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Immunization with a poly (lactide co-glycolide) encapsulated plasmid DNA expressing antigenic regions of HPV 16 and 18 results in an increase in the precursor frequency of T cells that respond to epitopes from HPV 16, 18, 6 and 11

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ABSTRACT

A phase II trial was conducted in subjects with human papillomavirus (HPV) associated high-grade cervical dysplasia testing the safety and efficacy of a microparticle encapsulated pDNA vaccine. Amolimogene expresses T cell epitopes from E6 and E7 proteins of HPV types 16 and 18. An analysis was performed on a subset of HLA-A2+ subjects to test whether CD8+ T cells specific to HPV 16, 18, 6 and 11 were increased in response to amolimogene immunization. Of the 21 subjects receiving amolimogene, 11 had elevated CD8+ T cell responses to HPV 16 and/or 18 peptides and seven of these also had increases to corresponding HPV 6 and/or 11 peptides. In addition, T cells primed and expanded *in vitro* with an HPV 18 peptide demonstrated cross-reactivity to the corresponding HPV 11 peptide. These data demonstrate that treatment with amolimogene elicits T cell responses to HPV 16, 18, 6 and 11.

1. Introduction

Human papilloma virus (HPV) infections are recognized as a primary risk factor in the development of cervical dysplasia and cervical cancer [1–4]. Over 30 different genetic subtypes of the virus have been associated with the disease progression, but most of high-grade dysplasia and cervical cancers are associated with a subset of "high risk" viral types; the most frequent being type 16 and type 18 and their closely related subtypes [3,5,6]. While the majority of HPV infections are cleared by the host, the probability of developing progressive disease is significantly increased in patients with moderately suppressed immune systems [2–4]. Therapeutic vaccination targeted towards viral antigens has been investigated as a means to enhance the host immune response to HPV and lead to disease resolution. This type of approach could be considered as an alternative to the surgical procedures that are currently used as standard of care [7]. HPV contains a double stranded circular DNA genome encoding six early expressing genes (E1, E2, E4, E5, E6 and E7) and two late expressing genes (L1 and L2). The L1 and L2 proteins form the viral capsid and are expressed late in infection [8]. The E1 and E2 viral proteins are required for viral DNA replication, while the E4 and E5 proteins are for virus assembly and amplification of the viral genome in the upper layers of the epithelium [9]. The E6 and E7 proteins of high-risk HPV are involved in disregulation of the cell cycle and therefore are oncogenic [10]. The E6 viral protein interacts with an E6-AP to target the p53 protein for degradation [11], whereas the E7 protein interferes with the normal function of the retinoblastoma protein [12]. Collectively, E6 and E7 disrupt the normal cell cycle checkpoint processes. As both proteins are required to maintain the transformed state, E6 and E7 represent potential anti-viral therapeutic drug targets.

Anogenital warts (condylomata acuminata) are caused by sexually transmitted, non-oncogenic types of HPV. The percentage of global population that develop these warts is somewhere in the range of 0.5–1%, with approximately one million new cases each year in the United States [13]. Over 90% of HPV infections resulting in anogenital warts are caused by HPV types 6 and 11. Although most individuals who contract the virus will clear the infection or remain asymptomatic, a number of people will develop condylomas. Anogenital warts are not life threatening however, there is a negative physical and mental impact on those affected with the disease. Since disease resolution is dependent upon cell mediated immune responses, a therapeutic vaccine that can prime and expand HPV 6 and 11 specific T-cells may be a useful treatment option for patients with the disease.

Amolimogene bepiplasmid (formerly ZYC101a) is an investigational immunotherapeutic that contains T cell epitopes from the E6 and E7 proteins of both HPV 16 and 18 that is being tested in

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the clinic as a treatment for high grade cervical dysplasia. Amolimogene consists of a plasmid DNA (pDNA) vector that is encapsulated within poly-lactide co-glycolide (PLG) microparticles (~2 micron mean diameter). Microparticle encapsulation increases the stability of the pDNA vaccine within the host by protecting the pDNA from enzymatic cleavage [14–17]. Once injected into the host, the particulate nature of the formulation will cause local inflammation to occur and the resulting influx of antigen presenting cells (APC) will phagocytose the microparticles [18]. The plasmid will then be released inside the activated APC resulting in direct transfection of professional antigen presenting cells that can express the HPV epitopes, in the context of MHC class I and II molecules, leading to T cell priming and expansion. Amolimogene has been tested for safety and efficacy in a randomized, double-blind, placebo controlled Phase II clinical trial in patients with high-grade cervical dysplasia [19]. The results demonstrated a favorable safety profile and, in a prospectively defined sub group of women under 25 years, clinical resolution of disease was significantly higher in amolimogene treated individuals compared to the placebo control group (70% versus 23%). It is of interest that although amolimogene comprises HPV 16 and 18 sequences, it was observed that resolution of lesions could occur in women with alternate (non 16 or 18) HPV subtypes.

