Tumor cells fail to present MHC-II restricted epitopes derived from oncogenes to CD4$^+$ T cells

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List of Abbreviations: HPV – Human papillomavirus, HLA – Human leukocyte antigen, TCR – T cell receptor, APC – Antigen-presenting cell, TIL – Tumor-infiltrating lymphocytes, ACT – Adoptive cellular therapy, HNSCC – Head and neck squamous cell carcinoma, PDAC – Pancreatic ductal adenocarcinoma

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Abstract

CD4⁺ T cells play a critical role in anti-tumor immunity via recognition of peptide antigens presented on MHC class II (MHC-II). Although some solid cancers can be induced to express MHC-II, the extent to which this enables direct recognition by tumor-specific CD4⁺ T cells is unclear. We isolated and characterized T cell antigen receptors (TCRs) from naturally primed CD4⁺ T cells specific for two oncoproteins, HPV16 E6 and the activating KRAS⁴¹²V mutation, from head and neck squamous cell carcinoma (HNSCC) and pancreatic ductal adenocarcinoma (PDAC) patients, respectively, and determined their ability to recognize autologous or human leukocyte antigen (HLA)-matched antigen-expressing tumor cells. We find in both cases that the TCRs are capable of recognizing peptide-loaded target cells expressing the relevant MHC-II or B cell antigen-presenting cells (APC) when the antigens are endogenously expressed and directed to the endosomal pathway but fail to recognize tumor cells expressing the source protein even after induction of surface MHC-II expression by IFN-γ or transduction with CIITA. These results suggest that priming and functional recognition of both a nuclear (E6) and a membrane-associated (KRAS) oncoprotein is predominantly confined to cross-presenting APC rather than via direct recognition of tumor cells induced to express MHC-II.
Introduction

Malignant transformation of somatic cells is initiated by the action of activated oncogenes which dysregulate cell growth pathways and lead to unchecked proliferation (1). In the case of human papillomavirus (HPV)-associated cancers, expression of the HPV early genes E6 and E7 contributes to oncogenesis by inhibiting the tumor suppressor genes p53 and Rb, respectively (2). Alternatively, constitutive oncogene activation may occur through somatic mutation in genes regulating cell growth and proliferation pathways, such as the RAS family. Indeed, activating KRAS mutations such as KRAS$^{G12V}$ are commonly found in patients with pancreatic, colon, and lung cancer (3).

While oncogenes promote disease, they can also provide targets for the host immune system. In recent years, the role of the immune system in controlling HPV-associated cancers has become widely recognized. CD8$^+$ and CD4$^+$ T cells recognizing HPV E6 and E7, as well as other HPV proteins, have been identified in both the peripheral blood and tumor-infiltrating lymphocytes (TIL) from patients with HPV-associated cancers (4–6). Immunotherapies targeting these antigens, including therapeutic cancer vaccines and adoptive cellular therapy (ACT) with TIL or T cell receptor (TCR)-engineered T cells, are an active area of preclinical and clinical investigation (2). T cell responses against oncogenic mutations have also been widely described (7–9). Furthermore, there is direct clinical evidence that ACT with TIL targeting mutant KRAS can promote objective responses (10).

While CD8$^+$ T cells recognizing epitopes derived from oncogenes have been shown to mediate direct cytotoxicity against tumor cells (11–13), the role of CD4$^+$ T cells in antitumor immunity is less well understood. Recent studies have highlighted a subset of CD4$^+$ T cells expressing cytotoxic markers, such as granzyme B (GrzmB), in human cancers (14, 15). The antigen specificities of these cells, however, and thereby their therapeutic potential, remains largely unknown. Furthermore, for these cytotoxic CD4$^+$ T cells to recognize and kill tumor cells, not only must the tumor cell express major histocompatibility class II (MHC-II), but the relevant antigens must also be directed to the appropriate presentation pathway.

