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HPV-specific T-cells can be generated from naïve T cells for use as immunotherapeutic strategy for immunocompromised patients

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Abstract

Human papilloma virus is a known cause of cervical cancer, squamous cell carcinoma, and laryngeal cancer. Although treatments exist for HPV-associated malignancies, patients unresponsive to these therapies have a poor prognosis. Recent findings from vaccine studies suggest that T cell immunity is essential for disease control. As EBV-specific T-cells have been highly successful in treating or preventing EBV-associated tumors, we hypothesized that the development of a manufacturing platform for HPV-specific T cells from healthy donors could be used in a third party setting to treat patients with high risk/relapsed HPV-associated cancers. Most protocols for generating virus-specific T-cells require prior exposure of the donor to the targeted virus, and as the seroprevalence of high-risk HPV types varies greatly by age and ethnicity, manufacturing of donor-derived HPV-specific T cells has proven challenging. We therefore made systematic changes to our current GMP-compliant protocols to improve antigen presentation, priming, and expansion for the manufacture of high efficacy HPV-specific T cells. Like others, we found that current methodologies fail to expand HPV-specific T cells from most healthy donors. By optimizing dendritic cell maturation and function with lipopolysaccharide (LPS) and IFN γ , adding IL-21 during priming, and depleting memory T-cells, we achieved reliable expansion of T-cells specific for oncoproteins E6 and E7 to clinically relevant amounts (mean 578-fold expansion, n=10), which were polyfunctional based on cytokine multiplex analysis. In the third-party setting,

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such HPV-specific T-cell products might serve as a potent salvage therapy for patients with HPV-associated diseases.

Keywords

Human papillomavirus; T-cell; immunotherapy; cervical cancer

INTRODUCTION

Human papilloma virus is the most common sexually transmitted infection in the US, and is a recognized oncovirus. Over 70% of cervical carcinomas are associated with HPV, and the virus is also frequently seen in penile, vulvar, anal and oropharyngeal cancers [1, 2]. Several serotypes of the human papillomavirus (HPV) are designated as high risk for oncogenesis. Among these, HPV types 16 and 18 are the most common and most associated with different malignancies: High risk HPV infections are responsible for approximately 5% of all human cancers (7.7% in developing countries, 2.2% in developed countries[2]) and are responsible for 7.5% of female cancer-related deaths worldwide each year [3].

The risks for HPV-associated infections and malignancies are particularly pronounced in immune compromised patients. Organ transplant recipients have twice the risk of developing cancer, and these malignancies are often more aggressive because of the prolonged immunosuppressive regimens required to prevent rejection [4]. Clearance of HPV is slow in patients with deficiencies in T cells or NK cells and severe HPV infection can occur in many forms of primary immunodeficiency [5]. In immunosuppressed populations, such as hematopoietic stem cell transplant (HSCT) recipients and HIV-infected patients, the incidence of HPV infection and associated malignancies is rising, even as treatments for these patients continue to increase their life expectancy [6]. In a recent study of over 28,000 allogeneic HSCT recipients, the risk of developing squamous cell carcinoma (SCC) was 5 times higher in patients with a history of chronic graft-vs-host disease (cGVHD) as compared to the general population [6]. The risk of an immunosuppressed patient developing squamous cell carcinoma (SCC) is 64 to 250 times higher than for an immunocompetent patient, and SCC in immunocompromised patients is more likely to be HPV-related [6, 7]. In patients with HIV, a low CD4+ T cell count is associated with multiple HPV infections, poor viral clearance and cervical dysplasia [8].

HPV vaccines are successful in preventing infections and the malignancies associated with the virus, but they have no impact on the risk of malignancies in patients with pre-existing chronic HPV infections and no therapeutic efficacy against malignancy [9-11]. Evidence from other opportunistic viral infections show that adoptive transfer of virus-specific T cells facilitates immune reconstitution and disease recovery [12], T cell immune therapies for these patients, notably in HSCT recipients, result in successful prevention and treatment of infections with viruses such as adenovirus, EBV, and CMV as well as the post-transplant lymphoproliferative disease associated with EBV [13-17]. In contrast to monoclonal antibodies, T cells can expand *in vivo* and persist long-term after infusion [18].

While T cell therapies are promising, generating a product for individual patients is costly and time-consuming, which limits its utility [19]. These limitations can be overcome by the use of third-party banks to deliver an “off the shelf,” partially-HLA matched T cell therapy. The seroprevalence of HPV is not universal, differing widely by age and ethnicity. This presents a challenge when manufacturing donor-derived HPV-specific T cells, as current protocols for *ex vivo* expansion depend on pre-existing donor immunity to the targeted virus. Ramos *et al.* successfully generated cytotoxic T lymphocytes (CTLs) from patients with HPV-associated cervical and oropharyngeal cancers, but generating them from healthy donors was unsuccessful [20].