Amolimogene was designed to induce and expand T cells specific to HPV 16 and 18 antigens. Therefore, quantitative analysis of enhanced HPV specific peripheral blood mononuclear cells (PBMC) from patients before, during, and after drug or placebo administration was performed to assess the immunological activity of the patient cohort. We used the IFN-γ ELISpot as a method to detect peripheral T cell activity in HLA-A2+ subjects following treatment with amolimogene or placebo control. The ELISpot assay has the ability to detect low frequency, antigen specific T-cells in the peripheral blood [20–24]. The work reported here was performed to test whether T cells specific to HPV 16 and 18 were induced by amolimogene immunization, as well as determine if increased levels of T cells specific for non 16/18 subtypes (HPV 6 and 11) were present in the peripheral blood of the patient cohort after immunization. An additional objective of our study was to explore whether T cell clones specific to an HPV 18 peptide can recognize and respond to target cells presenting the corresponding HPV 11 peptide. Although not evaluated in this study, the presence of higher levels of T cells with specificity for alternate HPV subtypes may help to explain the clinical observation of resolution of disease in women with non 16 and 18 HPV subtypes. Together, these efforts may provide the framework for future studies exploring the diversity and/or cross reactive nature of the T cell response after amolimogene immunization.

2. Materials and methods

2.1. Clinical trial subjects

Subjects with histologically confirmed high-grade lesions in the cervix (cervical intra-epithelial neoplasia II/III) were enrolled in the institutional review board (IRB) approved phase II clinical study. Amolimogene or placebo (saline) was administered to the subjects via intra-muscular injections in the lateral quadriceps. Injections occurred at weeks 0, 3 and 6. Subjects were closely monitored until the end of the 26 weeks trial, at which point they underwent a loop electrosurgical excision procedure (LEEP).

2.2. HPV typing

As described previously, all subjects enrolled in the study were HPV typed using residual sample from subjects' cytologic evaluation (ThinPrep® System) [25]. Gynecologic samples were collected using a cytobrush/spatula cervical sampling device. The sampling device was placed into transport medium and then sent to a central lab for analysis. HPV subtypes 16, 18, 31, 45 and 56 were detected by PCR (method adapted from Manos et al.) [26] at the Brigham and Women's Hospital (Boston, MA).

2.3. Isolation of peripheral blood mononuclear cells (PBMC)

Approximately 50 mL of heparinized venous blood samples were drawn at the clinical site during each subject visit and shipped overnight at ambient temperature to a central lab (Covance Laboratories, Indianapolis, IN) for PBMC processing. PBMC were isolated by sedimentation on a Ficoll-Paque gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and stored in a freezing medium containing 90% Fetal Calf Serum (FCS) (JRH Biosciences, Lenexa, KS), 10% DMSO (Sigma–Aldrich, St. Louis, MO). All PBMC samples were aliquoted at a concentration of 5×10^6/ml and stored frozen overnight at −80 °C before transfer to liquid nitrogen where they were maintained in the vapor phase. Aliquots of PBMC were shipped overnight on dry ice to Eisai Research Institute (Lexington, MA).

2.4. Peptides

All peptides for use in the immunological assays were acquired from Quality Control Biochemicals Inc. (Hopkinton, MA) or Neo-MPS Inc. (San Diego, CA). Lyophilized peptides, which were a minimum of 90% pure, were shipped from the manufacturer to Eisai Research Institute. The peptides were suspended in dimethyl sulfoxide (DMSO) (Sigma, Carlsbad, CA) to a final concentration of 20 mg/mL and pipetted into individual aliquots. The peptides were stored at −20 °C until the day of assay. Peptide selection was based on several criteria. First, only nonamer peptide sequences were considered for the immunological assays. Second, candidate peptide sequences were pre-selected based on HLA binding restriction such that only the HLA*0201 epitopes encoded within amolimogene with moderate to high affinity binding properties were considered (measured by competitive radioisotope labeled, peptide binding assays; data not shown). Third, a predictive algorithm was used to search for sequence homology between the drug encoded sequences and HPV 6 and 11 E6 and E7 amino acid sequences at positions P4, P5, P6 and P7 (the T cell receptor surface accessible side chain positions for a nonamer peptide bound within a HLA*0201 molecule) [27]. There were nine HPV peptides tested in total. Each of these peptides was grouped into one of three peptide sets (Table 1) made up of at least one HPV 16 or 18 peptide and its corresponding HPV 6 and/or 11 peptides. A pool of peptides derived from cytomegalovirus, Epstein Barr virus and influenza (CEF Pool; Anaspec, San Jose, CA) was used as a control in the ELISpot assay [28].

