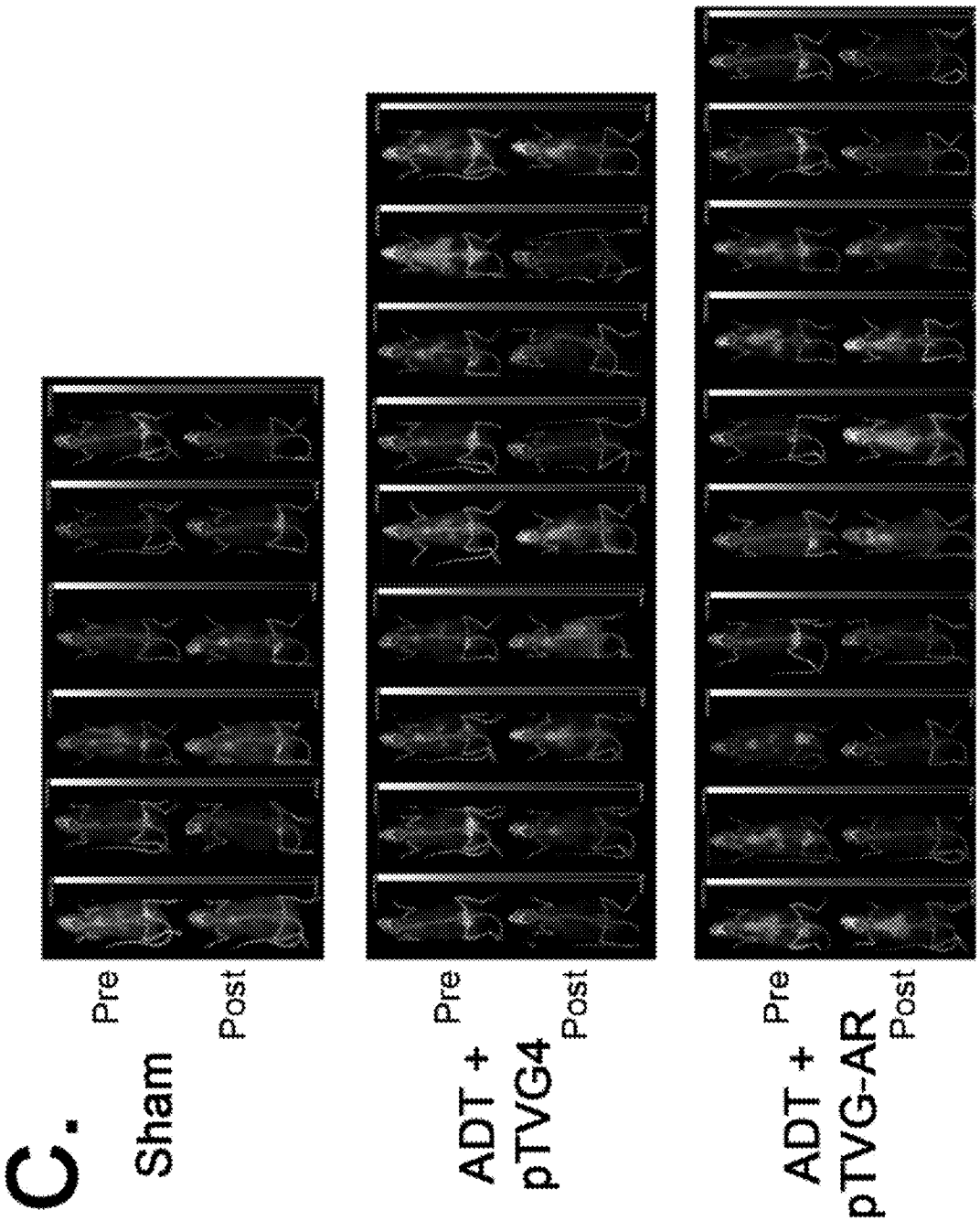
G




A.
IgG
FCS
CSS
Normalized To Mode
100
80
60
40
20
0
-10^3
0
10^3
10^4
10^5
AR
B.
Androgen Receptor MFI
1250
1000
750
500
250
0
PTEN-Cap8/ CSS
PTEN-Cap8/ FCS
AR Expression (delta OD450)
1.5
1.0
0.5
0.0
% AR+ Cells
100
80
60
40
20
0

FIGS. 15A-15B

FIG. 15C



FIGS. 15D-15H

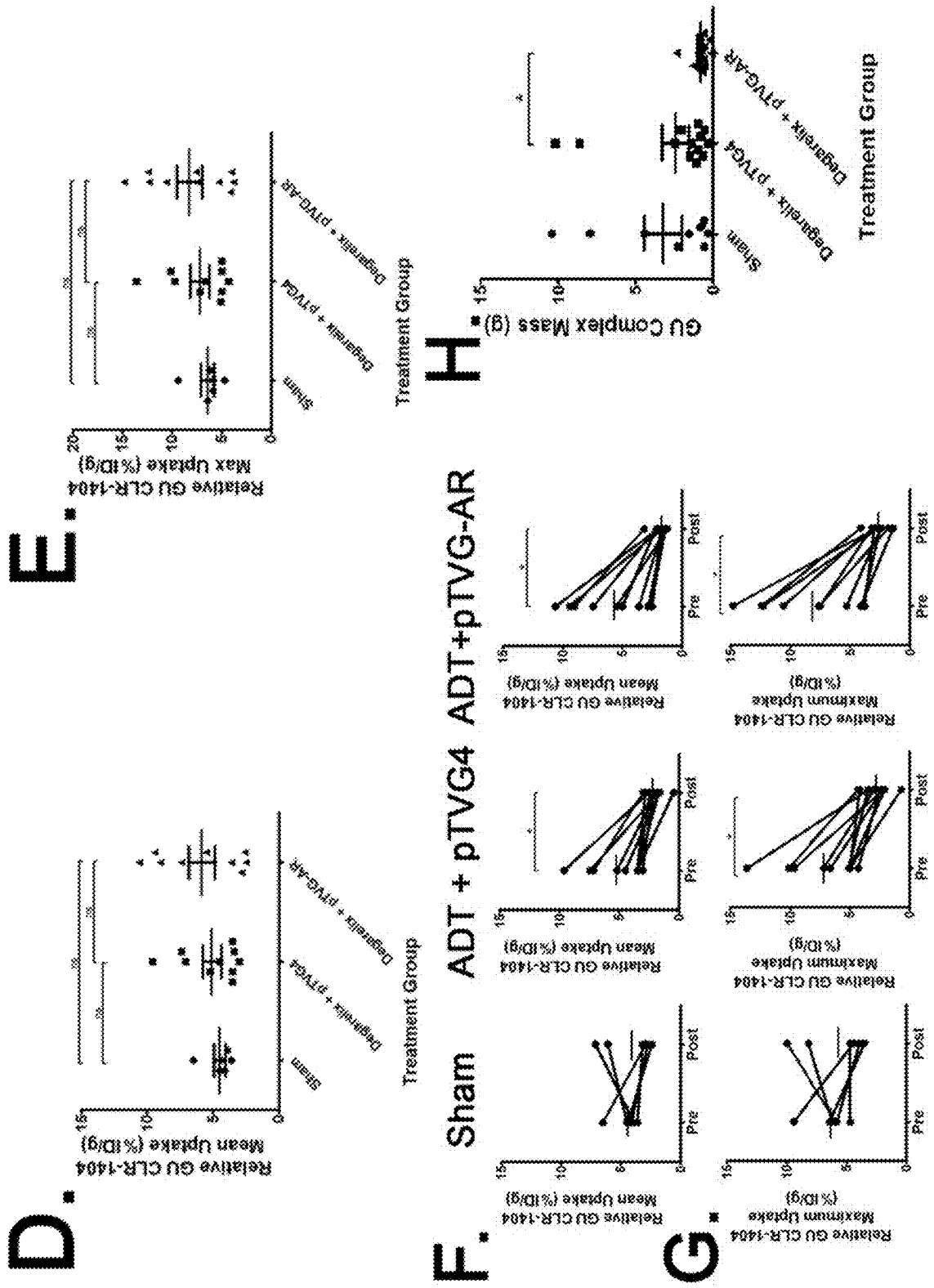
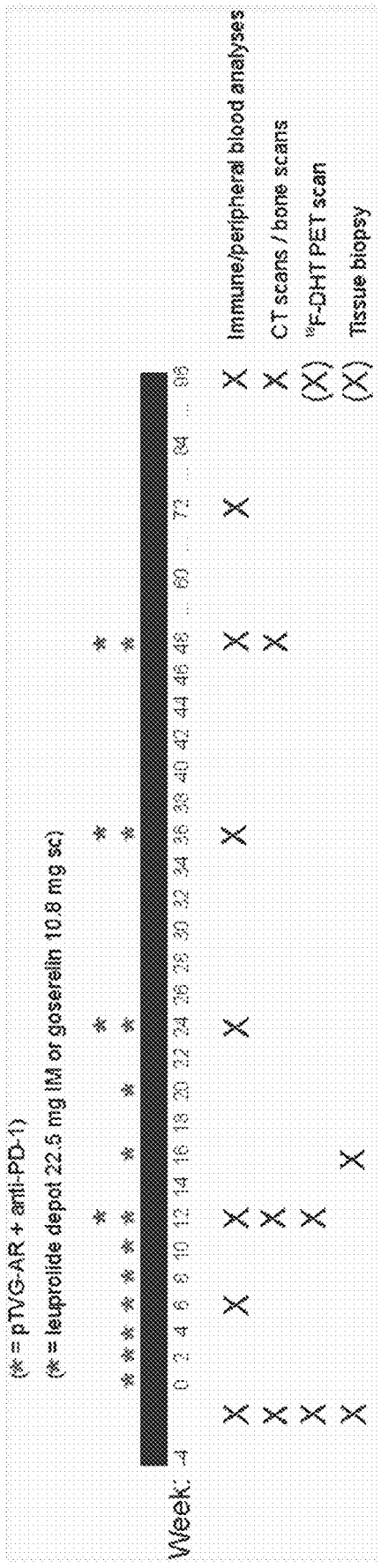

D.
Relative GU CLR-1404 Mean Uptake (% ID/g)
Sham
Degarelix + pTVG4
Degarelix + pTVG-AR
Treatment Group
E.
Relative GU CLR-1404 Max Uptake (% ID/g)
Sham
Degarelix + pTVG4
Degarelix + pTVG-AR
Treatment Group
H.
GU Complex Mass (g)
Sham
Degarelix + pTVG4
Degarelix + pTVG-AR
Treatment Group
F.
Sham
ADT + pTVG4
ADT+pTVG-AR
Relative GU CLR-1404 Mean Uptake (% ID/g)
Pre
Post
G.
Relative GU CLR-1404 Maximum Uptake (% ID/g)
Pre
Post

FIG. 16


(* = pTVG-AR + anti-PD-1)
(* = leuprolide depot 22.5 mg IM or goserelin 10.8 mg sc)
Week: -4 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 ... 60 ... 72 ... 84 ... 96
Immune/peripheral blood analyses
CT scans / bone scans
^{18}F-CHT PET scan
Tissue biopsy

COMBINATORIAL ANDROGEN DEPRIVATION WITH AN ANDROGEN RECEPTOR VACCINE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/347,646 filed on Jun. 9, 2016, the contents of which is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under CA142608 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Prostate cancer is a significant health risk for men over the age of 50, with about 200,000 newly diagnosed cases each year in the United States (Jemal A. et al., Cancer Statistics, 2005 (2005) CA Cancer J Clin, 55:10-30). It is the most common tumor diagnosed among men and the second leading cause of male cancer-related death in the United States (Jemal et al., Cancer Statistics, 2003 (2003) CA Cancer J Clin, 53:5-26). Despite advances in screening and early detection, approximately 30% of patients undergoing definitive prostatectomy or ablative radiation therapy will have recurrent disease at 10 years (Oefelein et al., 1997, J Urol, 158:1460-1465).

The androgen receptor (AR) is a steroid hormone receptor that plays a crucial role in the development of normal prostate tissue, as well as in the progression of prostate cancer. Patients with metastatic disease are initially treated with androgen deprivation therapy, and androgen deprivation typically is continued indefinitely once a patient has metastatic prostate cancer. Given its use in this context for over 60 years, androgen deprivation (AD) represents one of the first truly "targeted" therapies for a solid tumor, and there are few examples in the current armamentarium of novel cancer-targeting agents with as high a response rate. However, despite the initial response to this treatment in over 80% of patients, castration resistance usually emerges, with a median time of 2-3 years. Other groups have identified that amplification of the AR is a common, and perhaps the most common, mechanism of resistance to androgen deprivation therapy, with AR-activating mutations, overexpression and/or gene amplification occurring in over 50% of patients with castration-resistant disease. These findings underscore the importance of the AR to prostate cancer, and suggest that AR antigen-loss (a major means of resistance to immunotherapy) is less likely in human prostate cancer. Recent findings have demonstrated that AR-mediated signaling remains active in the majority of castrate-resistant tumors, and hence the preferred nomenclature is now "castrate resistant" rather than "androgen independent" as was formerly used. Because one of the central means of resistance to androgen deprivation is increased AR expression, in some cases through gene amplification, the pharmacological targeting of the AR can paradoxically cause the AR to remain a target in patients with advanced, castrate-resistant disease. Metastatic prostate cancer that is castration-resistant (mCRPC) is the lethal form of this disease. With a median life expectancy of less than 3 years for patients with mCRPC, treatments that can delay the establishment of castration resistance, or treat this stage of disease more effectively, are urgently needed.

DNA vaccines have recently been added to the arsenal of treatments against prostate cancer. Relative to other vaccine approaches, DNA vaccines are advantageous in being relatively easy and inexpensive to manufacture, and are "off-the-shelf" rather than individualized. Animal studies have demonstrated that DNA vaccines lead to antigen presentation through naturally processed MEW class I and II epitopes. Several DNA vaccines are being explored by academic and industry groups as novel treatments for different cancer types, and early stage clinical trials have shown DNA vaccines can augment immune responses and show evidence of clinical responses. Our laboratory has focused recent efforts on the ligand-binding domain of the androgen receptor (AR LBD) as a biologically relevant target protein, critical for the development and progression of prostate cancer. Our laboratory has demonstrated that many patients with prostate cancer have existing humoral and cellular immune responses specific for the AR LBD, and that cytolytic CD8+ T cells specific for the AR LBD can lyse human prostate cancer cells in an MHC class I-restricted fashion. We further demonstrated that a DNA vaccine encoding the AR LBD can elicit epitope-specific cytolytic CD8+ T cells in HLA-A2 transgenic mice, and used these mice as a tumor model system to assess DNA vaccines targeting AR LBD and other antigens. Immunizing tumor-bearing mice with AR LBD DNA vaccine elicited anti-tumor responses and significantly prolonged overall survival of mice.

There is a need for new and more effective treatment for prostate cancer, especially in treatment or prevention of castrate-resistant disease.

BRIEF SUMMARY

This disclosure is based on the surprising findings that the addition to androgen deprivation therapy of a vaccine directed toward the androgen receptor represses prostate tumor growth and delay onset or progression of metastatic disease.

Accordingly, in a first aspect, the disclosure encompasses a method for eliciting an anti-tumor response in a subject having prostate cancer comprising: a) administering to the subject androgen deprivation therapy (ADT or androgen suppression therapy); and b) administering to the subject a vaccine directed toward the androgen receptor, wherein the vaccine are administered in an amount effective to elicit an increased anti-tumor response to the prostate cancer. This results in an inhibition, delay or reduction in growth of prostate cancer or metastatic disease. The Examples demonstrate a significant delay in tumor growth when the vaccine was combined with standard ADT therapy. In one embodiment, the vaccine is a DNA vaccine comprising a polynucleotide that encodes for an androgen receptor or a fragment of the androgen receptor. In another embodiment, the vaccine is a polypeptide vaccine comprising the androgen receptor or fragments thereof.

Accordingly, in a second aspect, the disclosure encompasses a method for eliciting an anti-tumor response in a subject having prostate cancer comprising: a) administering to the subject ADT; and b) administering to the subject a recombinant DNA vaccine comprising a polynucleotide operably linked to a transcriptional regulatory element wherein the polynucleotide encodes a androgen receptor or a fragment of the androgen receptor, wherein the ADT and the recombinant DNA vaccine are administered in an

amount effective to elicit an increased anti-tumor response to the prostate cancer, and wherein the combination delays, reduced or inhibits prostate cancer cell growth or metastasis.

In a third aspect, the disclosure encompasses a method of increasing the efficacy of androgen deprivation therapy in a subject with prostate cancer comprising administering to the subject an effective amount of a recombinant DNA vaccine comprising a polynucleotide operably linked to a transcriptional regulatory element wherein the polynucleotide encodes an androgen receptor or a fragment of the androgen receptor, wherein the method inhibits, delays or reduces the growth of the prostate cancer.

In a fourth aspect, the disclosure encompasses a method of increasing the efficacy of ADT and/or augmenting or increasing the anti-tumor response of ADT treatment by administering an effective amount of a recombinant DNA vaccine and a PD-1 pathway inhibitor in an effective amount to increase the anti-tumor efficacy of ADT and/or increase or augment the anti-tumor response to ADT treatment. This triple combination therapy results in a significant delay in prostate tumor growth and metastasis.

In a fifth aspect, the disclosure encompasses a kit for treating prostate cancer comprising androgen deprivation therapy and a vaccine that elicits an anti-androgen receptor immune response.

In yet another aspect, the disclosure encompasses a kit for treating prostate cancer comprising androgen deprivation therapy, a vaccine that elicits an anti-androgen receptor immune response and a PD-1 pathway inhibitor.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

FIG. **1** shows that short- or long-term androgen withdrawal increases AR protein expression in 22Rv1 prostate cancer cells.

FIG. **2** shows that long-term androgen deprivation increases full-length AR expression, and short-term androgen deprivation induces a transient increase in AR-V7 expression. Top Panels: relative expression (normalized to β-actin control). Bottom panels: fold induction of expression over long-term FCS-cultured 22rv1 cells. * indicates $p<0.05$ by Student's t-test.