In this study, we investigated the capacity of CD4$^+$ T cells specific for the oncoproteins HPV-16 E6 and KRAS$^{G12V}$ to recognize antigen-expressing tumor cells. While both TCRs were able to directly recognize endogenously processed and presented antigen directed to the endosomal compartment of human leukocyte antigen (HLA)-matched B cells, neither was capable of recognizing antigen-expressing tumor cells with induced expression of MHC-II. Overall, our results suggest that only certain
antigens are presented directly on MHC-II+ tumor cells, which may limit the pool of CD4+ T cells capable of directly recognizing and eliminating tumor cells.
Results

Identification of HPV-16 E6-specific CD4⁺ and CD8⁺ T cell responses from TIL of HNSCC

We screened a series of individual TIL cultures isolated from 2-4mm tumor fragments from a patient (Hu-56) with HPV-16⁺ head and neck squamous cell carcinoma (HNSCC) (Supplemental Table S1) by IFN-γ ELISpot assays to identify T cell responses directed against HPV-16 viral oncoproteins E6 and E7 using peptide pools comprised of overlapping 15mers spanning the length of the E6 and E7 proteins. Notably, TIL fragments 12 and 29 generated a significant number of spots over background when restimulated with the E6 peptide pool and contained both CD4⁺ and CD8⁺ T cells (Figure 1A, Supplemental Figure S1A). To identify the relevant T cell subsets, we analyzed these TIL cultures using a cytokine secretion assay. This revealed TIL fragment 29 (F29) contained E6-reactive CD8⁺ T cells while fragment 12 (F12) contained both CD4⁺ and CD8⁺ T cells capable of recognizing the E6 peptide pool (Figure 1B). None of the CD4⁺ T cells produced IL-5 in response to E6 restimulation, confirming a Th1-like phenotype. To determine the T cell receptor (TCR) clonality of E6-specific TIL, we sorted single IFN-γ-secreting CD4⁺ T cells from F12 and CD8⁺ T cells from F12 and F29. TCR sequencing revealed a single TCR expressed by IFN-γ-secreting CD4⁺ T cells (31/31 cells sequenced), as well as a single TCR clone shared by IFN-γ-secreting CD8⁺ T cells from both F12 and F29 (40/41 and 37/38 cells sequenced, respectively) (Supplemental Table S2). These results indicate that monoclonal CD4⁺ and CD8⁺ T cells reactive to E6 are present within TIL from this patient.

HLA-A*02:01-restricted CD8⁺ T cells from TIL recognize E6 presented by a patient-derived tumor cell line

TIL from F29 produced IFN-γ when restimulated with the E629-38 peptide, suggesting that the F29 TCR recognized this previously described HLA-A*02:01-restricted epitope (Supplemental Figure S1B) (11). This was confirmed by tetramer-binding studies using E629-38/HLA-A*02:01 to label TCR-deficient J76 cells expressing human CD8 and using a previously described E628-38-specific TCR as a positive control (Figure 2A). Both TCRs exhibited similar levels of functional avidity as demonstrated by upregulation of the acute activation marker CD69 on J76 following stimulation with autologous B-LCLs pulsed with titrated concentrations of E629-38 peptide (Figure 2B, C). These data confirm the presence of a CD8⁺ T cell clone specific for a known epitope of E6 with similar functional characteristics to a TCR that has been tested in a clinical setting (16).
We next sought to determine whether the E6-specific CD8\(^+\) TCR could recognize endogenously-expressed antigen and thereby mediate anti-tumor cytotoxicity. To do this, we utilized a well-characterized HLA-A*02:01\(^+\) HPV16\(^+\) tumor line, CaSki, as well as an autologous tumor cell line (HNSCC-56) generated through cellular reprogramming of primary tumor tissue as previously described (17) for in vitro cytotoxicity assays. RNA-sequencing analysis confirmed expression of HPV-16 early genes, including E6, by both the primary tumor sample and the autologous cell line (Supplemental Figure S1C). Primary human CD8\(^+\) T cells expressing the F29 TCR were able to mediate cell killing of both CaSki and HNSCC-56 at a range of effector to target ratios in vitro (Figure 2D-E). Together, these data confirm that the F29 TCR recognizes endogenously-expressed E6 antigen.