2.5. HLA-typing

PBMC samples were sent to the American Red Cross (Dedham, MA) for HLA typing. The laboratory used a LABType SSO Typing Test (One Lambda, Inc., Canoga Park, CA) as a method to determine HLA A and B types from each patient.

2.6. In vitro immunization (IVI)

The following method was used to generate antigen specific T cells *in vitro*. On day 0, dendritic cells from three HLA-A2 donors were pulsed with the HPV16 E7 LLMGTLGIV or HPV18 E6 NLLIRCLRC peptides and were co-cultured with PBMC from the

Table 1
MHC class I HPV peptides tested in ELISpot assays.

Peptide ID	HPV source protein	HPV peptide sequence									HPV amino acid#
Set 1											
1	HPV 16 E6	K	L	P	***Q***	***L***	***C***	***T***	E	L	18–26
2	HPV 18 E6	K	L	P	***D***	***L***	***C***	***T***	E	L	13–21
3	HPV 11 E6	S	I	D	***Q***	***L***	***C***	***K***	T	F	12–20
4	HPV 6b E6	T	I	D	***Q***	***L***	***C***	***K***	T	F	12–20
Set 2											
5	HPV 16 E7	L	L	M	***G***	***T***	***L***	***G***	I	V	82–90
6	HPV 18 E7	L	F	L	***N***	***T***	***L***	***S***	F	V	89–97
7	HPV 6 E7/HPV 11 E7	L	L	L	***G***	***T***	***L***	***N***	I	V	82–90
Set 3											
8	HPV 18 E6	N	L	L	***I***	***R***	***C***	***L***	R	C	100–108
9	HPV 11 E6	K	V	L	***I***	***R***	***C***	***Y***	L	C	99–107

Nine HLA-A2+ peptides were selected for screening in all ELISpot assays. All peptides were nine amino acids in length. The HPV 16 and 18 peptides are encoded by amolimogene. A predictive algorithm was used to search for sequence homology between amolimogene encoded 16 and 18 E6 and E7 sequences, and HPV 6 and 11 E6 and E7 amino acid sequences at positions P4, P5, P6 and P7 (highlighted in italic bold), yielding the corresponding HPV 6 or 11 peptides used in these studies.

matching donor in a 24 well plate. The cultures were maintained for 21 days, and were restimulated with the priming peptide loaded onto autologous PBMC at days 7 and 14. IL-7 (10 ng/mL), IL-10 (10 ng/mL) and IL-2 (100 IU/mL) were added at days 1, 9, 11, 15 or 16. Culture media was replaced at days 11 and 15. At day 21, the antigen specific T cells were harvested, washed, counted and tested for peptide reactivity in the IFN-γ ELISpot assay. T cell reactivity to both the priming peptide and its corresponding HPV 6 or 11 peptide were tested.

2.7. ELISpot assay

An IFN-γ ELISpot assay was employed to enumerate the frequency of HPV specific effector cells within PBMC populations. This assay was designed to directly test for CD8+ T cell reactivity as previously described [24], or reactivity of antigen specific T cells generated from *in vitro* immunization cultures. Human IFN-γ ELISpot kits were purchased from R&D Systems Inc. (Minneapolis, MN). All steps involving plate development were performed according to the manufacturer's instructions. On the day of assay, either CD8+ T cells enriched from cryopreserved PBMC using CD8+ T-cell enrichment columns (R&D Systems Inc., Minneapolis, MN), or antigen specific T cells from IVI cultures were counted in trypan blue and adjusted to a concentration of 5×10^5/mL. The HLA-A2+ T2 cell line served as APC in the ELISpot assays [29]. T2 were adjusted to a final concentration of 1×10^6/mL and pulsed with 25 μg/mL of peptide for 3–4 h at 37 °C/5% CO_2 before being plated with the CD8+ T cells (100 μL of each per well). The negative control was ELISpot wells containing T cells and T2 cells with no peptide, and the positive control was T cells and T2 cells pulsed with CEF Pool (Anaspec, San Jose, CA).

Assays were set using complete PBMC medium [RPMI (JRH Lifesciences) with 10% human AB serum (C-Six Diagnostics, Germantown, WI), 1% HEPES buffer (Life Technologies, Grand Island, NY), 1% L-glutamine (Life Technologies), 1% Penicillin–Streptomycin (Life Technologies) and 0.1% 2-mercaptoethanol (Life Technologies)]. ELISpot plates were blocked with PBMC medium for 20 min at room temperature. Blocking medium was removed prior to the addition of cells to the plates. Duplicate test and control wells were set up for all assays performed in these experiments. Cells were incubated in plates for 24 h at 37 °C/5% CO_2. The plates were then developed as per the manufacturer's instructions. The developed plates were allowed to dry at RT and then shipped to Zellnet Consulting Inc. (Fort Lee, NJ) for counting via the ELISpot reader system (Carl Zeiss Vision, Germany) with KS ELISpot 4.0 software.