Here, we present a modified, currently GMP-compliant protocol for the manufacture of HPV-specific T cells with improved antigen presentation, priming, and T cell expansion. We used our previous success in expanding T cells from naïve cell sources [21, 22] as a template for expanding HPV T cells regardless of donor source. These HPV-specific T-cells represent a novel therapeutic option for patients with HPV-associated diseases. [19].

MATERIALS AND METHODS

Patients / Subjects

Blood was obtained after informed consent from healthy donors at Children’s National Medical Center (Washington D.C.), or from buffy coats provided by the National Institutes of Health, Bethesda, MD.

HPV16-E6 and HPV16-E7 peptide library

Overlapping libraries of 15-mer peptides encompassing HPV16-E6 (Protein ID P03126) and HPV16-E7 (P03129) were purchased from JPT Peptide Technologies (Berlin, Germany). Peptides were reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) at a working concentration of 200ng/uL.

Monocyte isolation and DC generation

Peripheral blood mononuclear cells (PBMCs) were isolated by lymphocyte gradient centrifugation (Lymphocyte Separation Medium; MP Biomedicals, Illkirch, France). Monocytes were isolated from PBMCs by CD14 selection using MACS Beads (Miltenyi Biotec, San Diego, CA) and cultured in 24-well plates in DC medium [CellGenix medium (CellGenix GmbH, Freiburg, Germany) and 1% alanyl-glutamine (GlutaMAX; Gibco Life Technologies, Grand Island, NY)], with 800 U/mL granulocyte/macrophage-colony stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) and 1200 U/mL IL-4 (R&D Systems). DCs were fed with GM-CSF and IL-4 on day 3. On day 5, DCs were pulsed with 1 µL peptide mixture (E6 and E7 mix) and matured by adding 1 mL of DC media containing 10 ng/mL Lipopolysaccharides from *E. coli* (LPS) ((Sigma-Aldrich, St. Louis, MO), 100 U/mL Recombinant Human Interferon- γ (IFN- γ) (R&D Systems), 100 ng/mL IL-6 (R&D Systems), 10 ng/mL IL-1 β (R&D Systems), 10 ng/mL TNF α (R&D Systems), 1 µg/mL prostaglandin E2 (R&D Systems), 800 U/mL GM-CSF and 1200 U/mL IL-4.

HPV16 E6/E7-specific T cell generation

After maturation, DCs were harvested and re-suspended in CTL medium [45% Click's medium (Irvine Scientific, Santa Ana, CA), 45% RPMI-1640 (HyClone, Logan, UT), 10% human AB serum (Gemini BioProducts, West Sacramento, CA) and 1% GlutaMAX]. CD14 negative PBMCs were thawed and underwent CD45RO depletion using MACS Beads (Miltenyi Biotec). These cells were stimulated with peptide-loaded DCs at a 1:4 ratio (DCs: depleted cells) in CTL medium with 60 ng/mL IL-21 (R&D Systems). Cell cultures were fed on day 3, 5 and 7 poststimulation with 5 ng/mL IL-7 (R&D Systems) and 5 ng/mL IL-15 (R&D Systems). Nine to twelve days after the first stimulation, T cells were harvested, counted and stimulated again with newly prepared peptide-loaded DCs (1:4 ratio) and 200 U/mL IL-2 (R&D Systems). Cell cultures were supplemented with 10 ng/mL IL15 every 2 days and split when confluent. Seven days after the second stimulation, T cells were harvested, counted, re-suspended in CTL medium and stimulated for a third time using newly prepared peptide-loaded DCs (1:4 ratio) with 200 U/mL IL-2. Cell cultures were fed with 10 ng/mL IL15 every 2 days and split when necessary.

Phenotyping of HPV-specific T-cells

CTL lines were analyzed with a panel of monoclonal antibodies to CD3, CD4, CD8, CD25, CCR7, CD45RA, CD45RO, CD28, CD62L, PD1, CD27, CD69, CD19, CD16, CD56, CD4, LAG3, and TIM3 (Becton Dickinson/Milltenyi Biotec). Samples were acquired on the BD Accuri C6 flow cytometer (Becton Dickinson), and the data were analyzed with the use of the BD C6 software (Becton Dickinson).