**Identification of an HLA-DQ-restricted E6-specific CD4\(^+\) T cell from TIL**

To expand E6-specific TIL for further functional analysis, we used the previously described rapid expansion culture protocol with OKT-3 and irradiated allogeneic PBMCs to expand TIL from F12. While the primary F12 culture originally contained both CD8\(^+\) and CD4\(^+\) T cells with E6 reactivity, the final cell product after rapid expansion contained 99% CD4\(^+\) T cells, of which roughly 38.33% demonstrated reactivity against E6 as demonstrated by intracellular cytokine staining for IFN-\(\gamma\) (Figure 3A). In addition to IFN-\(\gamma\), stimulated E6-specific CD4\(^+\) TIL were capable of secreting TNF-\(\alpha\), but not IL-2 (Supplemental Figure S2A). While the total frequency of GrzmB positive cells did not increase with antigen stimulation, gating on TNF-\(\alpha\)-secreting cells revealed that E6-specific CD4\(^+\) TIL contained more stored intracellular GrzmB than non-specific TIL (Supplemental Figure S2B). IFN-\(\gamma\)-secreting CD4\(^+\) TIL also expressed the coinhibitory marker PD-1, consistent with published signatures of tumor-reactive CD4\(^+\) TIL (Supplemental Figure S2C) (18, 19). E6-specific CD4\(^+\) TIL demonstrated a high functional avidity, as these cells were able to secrete cytokines at peptide concentrations less than 1 \(\mu\)g/ml (Figure 3B).

To determine specificity of the F12 TIL culture, we pulsed autologous B-LCLs with individual peptides corresponding to each of the 37 peptides contained in the original E6 pool (Supplemental Table S3). CD4\(^+\) TIL from F12 secreted IFN-\(\gamma\) when stimulated with peptides E6\(_{1-15}\) and E6\(_{5-19}\), which share an 11 amino acid core sequence (Supplemental Figure S2D). Coculture experiments in the presence of HLA-blocking antibodies revealed significantly diminished IFN-\(\gamma\) secretion by TIL when B-LCLs were blocked with anti-HLA-DQ antibodies but not anti-HLA-DR or isotype control antibodies (Figure 3C). To determine which HLA-DQ alleles are responsible for presentation of this epitope, we pulsed B-LCLs expressing known HLA-DQ alleles (Supplemental Table S4) with E6\(_{1-15}\) and found that
KAS011 cells expressing HLA-DQA1*01:02 and DQB1*05:02, but not Hu-195 B-LCLs expressing DQA1*05:05 and DQB1*03:01, were capable of presenting antigen to the TIL in a comparable manner to autologous Hu-56 B-LCLs (Figure 3D).

Finally, we sought to confirm that the TCR sequence identified previously was indeed responsible for E6 recognition. Expression of the F12 TCR in primary human healthy donor CD4+ T cells was sufficient to promote recognition of the E6_{1-15} peptide, as evidenced by upregulation of CD69 and PD-1 (Figure 3E). Taken together, these results demonstrate the identification of a novel HLA-DQ-restricted epitope of E6 recognized by monoclonal CD4+ TIL.

**E6-specific CD4+ T cells do not recognize or kill autologous tumor cells expressing MHC-II**

Recent studies have identified genetic signatures of cytotoxic CD4+ TIL in some human cancers (14, 15). However, whether cytotoxic CD4+ T cells play a role in HPV-driven cancers is unknown. To determine whether E6-specific CD4+ T cells from TIL were capable of directly recognizing HNSCC-56, we first investigated its MHC-II expression status. While the tumor cells expressed no detectable surface MHC-II in culture, treatment with IFN-γ for 72 hours was sufficient to induce upregulation of MHC-II (Figure 4A). Next, we cultured HNSCC-56 with CD4+ F12 TIL. Importantly, MHC-II+ tumor cells preconditioned with IFN-γ were unable to stimulate CD4+ TIL as measured by IFN-γ secretion (Figure 4B). However, IFN-γ-treated tumor cell lines pulsed with E6_{1-15} prior to the addition of TIL were able to stimulate a T cell response, indicating that surface expression of the restricting HLA-DQ alleles in a cell line that endogenously expresses E6 was not sufficient for tumor recognition but required exogenous loading of target peptide. The same requirement of exogenous target peptide loading was observed with CIITA-transduced tumor cells (HNSCC-56 CIITA), which constitutively express high levels of surface MHC-II (Figure 4B).