2.8. ELISpot analysis

A response to a peptide was considered positive if it was at least 50 spot forming cells (SFC) per million CD8+ T cells (with background subtracted) and 2-fold above the negative control (CD8+ T cells with T2 cells alone). A positive response after treatment initiation was defined as a 2-fold increase above the baseline response to the particular peptide.

3. Results

3.1. Peptides

Peptides were selected based on a combination of their experimentally determined binding affinity to HLA-A*0201 molecules (data not shown), and their potential cross reactivity as predicted by sequence homology to putative T cell receptor contact residues based on molecular modeling and crystallographic analysis of the T cell receptor/HLA*0201/peptide complex [27]. As shown in Table 1, each of the three peptide sets contains at least one peptide from HPV16 and/or 18 and their corresponding HPV 6 and/or 11 peptide. All peptides were stored frozen until the day of assay. After thawing, the peptides were pulsed onto APC (T2 cells) for use as stimulators in the ELISpot assay.

3.2. Trial design and subject selection

A multi-center, double-blind, randomized, placebo-controlled phase II trial was conducted in subjects with high grade cervical neoplasia to test the safety and efficacy of amolimogene and the results of this trial have been described previously [19]. Subjects were screened for confirmation of disease and then randomized into three groups. As shown in Fig. 1, the subjects were injected intra-muscularly with either 100 μg of encapsulated pDNA, 200 μg of encapsulated pDNA, or placebo control (saline) at timepoints week 0, 3 and 6. Subjects returned to the clinic for an observation period that included visits at weeks 10, 14, 18, 22 and 26.

For this immune response analysis, 26 subjects were selected from the phase II clinical trial based on HLA type (HLA-A2+) and sample availability from the PBMC archive. The subjects were assigned random identifying numbers (1–26) for these studies. There had to be at least one pre-treatment sample and one post-treatment sample from each subject. Most subjects in this analysis had a sample from baseline (week 0), week 14 (8 weeks post final immunization) and week 26 (20 weeks post final immunization)

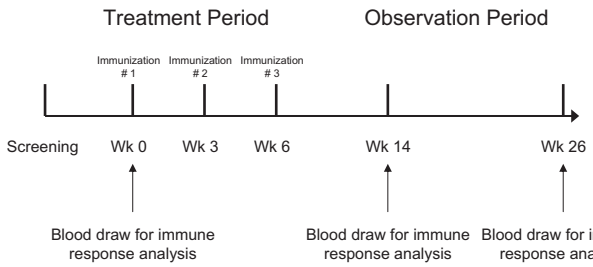

Fig. 1. Treatment timeline of the randomized, double-blind, placebo controlled phase II clinical trial testing amolimogene in HPV+ women with high grade cervical intraepithelial neoplasia. Patients were dosed at weeks 0, 3 and 6 with either 100 μg, 200 μg of encapsulated pDNA, or placebo control by intra-muscular administration. The patients returned to the clinic for observation at weeks 10, 14, 18, 22 and 26. Blood samples were collected at weeks 0, 14 and 26 for analysis of immune response to synthetic HPV peptides.

(Fig. 1). This provided PBMC from eight subjects that received the 100 μg dose, 13 subjects that received the 200 μg dose and five subjects that received the placebo control. Assays were performed in batch format in that all timepoints from one patient were tested on the same day.

3.3. CD8+ T cell responses to the HPV peptides

In order to detect low frequency HPV-specific cytotoxic T-lymphocytes (CTL), an IFN-γ ELISpot protocol that included a CD8+ T cell enrichment step prior to plating was used. CD8+ T cells were incubated with HLA-A2+ T2 cells pulsed with HPV peptides to test for recognition and reactivity to the antigenic peptides. In this analysis, enhanced HPV 16/18 specific CD8+ T-cell responses (2-fold greater than the pre-treatment value) were detected in 11 of 21 subjects receiving amolimogene. The results from the subjects that had increased HPV T cell responses post baseline are included in Table 2A–C. Also listed are those subjects that did not have a measurable increase in HPV immunity (Table 2 D). The frequency of HPV peptide responses in the placebo control group was low however, it should be noted that one subject (#22) had a response to the HPV 16 E7, LLMGTLGIV and HPV 18 E7, LFLNTLSFV peptides that was low in overall magnitude, but met the criteria for an enhanced response. Of the 11 subjects with increased HPV 16/18 T cell responses, all showed an increase to at least one of the amolimogene encoded HPV 16/18 peptides at the week 14 timepoint, 8 weeks after the final amolimogene immunization. At this timepoint, the most dominant epitopes were LLMGTLGIV (HPV 16 E7) and LFLNTLSFV (HPV 18 E7), with 8 of 11 and 7 of 11 subjects responding to them, respectively (Table 2, Peptide set 2). In most cases, these T cell responses were lower or not detectable at the week 26 timepoint.