Enzyme-linked immunospot assays

Seven days after the third stimulation, T cells were assessed for HPV16-E6 and -E7 specificity using Interferon-gamma (IFN γ) Enzyme-linked immunospot (ELISpot) analysis. T cell lines were utilized at 1-2 $\times$ 10⁵ cells/well of a 96-well plate. We measured the secretion of IFN γ in response to stimulation with E6 and E7 pepmixes and/or each of the 13 peptides from ISA Pharmaceuticals. Both negative (no-peptide) and positive (staphylococcal enterotoxin B, Fisher Scientific, Pittsburgh, PA) controls were included and all conditions were tested in duplicate. The Human Actin peptide (P68133, JPT) was used as an irrelevant antigen control. Millipore Multi Screen HTS filter plates (Millipore) were coated with IFN γ capture antibody (Mabtech) at a concentration of 10 μ g/mL for 4 hours or overnight at 4°C. Plates were washed with PBS and blocked for 1 hour at 37°C. For development, plates were washed in PBS/.05% Tween 20 (Sigma-Aldrich) and incubated with biotinylated IFN γ detection antibody (.5 μ g/mL; Mabtech) for 2 hours at 37°C, followed by incubation with streptavidin-coupled alkaline phosphatase complex (Vectastain; Vector Laboratories) for 1 hour at room temperature and spots were developed by incubation with 3-amino-9-ethylcarbazole substrate (Sigma) solution. IFN γ spot-forming-cells (SFC) were counted and evaluated by Zellnet Consulting using an automated plate reader system (Karl Zeiss). Since activated T cells also serve as antigen presenting cells in the ELISPOT assay [23], in order to determine the specific population responsible for the IFN γ secretion, blocking antibodies were used as previously described [24, 25]. Briefly, cells were plated in a 96 well plate and 15 μ L of anti-HLA (ABC or DP/DQ/DR) antibody (DAKO, Carpinteria, CA) was added to

the designated wells. After 45 minutes of incubation, pepmixes were added and the plating was completed as per the normal protocol. After overnight incubation, the plates were developed for IFN γ spots, dried overnight, and sent to ZellNet Consulting (Fort Lee, NJ) for analysis. The frequency of T cells specific to each antigen was expressed as spot-forming cells (SFCs) per number of cells per well ($1-2 \times 10^5$).

Bio-Plex Pro™ Human Cytokine 27-plex Assay

Supernatants were harvested from cell culture plates 24 hours after stimulation, and analyzed by Bio-Plex Pro™ 27-plex assay (Bio-Rad Laboratories, Inc., Hercules, CA) per manufacturer protocol. Undiluted human samples were analyzed in parallel. The Bio-Plex® MAGPIX™ instrument was utilized for the analysis.

RESULTS

Consistent generation of HPV specific T cells from naive T-cells is feasible.

The optimization schema (Supplementary Figure 1) involved the stepwise addition of different elements to identify the best conditions to expand HPV-specific T cells. Starting from a protocol used to expand T cells from patients and healthy donors (condition A), we successively added IL-21 (condition B); LPS and IFN γ (condition C); increased the antigen-presenting cell (APC) : effector ratio (condition D); depleted memory cells (condition E); and used higher doses of IL2 and IL15 for expansion (condition F). Condition F, incorporating modifications B, C, D, and E, achieved optimal expansion (Supplementary Figure 2) and specificity (Supplementary Figure 3). Despite the differences in the expansion method, all cell populations had the same phenotype (Supplementary Figure 4). Using this optimized approach, we expanded HPV-specific T cell products targeting HPV oncoproteins E6 and E7 to clinically relevant numbers (mean 578-fold expansion, $n=10$) (Figure 1A). HPV-specific T cells are also predominantly CD45RO+ (Figure 1B). These HPV-specific T cells specifically recognized E6 and E7 (137 ± 148 IFN γ SFC/ 1×10^5 cells and 68 ± 145 IFN γ SFC/ 1×10^5 cells respectively, compared to 7 ± 8 IFN γ SFC/ 1×10^5 cells against an irrelevant antigen) (Figure 1C). An analysis of their expression of exhaustion markers showed they express $38.2 \pm 5.73\%$ PD1, $71.14 \pm 1.53\%$ Tim3, and $22.3 \pm 6.43\%$ Lag3 ($n=5$) (Figure 1D).

HPV specific T cells are preferentially MHC class II restricted.

Most T cells expanded against HPV antigens were CD4+ (Figure 2A), with a mean of $68.6 \pm 23.3\%$ vs $18.2 \pm 20.9\%$ CD8+ T cells ($n=10$). As expected, expanded populations mostly recognized antigens through MHC class II confirmed by a decrease in responses to HPV antigen in the presence of HLA class II blocking antibodies (Figure 2B).

HPV-specific T cells are polyfunctional.