To test whether or not CD4+ TIL were capable of recognizing an endogenously processed and presented version of the E6 epitope on an APC, we retrovirally transduced autologous B-LCLs with an expression construct encoding the first 50 amino acids of E6 fused to the MHC-I transmembrane domain (E6-MITD), which induces localization of the fused amino acid sequence to the plasma membrane and supports presentation on MHC-II via endosomal trafficking (20). B-LCLs expressing the E6-MITD construct were capable of stimulating E6-specific CD4+ TIL, indicating that the peptide epitope recognized by these TIL can be naturally processed and presented on MHC-II when directed to the appropriate pathway (Figure 4C).
Next, we sought to determine whether the F12 E6-specific CD4+ TIL were capable of direct tumor cytotoxicity. Consistent with the data from our recognition assays, E6-specific CD4+ TIL were unable to limit the growth of HNSCC-56 tumor cells, which do not express MHC-II (Figure 4D). Furthermore, HNSCC-56 CIITA tumor cells also grew uninhibited in the presence of CD4+ TIL. However, growth of HNSCC-56 CIITA tumor cells pulsed with E6_{1-15} peptide was inhibited in an effector cell dose-dependent manner. Overall, these data suggest that while HNSCC-56 are capable of presenting E6 epitopes to CD8+ T cells, E6-specific CD4+ TIL are unable to directly recognize and lyse MHC-II+ autologous tumor cells.

It is known that genes involved in antigen presentation are frequently mutated in cancers, which can prevent recognition by T cells. To determine whether HNSCC-56 tumor cells contained any somatic mutations precluding antigen presentation on MHC-II, we performed whole exome sequencing of tumor cells and peripheral blood mononuclear cells (PBMCs) from Hu-56 as a reference sample. Of the 104 identified nonsynonymous somatic mutations, there were no apparent mutations in components of the MHC-II presentation pathway, including HLADA, HLADMB, and CD74 (Supplemental Table S5).

Identification of an HLA-DRB5*01:01-restricted KRAS^{G12V}-specific TCR from blood of a PDAC patient

Following these results, we set out to determine whether the inability of tumor cells to present antigen on MHC-II was true for other oncogenes. Given that circulating tumor-specific T cells have been identified in human cancer patients (8, 21), we stimulated PBMCs from a patient (Hu-66) with pancreatic ductal adenocarcinoma (PDAC) (Supplemental Table S1) with a pool of peptides corresponding to common oncogenic mutations (Supplemental Table S6) for 14 days, prior to restimulation with individual peptides to identify relevant T cell responses by ELISpot. These results indicated the presence of a T cell response directed against KRAS^{G12V}, as evidenced by an increased number of IFN-γ spots over background in response to KRAS^{G12V} amino acids 1-15 (Figure 5A). To identify the relevant TCRs associated with this response, we again employed the cytokine secretion assay to sort IFN-γ-secreting cells. The cytokine secretion assay revealed specific production of IFN-γ by CD4+ T cells in response to restimulation with KRAS^{G12V} (Figure 5B). Comparing the TCRβ chains from sorted IFN-γ-secreting cells with those present in unexpanded PBMC from this patient revealed a single TCR clonotype present at the highest frequency among both populations (Figure 5C, Supplemental Table S2). These results indicate that the frequency of this T cell clone was expanded at baseline in PBMC, suggestive of a naturally arising in vivo response rather than in vitro priming in our expansion culture.
To confirm the antigen specificity of this TCR, we expressed it in primary human CD4+ T cells by retroviral transduction and cultured these cells with autologous B-LCLs pulsed with either the mutant KRAS^{G12V} or corresponding wildtype peptide. Engineered T cells secreted IFN-γ when stimulated with the mutant, but not wildtype, KRAS peptide, confirming that this TCR is specific for KRAS^{G12V} (Figure 5D). Consistent with our results from the E6 TCR, engineered T cells expressing the KRAS^{G12V}-specific TCR were also able to recognize B-LCLs expressing a fragment of KRAS^{G12V}, but not wildtype KRAS, directed to the endosome by inclusion of an MITD, suggesting that this TCR recognizes endogenously processed and presented antigen (Figure 5D).