FIG. **3**A-D shows that HLA-A2-expressing, long-term androgen-deprived 22Rv1 cells have increased androgen receptor expression. 22Rv1/FCS (fetal calf serum-containing medium, containing androgen) and 22Rv1/CSS (charcoal stripped serum-containing medium, androgen depleted) cell lines (or non-HLA-A2-transfected 22 Rv1 controls) were evaluated for AR protein expression by ELISA (FIG. **3**A), RNA expression by qRT-PCR FIG. **3**B), HLA-A2 expression FIG. **3**C), and PD-L1 expression (FIG. **3**D) by flow cytometry (blue: 22 Rv1/FCS; red: 22Rv1/CSS; black: wild-type 22Rv1; grey: IgG-stained 22Rv1). * indicates $p<0.05$ by Student's t-test.

FIG. **4**A-B depict intracellular cytokine staining of splenocytes cultured with 22Rv1/FCS (4A) or 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells (mean fluorescent intensity quantified in inset—* indicates $p<0.05$ by Student's t-test).

FIG. **4**C depicts CD69 expression of splenocytes cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells (mean fluorescent intensity quantified in inset—* indicates p, 0.05 by Student's t-test).

FIG. **4**D depicts cytotoxicity of splenocytes cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells.

FIG. **5**A shows CD69 expression of splenocytes cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells (quantified in adjacent bar graph—* indicates $p<0.05$ by Student's t-test).

FIG. **5**B shows intracellular cytokine staining (IFNγ by TNFα) of T-cells cultured with 22Rv1/CSS (right panel) or 22Rv1/FCS (left panel) cells.

FIG. **5**C shows frequency of CD8+ T cells that express zero (blue), one (green), two (yellow), three (orange), or four (red) Th1 related molecules (IFNγ, TNFα, IL-2, and/or granzyme B).

FIG. **5**D shows granzyme B expression by CD8+ T cells following culturing with 22Rv1/FCS (red) or 22Rv1/CSS (blue) cells (quantified in adjacent bar graph—* indicates $p<0.05$ by Student's t-test). FIG. **5**E shows frequency of CD8+ T cells expressing the surface degranulation marker CD107a. FIG. **5**F shows cytotoxicity of T-cells cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells.

FIG. **6**A depicts a schematic of the timing of a clinical trial.

FIG. **6**B depicts the proposed dosage regimen of the vaccine in a clinical trial.

FIGS. **7**A and **7**B depict androgen-deprived 22Rv1 human prostate cancer cells and castrate-resistant MycCaP mouse prostate cancer cells have increased AR expression after ADT treatment.

FIG. **7**C depicts increased AR expression in tumors in the MycCaP prostate cancer model in vivo after chemical castration using the GnRH antagonist degarelix.

FIG. **7**D depicts T-cell activation by CD69 expression (left panel), IFNγ and TNFα cytokine expression (center panel), and cytotoxicity (right panel) that results from AR-specific CD8+ T-cell activation. In all panels, * indicates $p<0.05$ by Student's t-test.

FIG. **8**A depicts splenocytes collected and analyzed for immune responses against MycCaP/CR tumor cells by intracellular cytokine staining (left panel) and AR peptide-stimulated splenocytes from pTVG-AR-immunized mice were measured for ability to lyse MycCap/AS vs. MycCap/CR tumor cells (right panel).

FIG. **8**B depicts mice followed for tumor volume. In all panels, * indicates $p<0.05$ by Student's t-test.

FIG. **8**C depicts tumor volume post-challenge of control (sham), degarelix+pTVG4 and degarelix+pTVG-AR.

FIG. **9**A shows PBMC from patients previously immunized with a PAP-targeting vaccine were cultured in vitro for 72 hours with PAP in the presence of a PD-1-blocking antibody (or IgG control), and measured for IFNγ (left panel) or granzyme B (right panel) secretion by ELISA.

FIG. **9**B shows PBMC obtained from patients after immunization were injected into the footpads of NOD/SCID mice with PAP protein and PD-1 blocking antibody (or IgG control), and 24 hours later, footpad swelling was measured.

FIG. **9**C shows that PD-L1 expression was measured on circulating tumor cells from patients with persistent PAP-specific Th1-biased immune responses (R) vs non-responders (NR) following immunization with a DNA vaccine targeting PAP. The ratio PD-L1 MFI on post-treatment samples compared to pre-treatment samples is shown. In all panels, * indicates $p<0.05$ by Student's t-test.

FIG. **10**A shows that CD8+ T cells from MycCaP tumor-bearing animals treated with ADT and immunized with pTVG-AR have elevated PD-1 expression.

FIG. **10**B shows that recurrent tumors had elevated PD-L1 expression.

FIG. **10**C depicts delay in tumor growth in mice with AR-targeted immunization combined along with a PD-1 blocking antibody compared to immunization with pTVG-AR alone.

FIG. **10**D shows that combining AD with AR-directed immunization and PD-1 blockade further delayed tumor growth.

FIG. **11**A shows prostate cell lines (immortalized human epithelial lines: RWPE-1 and PrEC-E6; androgen-independent prostate cancer cell lines: DU-145 and PC-3; and androgen-dependent prostate cancer cell lines: LNCaP and 22Rv1) cultured in either androgen-replete (FCS) or androgen-deprived (CSS) medium for one to seven days (1d-7d) or for greater than six months (long-term: LT) analyzed for androgen receptor protein expression by quantitative ELISA (panel A).

FIG. **11**B shows prostate cell lines cultured long-term FCS (light grey) or CSS (dark grey). Cultured cell lines were analyzed for AR expression by intracellular staining using antibodies specific for the ligand-binding domain (top panels) or amino-terminal domain (lower panels).

FIG. **11**C shows quantified amplitude of AR expression in cultured prostate cell lines.

FIG. **11**D shows frequency of AR+ cells in cultured prostate cell lines.

FIG. **11**E shows RNA quantified from 22RV1/CSS cells cultured for different periods of time analyzed for the presence of full-length (dark grey) or AR-V7 (light grey) AR transcripts. In all panels, * indicates $p<0.05$ by student's t-test and data is representative of at least two independent experiments.

FIG. **11**F shows phenotypic validation of HLA-A2-transfected 22Rv1/FCS and 22Rv1/CSS cells. 22Rv1 cells cultured for greater than six months in androgen replete (22Rv1/FCS) or androgen deprived (22Rv1/CSS) medium were transfected with a lentiviral vector encoding HLA-A2. HLA-A2-expressing cells were then sorted by fluorescence-activated cell sorting, and expanded lines were evaluated for AR protein by quantitative ELISA. In all panels, * indicates $p<0.05$ by Student's t-test.

FIG. **11**G shows expanded lines of FIG. **11**F evaluated for AR protein by qRT-PCR.

FIG. **11**H shows cells of FIG. **11**G analyzed for the expression of HLA-A2.

FIG. **11**I shows cells of FIG. **11**G analyzed for the checkpoint ligand PD-L1 by flow cytometry.

FIG. **12**A shows AR-specific T cells have increased recognition and lysis of androgen-deprived tumor cells. AR805-specific human T-cell cultures incubated with HLA-A2-expressing 22Rv1/FCS or 22Rv1/CSS cells were measured for surface expression of CD69.

FIG. **12**B show AR805-specific human T-cell cultures incubated with HLA-A2-expressing 22Rv1/FCS or 22Rv1/CSS cells and measured for intracellular cytokine expression of IFNγ and/or TNFα.

FIG. **12**C demonstrates polyfunctional cytokine expression quantified in AR805-specific human T-cell cultures incubated with HLA-A2-expression 22Rvi/FCS or 22Rv1/CSS cells.

FIG. **12**D shows cytolytic and degranulation activity of AR-specific T cells measured by intracellular granzyme B expression.

FIG. **12**E shows cytolytic and degranulation activity of AR-specific T cells measured by surface CD107a expression.

FIG. **12**F shows tumor cell cytotoxicity of AR-specific T cells.

FIG. **12**G demonstrates AR-specific T cells obtained following peptide-immunization have increased recognition and lysis of androgen-deprived prostate tumor cells. Splenocytes from AR811 peptide-immunized HLA-A2 transgenic (HHDII-DR1) mice were co-cultured with HLA-A2-expressing 22Rv1/FCS or 22Rv1/CSS cells, and measured for intracellular cytokine expression of IFNg and TNFα.

FIG. **12**H demonstrates AR-specific T-cells of FIG. **12**G express CD69 on their surface.

FIG. **12**I demonstrates AR-specific T cells of FIG. **12**G are cytotoxic.

FIG. **13**A demonstrates that androgen deprivation increases AR expression in Myc-CaP tumor cells in vitro and in vivo. Androgen-sensitive (Myc-CaP/AS) and castrate-resistant (Myc-CaP/CR) cells were analyzed for AR protein expression by quantitative ELISA.

FIG. **13**B shows androgen-sensitive (Myc-CaP/AS) and castrate-resistant (Myc-CaP/CR) cells analyzed for AR protein expression by intracellular staining (quantified for amplitude and frequency of expression in side panels).

FIG. **13**C shows RNA samples from Myc-CaP/AS and Myc-CaP/CR cells analyzed by quantitative PCR for expression of full-length or splice variants mAR V2 or mAR V4.

FIG. **13**D shows Myc-CaP/AS tumor-bearing FVB mice with palpable tumors were treated with degarelix (n=4) or sham-treatment (n=3) and followed for tumor growth. Results are representative of two independent studies.

FIG. **13**E shows recurrent tumors collected and analyzed for AR expression by intracellular staining using an antibody directed against the ligand-binding domain (amplitude and frequency quantified in side panels). In all panels, * indicates $p<0.05$ by student's t-test.

FIG. **14**A shows androgen deprivation combined with immunization using pTVG-AR delayed the recurrence of castrate-resistant Myc-CaP tumors. Myc-CaP/AS tumor-bearing mice with palpable tumors were given a sham-treatment (n=3) or degarelix along with biweekly immunization with pTVG-AR (n=5) or empty vector (n=5) and followed for tumor growth (tumor volumes). Results are representative of three independent studies.

FIG. **14**B shows the Kaplan Meier curve for FIG. **14**A. Results are representative of three independent studies.

FIG. **14**C shows splenocytes from androgen-deprived animals immunized with pTVG4 or pTVG-AR cultured with Myc-CaP/AS or Myc-CaP/CR cells and assessed for CD4+ and CD8+ T-cell intracellular cytokine expression (with polyfunctional expression quantified in side pie charts).

FIG. **14**D shows splenocytes from androgen-deprived animals immunized with pTVG4 or pTVG-AR cultured with Myc-CaP/AS or Myc-CaP/CR cells and assessed for cytotoxicity.

FIG. **14**E demonstrates immunization with pTVG-AR delays tumor growth in the presence or absence of ADT, and results in increased tumor-infiltrating T cells. Myc-CaP/AS tumor-bearing mice were given degarelix or sham treatments, then immunized with either pTVG-AR or pTVG4 control, and assayed for tumor growth (tumor volumes).

FIG. **14**F shows Kaplan Meier plots for mice of FIG. **14**E.

FIG. **14**G shows analysis of tumor collected from FIG. **14**E for the frequency of infiltrating T cells by IHC. * indicates $p<0.05$ by Mann-Whitney U test.

FIG. **15**A shows androgen deprivation increases AR expression in PTEN-deficient tumors, and immunization with pTVG-AR delays the development of castrate-resistant prostate tumors in Pten–/– mice. PTEN-CaP8 tumor cells cultured in androgen-replete (FCS) or androgen-deprived (CSS) medium were analyzed for AR expression by intra-

cellular staining (quantified for amplitude and expression in side panels). * indicates $p<0.05$ by student's t-test.

FIG. **15**B shows the results of PTEN-CaP8 tumor cells cultured in androgen-replete (FCS) or androgen-deprived (CSS) medium analyzed for AR expression by quantitative ELISA. * indicates $p<0.05$ by student's t-test.

FIG. **15**C shows the results of twenty-week old $PbCre^{+}$ $PTEN^{fl/fl}$ mice given a sham treatment (n=9), or degarelix along with biweekly immunization with pTVG4 (n=13) or pTVG-AR (n=13), for five months. One week prior to initiation and completion of treatment, animals were administered ^{124}I-CLR1404 and PET/CT scanned 96 hr post intravenous injection (PET/CT images pre- and post-treatment). Signal greater than sixty percent of the max PET signal was used to calculate the mean and max percent injected dose (% ID/g_{tissue}) for tumor, and was normalized to background muscle uptake.

FIG. **15**D shows the pre-treatment mean ^{124}I-CLR1404 uptake for randomization of treatment groups. * indicates $p<0.05$ by Mann-Whitney U test.

FIG. **15**E shows the pre-treatment maximum ^{124}I-CLR1404 uptake for randomization of treatment groups. * indicates $p<0.05$ by Mann-Whitney U test.

FIG. **15**F shows changes in % ID/g_{mean} pre- to post-treatment calculated (mean values shown by solid horizontal bars). * indicates $p<0.05$ by Mann-Whitney U test.

FIG. **15**G shows % ID/g_{mean} pre- to post-treatment calculated (mean values shown by solid horizontal bars). * indicates $p<0.05$ by Mann-Whitney U test.

FIG. **15**H shows genitourinary complex masses collected during necropsy and analyzed. * indicates $p<0.05$ by Mann-Whitney U test.

FIG. **16** is a schematic of a treatment using an anti-androgen therapy leuprolide in combination with a DNA vaccine and an anti-PD1 therapy.

DETAILED DESCRIPTION

This disclosure provides compositions and methods related to combination therapies for treating prostate cancer. The specific combination of androgen therapy and vaccine against the androgen receptor (for example a DNA vaccine) unexpectedly and synergistically improves the efficacy ADT for treatment of prostate cancer, including metastatic or castrate-resistant disease. The combination therapy results in a significant decrease in tumor growth as compared to ADT alone.

In one embodiment, the method for eliciting an anti-tumor response in a subject having prostate cancer comprising: administering to the subject ADT and administering to the subject a vaccine directed toward the androgen receptor, wherein the vaccine is administered in an amount effective to elicit an increased anti-tumor response to the prostate cancer compared to ADT treatment alone. This increased anti-tumor response to prostate cancer results in the reduction, inhibition or delay of prostate cancer cell growth and/or metastasis, prolonging subject survival. In one embodiment, the anti-tumor response of the combination treatment results in a significant delay in tumor cell growth as compared to ADT treatment alone. In one embodiment, the vaccine is a DNA vaccine comprising a polynucleotide that encodes for an androgen receptor or a fragment of the androgen receptor. In another embodiment, the vaccine is a polypeptide vaccine comprising the androgen receptor or fragment thereof.

In one embodiment, the disclosure provides a method of eliciting an anti-tumor response in a subject having prostate cancer comprising: a. administering to the subject androgen deprivation therapy (ADT); and b. administering to the subject a recombinant DNA vaccine comprising a polynucleotide operably linked to a transcriptional regulatory element wherein the polynucleotide encodes a androgen receptor or a fragment of the androgen receptor, wherein the ADT and the recombinant DNA vaccine are administered in an amount effective to elicit an anti-tumor response to the prostate cancer.

Anti-tumor response in a subject includes the reducing, repressing, delaying or preventing tumor growth, reduction of tumor volume, and/or preventing, repressing, delaying or reducing metastasis of the tumor. Anti-tumor response includes the reduction of the number of tumor cells within the subject. In some embodiments, anti-tumor response includes an immune response to tumor cells expressing the androgen receptor, for example, a cytotoxic immune reaction against cells expressing androgen receptor. For example, an anti-tumor response may include lysis of tumor cells by AR-specific CD8+ T cells. Preferably, cellular immune reactions against androgen receptor are induced, with or without humoral immune reactions.

Androgen deprivation therapies (ADT) are therapies that reduce the levels of androgen hormones, or interfere with androgen receptor function/signaling, for example by use of androgen receptor-pathway targeting (e.g. antiandrogens) or chemical castration. Androgen deprivation therapy includes administering an effective amount of at least one androgen receptor pathway-targeting drug. Suitable drugs are known to one skilled in the art and include, but are not limited to, LHRH (or GnRH) analogues (agonists), LHRH (or GnRH) antagonists, AR antagonists, androgen synthesis inhibitors, other AR degrading or blocking agents, and combinations thereof. Suitable ADT include treatment with one or more of the following drugs:

(a) AR antagonists, including, but not limited to, flutamide, nilutamide, bicalutamide, enzalutamide, apalutamide, cyproterone acetate, megestrol acetate, chlormadinone acetate, spironolactone, canrenone, drospirenone, topilutamide (fluridil), cimetidine;

(b) androgen synthesis inhibitors, including, but not limited to, (i) 5α-reductase inhibitors, which include the non-limiting examples of finasteride, dutasteride, alfatradiol, and saw palmetto extract, (ii) CYP17A1 (17α-hydroxylase, 17,20-lyase) inhibitors, which include the non-limiting examples cyproterone acetate, spironolactone, danazol, gestrinone, ketoconazole, abiraterone acetate; (iii) 3β-Hydroxysteroid dehydrogenase inhibitors, which include the non-limiting examples danazol, gestrinone, abiraterone acetate; (iv) 17β-Hydroxysteroid dehydrogenase inhibitors, which include the non-limiting examples danazol, simvastatin; (v) CYP11A1 (cholesterol side-chain cleavage enzyme) inhibitors, which include the non-limiting examples aminoglutethimide, danazol; and (vi) HMG-CoA reductase inhibitors, which include the non-limiting example statins (e.g., atorvastatin, simvastatin);

(c) antigonadotropins including (i) progestogens, such as the non-limiting examples including progesterone, cyproterone acetate, medroxyprogesterone acetate, megestrol acetate, chlormadinone acetate, spironolactone, drospirenone; (ii) estrogens including the non-limiting examples of estradiol, ethinyl estradiol, diethylstilbestrol, conjugated equine estrogens; (iii) GnRH analogues, for example, GnRH agonists, including non-limiting examples buserelin, deslorelin, gonadorelin, goserelin, histrelin, leuprorelin, nafarelin, triptorelin; GnRH antagonists, including non-limiting examples abarelix, cetrorelix, degarelix, ganirelix; and combinations thereof. For example, suitable ADT

treatment include, but are not limited to, AR antagonists bicalutamide (Casodex, AstraZeneca®), apalutamide (ARN-509, Janssen), or enzalutamide (Xtandi, Astellas®), GnRH antanogist degarelix (Firmagon, Ferring Pharmaceuticals®), AR degrading agents such as galeterone (Tokai) and the like. In some embodiments, one or more suitable ADT drugs can be used, such as LHRH agonists or antagonists in combination with AR antagonists or degrading agents.