HPV specific T cells secreted multiple proinflammatory cytokines after three stimulations. In five evaluated products, T cells secreted high levels of IL6 (3792 ± 966 pg/mL), IL7 (18000 ± 0 pg/mL), IL8 (4766 ± 2271 pg/mL), IP10 (234 ± 220 pg/mL), MIP1b (4915 ± 2050 pg/mL) and TNFa (1945 ± 823 pg/mL).

HPV specific T cells derived from the naïve (CD45RO negative) population expand more robustly than their memory (CD45RO positive) counterparts.

With the assumption that the memory T cell fraction will only expand in individuals pre-exposed to the virus we compared expansion of T cells from the naïve fraction with the memory fraction. HPV-specific T cells were successfully expanded in 5/5 naïve populations but in only 3/5 from the memory fraction of the same donors. While the phenotype and specificity for all five donors were similar (Figure 4B and 4C), there was a much greater expansion rate of cells derived from naïve T cells (Figure 4A), (mean 675 ± 569 vs 207 ± 195 fold expansion for memory cells).

DISCUSSION

We successfully generated HPV16 specific T cells from healthy donors, and established the conditions for a robust and reproducible GMP-compliant protocol for T cell immunotherapy for HPV-positive malignancies. Other groups have previously developed T cell therapies for HPV targets, but most efforts have focused on generating them from patients with HPV malignancies [26-30]. Other studies using TCR gene modified T cells have been developed, but the strategy limits the HLA types that can be targeted [31-33]. Since immune dysfunction frequently accompanies HPV malignancies [34], “off the shelf” third-party HPV-specific T-cells could represent a more suitable and effective cell product [19].

In a study by Ramos *et al.*, genetically modified APC derived from B cells (B blasts) to stimulate T cells against E6 and E7 peptides were only able to expand cells from one of 20 healthy donors [20], suggesting that B blasts are less potent stimulators than DCs in priming immune responses *ex vivo*. We attribute our success in expanding HPV-naïve cells *ex vivo* to the use of DCs, which have been used to expand T cells against weakly immunogenic targets [24, 25], and the use of IL21 during priming.

IL21 co-stimulates T cells and improves T cell activation leading to antitumor activity [35]. Recently, IL21 was successfully used to expand melanoma-specific T cells [36, 37], and tumor-specific T cells generated in the presence of IL21 improved activity and long term persistence of infused cells in three patients at high risk for relapse [38]. The ability of IL21 to improve the persistence of T cell products (particularly from naïve T cells generated against self antigens in malignancies, or from seronegative individuals against viral antigens) may be related to its inhibition of FOXP3 induction in naïve CD4 T cells [39], enhanced generation of memory stem cells [40], and enhanced CD8+ cytotoxic T cell activity through increased activity of Tbet [41].

We also found that prior selection of naïve cells (CD45RO negative) increased the chances of expanding HPV specific T cells. The absence of contaminating memory cells allowed better priming to the novel antigen, resulting in a trend towards higher fold expansion (Figure 4A) and decreased exhaustion (Figure 4B). Interestingly, responses could still be generated from memory cells, suggesting that this expansion strategy could be applied irrespective of the donor serostatus. This approach may prove useful for generating T cells against weakly immunogenic antigens, or generating cells against antigens that the host has not previously been exposed to.

The improved culture conditions generated T cells secreting multiple proinflammatory cytokines, including IL-6, IL-7, IL-8, IFN γ , IL-1 β , and TNF α , which have prominent functions in stimulating cellular immune responses against malignancies. In vaccine recipients of bivalent and quadrivalent HPV vaccine CD4⁺ T-follicular helper cells were induced following vaccination [42], and a predominant Th1 CD4⁺ cellular response was similarly observed after SLP vaccine [30]. Thus, *ex-vivo* generated HPV-specific CTLs recapitulate the physiologic adaptive immune response to HPV vaccination.

A limitation of this study is the lack of knowledge of the HPV serostatus of the donors. However, given the low rates of HPV positivity in the US (varying from 7-45%), [43] our success in eliciting T cell expansions from all donors suggests that the protocol is effective in both exposed and unexposed individuals. Whether the resulting HPV-specific CTLs recognize the same epitopes within E6 and E7 versus those recognized during vaccination or infection is unknown and merit further study given the previously described differences in CMV epitopes recognized by CMV-specific CTLs generated from CMV-naïve donors [22].