In the 11 subjects that demonstrated an enhanced CD8+ T cell response to one of the amolimogene encoded HPV 16/18 peptides, seven showed increases in T cell responses to at least one of the corresponding HPV 6 or 11 peptides (Table 2). In all but two of these subjects (#13 and #2), the responses were highest in magnitude at week 14 and decreased markedly by their week 26 timepoint, as was the case in the responses to the HPV 16/18 T cell responses. Of note, T cell responses detected in subject #13 were higher than any other patient tested in this analysis, and the magnitude of the T cell response in this subject was maintained at the week 26 timepoint. The one subject in the placebo control group who had detectable increases in T cell responses to the HPV 16 and 18 in set #2 (#22) did not have detectable increases in her response to the corresponding HPV 6/11 peptide.

3.4. In vitro immunization (IVI) results

Three different HLA-A2 healthy donors PBMC were tested in cultures designed to prime and expand T cells specific to the HPV 18 E6 NLLIRCLRC peptide. At day 21, after three rounds of peptide stimulation, the T cell clones were collected and tested for cross reactivity by their ability to recognize and react to the corresponding HPV 11 E6 peptide. As shown in Fig. 2, T cells primed with the HPV 18 E6 peptide demonstrate cross reactive properties in 3/3 donors by responding to the HPV 11 E6 peptide. The magnitudes of responses to the HPV 11 E6 KVLIRCYLC peptide in all three cases were similar to or greater than the responses detected to the HPV 18 E6 priming peptide (Fig. 2). These results demonstrate that T cells specific for the HPV 18 peptide NLLIRCLRC are able to cross-react to the corresponding HPV11 peptide KVLIRCYLC.

4. Discussion

Amolimogene is an investigational therapeutic vaccine that consists of pDNA encoding antigenic regions of the E6 and E7 proteins from HPV 16 and 18 encapsulated in biodegradable PLG microparticles. The ability of amolimogene immunization to elicit T cells that recognize HPV 16 and 18 E6/E7 epitopes, as well as T cells that can recognize and respond to HLA-A2 restricted, HPV 6 and 11 peptides containing homologous T cell contact residues was investigated in this study. Peripheral blood CD8+ T cell responses were measured from HPV+ subjects with CIN 2/3 that were enrolled in a phase II clinical study [19]. The subjects in this trial were injected with 100 or 200 μg of encapsulated pDNA, or a placebo control. CD8+ T cells were enriched from the subject PBMC and IFN-γ ELISpot assays were performed to measure immune responses to the HPV peptide epitopes.

The HPV 16 and 18 peptides encoded by the amolimogene pDNA formulation were designed to be able to bind HLA-A2 molecules [30]. In our current study, increases in immune responses to HPV 16 and 18 peptides were detected in 11 of 21 HLA-A2+ subjects that had been immunized with amolimogene regardless of the dose. There was variation in the magnitude of response to the peptides among subjects however, there was consistency in the timepoint at which most responses were detected in that the strongest responses were detected at week 14 [8 weeks post the third (final) immunization]. By week 26, most effector T cell responses had decreased to levels that were near their baseline (week 0) responses. The week 14 T cell responses to the HPV 16 E7 LLMGTLGIV and HPV 18 E7 LFLNTLSFV peptides were highest in magnitude and frequency. It is not entirely clear why we did not observe increased T cell responses in 10/21 subjects that received amolimogene. Potential explanations are insufficient dose to prime and expand a measurable response, and/or in-correct blood collection timepoints. We were restricted to testing PBMC that were collected at weeks 0, 16 and 26 in this analysis, and it is plausible that peek immune responses occurred at timepoints far removed from these weeks.

T cell responses in HPV infected individuals to the LLMGTLGIV peptide are well characterized and this sequence has been used to develop additional HPV immunotherapeutics [31,32]. It should be noted that of the five placebo control subjects tested, only 1 (#22) demonstrated an increased response to the HPV peptides which was low in magnitude (i.e. $\leqslant$100 SFC/10e6 CD8+ T-cells). HPV typing was performed on the subjects in this analysis and only 5/24 had changes in their HPV status (by PCR) after their baseline visit. Of the five subjects (#9, #13, #15, #20 and #24) only two had elevated immune responses to the HPV peptide sets (#9 and #13). Of the remaining 19 subjects without changes in their HPV types, nine had elevated immune responses to HPV, suggesting that

Table 2
CD8+ T cell responses to HPV 16, 18, 6 and 11 peptides before and after dosing.