In some embodiments, androgen deprivation therapy (ADT) results in overexpression of the androgen receptor (AR) in the majority of prostate cancer patients' tumors. While this overexpression can promote the development of tumor escape variants, as the present disclosure discusses, it also causes prostate tumor cells to be more susceptible to lysis by AR-specific CD8+ T cells. The Examples demonstrate in vitro that increased AR expression in prostate tumor cells can occur following standard androgen deprivation, or following treatment with commercially available AR antagonists bicalutamide (Casodex, AstraZeneca®) or enzalutamide (Xtandi, Astellas®). Additionally, the Examples demonstrate in vivo that treatment with degarelix (Firmagon, Ferring Pharmaceuticals®), a GnRH antanogist used clinically for androgen deprivation therapy, results in increased AR expression in the MycCaP prostate tumor model. Significantly, while treatment with degarelix alone resulted in a delay in tumor growth, combining this treatment with a vaccine targeting the androgen receptor (pTVG-AR) resulted in a significant delay in tumor growth compared with degarelix treatment alone. Additionally, in animals that develop recurrent disease following combination degarelix and pTVG-AR treatment, tumors have a significant decrease in AR expression, suggesting this may be a biomarker of treatment failure.

Patients undergoing androgen deprivation using a variety of AR-targeting pharmaceutical agents may be immunized with DNA vaccine against the androgen receptor, for example, pTVG-AR, improving the response to these standard therapies. Improved response includes the inhibition or reduction in tumor cell growth or metastasis and/or delay in tumor cell growth or metastasis.

The ADT may be delivered by any suitable dosages and schedule known by one skilled in the art. For example, a non-limiting example includes an LHRH agonist alone or in combination with an antiandrogen (e.g., bicalutamide or enzalutamide). Another non-limiting example is the combination of LHRH agonist and abiraterone and apalutamide.

Non-limiting examples of suitable dosages for LHRH agonists include, for example, Leuprolide 20-25 mg (e.g. 22.5 mg) IM every three months; and/or Goserelin LHRH agonists are typically about 9-12 mg (e.g. 10.8 mg) sc every three months. Non-limiting examples of dosages for LHRH antagonist include Degarelix 240 mg sc once as first dose, and the 80 mg sc every 28 days subsequently; Abiraterone: 1000 mg by mouth daily; Apalutamide: 240 mg by mouth daily; Bicalutamide: 50 mg by mouth daily; and/or Enzalutamide: 160 mg by mouth daily.

In some embodiments, it is contemplated that the dosage or regimen of ADT which is known and understood in the art could possibly be varied from conventional parameters (e.g. reduced amount or frequency of dosage) when combined with the vaccine as described in the present invention.

The vaccine used in the methods of this disclosure may be a recombinant DNA vaccine that encodes the androgen receptor or fragments thereof or a peptide vaccine comprising a polypeptide androgen receptor or fragments thereof. The recombinant DNA vaccine used in the methods of this disclosure may comprise a polynucleotide that encodes a mammalian androgen receptor, the ligand binding domain of the androgen receptor, or a fragment of the androgen receptor. Suitable recombinant DNA vaccine for use is disclosed in U.S. Pat. Nos. 7,910,565 and 8,962,590, entitled "Prostate cancer vaccine," which are incorporated by reference in their entireties. In some embodiments, the recombinant DNA vaccine comprises a polynucleotide that encodes a mammalian androgen receptor, a fragment of the mammalian androgen receptor that comprises the ligand-binding domain, or certain fragments of the ligand-binding domain. The plasmid DNA vaccines, when directly introduced into subjects such as humans in vivo, induce the expression of encoded polypeptides within the subject, and cause the subjects' immune system to become reactive against the polypeptides. The vaccines may be any polynucleotides that are capable of eliciting immune responses to an encoded polypeptide.

In some embodiments, the DNA vaccine comprises pTVG-AR (pTVG-AR or pTVG-ARLBD are the same vector and used herein interchangeably). pTVG-AR is a vector comprising the coding sequence for the ligand-binding domain of the human androgen receptor gene inserted into the pTVG4 vector to create the immunization vector pTVG-AR, as disclosed in U.S. Pat. No. 7,910,565, incorporated by reference in its entirety.

The vaccine can be administered into a subject to elicit an immune response against androgen receptor in the subject. An "effective amount" or an "immunologically effective amount" means that the administration of that amount to a subject, either in a single dose or as part of a series, is effective for inducing an immune reaction against the androgen receptor (and, therefore, against cells expressing the androgen receptor). Further, an "effective amount" as contemplated in the present invention is an amount of vaccine that augments or increases the anti-tumor efficacy of ADT, resulting a delay or inhibitor of prostate tumor growth and metastasis.

Androgen receptor genes are known and have been cloned from many species. For example, the human, mouse, rat, dog, chimpanzee, macaque, and lemur androgen receptor mRNA that correspond to cDNA along with amino acid sequences can be found at GenBank Accession Nos. NM_000044 (cDNA-SEQ ID NO:1 and amino acid sequence-SEQ ID NO:2), NM_013476 (cDNA-SEQ ID NO:3 and amino acid sequence-SEQ ID NO:4), NM_012502 (cDNA-SEQ ID NO:5 and amino acid sequence-SEQ ID NO:6), NM_001003053, NM_001009012, U94179, and U94178, respectively. Androgen receptor genes from other species are also known. These species include but are not limited to *Sus scrofa, Astatotilapia burtoni, Gallus gallus, Kryptolebias marmoratus, Alligator mississippiensis, Leucoraja erinacea, Haplochromis burtoni, Pimephales promelas, Dicentrarchus labrax, Gambusia affinis, Micropogonias undulates, Oryzias latipes, Acanthopagrus schlegelii, Rana catesbeiana, Crocuta crocuta, Eulemur fulvus collaris*, and *Anguilla japonica* (see GenBank Accession Nos. NM_214314 (or AF161717), AY082342, NM_001040090, DQ339105, AB186356, DQ382340, AF121257, AY727529, AY647256, AB099303, AY701761, AB076399, AY219702, AY324231, AY128705, U94178, and AB023960, respectively). The ligand-binding domains of androgen receptors are well known in the art. For the purpose of the present invention, the ligand-binding domain of the human androgen receptor refers to a polypeptide that starts at any amino acid from amino acid positions 651 to 681 and ends at any amino acid from amino acid positions 900 to 920. For example, human androgen receptor or a fragment of the human androgen receptor that

comprises amino acids 681-900 as well as DNA vaccines containing a polynucleotide encoding the above are suitable vaccines. As will be readily recognized by one of ordinary skill in the art, any DNA sequence that encodes a ligand-binding domain or a larger fragment of an androgen receptor including the full-length receptor from one of the above species as well as other animals is suitable for the present invention.

Pharmaceutically acceptable carriers are well known to those of ordinary skill in the art (Amon, R. (Ed.) Synthetic Vaccines 1:83-92, CRC Press, Inc., Boca Raton, Fla., 1987). They include liquid media suitable for use as vehicles to introduce the peptide or polynucleotide into a patient but should not in themselves induce the production of antibodies harmful to the individual receiving the composition. An example of such liquid media is saline solution.

Moreover, the vaccine may also contain an adjuvant for stimulating the immune response and thereby enhancing the effect of the vaccine. Suitable adjuvants are known in the art and include, but are not limited to, GM-CSF, Montanide, or saponin-derivative adjuvants.

According to another embodiment, the DNA vaccine comprises a polynucleotide operatively linked to a transcriptional regulatory element (e.g., a promoter such as a heterologous promoter) wherein the polynucleotide encodes a member selected from (i) a mammalian androgen receptor (e.g., a human androgen receptor), (ii) a fragment of the androgen receptor that comprises the ligand-binding domain, (iii) a fragment of the ligand-binding domain defined by SEQ ID NO:9 (LLLFSIIPV, amino acids 811-819 of SEQ ID NO:2); (iv) a fragment of the ligand-binding domain defined by SEQ ID NO:10 (RMLYFAPDLV, amino acids 761-770 of SEQ ID NO:2), (v) a fragment of the ligand-binding domain defined by SEQ ID NO:11 (FLCMKALLL, amino acids 805-813 of SEQ ID NO:2), and (vi) a fragment of the ligand-binding domain defined by SEQ ID NO:12 (QLTKLLDSV, amino acids 859-867 of SEQ ID NO:2), wherein administration of said vaccine to a subject induces a cytotoxic immune reaction against cells expressing androgen receptor.

The DNA vaccine may comprise the polynucleotide directly linked to a transcriptional regulatory element that promotes the expression of the protein (e.g., androgen receptor or fragment thereof) within cells of the subject. Suitable transcriptional regulatory element (e.g., a promoter such as a heterologous promoter) are known in the art and include, but are not limited to, CMV promoter, Rous sarcoma virus (RSV) promoter, the simian virus 40 (SV40) promoter, the human elongation factor-1α (EF-1α) promoter, and the human ubiquitin C (UbC) promoter, among others.

The vaccine is suitably administered by intradermal, intramuscular, subcutaneous, or intravascular (including intravenous and intraarterial) administration to a mammal such as a human. In another aspect, the DNA vaccine is suitable for administration by muscle or skin electroporation to increase uptake of the DNA at the site of immunization.

The vaccines can be used in a prime-boost strategy in connection with the ADT therapy to induce robust and long-lasting immune response to androgen receptor. Priming and boosting vaccination protocols based on repeated injections of the same antigenic construct are well known and result in strong CTL responses. In general, the first dose may not produce protective immunity, but only "primes" the immune system. A protective immune response develops after the second or third dose.

The vaccine described herein may be provided in an effective amount to augment or increase the efficacy of ADT treatment, which can be seen by a delay, reduction or inhibition of prostate tumor cell growth or metastasis.

In one embodiment, the vaccines may be used in a conventional prime-boost strategy, in which the same antigen is administered to the animal in multiple doses. In a preferred embodiment, the DNA or peptide vaccine is used in one or more inoculations. These boosts are performed according to conventional techniques, and can be further optimized empirically in terms of schedule of administration, route of administration, choice of adjuvant, dose, and potential sequence when administered with another vaccine, therapy or homologous vaccine.

In one embodiment, the vaccine is administered every two weeks to every three months. In some embodiments, the vaccine is administered for at least 6 weeks, alternatively for at least 10 weeks, alternatively for at least 15 weeks, alternatively for at least 20 weeks, alternatively for at least 25 weeks, alternatively for at least 30 weeks, alternatively for at least 35 weeks, alternatively for at least 40 weeks, alternatively for at least 45 weeks, alternatively at least 48 weeks, alternatively for at least 50 weeks, alternatively for at least a year, alternatively for at least 18 months, alternatively for at least 20 months and can include any time in between (for example, 16 weeks 17 weeks, 18 weeks, 19 weeks, 24 weeks, etc). In some embodiments, the vaccine is administered biweekly for about 6 to about 14 weeks and subsequently administered quarterly for at least a year. In some embodiments, the vaccine is administered biweekly for about 6 to about 14 weeks and subsequently administered quarterly (i.e. every three months) for at least 18 months.

Suitable dosages of the DNA vaccine are known in the art, and include, but are not limited to, about 10 mcg to about 1 mg of DNA per dosage.

In some embodiments, the ADT and recombinant vaccine are administered concurrently. In other embodiments, the subject is treated with ADT and subsequently treated with the recombinant vaccine. The time period between the ADT and recombinant vaccine administration may be a short duration (e.g., hours or days) or may be of a longer duration (e.g. weeks or months). In some embodiments, the term concurrently means that the two components are administered in close timing to each other (e.g. within hours or on the same day), but may be administered by different routes of administration (e.g. ADT orally and vaccine by injection). In some embodiments, the administration are separate, e.g. separated by hours or days in between the vaccine and the ADT. In some embodiments, the vaccine and the ADT are administered over the same time period but using different regimens that require administration on different days. Suitable regimens are discussed more herein, for example, a regimen where the vaccine is administered for a period of time before beginning dose regimen for ADT.

In some embodiments, the recombinant DNA vaccine is administered prior to androgen deprivation therapy. In some embodiments, the DNA vaccine is administered every other week for 2-24 weeks before start of administration of ADT, and in some embodiments, the DNA vaccine administration is continued every 2-16 weeks during ADT therapy. Not to be bound by any theory, but the administration of the DNA vaccine prior to administration of androgen deprivation therapy may lead to preferred immune and anti-tumor responses as it has been shown that giving ADT prior to immunization may directly interfere with the priming of T

cell responses. One skilled in the art will be able to determine a preferred regimen of ADT and vaccine administration.

In some embodiments, the subject is a mammal, preferably a human.

In some embodiments, the methods of the disclosure further comprise administering to the subject an effective amount of a checkpoint pathway inhibitor in addition to the vaccine against androgen receptor to augment ADT treatment. In one example, the checkpoint pathway inhibitor is a PD-pathway inhibitor. Suitable PD-pathway inhibitors are known in the art. In some embodiments, the PD-pathway inhibitor is an anti-PD-1 blocking antibody or an anti-PD-L1 antibody.

Using different tumor antigen systems we have found that DNA vaccination can elicit PD-L1 expression in tumors as a result of tumor-specific T cells elicited that secrete IFNγ. Specifically, tumors expressing a model antigen had an increase in PD-L1 expression following immunization with a DNA vaccine encoding that antigen (Rekoske, B. T., H. A. Smith, B. M. Olson, B. B. Maricque, and D. G. McNeel. (2015). "PD-1 or PD-L1 Blockade Restores Antitumor Efficacy Following SSX2 Epitope-Modified DNA Vaccine Immunization." *Cancer Immunol Res.* 3:946-55). If the immunization was modified to elicit CD8+ T cells with higher PD-1 expression, this resulted in an inferior anti-tumor response.

The Examples below demonstrate that targeting the PD-1/PD-L1 pathway in combination with an AR-targeting vaccine may reduce or prevent tumor-mediated immune suppression. In MycCaP tumor-bearing animals treated with AD and immunized with pTVG-AR, CD8+ T cells were found to have elevated PD-1 expression (FIG. **10**A). Additionally, some recurrent tumors had elevated PD-L1 expression (FIG. **10**B). When AR-targeted immunization was combined along with a PD-1 blocking antibody, this treatment significantly delayed tumor growth compared to immunization with pTVG-AR alone (FIG. **10**C). Furthermore, combining ADT with AR-directed immunization and PD-1 blockade further delayed tumor growth (FIG. **10**D). Thus, the combination of vaccination and ADT with anti-PD-1 or anti-PD-L1 antibody treatment results in a greater anti-tumor response and may lead to the eradication of tumors. The addition of anti-PD-1 or anti-PD-L1 antibody treatment augments or increases the immune response to androgen receptor. For example, one non-limiting example of a method of treatment includes combination ADT comprising LHRH, abiraterone, apalutamide or a combination thereof with the AR vaccine and anti-PD-1 antibody.

Many different possible scenarios are envisioned for the methods of the present invention including treatment regimens using the double combination (DNA vaccine and ADT) and triple combination therapy (DNA Vaccine, PD-1 pathway inhibitor and ADT) contemplated in this invention. In these scenarios the double and triple combinations do not have to be co-administered and may just be administered over the same period of time in suitable dosage regimens. In other embodiments, the start of each therapy is staggered to provide the most efficacious treatment regimen (e.g. administration of at least 2 or more vaccine doses before the start of ADT therapy). In some embodiments, the method of treating includes administering the triple combination for a period of time followed by administration of the double combination for a second period of time. In other embodiments, the method of treating includes a period of time administering the double combination followed by a period of time administering the triple combination therapy. In other embodiments, the method of treating includes a period of time administering the double or triple combination which includes within that time a period in which one or more of the treatments is not administered while the other therapies are maintained. For example, in the DNA vaccine and PD-1 pathway inhibitor may be administered weekly or biweekly for 2-12 weeks before ADT is administered, whereby DNA vaccine, PD-1 pathway inhibitor and ADT are all administered for at least 12-48 additional weeks, after which time ADT therapy may be stopped for a period of time during with booster administration of the vaccine may continue with or without the PD-1 pathway inhibitor. In some instances, ADT therapy may be re-initiated weeks to months later. Other suitable combinations of the therapies, treatment times and dosing regimens are contemplated to be determined by one skilled in the art.

In some embodiments, the double combination therapy of DNA vaccine and ADT are contemplated. In some embodiments, the DNA vaccine is administered at least once (i.e. from 1-12 times) weekly or bi-weekly before the beginning of ADT. Starting the vaccine before ADT treatment may have some advantages in priming the immune system to be activated against cells expressing androgen receptor, which we have found is overexpressed in tumor cells after ADT treatment. This allows for a more robust and increased immune response to tumor cells resulting in a delay or decreased tumor growth. In some embodiments, ADT therapy is started before the administration of the DNA vaccine, e.g. for at least one month or more before DNA vaccine is administered.

In some embodiments, the ADT administration can also be intermittent. During intermittent ADT administration, the PSA number in a subject may be monitored to determine if ADT therapy should be stopped and/or started again. For example, ADT is stopped once the PSA number is lowered to a suitable level and stabilized; and ADT is restarted when the PSA number increases again (sometimes months, maybe years later). Further, the use of the DNA vaccine may include dosages where the vaccine is given periodically (e.g. every 2 week to every 3 months) over the first year to elicit an anti-tumor response and then administered occasionally (e.g. 3 months or more) as maintenance boosters to maintain an anti-tumor immune response in combination with continuous or intermittent ADT administration.