To identify the restricting HLA allele, we repeated these peptide stimulation experiments using B-LCLs expressing different combinations of HLA genes. B-LCLs expressing the common HLA-B7-DR15-DQ6 haplotype were all able to stimulate the engineered T cells, while B-LCLs without these alleles were not (Figure 5E). Ultimately, stimulation of these cells by peptide pulsed IHW03304, a mouse DAP3 cell line transfected with human HLA-DRB5*01:01, confirmed this as the restricting HLA molecule (Figure 5F).

**MHC-II+ NCI-H2444 and DAN-G tumor cells do not present KRAS^{G12V}-derived epitope to CD4+ T cells**

Next, we investigated the capacity of tumor cells to directly present KRAS^{G12V}-derived epitopes on MHC-II. To generate HLA-matched, MHC-II+ cell lines for these studies, we transduced the KRAS^{G12V+} cell lines NCI-H2444 and DAN-G, derived from human lung and pancreatic cancers respectively, with CIITA and a construct encoding HLA-DRB5*01:01 with a truncated CD34 reporter gene (Figure 6A). Consistent with our previous results, MHC-II+ tumor cells expressing the restricting HLA allele were unable to stimulate TCR-engineered T cells unless target cells were first pulsed with exogenous peptide (Figure 6B). Importantly, there were no listed somatic mutations in MHC-II presentation genes (HLADMA, HLADMB, CD74) for either NCI-H2444 or DAN-G in publicly available sequencing data (22). NCI-H2444 CIITA cells grew uninhibited in the presence of TCR-engineered T cells, while NCI-H2444 CIITA cells pulsed with KRAS^{G12V} peptide experienced delayed tumor growth (Figure 6C). Consistent with the results obtained for HNSCC-56, CD8+ T cells expressing an HLA-A*03:01-restricted KRAS^{G12V}-specific TCR (23) were capable of recognizing NCI-H2444 cells, but not CaSki cells, which express HLA-A*03:01 but not KRAS^{G12V} (Figure 6D). These results demonstrate that a distinct oncogene present in the cytosol is also unable to be presented by MHC-II+ tumor cells despite being effectively presented on MHC-I.
Expression of coinhibitory ligands such as PD-L1 by tumor cells is known to limit TCR signaling in T cells. To determine whether this mechanism may be preventing recognition of MHC-II+ tumor cells by CD4+ T cells, we cultured CD4+ T cells expressing our KRASG12V-specific TCR or the E6-specific F12 TCR with NCI-H2444 CIITA and HNSCC-56 CIITA, respectively, with or without anti-PD-L1 blocking antibodies. Blocking of PD-L1/PD-1 interactions was not sufficient to promote tumor cell recognition (Supplemental Figure S3). Similarly, addition of anti-CD28 agonist antibody did not modulate tumor cell recognition. These results suggest that a lack of antigen presentation rather than the presence or absence of coinhibitory or costimulatory signals, respectively, is responsible for this phenotype.

Some studies suggest that endogenous proteins internalized from the plasma membrane are preferentially loaded onto MHC-II for presentation compared to other subcellular compartments (24). While KRAS proteins are frequently associated with membranes via lipid modifications, they do not express a transmembrane domain and these interactions are not stable (3, 25). We therefore reasoned that anchoring the immunogenic fragment of KRASG12V to the plasma membrane of tumor cells may enable direct antigen recognition by CD4+ T cells. However, tumor cells expressing the KRASG12V-MITD construct, as evidenced by expression of a P2A-linked eGFP reporter, were similarly unable to stimulate TCR-engineered CD4+ T cells (Supplemental Figure S4). These results suggest that subcellular oncogene localization is not the sole determinant of direct presentation on MHC-II by tumor cells. In addition, these results suggest that overexpression of the antigen is not sufficient to promote presentation on MHC-II.