Treatment		Peptide 1: HPV 16 E6; KLPQLCTEL			Peptide 2: HPV 18 E6; KLPDLCTEL			Peptide 3: HPV 11 E6; SIDQLCKTF			Peptide 4: HPV 6b E6; TIDQLCKTF		
Group	Subject	Wk 0	Wk 14	Wk 26	Wk 0	Wk 14	Wk 26	Wk 0	Wk 14	Wk 26	Wk 0	Wk 14	Wk 26
(A) Increase in HPV T cell response													
100 μg	2	0	30	90	0	100	0	0	260	90	140	200	180
100 μg	4	0	140	20	0	30	20	0	200	20	0	210	70
200 μg	13	10	550	730	30	650	440	20	1020	910	70	890	1030
200 μg	14	20	0	40	20	160	100	230	40	30	370	50	40

Treatment		Peptide 5: HPV 16 E7; LLMGTLGIV			Peptide 6: HPV 18 E7; LFLNTLSFV			Peptide 7: HPV 6/11 E7; LLLGTLNIV		
Group	Subject	Wk 0	Wk 14	Wk 26	Wk 0	Wk 14	Wk 26	Wk 0	Wk 14	Wk 26
(B) Increase in HPV T cell response										
100 μg	1	10	80	80	20	70	50	10	40	20
100 μg	2	130	490	130	0	320	70	0	70	70
100 μg	3	30	60	10	20	80	0	10	100	0
100 μg	4	0	260	20	0	60	20	0	50	10
100 μg	5	50	30	40	10	50	110	10	10	30
200 μg	9	0	240	10	0	140	10	0	170	20
200 μg	11	50	160	0	70	0	0	200	130	160
200 μg	12	0	110	30	0	0	40	0	0	20
200 μg	13	120	810	550	20	600	530	0	440	450
Placebo	22	50	60	100	30	90	60	60	90	10

Treatment		Peptide 8: HPV 18 E6; NLLIRCLRC			Peptide 9: HPV 11 E6; KVLIRCYLC		
Group	Subject	Wk 0	Wk 14	Wk 26	Wk 0	Wk 14	Wk 26
(C) Increase in HPV T cell response							
100 μg	3	20	180	40	0	80	0
200 μg	9	0	50	10	0	80	20
200 μg	10	0	70	0	0	50	0
200 μg	11	60	290	80	260	0	100
200 μg	13	0	1090	1040	0	600	810

Treatment group	Subjects
(D) No increase in HPV T cell response	
100 μg	0
200 μg	15, 16, 17, 18, 19, 20 and 21
Placebo	23, 24, 25 and 26

Subjects shown in (A) through (C) had elevated post baseline T cell responses to at least one HPV 16 and/or 18 peptide. Responses to the corresponding HPV 6 and/or 11 peptide are also shown. Subjects listed in section D did not demonstrate an elevated HPV T cell response post baseline. Values represent IFN-γ ELISpot responses to HLA-A2 restricted HPV peptides (all HPV 16 and 18 peptides are encoded by the amolimogene pDNA construct). A: peptide set #1. B: peptide set #2. C: peptide set #3. Timepoints tested include week 0 (pre-injection baseline), week 14 (8 weeks post final injection) and week 26 (20 weeks post final injection). Values are reported as IFN-γ spot forming cells (SFC) per 10e6 CD8+ T-cells. Positive responses are highlighted and defined as greater than or equal to 50 SFC and at least 2-fold greater than the Wk 0 timepoint. Assays were set up in batch format to minimize variability (i.e. wk 0, wk 14 and wk 26 samples from each subject were evaluated on the same day in the same assay plate by the same operator).