In some embodiments, the DNA vaccine, PD-1 pathway inhibitor, and ADT are each administered separately and each are administered over different overlapping time periods. In some instances, all three are administered over the same time period. In some embodiments, all three treatments are administered over the same time period constantly but at different timing intervals. In some embodiments, all three treatments are not constantly administered (e.g. there is period of time in which one or more of the treatments are not administered). In some embodiments, each of the treatments is provided in different dosages that are spaced out at different times after the start of treatment. For example, the DNA vaccine may be administered before the start of treatment with the PD-1 pathway inhibitor and before the start of ADT treatment. In some examples, the DNA vaccine may be administered once every 1-12 weeks, for at least 6 weeks or more, for example once every week or once every other week for 1-24 weeks followed by once every 3-8 weeks for at least an additional 24 weeks or more. In another example, the DNA vaccine and PD-1 pathway inhibitor may be administered on the same schedule of administration before the beginning of ADT treatment (e.g. every 2 weeks for 6-36 week, followed by every 4-6 weeks for at least an

additional 6-36 weeks, followed by booster administration every 12-24 weeks for at least an additional year). Other suitable combinations of dosing schedules is contemplated.

In some embodiments, the length of each treatment (DNA vaccine, PD-1 pathway inhibitor and ADT) and period over which treatment is provided for at least a portion of time. In some embodiments, one or more of the treatments are administered over the same time period. For example, the DNA vaccine, PD-1 pathway inhibitor may be administered at different dosages and at different times over the course of months to years, while the ADT can be administered by known protocols over some or all of the same time period of months or years.

In some embodiments, the DNA vaccine and the PD-1 pathway inhibitor are administered in multiple dosages prior to the start of the ADT and continue during ADT administration. In some embodiments, the combination of treatment is administered as follows:

vaccine and PD-1 pathway inhibitor administered every 2-4 weeks (e.g. 2 weeks) for at least 8 to 16 weeks, followed by administration of the vaccine and PD-1 pathway inhibitor every 4 weeks for at least 8 to 16 additional weeks, followed by administration of the vaccine and PD-1 pathway inhibitor every 12 weeks (or alternatively every 3 months) for at least an additional 24 weeks; and

ADT is administered beginning between week 10 and week 14 after start of the vaccine and PD-1 pathway inhibitor initial administration, and is administered every 12 weeks for at least 4 additional treatment times (i.e. for at least 48 weeks).

In a preferred embodiment, the vaccine is a DNA vaccine against an androgen receptor and the PD-1 pathway inhibitor is an anti-PD-1 antibody, and the ADT is leuprolide depot 22.5 mg intramuscular administration or goserelin 10.8 mg subcutaneous administration. A suitable dosage regimen is found in FIG. **16**, wherein the DNA vaccine and PD-1 pathway inhibitor is administered every 2 weeks for the first 12 weeks, followed by administration every 4 weeks for an additional 12 weeks, and subsequently followed by administration every 12 weeks for at least an additional 24-48 weeks. In this dosage regimen, the ADT is administered at 12 weeks, 24 weeks, 36 weeks and 48 weeks after the initial vaccine/PD-1 inhibitor treatment. This regimen can be extended to at least a year or more in order to treat the tumor.

In some embodiments, the disclosure provides a method of increasing the efficacy of androgen deprivation therapy in a subject with prostate cancer comprising administering to the subject an effective amount of a recombinant DNA vaccine comprising a polynucleotide operably linked to a transcriptional regulatory element wherein the polynucleotide encodes an androgen receptor or a fragment of the androgen receptor, wherein the method inhibits, delays or reduces the growth of the prostate cancer. In some embodiments, the method further comprises administering to the subject an effective amount of a PD-pathway inhibitor.

The subject may have previously been diagnosed as having prostate cancer. In some embodiments, the prostate cancer may be in any stage, for example, early stage prostate cancer or newly diagnosed prostate cancer. In some embodiments, the prostate cancer may be metastatic prostate cancer. In another embodiment, the prostate cancer is castration resistant prostate cancer (mCRPC).

Some embodiments provide a kit for treating prostate cancer. The kit comprises androgen deprivation therapy and a vaccine (for example a DNA vaccine) that elicits an anti-androgen receptor immune response. A set of instructions on the dosages and regiments for administering the ADT and recombinant DNA vaccine may also be provided. In some embodiments, the androgen receptor therapy consists of one or more drugs that target the AR pathway by interfering with AR expression or signaling. Suitable vaccines and drugs are discussed above.

In another embodiment the kit comprises androgen deprivation therapy, a vaccine (for example a DNA vaccine) that elicits an anti-androgen receptor immune response and a PD-1 pathway inhibitor (such as a PD-1 antibody). A set of instructions on the dosages and regimen for each treatment may be provided.

The invention will be more fully understood upon consideration of the following non-limiting examples. Each publication, patent, and patent publication cited in this disclosure is incorporated in reference herein in its entirety.

Example 1

Androgen Deprivation Increases Androgen Receptor (AR) Expression and Enhances Tumor Cell Susceptibility to AR-Specific T-Cell Responses

This Example demonstrates that Androgen deprivation results in increased full length AR expression in tumor cells (22Rv1 cells), whether deprivation was for a short or extended period of time. Co-culture of AR peptide-specific CD8+ T cells with HLA-A2-expressing tumor cells resulted in increased T-cell activation, cytokine expression, and cytotoxicity assays.

Materials and Methods

Cell Culture

22Rv1, LNCaP, PC3, and DU145 cells were obtained from ATCC, and their identity and lack of *mycoplasma* contamination was confirmed by DDC Medical. Cells were cultured in RPMI-1640 medium with 200 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1 mM β-mercaptoethanol. This base medium was supplemented with either 10% complete FCS (RPMI/FCS), or 10% charcoal-stripped serum (RPMI/CSS) to generate androgen-deprived culture medium. Charcoal-stripped serum was generated by incubating dextran-coated charcoal with heat-inactivated FCS and incubating overnight at 4° C., followed by centrifugation and sterile filtration, followed by analysis for testosterone by Testosterone AccuBind ELISA (Monobind).

Androgen Receptor Enzyme-Linked Immunosorbent Assay (ELISA)

Cultured prostate cancer cells were collected, cell lysates prepared, and analyzed for protein expression using the PathScan androgen receptor (AR) ELISA per manufacturer's instructions (Cell Signaling Technology). Briefly, microwell strips (pre-coated with anti-AR antibody) were coated with 2 mg/mL protein lysates in triplicate, and incubated overnight at 4° C. The following day, AR was detected using a detection antibody followed by HRP-linked secondary antibody and TMB substrate development. A standard curve using purified AR LBD protein (Invitrogen) was generated, and used to obtain relative AR concentration per mg cell lysate.

Androgen Receptor Quantitative Real-Time PCR

Cultured prostate cancer cells were collected, RNA was prepared using Qiagen RNeasy RNA purification system, common concentrations of RNA was used to synthesize cDNA using iScript cDNA synthesis kid (BioRad) and used as a template for qPCR reactions using SsoFast qPCR supermix (BioRad). Reactions were run a Bio-Rad MyiQ thermocycler, using an annealing temperature of 60° C. and 40 cycles. Primer sets:

AR-FL_Fwd (SEQ ID NO: 7)
(ACATCAAGGAACTCGATCGTATCATTGC);

AR-FL_Rev (SEQ ID NO: 8)
(TTGGGCACTTGCACAGAGAT);

AR-V7_Fwd (SEQ ID NO: 13)
(CCATCTTGTCGTCTTCGGAAATGTTATGAAGC);

AR-V7_Rev (SEQ ID NO: 14)
(TTTGAATGAGGCAAGTCAGCCTTTCT);

β-actin_Fwd (SEQ ID NO: 15)
(TCATGAAGTGTGACGTTGACATCCGT);

β-actin_Rev (SEQ ID NO: 16)
(CTTAGAAGCATTTGCGGTGCACGATG).

Results were analyzed by the $2^{-\Delta Ct}$ method relative to β-actin as a control gene, and fold induction over FCS-treated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, as published [6].

Generation and Validation of HLA-A2-Expressing 22Rv1 Cells

22Rv1 cells cultured greater than six months in RPMI/FCS or RPMI/CSS were diluted into 96-well flat bottom plates at a concentration of 50 cells/well, and transfected with a lentivirus encoding the human HLA-A2 complex. Cells were expanded, stained with HLA-A2-FITC (Biolegend), and sorted for HLA-A2+ events (FACSAria Cell Sorter, BD Biosciences). HLA-A2+22Rv1/FCS and 22Rv1/CSS cells were expanded, and AR protein and mRNA expression was validated as above, and HLA-A2 and PD-L1 expression was evaluated by flow cytometry.

Mouse Immunology Assays

For mouse immunology studies, HHDII-DR1 heterozygous mice were immunized subcutaneously on the right hind flank with 100 μg of the AR811 peptide given with 200 μl Complete Freund's Adjuvant (Sigma), as published (Olson et al., *Cancer Immunol, Immunother.* (2011), 33: 639-647). Seven days later, splenocytes were collected, restimulated for six days with AR811 peptide, and used for intracellular cytokine staining assays and cytotoxicity assays. For intracellular cytokine staining, 200,000 splenocytes were stimulated for 18 hours with media alone, 2000 22Rv1/FCS cells, 2000 22Rv1/CSS cells, or a PMA/Ionomycin positive control. Cells were treated with monensin (GolgiStop, 2 μM, BD Biosciences) for four hours at 37° C./5% CO_2. Cells were then stained with fluorescently-labeled CD3, CD4, CD8, and CD69 antibodies, and after fixation and permeabilization, intracellular staining was conducted using fluorescently-labeled antibodies for IFNγ and TNFα (BD Biosciences), or the corresponding isotype controls. Cells were subsequently analyzed using an LSR II flow cytometer (BD Biosciences), and events were analyzed by gating CD3+CD8+ splenocytes and analyzing this population for expression of IFNγ and/or TNFα, as well as surface CD69 expression. Cytotoxicity assays were performed as has been previously described (Smith et al., Canc. Res. (2011), 71: 6785-6795). Briefly, restimulated splenocytes were cultured with 22Rv1/FCS or 22Rv1/CSS target cell lines for 4-6 hours, after which LDH release was calculated using a variation of the Cytotox 96 Assay kid (Promega). The optical density (OD) signal contributed by the media alone was subtracted from all values. All sample conditions were evaluated in triplicate, with the standard error shown.

Human Immunology Assays

For human immunology studies, human T-cell cultures were generated as has been previously described (Olson et al., *Cancer Immunol, Immunother.* (2011), 33: 639-647). Briefly, PBMC samples from HLA-A2+ prostate cancer patients were cultured with irradiated peptide-pulsed antigen-presenting cells (either autologous DCs, PBMC, or lymphoblastoid B-cell lines). After 24 hours, cells were treated with 10 U/mL IL-2, and restimulated weekly with irradiated peptide-pulsed APCs, and after 2-8 in vitro stimulations, cultures were tested for cytotolytic activity using cytotoxicity assays. AR805 peptide-specific T cells were subsequently used for intracellular cytokine staining assays and cytotoxicity assays. For intracellular cytokine staining, assays were conducted as above, but using fluorescently-labeled antibodies for intracellular IFNγ, TNFα, IL-2, and granzyme B (GrB), or the corresponding isotype controls. Cells were subsequently analyzed using an LSR II flow cytometer, and events were analyzed by gating CD3+CD8+ T-cells and analyzing this population for expression of IFNγ, TNFα, IL-2, and/or GrB, as well as surface CD69 and CD107a expression. Cytotoxicity assays were performed as above and has been previously described (Smith et al., Canc. Res. (2011), 71: 6785-6795).

Results

Androgen Deprivation Increases AR Protein Expression in Some Prostate Cancer Cell Lines In Vitro.

DU145, PC3, LNCaP, and 22Rv1 cells were cultured under androgen-replete (FCS; media supplemented with complete FCS) or androgen-deprived (CSS; charcoal-stripped serum) conditions for 1, 3, 5, or 7 days or for at least three months (LT; long-term). Protein lysates were collected and analyzed for AR protein expression by ELISA as shown in FIG. **1**A-D. * indicates $p<0.05$ by Student's t-test.

Androgen Deprivation Induces a Transient Increase in AR-V7 mRNA Expression and Sustained Overexpression of Full-Length AR mRNA.

22Rv1 cells were cultured under androgen-replete or androgen-deprived conditions for 1, 3, 5, or 7 days or for at least three months. RNA was isolated and used to synthesize cDNA, and cDNA was used as the template for qRT-PCR reactions for either full-length AR (FIGS. **2**A and **2**C, left panels) or AR-V7 (FIGS. **2**B and **2**D, right panels) expression. Top Panels: relative expression (normalized to β-actin control). Bottom panels: fold induction of expression over long-term FCS-cultured 22rv1 cells. * indicates $p<0.05$ by Student's t-test.

ARLBD Peptide-Specific T-Cells have Increased Levels of T-Cell Activation, Th1 Polyfunctional Cytokine Expression, and Cytotoxicity Against Androgen-Deprived Prostate Cancer Cells than Cells Cultured in Androgen-Replete Conditions.

22Rv1 cells were cultured greater than six months in androgen-replete (FCS) or androgen-deprived (CSS) conditions, transfected with a lentivirus encoding HLA-A2, and sorted for HLA-A2-expressing cells by flow cytometry. Subsequent 22Rv1/FCS and 22Rv1/CSS cell lines (or non-HLA-A2-transfected 22 Rv1 controls) were evaluated for AR protein expression by ELISA (FIG. **3**A), RNA expression by qRT-PCR (FIG. **3**B), HLA-A2 expression (FIG. **3**C), and PD-L1 expression (FIG. **3**D) by flow cytometry (blue: 22 Rv1/FCS; red: 22Rv1/CSS; black: wild-type 22Rv1; grey: IgG-stained 22Rv1). * indicates $p<0.05$ by Student's t-test.

AR811 Peptide-Immunized Mice have Increased Cytokine Expression, T-Cell Activation, and Cytotoxicity when Exposed to Androgen-Deprived 22Rv1 Cells.

Splenocytes from AR811 peptide-immunized HHDII-DR1 heterozygous mice were evaluated for immune responses against HLA-A2-expressing 22Rv1/FCS or 22Rv1/CSS cells. FIG. **4**A-B shows intracellular cytokine staining of splenocytes cultured with 22Rv1/FCS (FIG. **4**A) or 22Rv1/CSS (FIG. **4**B). FIG. **4**C depicts CD69 expression of splenocytes cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells (mean fluorescent intensity quantified in inset—* indicates $p<0.05$ by Student's t-test). FIG. **4**D shows cytotoxicity of splenocytes cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells.

Human AR805 peptide-specific T-cells are shown to have increased levels of T-cell activation, Th1 polyfunctional cytokine expression, and cytotoxicity when exposed to androgen-deprived 22Rv1 prostate cancer cells. AR805 peptide-specific T-cells (previously cultured from the peripheral blood of HLA-A2+ prostate cancer patients (Smith et al., Canc. Res. (2011), 71: 6785-6795) were evaluated for immune responses against HLA-A2-expressing 22Rv1/FCS or 22Rv1/CSS cells. FIG. **5**A shows CD69 expression of splenocytes cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells (quantified in adjacent bar graph—* indicates $p<0.05$ by Student's t-test). FIG. **5**B shows intracellular cytokine staining (IFNγ by TNFα) of T-cells cultured with 22Rv1/CSS (right panel) or 22Rv1/FCS (left panel) cells. FIG. **5**C shows frequency of CD8+ T cells that were found to express zero (blue), one (green), two (yellow), three (orange), or four (red) Th1 related molecules (IFNγ, TNFα, IL-2, and/or granzyme B). FIG. **5**D shows granzyme B expression by CD8+ T cells following culturing with 22Rv1/FCS (red) or 22Rv1/CSS (blue) cells (quantified in adjacent bar graph—* indicates $p<0.05$ by Student's t-test). FIG. **5**E shows frequency of CD8+ T cells expressing the surface degranulation marker CD107a. FIG. **5**F shows cytotoxicity of T-cells cultured with 22Rv1/CSS (blue) or 22Rv1/FCS (red) cells.

Thus, androgen deprivation increases androgen receptor expression and causes prostate tumor cells to have increased susceptibility to AR-specific T-cells.

Example 2

Phase I Clinical Trial Evaluating a DNA Vaccine Encoding the AR-LBD in Combination with Androgen Deprivation Therapy

FIG. **6**A depicts a clinical trial. Men with metastatic prostate cancer who have recently (within 1-6 months) initiated androgen deprivation therapy (ADT) are enrolled in a clinical trial evaluating the safety and immunogenicity of a DNA vaccine encoding the AR LBD (pTVG-AR). This trial seeks to capitalize on targeting one of the most common mechanisms of resistance to ADT (overexpression of the AR) by combining ADT with pTVG-AR, ideally resulting in delayed time to progression (dashed blue line) to castrate-resistant disease (CRPC). FIG. **6**B depicts the different ARMS of the study. Patients receive either six biweekly immunizations followed by quarterly boosters, or two biweekly immunizations every ten weeks, either alone or in combination with GM-CSF. Immunizations are continued 18 months or to disease progression. Primary endpoints are safety and ARLBD-specific immunity. Secondary objectives of this trial include evaluating which schedule of immunization is best able to elicit long-lived ARLBD-specific T-cell responses, the effect of GM-CSF in generating immune responses, and to determine the median time to PSA progression and 18-month PSA progression-free survival.

Example 3

AR Overexpression Occurs in Human and Mouse Prostate Tissue Following AD Therapies and Enhances their Recognition by AR-Specific T-Cells

Androgen-deprived 22Rv1 human prostate cancer cells and castrate-resistant MycCaP mouse prostate cancer cells have increased AR expression (FIGS. **7**A and **7**B) after ADT treatment. We also showed this occurs in the MycCaP prostate cancer model in vivo, as chemical castration using the GnRH antagonist degarelix resulted in increased AR expression in tumors (FIG. **7**C). This increased AR expression also caused these tumor cells to be better recognized by AR-specific CD8+ T-cells, as T-cells have higher levels of activation, cytokine expression, and cytotoxicity when cultured with androgen-deprived tumor cells (FIG. **7**D).