In conclusion, this improved method to produce HPV-specific T cells should pave the way for the creation of third-party banks of GMP-compliant HPV specific T cell products to use as an “off the shelf” therapeutic to treat patients with HPV-associated diseases, similar to that seen in the 3rd party EBV-specific T cell and multi-virus specific T cell settings for post transplant lymphoma and post transplant infections respectively [44][45]. This approach could be utilized as a platform for targeted cellular therapy for treatment of HPV+ malignancies alongside standard therapy, and may improve survival rates for these devastating forms of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- [1]. Stanley M, Immunobiology of HPV and HPV vaccines, Gynecologic oncology 109(2 Suppl) (2008) S15–21. [PubMed: 18474288]
- [2]. Tota JE, Chevarie-Davis M, Richardson LA, Devries M, Franco EL, Epidemiology and burden of HPV infection and related diseases: implications for prevention strategies, Preventive medicine 53 Suppl 1 (2011) S12–21. [PubMed: 21962466]
- [3]. Tummers B, Burg SH, High-risk human papillomavirus targets crossroads in immune signaling, Viruses 7(5) (2015) 2485–506. [PubMed: 26008697]
- [4]. Reusser NM, Downing C, Guidry J, Tying SK, HPV Carcinomas in Immunocompromised Patients, Journal of clinical medicine 4(2) (2015) 260–81. [PubMed: 26239127]
- [5]. Leiding JW, Holland SM, Warts and all: human papillomavirus in primary immunodeficiencies, The Journal of allergy and clinical immunology 130(5) (2012) 1030–48. [PubMed: 23036745]
- [6]. Tedeschi SK, Savani BN, Jagasia M, Engelhardt B, Anasetti C, Barrett AJ, Lee S, Time to consider HPV vaccination after allogeneic stem cell transplantation, Biology of blood and marrow

transplantation : journal of the American Society for Blood and Marrow Transplantation 16(8) (2010) 1033–6.

- [7]. Ojha RP, Tota JE, Offutt-Powell TN, Klosky JL, Minniear TD, Jackson BE, Gurney JG, Human papillomavirus-associated subsequent malignancies among long-term survivors of pediatric and young adult cancers, *PloS one* 8(8) (2013) e70349. [PubMed: 23940566]
- [8]. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, Kenter G, Offringa R, van der Burg SH, Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6, *Cancer research* 64(15) (2004) 5449–55. [PubMed: 15289354]
- [9]. Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J, Edwards L, Parker RL, Denny L, Giffear M, Brown AS, Marcozzi-Pierce K, Shah D, Slager AM, Sylvester AJ, Khan A, Broderick KE, Juba RJ, Herring TA, Boyer J, Lee J, Sardesai NY, Weiner DB, Bagarazzi ML, Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial, *Lancet* 386(10008) (2015) 2078–88. [PubMed: 26386540]
- [10]. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, Giffear M, Pankhong P, Khan AS, Broderick KE, Knott C, Lin F, Boyer JD, Draghia-Akli R, White CJ, Kim JJ, Weiner DB, Sardesai NY, Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses, *Science translational medicine* 4(155) (2012) 155ra138.
- [11]. van der Sluis TC, van der Burg SH, Arens R, Melief CJ, New approaches in vaccine-based immunotherapy for human papillomavirus-induced cancer, *Current opinion in immunology* 35 (2015) 9–14. [PubMed: 26001120]
- [12]. Fujita Y, Rooney CM, Heslop HE, Adoptive cellular immunotherapy for viral diseases, *Bone marrow transplantation* 41(2) (2008) 193–8. [PubMed: 17982497]
- [13]. Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, Carrum G, Krance RA, Chang CC, Molldrem JJ, Gee AP, Brenner MK, Heslop HE, Rooney CM, Bollard CM, Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals, *Nature medicine* 12(10) (2006) 1160–6.
- [14]. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, Leung K, Carrum G, Gee AP, Vera JF, Krance RA, Brenner MK, Rooney CM, Heslop HE, Leen AM, Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT, *Science translational medicine* 6(242) (2014) 242ra83.
- [15]. Melenhorst JJ, Leen AM, Bollard CM, Quigley MF, Price DA, Rooney CM, Brenner MK, Barrett AJ, Heslop HE, Allogeneic virus-specific T cells with HLA alloreactivity do not produce GVHD in human subjects, *Blood* 116(22) (2010) 4700–2. [PubMed: 20709906]
- [16]. Leen AM, Christin A, Myers GD, Liu H, Cruz CR, Hanley PJ, Kennedy-Nasser AA, Leung KS, Gee AP, Krance RA, Brenner MK, Heslop HE, Rooney CM, Bollard CM, Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation, *Blood* 114(19) (2009) 4283–92. [PubMed: 19700662]
- [17]. Gerdemann U, Katari UL, Papadopoulou A, Keirnan JM, Craddock JA, Liu H, Martinez CA, Kennedy-Nasser A, Leung KS, Gottschalk SM, Krance RA, Brenner MK, Rooney CM, Heslop HE, Leen AM, Safety and clinical efficacy of rapidly-generated trivirus-directed T cells as treatment for adenovirus, EBV, and CMV infections after allogeneic hematopoietic stem cell transplant, *Molecular therapy : the journal of the American Society of Gene Therapy* 21(11) (2013) 2113–21. [PubMed: 23783429]
- [18]. Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, Bollard CM, Liu H, Wu MF, Rochester RJ, Amrolia PJ, Hurwitz JL, Brenner MK, Rooney CM, Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients, *Blood* 115(5) (2010) 925–35. [PubMed: 19880495]
- [19]. Leen AM, Bollard CM, Mendizabal AM, Shpall EJ, Szabolcs P, Antin JH, Kapoor N, Pai SY, Rowley SD, Kebriaei P, Dey BR, Grilley BJ, Gee AP, Brenner MK, Rooney CM, Heslop HE, Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation, *Blood* 121(26) (2013) 5113–23. [PubMed: 23610374]