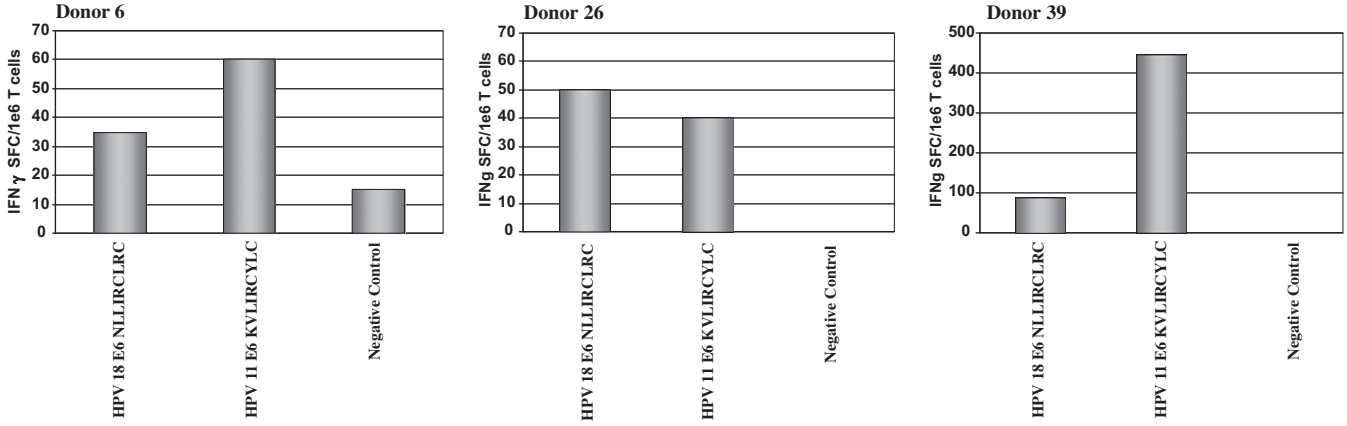

Fig. 2. Results from *in vitro* immunization (IVI) cultures. T cells generated from PBMC of three HLA-A2+ healthy donors were primed with HPV 18 E6 peptide (NLLIRCLRC). The cells were stimulated with the priming peptide at days 0, 7 and 14. At day 21, the cells were harvested and tested using IFN-γ ELISpot for reactivity to the priming peptide and its corresponding HPV 11 E6 peptide (KVLIRCYLC). The negative control peptide was a known HLA-A2 binding peptide from the CYP1B1 protein, FLDPRPLTV [42]. The peptide sequences are listed on the *X*-axis. Results are reported as IFN-γ SFC/10^6 T cells.

elevated immune responses are likely not due to new HPV infections but rather due to immunization with amolimogene.

Next we were interested to study whether T cells with specificity for other HPV types were induced by amolimogene immuniza-

tion. Subject T cells were tested for their ability to recognize and respond to peptides from HPV 6 and 11, the HPV types associated with genital warts. HPV 6 or 11 peptides were selected based on several criteria, one of which included a predictive algorithm that selected peptides based on sequence homology at the P4, P5, P6 and P7 positions. Each HPV 6 or 11 nonomer peptide had as few as one and as many as six amino acid substitutions. Increases in T cell responses to HPV 6 and/or 11 were detected in 7 of 21 subjects that had received amolimogene. As was the case in the responses to the HPV 16/18 peptides, most responses to the HPV 6/11 peptides peaked at week 14 and were reduced at the week 26 timepoint. These data offer evidence that amolimogene immunization can yield increases in CD8+ T cells with specificity for HPV 6 and 11 subtypes. As previously mentioned, we observed increases in HPV 16/18 T-cell responses at week 14 and 26 in one placebo control subject. Interestingly, the T cell response to the corresponding HPV 6/11 peptide in this patient did not increase over time, resulting in 70, 90 and 10 SFC at weeks 0, 14 and 26, respectively. As shown in Table 3, a correlation exists between T cells generated to HPV 16/18 peptides and HPV 6/11 peptides. In most cases (7/11 subjects), when there was a detectable increase in T cell responses to HPV 16 and/or 18 peptides, there was also an increase to the corresponding HPV 6 and/or 11 peptides. This correlation suggests that the same T cell clones that are induced by amolimogene immunization are responding to two different, but homologous, peptides. The one exception was subject #21. This subject had a history of genital warts which may explain why there were detectable responses to the HPV 6b E6 TIDQLCKTF peptide (60 SFC at Week 14) with no corresponding response to the HPV 16 E6 KLPQLCTEL (0 SFC at Week 14) or HPV 18 E6 KLPDLCTEL (10 SFC at Week 14) peptides. Also shown in Table 3 is whether the subjects experienced resolution of their disease. We did not observe a correlation between induction of peripheral HPV immune responses to the peptides selected for this study and CIN II/III resolution. It has been previously reported that patients with CIN III have measurable CTL responses to HPV 16 antigens but do not clear their disease [33]. One potential explanation for this observation is that regulatory T cells could have interfered with or suppressed the effector T cells from effectively targeting the dysplastic tissue. It is known that regulatory T cells are present in and play a key role in HPV associated cervical lesions (persistence and progression to carcinoma) [34,35]. Our analysis on this small number of trial subjects was not designed to further explore this observation but rather to study the induction of HPV T cell responses and their cross reactive properties following immunization with amolimogene.

The results of this study indicated that amolimogene can induce T cell responses to multiple T cell epitopes, a phenomenon that has been well documented with other DNA vaccines [36–38]. Enhanced CD8+ T cell responses were detected at week 14 (8 weeks after final immunization) to multiple HLA-A2 restricted peptides from HPV 16 and 18. Not only were the CD8+ T cell responses directed towards HPV 16 and 18, they were also reactive with peptides from HPV 6 and 11 that had sequence homology. Based on our data set from the clinical trial subjects it is not clear if the responses we detected were generated from HPV 16/18 specific CD8+ T cells that were cross reacting to HPV 6/11 epitopes, or by distinct CD8+ T cell populations that are specific to unique epitopes from HPV 16/18 and 6/11. We initiated experiments to test whether a T cell line specific for an HPV 18 epitope, encoded by the amolimogene pDNA construct, could react to an HPV 11 epitope with similar sequence homology. The results from this demonstrate that T cell lines primed to a single HPV 18 peptide can recognize and respond to an epitope from HPV 11. This offers evidence that

Table 3
Summary of HPV immune responses and clinical histopathology.