Briefly, Human 22Rv1 prostate cancer cells cultured in androgen replete (FCS) or—deprived (CSS) conditions (FIG. **7**A), or mouse MycCaP cells serially passaged in untreated (MycCaP/AS) or castrate (MycCaP/CR) mice (FIG. **7**B) were collected and analyzed for AR expression by qPCR (left panels), ELISA (center panels), and intracellular staining (ICS, right panels). FIG. **7**C, FVB mice were challenged with MycCaP/AS tumor cells, and given degarelix or a sham treatment. At the time of outgrowth, tumors were collected and analyzed for AR expression by intracellular flow cytometry (example histogram with samples stained with IgG or AR-intracellular antibodies; quantified in right panels). FIG. **7**D, AR-specific CD8+ T cells were cultured with 22Rv1/FCS or 22Rv1/CSS cells, and evaluated for T-cell activation by CD69 expression (left panel), IFNγ and TNFα cytokine expression (center panel), and cytotoxicity (right panel). In all panels, * indicates $p<0.05$ by Student's t-test.

Example 4

AD with AR-Directed Immunization Increases Anti-Tumor Immune Responses and Delays Tumor Recurrence

This Experiment demonstrates that the combination of AD with AR-directed immunization increases anti-tumor immune response. MycCaP tumor-bearing FVB mice treated with degarelix (ADT treatment) followed by immunization at weekly intervals with the pTVG-AR DNA vaccine had enhanced immune responses against androgen-deprived tumor cells and a delay in prostate cancer regrowth compared to controls (FIG. **8**). Briefly, male FVB mice (n=8) were implanted with MycCaP tumors, treated with degarelix (or sham), and immunized weekly with pTVG-AR or control pTVG4. FIG. **8**A, splenocytes were collected and analyzed for immune responses against MycCaP/CR tumor cells by intracellular cytokine staining (left panel) and AR peptide-stimulated splenocytes from pTVG-AR-immunized mice were measured for ability to lyse MycCap/AS vs. MycCap/CR tumor cells (right panel). FIG. **8**B depicts mice followed for tumor volume. In all panels, * indicates $p<0.05$ by Student's t-test. FIG. **8**C depicts tumor volume post-challenge of control (sham), degarelix+pTVG4 and degarelix+pTVG-AR.

Example 5

Increased Androgen Receptor Expression in Prostate Cancer Cells Following Androgen Deprivation Increases Recognition by Androgen Receptor-Specific T Cells

This example again demonstrates that androgen deprivation increases AR expression in human and murine prostate tumor cells in vivo and in vitro that persisted over time. Increased AR expression was associated with increased recognition and cytolytic activity by AR-specific T cells. Further, ADT combined with vaccination, using a DNA vaccine encoding the ligand-binding domain of the AR, led to improved anti-tumor responses as measured by tumor volumes and delays in the emergence of castrate-resistant prostate tumors in two murine prostate cancer models (Myc-CaP and prostate-specific PTEN-deficient mice). This data supports the benefits of combining ADT with AR-directed immunotherapy over ADT combined with other immunotherapeutic approaches by specifically targeting a major mechanism of resistance, overexpression of AR.

Materials and Methods of Example 5

Mice and Cell Lines

Human prostate cancer cells were obtained from ATCC, and cultured in RPMI-1640 medium with 200 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1 mM β-mercaptoethanol. Cell identity and *mycoplasma* testing was confirmed by DDC Medical (Fairfield, Ohio). Myc-CaP/AS or Myc-CaP/CR cells (androgen-sensitive and castrate-resistant variants of the Myc-CaP parental line originally generated by Charles Sawyers) and culture conditions have been previously described (22). Both human and mouse cell lines were maintained in either 10% complete fetal calf serum (FCS) or charcoal-stripped serum (CSS) for androgen-replete or androgen-deprived conditions.

Tumor studies using Myc-CaP tumor cells were conducted in wild-type male FVB mice (Jackson Laboratory, Bar Harbor, Me.). PTEN knock-out mice were generated by crossing Pten foxed (loxp/loxp) animals with Probasin-Cre (PB-Cre4+) as has been described (23). Mice were screened by PCR for the floxed or wild-type PTEN alleles (forward primer: CAA GCA CTC TGC GAA CTG AG; reverse primer: AAG TTT TTG AAG GCA AGA TGC) and PB-Cre transgene (forward primer: CTG AAG AAT GGG ACA GGC ATT G; reverse primer: CAT CAC TCG TTG CAT CGA CC). Mice were maintained under aseptic conditions and all experiments were conducted under an IACUC-approved protocol.

Tumor Studies

FVB mice were inoculated subcutaneously with 106 Myc-CaP/AS tumor cells, and followed daily for the presence of palpable tumors. Once tumors were palpable, mice were treated subcutaneously with either degarelix (25 mg/kg) or a vehicle sham treatment every four weeks. For immunization studies, degarelix-treated animals were randomized to weekly immunization with 100 μg pTVG4 or pTVG-AR beginning one day after receiving degarelix. Tumor growth was measured at least three times weekly, and tumor volumes calculated as we've published (19). At the time of euthanasia, tumors and spleens were collected. For studies using PTEN-deficient mice, animals began receiving degarelix (25 mg/kg) at 20 weeks (+/−two weeks) of age, followed by biweekly immunization with 100 μg pTVG4 or pTVG-AR beginning one day after ADT. Animals were treated until 40 weeks of age (+/−two weeks) before tissue collection.

Androgen Receptor Enzyme-Linked Immunosorbent Assay (ELISA)

Cultured prostate cancer cells were collected, cell lysates prepared, and analyzed for protein expression using the PathScan androgen receptor ELISA per manufacturer's instructions (Cell Signaling Technology, Danvers, Mass.). Briefly, microwell strips (pre-coated with anti-AR antibody) were coated with 2 mg/mL protein lysates in triplicate, and incubated overnight. AR was detected using a detection antibody followed by HRP-linked secondary antibody and TMB substrate development. A standard curve using purified AR LBD protein (Invitrogen, Carlsbad, Calif.) was generated, and used to determine relative AR concentration per mg cell lysate.

Flow Cytometry

For androgen receptor intracellular staining, cells were stained with a Live/Dead GhostDye 780 Live/Dead Stain (Tonbo Biosciences, San Diego, Calif.) and CD45 (clone 30-F11, Tonbo Biosciences) for dissociated tumor samples, and intracellularly stained with antibodies directed against the androgen receptor ligand-binding domain (clone EP670Y, Abcam, Cambridge, United Kingdom) and amino terminal domain (clone D6F11, Cell Signaling Technologies), or isotype controls. For HLA-A2 and PD-L1 expression, cells were stained with HLA-ABC (clone W6/32, eBioscience, San Diego, Calif.) and PD-L1 (clone MIH-5, eBioscience) antibodies.

Androgen Receptor Quantitative Real-Time PCR

Prostate tumor cells (cell lines or dissociated tumors) were collected, RNA was prepared (RNeasy RNA purification system; Qiagen, Hilden, Germany), used to synthesize cDNA (iScript cDNA synthesis kit; BioRad, Hercules, Calif.), and used as a template for qPCR reactions using SsoFast qPCR supermix (BioRad). Reactions were performed using a Bio-Rad MyiQ thermocycler, using an annealing temperature of 60° C. and 40 cycles. Primer sets:

full-length human androgen receptor

forward: SEQ ID NO 7
ACATCAAGGAACTCGATCGTATCATTGC,;

reverse: SEQ ID NO: 8
TTGGGCACTTGCACAGAGAT,,

AR-V7

forward: SEQ ID NO: 13
CCATCTTGTCGTCTTCGGAAATGTTATGAAGC,;

reverse: SEQ ID NO: 14
TTTGAATGAGGCAAGTCAGCCTTTCT,,

full length mouse AR

forward: SEQ ID NO: 17
GGACCATGTTTTACCCATCG,;

reverse: SEQ ID NO: 18
ATCTGGTCATCCACATGCAA,,

mouse AR-V2

forward:
SEQ ID NO: 17
GGACCATGTTTTACCCATCG,;

reverse:
SEQ ID NO: 19
TTGTTGTGGCAGCAGAGTTC,,

mouse AR-V4

forward:
SEQ ID NO: 17
GGACCATGTTTTACCCATCG,;

reverse:
SEQ ID NO: 20
AAGTGGGGAACCACAGCAT,,
and

β-actin

forward:
SEQ ID NO: 15
TCATGAAGTGTGACGTTGACATCCGT,;

reverse:
SEQ ID NO: 16)
CTTAGAAGCATTTGCGGTGCACGATG, (24-26).

Results were analyzed by the 2-ΔCt method relative to β-actin as a control gene, as published (26).

Immunology Assays

To study immune responses, human T-cell lines or splenocytes were collected as previously described (20), and used for intracellular cytokine staining assays and cytotoxicity assays. For intracellular cytokine staining, cells were stimulated for 18 hours with media alone, an ARLBD peptide pool (a pool of 15-mer peptides, overlapping by 11 residues, and covering the entire sequence of the AR LBD; LifeTein, Somerset, N.J.), tumor cells, or a PMA/Ionomycin positive control. Cells were stained using a fixable live/dead marker (Tonbo Bioscience) and extracellular and intracellular antibodies. Human antibodies: CD3 (clone UCHT1, BD Biosciences), CD4 (clone RPA-T4, BD Biosciences), CD8 (clone RPA-T8, eBioscience), CD69 (clone FN50, BD Biosciences), CD107a (clone H4-A3, BD Biosciences), IL2 (clone MQ1-17H12, eBioscience), IFNγ(clone 4S.B3, BioLegend, San Diego, Calif.), TNFα (clone MAb11, BD Biosciences), GrB (clone GB11, BD Biosciences). Mouse antibodies: CD3 (clone 17A2, BD Biosciences), CD4 (clone GK1.5, BD Biosciences), CD8 (clone 53-6.7, BD Biosciences), CD45 (clone 30-F11, BD Biosciences), CD69 (clone H1.2F3, eBioscience), IFNγ(clone XMG1.2, BD Biosciences), TNFα (clone MP6-XT22, BD Biosciences). Cells were subsequently analyzed using an LSR II or Fortessa flow cytometer (BD Biosciences), and events were analyzed by gating CD3+CD4+ or CD3+CD8+ cells and analyzing this population for expression of CD69, CD107a, IFNγ, TNFα, IL2, and/or GrB. Cytotoxicity assays were performed as has been previously described (20). Briefly, splenocytes were restimulated for five days with an ARLBD peptide pool, and were cultured with tumor cell lines, after which LDH release was calculated using the Cytotox 96 Assay kit (Promega, Madison, Wis.), as previously published (19).

Immunohistochemistry

Paraffin-embedded MycCaP tumors were stained for CD3 expression by immunohistochemistry as has been described (20). Sections were stained with primary antibodies (CD3: clone SP7, Abcam), developed using the LSAB+ System-HRP (Agilent Technologies, Santa Clara, Calif.) and Metal Enhanced DAB Substrate Kit DAB metal concentration (Thermo Fisher Scientific, Waltham, Mass.), imaged using an Olympus BX51 fluorescent microscope (Olympus, Lombard, Ill.) in combination with SPOT RT analysis software (SPOT Imaging Solutions, Sterling Heights, Mich.), and quantified by the frequency of CD3+ cells per 10× field, counting at least five fields per tumor section per animal by a blinded investigator.

Positron Emission Tomography/Computed Tomography Imaging

All mice were intravenously administered between 5-8 MBq of 124I-CLR1404 and then micro positron emission tomography/computed tomography (PET/CT) scanned 96 hrs post-injection. During scanning, mice were anesthetized with 2% isoflurane inhalation gas mixed with 1 L/min of pure oxygen (27). Mice were scanned with the Siemens Inveon Hybrid microPET/CT (Siemens Medical Solutions, Knoxville, Tenn.) in the prone position. Forty-million counts per mouse were collected for the PET scan to obtain adequate signal-to-noise. PET data were histogrammed into one static frame and subsequently reconstructed using ordered-subset expectation maximization (OSEM) of three dimensions followed by the maximum a posteriori algorithm, and CT attenuation and scatter correction were applied based on the NEMA NU 4 image-quality parameters (28).

All PET and CT images were co-registered. Image data were analyzed using the General Analysis tools provided by Siemens Inveon Research Workplace (Siemens Medical Solutions). Data were identically window/leveled and scaled according to each animal's decay corrected injection activity. Based on the PET and CT images, a reference volume of interest (VOI) was drawn around each tumor and a separate background tissue VOI was drawn on muscle and liver. VOI thresholding within the reference tumor VOI was adjusted to include all signal greater than sixty percent of the maximum signal. Data were reported as percent injected dose normalized by the mass of the tissue VOI (% ID/g tissue), with the assumption that all tissue density is akin to water (1 g/mL). Data were then averaged within pre- and post-treatment groups and normalized to background tissue values.

Results

Androgen Deprivation Increases Androgen Receptor Expression and Enhances AR-Specific T Cell Responses to Androgen-Deprived Prostate Tumor Cell Lines

In this Example, a panel of six prostate cell lines (two immortalized prostate epithelial lines, two androgen-independent prostate cancer lines, and two androgen-dependent prostate cancer lines) were cultured for short (one to seven days) or extended periods (greater than six months) in androgen-deprived medium and analyzed for AR expression. Androgen deprivation was found to result in an increase in AR protein expression in androgen-dependent prostate tumor cells by quantitative ELISA (FIG. **11**A), as well as by intracellular staining using antibodies directed against both the ligand-binding domain as well as the amino-terminal domain (FIG. **11**B, with the amplitude and frequency of AR expression quantified in FIGS. **11**C and **11**D, respectively). Analysis of 22Rv1 cells (which are known to express AR-V7, an LBD-loss splice variant) showed that androgen deprivation led to a steadily increas-

ing expression in full-length AR as well as a transient increase in AR-V7 (FIG. **11**E), with no detectable expression of AR-V1, AR567es, or other splice variants). However, AR-V7 expression was at significantly lower levels compared to full-length AR transcripts.

To determine whether this increase in AR expression following androgen deprivation resulted in enhanced AR-specific T-cell effector function against these tumor cells, 22Rv1 cells were first transfected to express HLA-A2 as a model MHC molecule, and one for which AR-restricted epitopes have been previously identified (19). After generating this cell line, increased AR protein and RNA expression following androgen-deprivation observed in the parental cell lines was confirmed in these HLA-A2-expressing lines (FIG. **11**F-G). These 22Rv1/FCS and 22Rv1/CSS cells were then incubated with T cell lines specific for the HLA-A2-restricted AR805 epitope. T cells cultured with the 22Rv1/CSS cell line were shown to have higher levels of T-cell activation (as measured by CD69 expression—FIG. **12**A), as well as increased expression of Th1 cytokines (FIG. **12**B), including CD8+ T cells with polyfunctional cytokine expression (FIG. **12**C), compared to T cells that had been stimulated with 22Rv1 cells cultured under androgen-replete conditions. Co-culture with 22Rv1/CSS cells also resulted in higher expression of granzyme B (FIG. **12**D), the degranulation marker CD107a (FIG. **12**E), as well as increased cytotoxicity (FIG. **12**F) compared to co-culture with 22Rv1/FCS cells. Similar studies, using splenocytes from HLA-A2 transgenic mice that were directly immunized with another HLA-A2 restricted epitope, AR811, replicated these results in terms of increased cytokine expression, T-cell activation, and cytotoxicity when cultured with androgen-deprived HLA-A2-expressing 22Rv1 cells (FIG. **12**G). These differences in T-cell recognition and cytotoxicity were likely not due to altered MHC class I nor PD-L1 expression, as 22Rv1/CSS and 22Rv1/FCS cells expressed identical levels of both HLA-A2 and PD-L1 (FIG. **11**H-I).

Androgen Deprivation Increases AR Expression in Myc-CaP Tumor Cells In Vitro and in Vivo

We have previously used the TRAMP mouse model to study the impact of vaccines targeting AR on tumor development and progression (20). However, it has previously been reported that androgen deprivation of TRAMP mice, and many other murine prostate tumor models, results in AR loss and the development of neuroendocrine tumors (29). Consequently, we sought to evaluate other models more representative of human prostate cancer that continue to express AR following androgen deprivation. One such model is the Myc-CaP cell line, which mimics the human disease in that it maintains AR expression following castration (22). To confirm this, androgen-sensitive Myc-CaP cells (Myc-CaP/AS, generated from untreated FVB mice), and castration-resistant Myc-CaP cells (Myc-CaP/CR, generated from serial passaging of the Myc-CaP/AS cell line through castrated mice) were studied. Similar to what was observed in the human prostate cancer cell lines, the Myc-CaP/CR cell line was found to have increased full-length AR expression by both quantitative ELISA (FIG. **13**A) and intracellular staining compared to the Myc-CaP/AS cell line (FIG. **13**B). While analysis of RNA transcripts showed an increase in the murine AR splice variants mAR-V2 and mAR-V4, these splice variants were similarly several fold lower in expression than the full-length AR (FIG. **13**C). To study the expression of AR in vivo, FVB mice were inoculated with Myc-CaP/AS cells, and then given either a sham treatment or castration by administration of a GnRH antagonist (degarelix). Animals were followed for tumor growth (FIG. **13**D), and recurrent tumors were collected and CD45− cells were analyzed for AR expression by intracellular staining. Tumors that recurred following androgen deprivation were found to have increased AR expression, both in terms of frequency of CD45− cells with detectable expression of the AR, as well as the amplitude of AR expression within these cells (FIG. **13**E).

Immunization with pTVG-AR Delays the Growth of Castration-Resistant Prostate Tumors Following Androgen Deprivation

This Example also demonstrates that androgen deprivation in combination with AR-targeted vaccination delays the outgrowth of castration-resistant tumors by specifically targeting cells overexpressing AR. Mice were implanted with Myc-CaP/AS tumors, and mice with established tumors were given either a sham treatment or degarelix. Mice treated with degarelix were then randomized to immunization with either a DNA vaccine encoding the AR LBD (pTVG-AR), or an empty vector control (pTVG4). The combination treatment with degarelix and pTVG-AR was found to delay tumor growth compared to treatment with degarelix and control vaccine (FIG. **14**A-B). Additionally, when animals were evaluated for evidence of immune responses against the Myc-CaP/AS or Myc-CaP/CR cell lines, animals immunized with pTVG-AR were found to have increased immune responses against the castration-resistant cell line, both in terms of cytokine expression (FIG. **14**C) as well as cytotoxicity (FIG. **14**D). In parallel studies, we found that immunization of Myc-CaP/AS-bearing mice with pTVG-AR resulted in an increased frequency of tumor-infiltrating CD3+ T cells, and this was further increased when vaccination was combined with degarelix treatment (FIG. **14**E).