- [20]. Ramos CA, Narala N, Vyas GM, Leen AM, Gerdemann U, Sturgis EM, Anderson ML, Savoldo B, Heslop HE, Brenner MK, Rooney CM, Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes for adoptive immunotherapy of HPV-associated malignancies, *J Immunother* 36(1) (2013) 66–76. [PubMed: 23211628]
- [21]. Hanley PJ, Cruz CR, Savoldo B, Leen AM, Stanojevic M, Khalil M, Decker W, Molldrem JJ, Liu H, Gee AP, Rooney CM, Heslop HE, Dotti G, Brenner MK, Shpall EJ, Bollard CM, Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes, *Blood* 114(9) (2009) 1958–67. [PubMed: 19443656]
- [22]. Hanley PJ, Melenhorst JJ, Nikiforow S, Scheinberg P, Blaney JW, Demmler-Harrison G, Cruz CR, Lam S, Krance RA, Leung KS, Martinez CA, Liu H, Douek DC, Heslop HE, Rooney CM, Shpall EJ, Barrett AJ, Rodgers JR, Bollard CM, CMV-specific T cells generated from naive T cells recognize atypical epitopes and may be protective in vivo, *Science translational medicine* 7(285) (2015) 285ra63.
- [23]. McLaughlin LP, Lang H, Williams E, Wright KE, Powell A, Cruz CR, Colberg-Poley AM, Barese C, Hanley PJ, Bollard CM, Keller MD, Human parainfluenza virus-3 can be targeted by rapidly ex vivo expanded T lymphocytes, *Cytotherapy* 18(12) (2016) 1515–1524. [PubMed: 27692559]
- [24]. Weber G, Gerdemann U, Caruana I, Savoldo B, Hensel NF, Rabin KR, Shpall EJ, Melenhorst JJ, Leen AM, Barrett AJ, Bollard CM, Generation of multi-leukemia antigen-specific T cells to enhance the graft-versus-leukemia effect after allogeneic stem cell transplant, *Leukemia* 27(7) (2013) 1538–47. [PubMed: 23528871]
- [25]. Weber G, Caruana I, Rouse RH, Barrett AJ, Gerdemann U, Leen AM, Rabin KR, Bollard CM, Generation of tumor antigen-specific T cell lines from pediatric patients with acute lymphoblastic leukemia--implications for immunotherapy, *Clinical cancer research : an official journal of the American Association for Cancer Research* 19(18) (2013) 5079–91. [PubMed: 23838315]
- [26]. Bellone S, El-Sahwi K, Cocco E, Casagrande F, Cargnelutti M, Palmieri M, Bignotti E, Romani C, Silasi DA, Azodi M, Schwartz PE, Rutherford TJ, Pecorelli S, Santin AD, Human papillomavirus type 16 (HPV-16) virus-like particle L1-specific CD8+ cytotoxic T lymphocytes (CTLs) are equally effective as E7-specific CD8+ CTLs in killing autologous HPV-16-positive tumor cells in cervical cancer patients: implications for L1 dendritic cell-based therapeutic vaccines, *Journal of virology* 83(13) (2009) 6779–89. [PubMed: 19386711]
- [27]. Santin AD, Hermonat PL, Ravaggi A, Chiriva-Internati M, Zhan D, Pecorelli S, Parham GP, Cannon MJ, Induction of human papillomavirus-specific CD4(+) and CD8(+) lymphocytes by E7-pulsed autologous dendritic cells in patients with human papillomavirus type 16- and 18-positive cervical cancer, *Journal of virology* 73(7) (1999) 5402–10. [PubMed: 10364287]
- [28]. van Poelgeest MI, Visconti VV, Aghai Z, van Ham VJ, Heusinkveld M, Zandvliet ML, Valentijn AR, Goedemans R, van der Minne CE, Verdegaal EM, Trimbos JB, van der Burg SH, Welters MJ, Potential use of lymph node-derived HPV-specific T cells for adoptive cell therapy of cervical cancer, *Cancer immunology, immunotherapy : CII* 65(12) (2016) 1451–1463. [PubMed: 27619514]
- [29]. Thomas KJ, Smith KL, Youde SJ, Evans M, Fiander AN, Borysiewicz LK, Man S, HPV16 E6 29-38-specific T cells kill cervical carcinoma cells despite partial evasion of T-cell effector function, *International journal of cancer* 122(12) (2008) 2791–9. [PubMed: 18366058]
- [30]. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, Drijfhout JW, Valentijn AR, Wafelman AR, Oostendorp J, Fleuren GJ, Offringa R, Melief CJ, van der Burg SH, Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine, *Clinical cancer research : an official journal of the American Association for Cancer Research* 14(1) (2008) 178–87. [PubMed: 18172269]
- [31]. Draper LM, Kwong ML, Gros A, Stevanovic S, Tran E, Kerkar S, Raffeld M, Rosenberg SA, Hinrichs CS, Targeting of HPV-16+ Epithelial Cancer Cells by TCR Gene Engineered T Cells Directed against E6, *Clinical cancer research : an official journal of the American Association for Cancer Research* 21(19) (2015) 4431–9. [PubMed: 26429982]