Subject ID	Treatment group	Age	Elevated immune Response to HPV 16/18	Elevated immune Response to HPV 6/11	CIN II/III resolution
1	100 μg	35	Yes	No	Yes
2	100 μg	25	Yes	Yes	No
3	100 μg	45	Yes	Yes	NA
4	100 μg	32	Yes	Yes	NA
5	100 μg	23	Yes	No	No
6	100 μg	22	No	No	NA
7	100 μg	20	No	No	No
8	100 μg	32	No	No	Yes
9	200 μg	23	Yes	Yes	Yes
10	200 μg	28	Yes	Yes	No
11	200 μg	23	Yes	Yes	No
12	200 μg	22	Yes	No	No
13	200 μg	19	Yes	Yes	NA
14	200 μg	27	Yes	No	Yes
15	200 μg	20	No	No	Yes
16	200 μg	36	No	No	No
17	200 μg	28	No	No	Yes
18	200 μg	32	No	No	NA
19	200 μg	33	No	No	No
20	200 μg	30	No	No	No
21	200 μg	47	No	Yes	No
22	Placebo	32	Yes	No	NA
23	Placebo	32	No	No	No
24	Placebo	33	No	No	Yes
25	Placebo	25	No	No	Yes
26	Placebo	23	No	No	Yes

All subjects evaluated in this study are listed in the table. Elevated immune responses are defined as greater than or equal to 50 SFC and at least 2-fold greater than the Wk 0 timepoint to any of the HPV peptides. A panel of three pathologists determined the consensus diagnosis at study entry and exit. CIN II/III resolution was classified as CIN II/III clinical diagnosis at study entry and any one of the following clinical diagnosis at the end of study: normal, atypical squamous cells or low grade squamous intraepithelial lesions. NA: not applicable to this analysis.

suggests the peripheral blood HPV 16/18 T cells that are expanded by amolimogene immunization are capable of cross reacting to HPV 6/11 epitopes.

Other studies have examined cross reactivity in mouse and man. Kreijtz et al. [39] demonstrated a considerable ability of seasonal CTL specific to the H3N2 influenza virus to recognize epitopes from the avian H5N1 influenza virus. This observation is important when assessing the potential impact of a pandemic outbreak caused by H5N1, and suggests that the presence of these cross reactive CTL may benefit the general population. In another study, Nilges et al. [40], showed that CTL reactive to the HPV16 $E7_{11-19/20}$ epitope, TMDLQPET, would also recognize the HLA-A2 peptide from the human coronavirus NS2. The peptides have 66% sequence homology and the data support the notion that cross reactive CD8+ T cells can recognize immunogenic regions of HPV16 as well as regions of a common pathogen. In work performed by McCarthy and colleagues [41], the authors show that immunization of HLA-A2/K^b transgenic mice with HPV 18 and 45 E6 DNA yields cross reactive T-cells that recognize other related HPV types. Finally, the work reported by Williams et al. [42], demonstrate CD4+ T cell lines, specific to HPV 11 L1, generated from 21 unrelated healthy donors have the ability to recognize and respond to alternate HPV types. Our observations of CD8+ T cells capable of cross reactivity offer a potential explanation of how amolimogene induced HPV 16/18 specific T cells could recognize peptide epitopes from other HPV strains.

The presence of a cell mediated immune response is helpful in clearing HPV infections [43]. Enhancing the number of functional T cells specific to HPV 16, 18, 6 and 11 epitopes within the host may provide a clinical benefit for patients with HPV-mediated diseases. In 21 subjects receiving amolimogene, 11 were found to have a T cell response to the tested HLA-A2 restricted HPV 16/18 peptides at either week 14 or week 26 timepoints that was at least 2-fold greater than the baseline value. Seven of these 11 subjects had increased T cell responses to the tested HLA-A2 restricted HPV 6/11 peptides. The results demonstrate that immunization with amolimogene, an investigational therapeutic vaccine based on HPV 16/18 E6/E7 sequences, elicits T cell responses that also recognize HPV 6/11 epitopes. Furthermore, we showed that a T cell line specific to an HPV 18 peptide can recognize the HPV 11 peptide used in our studies. These data, combined with the clinical observation that disease resolution occurred in women that were not HPV 16 or 18 positive, suggest that additional studies designed to further characterize the ability of amolimogene to induce CD8+ and/or CD4+ T cells that react with other HPV types would be of interest.

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