Androgen Deprivation Increases AR Expression in PTEN-Deficient Tumors, and Immunization with pTVG-AR, Combined with ADT, Decreased Growth of Castration-Resistant Tumors

As an additional, relevant model of human prostate cancer, this Example utilized the PbCre PTENfl/fl mice, in which prostate-specific expression of the Cre recombinase drives deletion of the PTEN tumor suppression and the formation of autochthonous prostate tumors. The PTEN-CaP8 cell line (derived from one of these autochthonous tumors) was similarly cultured in androgen-replete or androgen-deprived medium. As shown in FIG. **15**A-B, androgen-deprivation resulted in a significant increase in AR protein expression, similar to the human prostate cancer cell lines and Myc-CaP cell lines above. Twenty-week old PbCre+ PTENfl/fl mice were then given either a sham treatment, or degarelix in combination with pTVG-AR vaccine or vector control. To non-invasively monitor tumor growth, as well as to randomize animals prior to treatment, we utilized micro-PET/CT imaging, employing the novel radiotracer 124I-CLR1404, which is a radioiodinated alkylphosphocholine (APC) analog that has shown selective tumor uptake in >95% of malignant models to date (30). Animals were intravenously administered 124I-CLR1404 and subsequently PET/CT scanned within one week prior to initiation and completion of therapy (FIG. **15**C), and imaging results were analyzed for mean and maximum tumor uptake. Analysis of tumors pre-treatment showed no difference between mean and maximum tumor uptake (FIG. **15**D-E). While some animals with large tumors died prior to the last imaging session, and hence not all animals underwent post-treatment imaging, notwithstanding, androgen deprivation was shown to result in decreased 124I-CLR1404 mean and max tumor uptake, as shown in FIG. **15**F and FIG. **15**G,

respectively. No significant difference in % ID/gmean or % ID/gmax was detected post-treatment between animals receiving ADT and control vaccine versus animals receiving ADT and AR-targeted vaccine. However, as measured during necropsy, animals treated with degarelix and pTVG-AR had significantly smaller tumor volumes, as determined by genitourinary complex weight, compared to animals receiving degarelix and pTVG4 (FIG. **15**H).

Discussion

This Example demonstrates that androgen deprivation results in increased full-length AR expression in vitro and in vivo that persists over time, and that this increased AR expression is associated with these cells being better targets for AR-specific T cells. Furthermore, a DNA vaccine encoding the AR LBD enhanced immune responses that preferentially recognized and lysed castrate-resistant prostate cancer cells, and delayed the recurrence of castrate-resistant disease when combined with ADT. A vaccine targeting the AR may be preferred over other antigen-specific vaccines when specifically combined with ADT by targeting a major mechanism of resistance that drives castrate-resistant tumor growth.

In summary, this Example shows that increased AR expression in prostate cancer cells following ADT results in enhanced recognition and lysis by AR-specific T cells. The combination of ADT and AR-specific immunization in vivo enhanced anti-tumor T cell immunity, as well as delayed the recurrence of castrate-resistant tumors. These studies provide a rationale for combining ADT with AR-targeted immunization, an approach that is being evaluated in a Phase I clinical trial (NCT02411786).

Example 6

Immunization Elicits PD-L1 Expression in Tumors and PD-1/PD-L1 Blockage can Increase the Anti-Tumor Efficacy of DNA Vaccination

Using different tumor antigen systems we have found that DNA vaccination can elicit PD-L1 expression in tumors as a result of tumor-specific T cells elicited that secrete IFNγ. Specifically, we have reported that tumors expressing a model antigen had an increase in PD-L1 expression following immunization with a DNA vaccine encoding that antigen (Rekoske, B. T., H. A. Smith, B. M. Olson, B. B. Maricque, and D. G. McNeel. (2015). "PD-1 or PD-L1 Blockade Restores Antitumor Efficacy Following SSX2 Epitope-Modified DNA Vaccine Immunization." *Cancer Immunol Res.* 3:946-55). If the immunization was modified to elicit CD8+ T cells with higher PD-1 expression, this resulted in an inferior anti-tumor response. Combining vaccination with anti-PD-1 or anti-PD-L1 antibody treatment resulted in a greater anti-tumor response and eradication of tumors in some animals (Rekoske, B. T., H. A. Smith, B. M. Olson, B. B. Maricque, and D. G. McNeel. (2015). "PD-1 or PD-L1 Blockade Restores Antitumor Efficacy Following SSX2 Epitope-Modified DNA Vaccine Immunization." *Cancer Immunol Res.* 3:946-55). We have recently identified that this also occurs following human immunization using cryopreserved blood samples collected from patients with advanced prostate cancer treated with a DNA vaccine encoding prostatic acid phosphatase (PAP).

Using in vitro and trans vivo methods, we found that immune responses to PAP were detected and/or augmented when combined with PD-1 blockade (FIG. **9**A, B). Moreover, we detected increased expression of PD-L1 on circulating tumor cells following DNA vaccination, and we found that higher expression correlated with the development of persistent antigen-specific IFNγ-secreting T cell immune responses (FIG. **9**C). We observed similar results in blood samples from patients treated with sipuleucel-T, an FDA-approved vaccine for prostate cancer which targets the same PAP antigen (data not shown). Together, these data provide evidence to support combining anti-tumor vaccines with a PD-1 pathway inhibitor. Briefly, FIG. **9**A shows PBMC from patients previously immunized with a PAP-targeting vaccine were cultured in vitro for 72 hours with PAP in the presence of a PD-1-blocking antibody (or IgG control), and measured for IFNγ (left panel) or granzyme B (right panel) secretion by ELISA. FIG. **9**B shows PBMC obtained from patients after immunization were injected into the footpads of NOD/SCID mice with PAP protein and PD-1 blocking antibody (or IgG control), and 24 hours later, footpad swelling was measured. FIG. **9**C, PD-L1 expression was measured on circulating tumor cells from patients with persistent PAP-specific Th1-biased immune responses (R) vs. non-responders (NR) following immunization with a DNA vaccine targeting PAP. The ratio of PD-L1 MFI on post-treatment samples compared to pre-treatment samples is shown. In all panels, * indicates $p<0.05$ by Student's t-test.

More recent preliminary data also suggest that targeting the PD-1/PD-L1 pathway in combination with an AR-targeting vaccine is a rationale combination to circumvent this means of tumor-mediated immune suppression. In MycCaP tumor-bearing animals treated with AD and immunized with pTVG-AR (as in FIG. **8**), CD8+ T cells were found to have elevated PD-1 expression (FIG. **10**A). Additionally, it was observed in other models following the generation of antigen-specific immune responses following immunization, some recurrent tumors had elevated PD-L1 expression (FIG. **10**B). When AR-targeted immunization was combined along with a PD-1 blocking antibody, this treatment significantly delayed tumor growth compared to immunization with pTVG-AR alone (FIG. **10**C). Furthermore, combining ADT with AR-directed immunization and PD-1 blockade further delayed tumor growth (FIG. **10**D). Together, these finding suggest that PD-1 pathway inhibitors would be an effective means to target resistance to combined ADT and AR-directed immunization and could prevent (or significantly delay) the formation of the lethal, castrate-resistant form of prostate cancer.

Briefly, FVB mice were implanted subcutaneously with MycCaP tumor cells, treated the following day with degarelix, and the following day were immunized with pTVG4 (vector control) or pTVG-AR. At the time of tumor outgrowth, animals were analyzed for PD-1 expression on splenic CD8+ T-cells (FIG. **10**A) and PD-L1 expression on CD45− tumor cells (FIG. **10**B). For FIG. **10**C, FVB mice (n=5) were implanted with MycCaP tumors, and the following day immunized with pTVG-AR (repeated weekly), without castration, and each day following vaccination were treated with a PD-1-blocking antibody or control, and followed for tumor growth. For FIG. **10**D, MycCaP tumor-bearing FVB mice were treated with degarelix, pTVG-AR, and anti-PD-1 (n=5) or IgG control (n=9), and followed for tumor growth. In all panels, * indicates $p<0.05$ by Student's t-test.

Example 7

Clinical Trial Design Using AR-Targeted Vaccination in Combination with Androgen Deprivation and T-Cell Checkpoint Blockade

An open-label, randomized pilot clinical trial is to be performed, with a maximum of 50 patients with newly

diagnosed prostate cancer. The patients are randomly assigned to one of three treatment arms below.

Study Objectives:

The primary clinical objectives of the trial is safety and pathological complete response rate per study arm. Primary objectives include evaluating the safety of combination androgen deprivation, alone or in combination pTVG-AR DNA vaccine with or without pembrolizumab, in patients with newly diagnosed prostate cancer; and to determine the pathological complete response rate in patients with prostate cancer treated with combination androgen deprivation (LHRH agonist, abiraterone acetate, and apalutamide) or with pTVG-AR, with or without pembrolizumab, prior to radical prostatectomy.

Secondary objectives include determining one year PSA progression-free survival rates; determining whether treatment with pTVG-AR, with or without pembrolizumab, elicits persistent systemic AR-specific Th1-biased T-cell responses, and determining whether treatment with pTVG-AR, with or without pembrolizumab, elicits increased prostate tissue-infiltrating CD8+ T cells.

Subject Population:

Eligible subjects are patients with newly diagnosed prostate cancer who are planning to undergo radical prostatectomy as extirpative treatment. Subjects need not be HLA-A2 positive, however serological typing is performed to identify patients for epitope-specific T-cell analyses. In previous trials at our institution, we have found ~50% of patients were HLA-A2-expressing, consequently we anticipate ~50% of patients are available for these analysis.

Trial Design:

This will be a randomized, open-label, multi-institution pilot trial designed to evaluate the immunological and clinical effect of a DNA vaccine encoding AR with rhGM-CSF adjuvant given with or without pembrolizumab. Study arms will be defined as follows:

Arm 1: Leuprolide depot (or equivalent) 22.5 mg intramuscular injection day 1, day 85
- Abiraterone acetate 1000 mg p.o. daily, beginning day 1 until day prior to surgery
- Prednisone 5 mg p.o. daily, beginning day 1 until 1 week after surgery, then taper
- Apalutamide 240 mg p.o. daily, beginning day 1 until day prior to surgery

Arm 2: Leuprolide depot (or equivalent) 22.5 mg intramuscular injection day 1, day 85
- Abiraterone acetate 1000 mg p.o. daily, beginning day 1 until day prior to surgery
- Prednisone 5 mg p.o. daily, beginning day 1 until 1 week after surgery, then taper
- Apalutamide 240 mg p.o. daily, beginning day 1 until day prior to surgery
- pTVG-AR (100 μg) with rhGM-CSF (208 μg) administered intradermally (i.d.) biweekly 6 times, beginning day 1

Arm 3: Leuprolide depot (or equivalent) 22.5 mg intramuscular injection day 1, day 85
- Abiraterone acetate 1000 mg p.o. daily, beginning day 1 until day prior to surgery
- Prednisone 5 mg p.o. daily, beginning day 1 until 1 week after surgery, then taper
- Apalutamide 240 mg p.o. daily, beginning day 1 until day prior to surgery
- pTVG-AR (100 μg) with rhGM-CSF (208 μg) administered intradermally (i.d.) every 3 weeks 8 times, beginning day 1
- Pembrolizumab 2 mg/kg, administered intravenously over 30 minutes, every 3 weeks 8 times, beginning day 1, each dose following pTVG-AR vaccination

A total of 50 eligible patients (10 in Arm 1, 20 in Arm 2 and 20 in Arm 3) will be randomized. All subjects are followed for adverse events; if adverse events attributed to study treatment exceed the tolerability limit (≥33% grade>2 toxicity, or ≥10% grade>3 toxicity), further accrual would halt.

Measurement of Effect:

Patients eligible for this trial will not have metastatic disease at the time of enrollment.

Pathological evaluation: Prostate tissues obtained by biopsy pre-treatment and at the time of prostatectomy, will be reviewed and graded by a single pathologist (Dr. Jiaoti Huang, MD PhD or designee) as per standard clinical pathology review. The absence of identifiable prostate cancer at the time of prostatectomy will be used to define a pathological complete response.

Serum PSA evaluation: Serum PSA is expected to be undetectable following prostatectomy in the absence of residual/recurrent disease. Hence PSA progression will be defined as a detectable PSA (above the clinical lab's lower limit of detection) at any point after 3 months after the date of prostatectomy, and confirmed by a second reading at least 2 weeks later.

Safety:

All subjects are observed at every visit during the period of treatment for symptoms assessment. Laboratory analyses is performed at regular intervals for evidence of adverse events. These clinical laboratory studies include complete blood counts, creatinine, liver function tests, PSA, serum aldolase (for muscle-related toxicity assessment), and antinuclear antibodies. Adverse events are graded by the current version (4.0) of the NCI Common Terminology Criteria. The number and severity of toxicity incidents are analyzed descriptively in tabular format.

Immunological Monitoring:

Blood will be collected by either peripheral blood draw (up to 210 mL) or leukapheresis (50-100 mL) pre-immunization, after 3 months of treatment, at the time of prostatectomy, and at 3 months, 6 months, and 12 months after prostatectomy, for immunological monitoring. From the heparinized blood, peripheral blood mononuclear cells (PBMC) will be prepared by density centrifugation over Ficoll-Paque using standard techniques. PBMC will be used directly for analysis, and residual material cryopreserved in liquid nitrogen using 90% autologous serum collected at the time of blood draw, or 90% fetal calf serum, and 10% DMSO. Sera will be prepared from the red-top tubes and stored in aliquots at −80° C. for antibody analyses. IFNγ and granzyme B ELISPOT analysis, and ELISA tests for antigen-specific antibodies, will be the primary methods of analysis. The primary antigens tested will be AR (experimental), PSA (negative control), and tetanus toxoid (positive control). The primary immune analysis will be conducted at the 6-month post-surgery time point, and compared with the pre-treatment time point, and for patients to be evaluable for immune response (primary endpoint), blood (PBMC and serum) from this time point must be available for analysis. However, immune monitoring will be conducted at the other time points indicated in secondary analyses to evaluate kinetic measures of immunity, and evaluate whether durable immune responses of particular phenotypes are elicited and/or maintained. Assays may be conducted at the time of sample collection (fresh) and/or batched and performed at one time from multiple cryopreserved samples collected at

different time points. Other methods of effector and regulatory T-cell response to AR and other human tissue antigens may be used.

Quantitative Assessment of AR-Specific CD8+ T-Cell Effector Immunity AR-Specific IFNγ- and Granzyme B-Secreting T-Cell Precursor Frequency Quantification by ELISPOT:

ELISPOT will be used as the preferred methodology, as it permits analysis of low-frequency events (LOD~1:100,000 cells) and also permits simultaneous analysis of cryopreserved batched specimens [22]. IFNγ and granzyme B will be preferred analytes evaluated, as these are specifically associated with inflammatory/tissue-destructive (Th1-type, cytolytic) immune responses. Specifically, cryopreserved PBMC from subjects at the various time points will be thawed, rested, and then transferred to 96-well nitrocellulose microtiter (ELISPOT) plates previously coated with monoclonal capture antibodies specific for IFNγ or granzyme B. 10^5 cells per well will be cultured in the presence of media (RPMI 1640 supplemented with L-glutamine, penicillin/streptomycin, β-mercaptoethanol and 10% human AB serum) only (no antigen), 2 μg/ml AR protein, 2 μg/ml PSA protein (negative control), 2 μg/ml of peptide libraries specific for AR or control, 250 ng/ml tetanus toxoid, or 2.5 μg/ml PHA (positive mitogenic control) for 24-48 hours. Plates will then be washed with PBS containing 0.05% Tween-20 and incubated for 2.5 hours at room temperature with 50 μl/well PBS containing 5 μg/ml biotinylated detection antibodies for either IFNγ or granzyme B. After incubation, wells will be washed with PBS, and further incubated with 100 μl/well streptavidin-labeled alkaline phosphatase (BioRad, Hercules, Calif.) and then developed with 100 μl/well BCIP/NBT colorimetric substrate (Bio-Rad). The colorimetric reaction will be stopped by rinsing the plates under cool tap water, and wells will be allowed to dry completely before spots are enumerated with an ELISPOT automatic plate reader.

Reporting and Response Definition:

Results will be presented as previously reported as the mean (+/−standard deviation) number of spot-forming-units (sfu) per 10^6 cells (frequency), calculated by subtracting the mean number of spots obtained from the no antigen control wells from the mean number obtained in the experimental wells, normalized to 10^6 starting PBMC, from 8-well replicate assays [23]. Comparison of experimental wells with control, no antigen, wells will be performed using a two-sample t-test, with $p<0.05$ (two-sided) defined as a significant antigen-specific T-cell response. A significant antigen-specific response resulting from immunization will then be defined as a AR-specific response detectable at the 6-month post-surgery time point (or other post-treatment time point evaluated) that is significantly higher than to media only (as above), at least 3-fold higher than the mean baseline value, and with a frequency>10 per 10^6 PBMC.

Assessment of Antigen-Specific Antibody Immunity: Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Antibodies Responses to AR:

The presence of a coexisting humoral immune response to AR (or other antigens) will be evaluated by ELISA using an indirect method similar to that described previously [61]. Specifically, Immulon-4 ELISA plates (Dynex Technologies Inc.) will be coated with 2 μg/ml purified AR LBD protein (Research Diagnostics, Inc., or other antigens or commercial sources) in 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) overnight at 4° C. After blocking with PBS/1% BSA for 1 hour at room temperature, wells will be washed with PBS+0.05% Tween-20 (PBS-Tween) and then incubated for 1 hour with human sera diluted 1:25, 1:50, 1:100 and 1:200. After washing, plates will then be sequentially incubated with a peroxidase-conjugated anti-human IgG detection antibody (Amersham), followed by peroxidase enzyme TMB substrate (Kierkegaard and Perry Laboratories). The color reaction will be stopped with 1N H2504 and the optical density measured at 450 nm. Antibody titers for AR-specific IgG antibodies will be determined as previously described [61].