- [32]. Scholten KB, Schreurs MW, Ruizendaal JJ, Kueter EW, Kramer D, Veenbergen S, Meijer CJ, Hooijberg E, Preservation and redirection of HPV16E7-specific T cell receptors for immunotherapy of cervical cancer, *Clin Immunol* 114(2) (2005) 119–29. [PubMed: 15639645]
- [33]. Scholten KB, Turksma AW, Ruizendaal JJ, van den Hende M, van der Burg SH, Heemskerk MH, Meijer CJ, Hooijberg E, Generating HPV specific T helper cells for the treatment of HPV induced malignancies using TCR gene transfer, *Journal of translational medicine* 9 (2011) 147. [PubMed: 21892941]
- [34]. Andersen AS, Koldjaer Solling AS, Ovesen T, Rusan M, The interplay between HPV and host immunity in head and neck squamous cell carcinoma, *International journal of cancer* 134(12) (2014) 2755–63. [PubMed: 23913554]
- [35]. Croce M, Rigo V, Ferrini S, IL-21: a pleiotropic cytokine with potential applications in oncology, *Journal of immunology research* 2015 (2015) 696578. [PubMed: 25961061]
- [36]. Zoon CK, Wan W, Graham L, Bear HD, Addition of interleukin-21 for expansion of T-cells for adoptive immunotherapy of murine melanoma, *International journal of molecular sciences* 16(4) (2015) 8744–60. [PubMed: 25903148]
- [37]. Yu X, He J, Mongkhoune S, Peng Y, Xie Y, Su J, Zhou SF, Xie XX, Luo GR, Fang Y, Li X, Zhou N, Zhao YX, Lu XL, Artificial antigen-presenting cells plus IL-15 and IL-21 efficiently induce melanoma-specific cytotoxic CD8+ CD28+ T lymphocyte responses, *Asian Pacific journal of tropical medicine* 6(6) (2013) 467–72. [PubMed: 23711708]
- [38]. Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, Duerkopp N, Roberts IM, Pogosov GL, Ho WY, Ochsenreither S, Wolf M, Bar M, Radich JP, Yee C, Greenberg PD, Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients, *Science translational medicine* 5(174) (2013) 174ra27.
- [39]. Kannappan V, Butcher K, Trela M, Nicholl I, Wang W, Attridge K, Interleukin 21 inhibits cancer-mediated FOXP3 induction in naive human CD4 T cells, *Cancer immunology, immunotherapy* : CII (2017).
- [40]. Alvarez-Fernandez C, Escriba-Garcia L, Vidal S, Sierra J, Briones J, A short CD3/CD28 costimulation combined with IL-21 enhance the generation of human memory stem T cells for adoptive immunotherapy, *Journal of translational medicine* 14(1) (2016) 214. [PubMed: 27435312]
- [41]. Sutherland AP, Joller N, Michaud M, Liu SM, Kuchroo VK, Grusby MJ, IL-21 promotes CD8+ CTL activity via the transcription factor T-bet, *J Immunol* 190(8) (2013) 3977–84. [PubMed: 23479229]
- [42]. Matsui K, Adelsberger JW, Kemp TJ, Baseler MW, Ledgerwood JE, Pinto LA, Circulating CXCR5(+)CD4(+) T Follicular-Like Helper Cell and Memory B Cell Responses to Human Papillomavirus Vaccines, *PloS one* 10(9) (2015) e0137195. [PubMed: 26333070]
- [43]. Liu G, Markowitz LE, Hariri S, Panicker G, Unger ER, Seroprevalence of 9 Human Papillomavirus Types in the United States, 2005–2006, *The Journal of infectious diseases* 213(2) (2016) 191–8. [PubMed: 26320259]
- [44]. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, Carrum G, Sasa G, Lulla P, Watanabe A, Kuvalekar M, Gee AP, Wu MF, Liu H, Grilley BJ, Krance RA, Gottschalk S, Brenner MK, Rooney CM, Heslop HE, Leen AM, Omer B, Off-the-Shelf Virus-Specific T Cells to Treat BK Virus, Human Herpesvirus 6, Cytomegalovirus, Epstein-Barr Virus, and Adenovirus Infections After Allogeneic Hematopoietic Stem-Cell Transplantation, *J Clin Oncol* 35(31) (2017) 3547–3557. [PubMed: 28783452]
- [45]. Barker JN, Doubrovina E, Sauter C, Jaroscak JJ, Perales MA, Doubrovin M, Prockop SE, Koehne G, O'Reilly RJ, Successful treatment of EBV-associated posttransplantation lymphoma after cord blood transplantation using third-party EBV-specific cytotoxic T lymphocytes, *Blood* 116(23) (2010) 5045–9. [PubMed: 20826724]
- [46]. Llosa NJ, Cooke KR, Chen AR, Gamper CJ, Klein OR, Zambidis ET, Luber B, Rosner G, Siegel N, Holuba MJ, Robey N, Hayashi M, Jones RJ, Fuchs E, Holdhoff M, Loeb DM, Symons HJ, Reduced-Intensity Haploidentical Bone Marrow Transplantation with Post-Transplant Cyclophosphamide for Solid Tumors in Pediatric and Young Adult Patients, *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* (2017).

Figure 1. HPV-specific T cells can only be expanded from healthy donors in the presence of IL21 and LPS and IFN γ -matured dendritic cells.

A. Combined fold expansion from 10 healthy donors, with average expansion normalized to the initial number of cells and increases during each stimulation depicted. Vertical lines indicate standard deviation. B. Phenotype of cells after three stimulations, black bars show median expression as a percentage of CD3+ T cells, and error bars show standard deviation. C. Specificity of T cells from 10 donors against HPV antigens E6 and E7. Each symbol depicts a healthy donor, and the line depicts the median. All 10 donors were specific for at least one antigen, as measured by counts at least two times more than background. D. Expression of exhaustion markers PD1, Tim3, and Lag3 in 5 evaluated donors. Black bars show median expression as a percentage of CD3+ T cells, and error bars show standard deviation.

Figure 2. HPV-specific T cells recognize antigens via MHC class II presentation.

A. Percentage of cells expressing markers of different subsets: circles denote individual donors while the black horizontal line depicts the median. B. ELISPOT showing inhibition of specific responses upon addition of HLA-blocking antibodies, in an evaluated healthy donor. Black bars denote number of spot forming cells per 1×10^5 cells in each patient, and error bars depict standard deviation from duplicates.

Figure 3. HPV-specific T cells secrete multiple pro-inflammatory cytokines.

Five evaluated T cell lines were tested for secretion of cytokines in response to stimulation with HPV E6 and E7 antigens. Black bars depict concentration of cytokine tested in pg/mL; error bars depict standard deviation.

Figure 4. HPV-specific T cells can be generated from both the naive and memory compartments.

A. Combined fold expansion from 5 healthy donors, with average expansion normalized to the initial number of cells and increases during each stimulation depicted. Light gray lines show T cells derived from the naive (CD45RO^{negative}) compartment, while black lines show T cells derived from the memory (CD45RO⁺) compartment. Vertical lines indicate standard deviation. B. Specificity of T cells from 5 donors against HPV antigens E6 and E7; each symbol depicts a T cell derived from the naive fraction (light gray circles) or the memory fraction (black squares); line depicts the median. C. Phenotype of cells after three stimulations, light gray circles (naive fraction-derived T cells) and black squares (memory fraction-derived T cells) show individual patient expression of markers as a percentage of CD3⁺ T cells; horizontal lines show the median.