Reporting and Response Definition:

These are not strictly quantitative assays. IgG response will be reported graphically demonstrating sera dilution curves, and by titer—defined as the highest sera dilution at which IgG responses are detectable above the mean+3 standard deviations of the negative control. A positive IgG response resulting from immunization will be defined as an antigen-specific (anti-AR) IgG titer at least 4-fold higher than the baseline titer detectable at the 6-month post-treatment time point (or other post-treatment time point evaluated).

Histopathology Evaluation:

Tissue biopsies obtained pre-treatment and at the time of prostatectomy will be available from all subjects. The purpose of these studies is first to determine whether treatment with androgen deprivation alone (ARM 1) results in an increase in CD8+ T cells, and whether this is further increased by the use of an AR-targeting vaccine (ARM 2) and further yet with pembrolizumab (ARM 3). This will be determined by standard immunohistochemistry, and by quantitative flow cytometry (when feasible with fresh tissue). As an exploratory method, the frequency of CD8+ T cells, relative to other populations, will also be determined by mRNA analysis of frozen or paraffin-embedded tissue samples. As a further exploratory method, the frequency of specific CD8+ T cell populations will be determined by TCR sequencing using frozen tissue samples (Adaptive Biotechnologies, Seattle, Wash.).

Secondary goals of the histopathology evaluation will be to determine whether immunization with pTVG-AR affects PD-L1 expression in the tumor (likely by eliciting tumor antigen-specific T cells secreting IFNγ), and whether treatment increases expression of other T-cell regulatory ligands on T cells (PD-1, CTLA-4, TIM-3, BTLA, VISTA, LAG-3) or tumors (e.g. HVEM, phosphatidyl serine, PD-L2). Consequently, biopsy specimens obtained pre-treatment and after 12 weeks will be stained with antibodies specific for CD3, CD4, CD8, FoxP3, PD-1, CTLA-4, TIM3, BTLA, VISTA, LAG-3, PD-L1, PD-L2, phosphatidyl serine, HVEM and potentially other markers. Staining and quantification will be reviewed by a pathologist blinded to the treatment groups to determine CD8+ T cells per field, CD4+FoxP3+ (Treg):CD8+ T cell ratio, PD-L1 expression, and whether these or the expression of CD8+ T cells expressing one regulatory receptors (or tumor cells expressing one or more regulatory ligands) change from pre-treatment to the prostatectomy time point.

At the University of Wisconsin, samples will be transported to the UWCCC TRIP (Translational Research Initiatives in Pathology) lab for formalin fixation, paraffin embedding, sectioning, H&E staining, and ultimately for IHC analysis as described above.

Example 8

Similar to Example 7, a suitable dosage regimen for the combination treatment using vaccine, ADT and a PD-1 pathway blockage is diagramed in FIG. **16**. Suitable testing

parameters are shown under the timeline and will be performed as discussed in Example 7.

Each publication, patent, and patent publication cited in this disclosure is incorporated in reference herein in its entirety. The present invention is not intended to be limited to the foregoing examples, but encompasses all such modifications and variations as come within the scope of the appended claims.

REFERENCES FROM EXAMPLE 5, each is incorporated by reference in its entirety

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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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Val Ile Gln Asn Pro Gly Pro Arg His Pro Glu Ala Ala Ser Ala Ala
        35                  40                  45

Pro Pro Gly Ala Ser Leu Leu Leu Leu Gln Gln Gln Gln Gln Gln Gln
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Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
65                  70                  75                  80

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Pro Gln Ala His Arg Arg Gly Pro Thr Gly Tyr Leu Val Leu Asp Glu
            100                 105                 110

Glu Gln Gln Pro Ser Gln Pro Gln Ser Ala Leu Glu Cys His Pro Glu
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Arg Gly Cys Val Pro Glu Pro Gly Ala Ala Val Ala Ala Ser Lys Gly
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Pro Ser Thr Leu Ser Leu Leu Gly Pro Thr Phe Pro Gly Leu Ser Ser
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Cys Ser Ala Asp Leu Lys Asp Ile Leu Ser Glu Ala Ser Thr Met Gln
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Leu Leu Gln Gln Gln Gln Gln Glu Ala Val Ser Glu Gly Ser Ser Ser
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Gly Arg Ala Arg Glu Ala Ser Gly Ala Pro Thr Ser Ser Lys Asp Asn
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Tyr Leu Gly Gly Thr Ser Thr Ile Ser Asp Asn Ala Lys Glu Leu Cys
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Lys Ala Val Ser Val Ser Met Gly Leu Gly Val Glu Ala Leu Glu His
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Leu Ser Pro Gly Glu Gln Leu Arg Gly Asp Cys Met Tyr Ala Pro Leu
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Ser Ser Trp His Thr Leu Phe Thr Ala Glu Glu Gly Gln Leu Tyr Gly
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Tyr Gly Tyr Thr Arg Pro Pro Gln Gly Leu Ala Gly Gln Glu Ser Asp
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Phe Thr Ala Pro Asp Val Trp Tyr Pro Gly Gly Met Val Ser Arg Val
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Pro Tyr Pro Ser Pro Thr Cys Val Lys Ser Glu Met Gly Pro Trp Met
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Asp Ser Tyr Ser Gly Pro Tyr Gly Asp Met Arg Leu Glu Thr Ala Arg
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Asp His Val Leu Pro Ile Asp Tyr Tyr Phe Pro Pro Gln Lys Thr Cys
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Leu Ile Cys Gly Asp Glu Ala Ser Gly Cys His Tyr Gly Ala Leu Thr
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Cys Gly Ser Cys Lys Val Phe Phe Lys Arg Ala Ala Glu Gly Lys Gln
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Lys Tyr Leu Cys Ala Ser Arg Asn Asp Cys Thr Ile Asp Lys Phe Arg
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Arg Lys Asn Cys Pro Ser Cys Arg Leu Arg Lys Cys Tyr Glu Ala Gly
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Gln Glu Glu Gly Glu Ala Ser Ser Thr Thr Ser Pro Thr Glu Glu Thr
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Thr Gln Lys Leu Thr Val Ser His Ile Glu Gly Tyr Glu Cys Gln Pro
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Ile Phe Leu Asn Val Leu Glu Ala Ile Glu Pro Gly Val Val Cys Ala
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Gly His Asp Asn Asn Gln Pro Asp Ser Phe Ala Ala Leu Leu Ser Ser
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Lys Ala Leu Pro Gly Phe Arg Asn Leu His Val Asp Asp Gln Met Ala
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Leu Val Phe Asn Glu Tyr Arg Met His Lys Ser Arg Met Tyr Ser Gln
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Cys Val Arg Met Arg His Leu Ser Gln Glu Phe Gly Trp Leu Gln Ile
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Thr Pro Gln Glu Phe Leu Cys Met Lys Ala Leu Leu Leu Phe Ser Ile
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Ile Pro Val Asp Gly Leu Lys Asn Gln Lys Phe Phe Asp Glu Leu Arg
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Met Asn Tyr Ile Lys Glu Leu Asp Arg Ile Ile Ala Cys Lys Arg Lys
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Asn Pro Thr Ser Cys Ser Arg Arg Phe Tyr Gln Leu Thr Lys Leu Leu
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Asp Ser Val Gln Pro Ile Ala Arg Glu Leu His Gln Phe Thr Phe Asp
865                 870                 875                 880

Leu Leu Ile Lys Ser His Met Val Ser Val Asp Phe Pro Glu Met Met
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tttcattgaa aatgcctagg aattgcaata gggagaaatc cagatgtggg gccaggtgcc 8820

cacccaaagg actggccagc agcctcttca tgggatctga ggcattggga aaaggaaggc 8880

tatttccttg gttttcacca tccttgttag agaagggcag ttgcctggtc ttgggaacct 8940

ggagcaaacg ctccttctgt cacatcaatt ctttcccctg caattgaggt gctcttgcta 9000

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gctaatgtat ttttctctgt taaaaatttg ttagtgtgtt agaagtcata tctctgtagg 9120

tacagatcct ttgctaccca tgagtagagg gatttttttt cttcaattaa gagtttgacc 9180

ctggggtctg ttgcccagag cccatccaga aaaaaaaatc cacatttgtc acaatttttc 9240

tgaaatttca gtcaaggtaa cagatcgctg ggagttctct ttacccccc aaaaaagcag 9300

ataattgaat ttagcaggtg gtgttttaga gcaaaaaaca aaacagcctt tgacccagct 9360

ttaatatgac ccaatttaat ctggccagga agcaggtaat gtgtattaat tggcttccaa 9420

tcctggttga gtgtagcaag gttctacttt gtttcctagt tccttttgtt acatggcctt 9480

tcacagaaag gattgactgg gtttgcagta tatcttatgg ccttagcacc tattgctaac 9540

tgtcctgaag ggaattgcct atggggttgt cctataagcc acttctatca ttaaaagcag 9600

ccaccaatgg aatctcccag gtttgaaaaa aaaaaaaaaa aacagatggt cctttaccat 9660

tcattgacac acatccctgc tttcctgtag acagattgac tggacattga ttagggaata 9720

catggcaaat gacatgctta cactaccctg gagattaatt tggcagtagg agggaataga 9780

caatgtaacc aagaatgtaa tgtaattctt atagagataa gaattaaatc tggatgtgga 9840

gagagcaaag agagaaagca ttcaatttttt ttttcaaaag aaaccaattt attttgcttg 9900

aaacttcttt cgctggggct tcagttctca cagcggctct tggtctccac tgggcagcag 9960

gaccagcccc aagcgctagt gttctgttct ctttttgtaa tcttggaatc ttttgttgct 10020

ctaaatacaa ttaaaaatgg cagaaacttg tttgttggaa tac 10063

<210> SEQ ID NO 4
<211> LENGTH: 899
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 4

Met Glu Val Gln Leu Gly Leu Gly Arg Val Tyr Pro Arg Pro Pro Ser
1 5 10 15

Lys Thr Tyr Arg Gly Ala Phe Gln Asn Leu Phe Gln Ser Val Arg Glu
20 25 30

-continued

```
Ala Ile Gln Asn Pro Gly Pro Arg His Pro Glu Ala Ala Asn Ile Ala
        35                  40                  45

Pro Pro Gly Ala Cys Leu Gln Gln Arg Gln Glu Thr Ser Pro Arg Arg
    50                  55                  60

Arg Arg Arg Gln Gln His Thr Glu Asp Gly Ser Pro Gln Ala His Ile
65                  70                  75                  80

Arg Gly Pro Thr Gly Tyr Leu Ala Leu Glu Glu Glu Gln Gln Pro Ser
                85                  90                  95

Gln Gln Gln Ala Ala Ser Glu Gly His Pro Glu Ser Ser Cys Leu Pro
            100                 105                 110

Glu Pro Gly Ala Ala Thr Ala Pro Gly Lys Gly Leu Pro Gln Gln Pro
        115                 120                 125

Pro Ala Pro Pro Asp Gln Asp Asp Ser Ala Ala Pro Ser Thr Leu Ser
    130                 135                 140

Leu Leu Gly Pro Thr Phe Pro Gly Leu Ser Ser Cys Ser Ala Asp Ile
145                 150                 155                 160

Lys Asp Ile Leu Asn Glu Ala Gly Thr Met Gln Leu Leu Gln Gln Gln
                165                 170                 175

Gln Gln Gln Gln Gln His Gln Gln Gln His Gln Gln His Gln Gln Gln
            180                 185                 190

Gln Glu Val Ile Ser Glu Gly Ser Ser Ala Arg Ala Arg Glu Ala Thr
        195                 200                 205

Gly Ala Pro Ser Ser Ser Lys Asp Ser Tyr Leu Gly Gly Asn Ser Thr
    210                 215                 220

Ile Ser Asp Ser Ala Lys Glu Leu Cys Lys Ala Val Ser Val Ser Met
225                 230                 235                 240

Gly Leu Gly Val Glu Ala Leu Glu His Leu Ser Pro Gly Glu Gln Leu
                245                 250                 255

Arg Gly Asp Cys Met Tyr Ala Ser Leu Leu Gly Gly Pro Pro Ala Val
            260                 265                 270

Arg Pro Thr Pro Cys Ala Pro Leu Pro Glu Cys Lys Gly Leu Pro Leu
        275                 280                 285

Asp Glu Gly Pro Gly Lys Ser Thr Glu Glu Thr Ala Glu Tyr Ser Ser
    290                 295                 300

Phe Lys Gly Gly Tyr Ala Lys Gly Leu Glu Gly Glu Ser Leu Gly Cys
305                 310                 315                 320

Ser Gly Ser Ser Glu Ala Gly Ser Ser Gly Thr Leu Glu Ile Pro Ser
                325                 330                 335

Ser Leu Ser Leu Tyr Lys Ser Gly Ala Leu Asp Glu Ala Ala Ala Tyr
            340                 345                 350

Gln Asn Arg Asp Tyr Tyr Asn Phe Pro Leu Ala Leu Ser Gly Pro Pro
        355                 360                 365

His Pro Pro Pro Pro Thr His Pro His Ala Arg Ile Lys Leu Glu Asn
    370                 375                 380

Pro Leu Asp Tyr Gly Ser Ala Trp Ala Ala Ala Ala Ala Gln Cys Arg
385                 390                 395                 400

Tyr Gly Asp Leu Gly Ser Leu His Gly Gly Ser Val Ala Gly Pro Ser
                405                 410                 415

Thr Gly Ser Pro Pro Ala Thr Thr Ser Ser Ser Trp His Thr Leu Phe
            420                 425                 430

Thr Ala Glu Glu Gly Gln Leu Tyr Gly Pro Gly Gly Gly Gly Gly Ser
        435                 440                 445
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Ser Ser Pro Ser Asp Ala Gly Pro Val Ala Pro Tyr Gly Tyr Thr Arg
    450                 455                 460

Pro Pro Gln Gly Leu Thr Ser Gln Glu Ser Asp Tyr Ser Ala Ser Glu
465                 470                 475                 480

Val Trp Tyr Pro Gly Gly Val Val Asn Arg Val Pro Tyr Pro Ser Pro
                485                 490                 495

Asn Cys Val Lys Ser Glu Met Gly Pro Trp Met Glu Asn Tyr Ser Gly
            500                 505                 510

Pro Tyr Gly Asp Met Arg Leu Asp Ser Thr Arg Asp His Val Leu Pro
        515                 520                 525

Ile Asp Tyr Tyr Phe Pro Pro Gln Lys Thr Cys Leu Ile Cys Gly Asp
    530                 535                 540

Glu Ala Ser Gly Cys His Tyr Gly Ala Leu Thr Cys Gly Ser Cys Lys
545                 550                 555                 560

Val Phe Phe Lys Arg Ala Ala Glu Gly Lys Gln Lys Tyr Leu Cys Ala
                565                 570                 575

Ser Arg Asn Asp Cys Thr Ile Asp Lys Phe Arg Arg Lys Asn Cys Pro
            580                 585                 590

Ser Cys Arg Leu Arg Lys Cys Tyr Glu Ala Gly Met Thr Leu Gly Ala
        595                 600                 605

Arg Lys Leu Lys Lys Leu Gly Asn Leu Lys Leu Gln Glu Glu Gly Glu
    610                 615                 620

Asn Ser Asn Ala Gly Ser Pro Thr Glu Asp Pro Ser Gln Lys Met Thr
625                 630                 635                 640

Val Ser His Ile Glu Gly Tyr Glu Cys Gln Pro Ile Phe Leu Asn Val
                645                 650                 655

Leu Glu Ala Ile Glu Pro Gly Val Val Cys Ala Gly His Asp Asn Asn
            660                 665                 670

Gln Pro Asp Ser Phe Ala Ala Leu Leu Ser Ser Leu Asn Glu Leu Gly
        675                 680                 685

Glu Arg Gln Leu Val His Val Val Lys Trp Ala Lys Ala Leu Pro Gly
    690                 695                 700

Phe Arg Asn Leu His Val Asp Asp Gln Met Ala Val Ile Gln Tyr Ser
705                 710                 715                 720

Trp Met Gly Leu Met Val Phe Ala Met Gly Trp Arg Ser Phe Thr Asn
                725                 730                 735

Val Asn Ser Arg Met Leu Tyr Phe Ala Pro Asp Leu Val Phe Asn Glu
            740                 745                 750

Tyr Arg Met His Lys Ser Arg Met Tyr Ser Gln Cys Val Arg Met Arg
        755                 760                 765

His Leu Ser Gln Glu Phe Gly Trp Leu Gln Ile Thr Pro Gln Glu Phe
    770                 775                 780

Leu Cys Met Lys Ala Leu Leu Leu Phe Ser Ile Ile Pro Val Asp Gly
785                 790                 795                 800

Leu Lys Asn Gln Lys Phe Phe Asp Glu Leu Arg Met Asn Tyr Ile Lys
                805                 810                 815

Glu Leu Asp Arg Ile Ile Ala Cys Lys Arg Lys Asn Pro Thr Ser Cys
            820                 825                 830

Ser Arg Arg Phe Tyr Gln Leu Thr Lys Leu Leu Asp Ser Val Gln Pro
        835                 840                 845

Ile Ala Arg Glu Leu His Gln Phe Thr Phe Asp Leu Leu Ile Lys Ser
    850                 855                 860

His Met Val Ser Val Asp Phe Pro Glu Met Met Ala Glu Ile Ile Ser
```

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865 870 875 880

Val Gln Val Pro Lys Ile Leu Ser Gly Lys Val Lys Pro Ile Tyr Phe
885 890 895

His Thr Gln

<210> SEQ ID NO 5
<211> LENGTH: 4137
<212> TYPE: DNA
<213> ORGANISM: Rattus norvegicus

<400> SEQUENCE: 5

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agggcatcag aggggaaaag actgagttag ccactccagt gccatacaga agcttaaggg 120

acataccacg ccagccccag cccagcgaca gccaacgcct gttgcagagc ggcggcttcg 180

aagccgccgc ccagaagctg ccctttcctc ttcggtgaag tttctaaaag ctgcgggaga 240

ctcggaggaa gcgaagaaag tgtccggtag gactacgact gcctttgtcc tcctccctcc 300

taccctacc cctcctgggt cccctctccc tgagcggact aggcaggctt cctggccagc 360

cctctcccct acaccaccag ctctgccagc cagtttgcac agaggtaact ccctttggct 420

gaaagcagac gagcttgttg cccattggaa gggaggcttt tgggagccca gagactgagg 480

agcaacagca cgctggagag tccctgattc caggttctcc cccctgcacc tcctactgcc 540

cgcccctcac cctgtgtgtg cagctagaat tgaaaagatg aaaagacagt tggggcttca 600

gtagtcgaaa gcaaaacaaa agcaaaaaga aaacaaaaag aaaatagccc agttcttatt 660

tgcacctgct tcagtggaca ttgactttgg aaggcagaga attttccttc cccccagtca 720

agctttgagc atcttttaat ctgttcttca agtatttagg gacaaactgt gaaactagca 780

gggcagatcc tgtctagcgc gtgccttcct ttacaggaga ctttgaggct atctgggcgc 840

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agatactaca tcatcagtca gtagaactct tcagagcaag agacgaggag gcaggataag 960

ggaattcggt ggaagctaga gacaagctaa aggatggagg tgcagttagg gctgggaagg 1020

gtctacccac ggcccccgtc caagacctat cgaggagcgt tccagaatct gttccagagc 1080

gtgcgcgaag cgatccagaa cccgggcccc aggcaccctg aggccgctag catagcacct 1140

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caccctgagg atggctctcc tcaagcccac atcagaggca ccacaggcta cctggccctg 1260

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gctcctccag atcaggatga ctcagctgcc ccatccacgt tgtccctact gggccccact 1440

ttcccaggct taagcagctg ctccgcagac attaaagaca tcctgagcga ggccggcacc 1500

atgcaacttc ttcagcagca gcagcaacag caacagcagc agcagcagca gcagcagcag 1560

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ggaggtccac ccgccgtgcg tcccactcct tgtgcgcctc tggccgaatg caaaggtctt 1860

tccctggacg aaggcccggg caaaggcact gaagagactg ctgagtattc ctctttcaag 1920

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ggaggttacg ccaaagggtt ggaaggtgag agtctgggct gctctggcag cagtgaagca 1980

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gacgaggcag cagcatacca gaatcgcgac tactacaact ttccgctcgc tctgtccggg 2100

ccgccgcacc ccccgccccc tacccatcca cacgcccgca tcaagctgga gaacccgtcg 2160

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ggcagcagta gcccaagcga tgctgggcct gtagcccccct atggctacac tcggcccccct 2400

caggggctgg caagccagga gggtgacttc tctgcctctg aagtgtggta tcctggtgga 2460

gttgtgaaca gagtccccta tcccagtccc agttgtgtta aaagtgaaat gggaccttgg 2520

atggagaact actccggacc ttatggggac atgcgtttgg acagtaccag ggaccacgtt 2580

ttacccatcg actattactt cccaccccag aagacctgcc tgatctgtgg agatgaagct 2640

tctggttgtc actacggagc tctcacttgt ggcagctgca aggtcttctt caaaagagct 2700

gcggaaggga aacagaagta tctatgtgcc agcagaaatg attgcaccat tgataaattt 2760

cggaggaaaa attgtccatc gtgtcgtctc cggaaatgtt atgaagcagg gatgactctg 2820

ggagctcgta agctgaagaa acttggaaat ctcaaactac aggaagaagg agaaaactcc 2880

agtgctggta gccccactga ggacccatcc cagaagatga ctgtatcaca cattgaaggc 2940

tatgaatgtc aacctatctt tcttaatgtc ctggaagcca ttgagccagg agtggtgtgt 3000

gccggacatg acaacaacca gcctgattcc tttgctgcct tgttatctag tctcaacgag 3060

cttggcgaga gacagcttgt acatgtggtc aagtgggcca aggccttgcc tggcttccgc 3120

aacttgcatg tggatgacca gatggcagtc attcagtatt cctggatggg actgatggta 3180

tttgccatgg gttggcggtc cttcactaat gtcaactcta ggatgctcta ctttgcacct 3240

gacctggttt tcaatgagta tcgcatgcac aagtctcgaa tgtacagcca gtgcgtgagg 3300

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aaaaatccca catcctgctc aaggcgcttc taccagctca ccaagctcct ggattctgtg 3540

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<210> SEQ ID NO 6
<211> LENGTH: 902
<212> TYPE: PRT
<213> ORGANISM: Rattus norvegicus

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<400> SEQUENCE: 6

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Met Glu Val Gln Leu Gly Leu Gly Arg Val Tyr Pro Arg Pro Pro Ser
1               5                  10                  15

Lys Thr Tyr Arg Gly Ala Phe Gln Asn Leu Phe Gln Ser Val Arg Glu
            20                  25                  30

Ala Ile Gln Asn Pro Gly Pro Arg His Pro Glu Ala Ala Ser Ile Ala
        35                  40                  45

Pro Pro Gly Ala Cys Leu Gln Gln Arg Gln Glu Thr Ser Pro Arg Arg
    50                  55                  60

Arg Arg Arg Gln Gln His Pro Glu Asp Gly Ser Pro Gln Ala His Ile
65                  70                  75                  80

Arg Gly Thr Thr Gly Tyr Leu Ala Leu Glu Glu Glu Gln Gln Pro Ser
                85                  90                  95

Gln Gln Gln Ser Ala Ser Glu Gly His Pro Glu Ser Gly Cys Leu Pro
            100                 105                 110

Glu Pro Gly Ala Ala Thr Ala Pro Gly Lys Gly Leu Pro Gln Gln Pro
        115                 120                 125

Pro Ala Pro Pro Asp Gln Asp Asp Ser Ala Ala Pro Ser Thr Leu Ser
    130                 135                 140

Leu Leu Gly Pro Thr Phe Pro Gly Leu Ser Ser Cys Ser Ala Asp Ile
145                 150                 155                 160

Lys Asp Ile Leu Ser Glu Ala Gly Thr Met Gln Leu Leu Gln Gln Gln
                165                 170                 175

Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
            180                 185                 190

Gln Gln Gln Glu Val Ile Ser Glu Gly Ser Ser Ser Val Arg Ala Arg
        195                 200                 205

Glu Ala Thr Gly Ala Pro Ser Ser Ser Lys Asp Ser Tyr Leu Gly Gly
    210                 215                 220

Asn Ser Thr Ile Ser Asp Ser Ala Lys Glu Leu Cys Lys Ala Val Ser
225                 230                 235                 240

Val Ser Met Gly Leu Gly Val Glu Ala Leu Glu His Leu Ser Pro Gly
                245                 250                 255

Glu Gln Leu Arg Gly Asp Cys Met Tyr Ala Ser Leu Leu Gly Gly Pro
            260                 265                 270

Pro Ala Val Arg Pro Thr Pro Cys Ala Pro Leu Ala Glu Cys Lys Gly
        275                 280                 285

Leu Ser Leu Asp Glu Gly Pro Gly Lys Gly Thr Glu Glu Thr Ala Glu
    290                 295                 300

Tyr Ser Ser Phe Lys Gly Gly Tyr Ala Lys Gly Leu Glu Gly Glu Ser
305                 310                 315                 320

Leu Gly Cys Ser Gly Ser Ser Glu Ala Gly Ser Ser Gly Thr Leu Glu
                325                 330                 335

Ile Pro Ser Ser Leu Ser Leu Tyr Lys Ser Gly Ala Val Asp Glu Ala
            340                 345                 350

Ala Ala Tyr Gln Asn Arg Asp Tyr Tyr Asn Phe Pro Leu Ala Leu Ser
        355                 360                 365

Gly Pro Pro His Pro Pro Pro Pro Thr His Pro His Ala Arg Ile Lys
    370                 375                 380

Leu Glu Asn Pro Ser Asp Tyr Gly Ser Ala Trp Ala Ala Ala Ala Ala
385                 390                 395                 400

Gln Cys Arg Tyr Gly Asp Leu Ala Ser Leu His Gly Gly Ser Val Ala
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                405                 410                 415

Gly Pro Ser Thr Gly Ser Pro Pro Ala Thr Ala Ser Ser Ser Trp His
            420                 425                 430

Thr Leu Phe Thr Ala Glu Glu Gly Gln Leu Tyr Gly Pro Gly Gly Gly
        435                 440                 445

Gly Gly Ser Ser Ser Pro Ser Asp Ala Gly Pro Val Ala Pro Tyr Gly
    450                 455                 460

Tyr Thr Arg Pro Pro Gln Gly Leu Ala Ser Gln Glu Gly Asp Phe Ser
465                 470                 475                 480

Ala Ser Glu Val Trp Tyr Pro Gly Gly Val Val Asn Arg Val Pro Tyr
                485                 490                 495

Pro Ser Pro Ser Cys Val Lys Ser Glu Met Gly Pro Trp Met Glu Asn
            500                 505                 510

Tyr Ser Gly Pro Tyr Gly Asp Met Arg Leu Asp Ser Thr Arg Asp His
        515                 520                 525

Val Leu Pro Ile Asp Tyr Tyr Phe Pro Pro Gln Lys Thr Cys Leu Ile
    530                 535                 540

Cys Gly Asp Glu Ala Ser Gly Cys His Tyr Gly Ala Leu Thr Cys Gly
545                 550                 555                 560

Ser Cys Lys Val Phe Phe Lys Arg Ala Ala Glu Gly Lys Gln Lys Tyr
                565                 570                 575

Leu Cys Ala Ser Arg Asn Asp Cys Thr Ile Asp Lys Phe Arg Arg Lys
            580                 585                 590

Asn Cys Pro Ser Cys Arg Leu Arg Lys Cys Tyr Glu Ala Gly Met Thr
        595                 600                 605

Leu Gly Ala Arg Lys Leu Lys Lys Leu Gly Asn Leu Lys Leu Gln Glu
    610                 615                 620

Glu Gly Glu Asn Ser Ser Ala Gly Ser Pro Thr Glu Asp Pro Ser Gln
625                 630                 635                 640

Lys Met Thr Val Ser His Ile Glu Gly Tyr Glu Cys Gln Pro Ile Phe
                645                 650                 655

Leu Asn Val Leu Glu Ala Ile Glu Pro Gly Val Val Cys Ala Gly His
            660                 665                 670

Asp Asn Asn Gln Pro Asp Ser Phe Ala Ala Leu Leu Ser Ser Leu Asn
        675                 680                 685

Glu Leu Gly Glu Arg Gln Leu Val His Val Val Lys Trp Ala Lys Ala
    690                 695                 700

Leu Pro Gly Phe Arg Asn Leu His Val Asp Asp Gln Met Ala Val Ile
705                 710                 715                 720

Gln Tyr Ser Trp Met Gly Leu Met Val Phe Ala Met Gly Trp Arg Ser
                725                 730                 735

Phe Thr Asn Val Asn Ser Arg Met Leu Tyr Phe Ala Pro Asp Leu Val
            740                 745                 750

Phe Asn Glu Tyr Arg Met His Lys Ser Arg Met Tyr Ser Gln Cys Val
        755                 760                 765

Arg Met Arg His Leu Ser Gln Glu Phe Gly Trp Leu Gln Ile Thr Pro
    770                 775                 780

Gln Glu Phe Leu Cys Met Lys Ala Leu Leu Leu Phe Ser Ile Ile Pro
785                 790                 795                 800

Val Asp Gly Leu Lys Asn Gln Lys Phe Phe Asp Glu Leu Arg Met Asn
                805                 810                 815

Tyr Ile Lys Glu Leu Asp Arg Ile Ile Ala Cys Lys Arg Lys Asn Pro
            820                 825                 830
```

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```
Thr Ser Cys Ser Arg Arg Phe Tyr Gln Leu Thr Lys Leu Leu Asp Ser
        835                 840                 845

Val Gln Pro Ile Ala Arg Glu Leu His Gln Phe Thr Phe Asp Leu Leu
    850                 855                 860

Ile Lys Ser His Met Val Ser Val Asp Phe Pro Glu Met Met Ala Glu
865                 870                 875                 880

Ile Ile Ser Val Gln Val Pro Lys Ile Leu Ser Gly Lys Val Lys Pro
                885                 890                 895

Ile Tyr Phe His Thr Gln
            900


<210> SEQ ID NO 7
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 7

acatcaagga actcgatcgt atcattgc                                        28


<210> SEQ ID NO 8
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 8

ttgggcactt gcacagagat                                                 20


<210> SEQ ID NO 9
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Leu Leu Leu Phe Ser Ile Ile Pro Val
1               5


<210> SEQ ID NO 10
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

Arg Met Leu Tyr Phe Ala Pro Asp Leu Val
1               5                   10


<210> SEQ ID NO 11
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Phe Leu Cys Met Lys Ala Leu Leu Leu
1               5


<210> SEQ ID NO 12
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
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<400> SEQUENCE: 12

Gln Leu Thr Lys Leu Leu Asp Ser Val
1 5

<210> SEQ ID NO 13
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 13

ccatcttgtc gtcttcggaa atgttatgaa gc 32

<210> SEQ ID NO 14
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 14

tttgaatgag gcaagtcagc ctttct 26

<210> SEQ ID NO 15
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 15

tcatgaagtg tgacgttgac atccgt 26

<210> SEQ ID NO 16
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 16

cttagaagca tttgcggtgc acgatg 26

<210> SEQ ID NO 17
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 17

ggaccatgtt ttacccatcg 20

<210> SEQ ID NO 18
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 18

atctggtcat ccacatgcaa 20

<210> SEQ ID NO 19

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<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 19

ttgttgtggc agcagagttc 20

<210> SEQ ID NO 20
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 20

aagtggggaa ccacagcat 19

We claim:

1. A method of eliciting an anti-tumor response in a subject having prostate cancer comprising:

a. administering to the subject androgen deprivation therapy (ADT) comprising degarelix; and

b. administering to the subject a recombinant DNA vaccine comprising a polynucleotide operably linked to a transcriptional regulatory element wherein the polynucleotide encodes an androgen receptor or a fragment of the androgen receptor selected from the group consisting of (i) a mammalian androgen receptor, (ii) a fragment of the androgen receptor that comprises a ligand-binding domain, (iii) a fragment of the ligand-binding domain defined by SEQ ID NO:9, (iv) a fragment of the ligand-binding domain defined by SEQ ID NO:10, (v) a fragment of the ligand-binding domain defined by SEQ ID NO:11, and (vi) a fragment of the ligand-binding domain defined by SEQ ID NO:12, whereby the DNA vaccine elicits an immune response in the subject against the androgen receptor,

wherein the recombinant DNA vaccine is administered in an amount effective to elicit an increased anti-tumor response to the prostate cancer, wherein the combination treatment of ADT and the recombinant DNA vaccine inhibits, delays or reduces the growth of the prostate cancer.

2. The method of claim **1**, wherein the DNA vaccine comprises the polynucleotide that encodes a fragment of the androgen receptor ligand binding fragment.

3. The method of claim **1**, wherein the DNA vaccine is administered every two weeks to every three months.

4. The method of claim **1**, wherein the DNA vaccine is administered biweekly for about 6 to about 10 weeks and subsequently administered quarterly for at least a year.

5. The method of claim **1**, wherein the vaccine is administered in a dosage of about 10 mcg to 1 mg.

6. The method of claim **1**, wherein the ADT and recombinant vaccine are administered concurrently.

7. The method of claim **1**, wherein the ADT is administered before administering the recombinant vaccine.

8. The method of claim **1**, wherein the ADT is administered after administering at least one dosage of the recombinant vaccine.

9. The method of claim **1**, wherein the DNA vaccine is administered intradermally, intramuscularly, subcutaneously, intravenously or intra-arterially, with or without electroporation.

10. The method of claim **1**, wherein the DNA vaccine comprises pTVG-ARLBD.

11. The method of claim **1**, wherein the method further comprises administering to the subject an effective amount of a PD-pathway inhibitor.

12. The method of claim **1**, wherein the prostate cancer is castration resistant prostate cancer (mCRPC).

13. The method of claim **1**, wherein the prostate cancer is recurrent or metastatic prostate cancer.

14. A method of eliciting an anti-tumor response in a subject having prostate cancer comprising:

a. administering to the subject androgen deprivation therapy (ADT) comprising degarelix;

b. administering to the subject a recombinant DNA vaccine comprising a polynucleotide operably linked to a transcriptional regulatory element wherein the polynucleotide encodes an androgen receptor or a fragment of the androgen receptor selected from the group consisting of (i) a mammalian androgen receptor, (ii) a fragment of the androgen receptor that comprises a ligand-binding domain, (iii) a fragment of the ligand-binding domain defined by SEQ ID NO:9, (iv) a fragment of the ligand-binding domain defined by SEQ ID NO:10, (v) a fragment of the ligand-binding domain defined by SEQ ID NO:11, and (vi) a fragment of the ligand-binding domain defined by SEQ ID NO:12, whereby the DNA vaccine elicits an immune response in the subject against the androgen receptor, and

c. administering to the subject an effective amount of a PD-pathway inhibitor,

wherein the recombinant DNA vaccine and PD-pathway inhibitor are administered in an amount effective to elicit an increased anti-tumor response to the prostate cancer, wherein the combination treatment of ADT, the recombinant DNA vaccine and PD-pathway inhibitor inhibits, delays or reduces the growth of the prostate cancer.

15. The method of claim **14**, wherein the DNA vaccine comprises the polynucleotide that encodes a fragment of the androgen receptor ligand binding fragment.

16. The method of claim **14**, wherein the DNA vaccine is administered every two weeks to every three months.

17. The method of claim **14**, wherein the DNA vaccine is administered biweekly for about 6 to about 10 weeks and subsequently administered quarterly for at least a year.

18. The method of claim **14**, wherein the vaccine is administered in a dosage of about 10 mcg to 1 mg.

19. The method of claim **14**, wherein the DNA vaccine comprises pTVG-ARLBD.

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