Our results thus far suggest that tumor-specific CD4+ T cells may be more likely to recognize antigen presented by APCs than tumor cells themselves. Therefore, we assessed the capacity of HLA-DRB5*01:01+ dendritic cells (DCs) to present antigen derived from exogenous protein. HLA-matched DCs generated in vitro were capable of stimulating TCR-engineered CD4+ T cells expressing the KRASG12V-specific TCR when immature DCs were either pulsed with the KRASG12V 20mer peptide or fed KRASG12V whole protein, but not KRAS wildtype protein (Figure 6D). These results suggest that while the TCR is unable to recognize endogenous antigen presented by tumor cells, it can recognize cross-presented exogenous antigen in the context of DCs.
Discussion

The goal of this study was to determine whether TCRs isolated from naturally primed T cells obtained from cancer patients could recognize tumor cells expressing oncogenes. With respect to the class I (HLA-A*02:01)-restricted HPV16 E629-38 TCR obtained from HNSCC TIL, recognition was - perhaps unsurprisingly - affirmed for both an autologous tumor cell line and a well-characterized HLA-matched and E6-expressing tumor cell, CaSki (Figure 2). The same TIL (Figure 1) also contained significant numbers of IFN-γ-producing E6-specific CD4+ T cells (Figure 3) which, in contrast, failed to recognize E6-expressing tumor cells, even when they were induced to express ample surface MHC class II of the correct presenting allele (DQA1*01:02/DQB1*05:02) by IFN-γ treatment or CIITA transduction. (Figure 4). Importantly, CIITA expression is known to induce not only the surface expression of MHC-II proteins, but also HLA-DM and invariant chain (Ii) molecules which are required for antigen processing and presentation (26). HLA-matched B cells, in contrast, were recognized by this TCR when the antigen was directed to the MHC-II pathway. A similar situation was observed in the case of a KRASG12V-specific HLA-DRB5*01:01-restricted TCR which could recognize HLA-matched B cells, but not MHC-II-expressing tumor cells when antigen was directed to the MHC-II presentation pathway. Both TCRs were found to possess sufficient functional avidity to mediate recognition of endogenously expressed antigen under the conditions described above, and both were capable of mediating cytotoxicity of peptide-loaded target cells. This latter observation mirrors the circumstance presented in several reports on cytotoxic CD4+ T cells, although our data extends the context of this observation by showing that endogenous expression of the source antigen and induction of MHC-II by IFN-γ is unlikely to yield a physiologic target on epithelial-derived tumor cells for TCRs against nuclear or membrane-associated antigens.

Recent studies from human bladder cancer and melanoma have identified genetic signatures corresponding to cytotoxic CD4+ T cells among TIL (14, 15). Functionally, these cytotoxic CD4+ TIL are capable of direct MHC-II-dependent tumor recognition, ultimately leading to target lysis by granzymes in a manner similar to their CD8+ counterparts. However, therapeutic translation of these findings may be limited by the fact that few solid tumors express MHC-II. Indeed, two independent studies suggest that only around a third of melanoma patients exhibit any MHC-II+ tumor cells, and the frequency of these cells within the tumor is often less than 10% (15, 27). Instead, the predominant cellular source of MHC-II in the tumor microenvironment appears to be infiltrating leukocytes (24).
Although some solid tumor cells do express either constitutive or inducible MHC-II, our results indicate that despite the presence of oncogene-specific CD4+ T cells among TIL and PBMC, these cells are unable to directly recognize and lyse antigen-expressing MHC-II+ tumor cells, whereas epitopes from the same protein may be effectively presented to CD8+ T cells on MHC-I (13). This suggests that simply expressing a target antigen may not be sufficient to confer recognition by CD4+ T cells for the subset of tumors that express MHC-II. Furthermore, these results appear to be consistent across multiple tumor types, as the cell lines queried in this study include those derived from HNSCC, lung adenocarcinoma, and pancreatic cancer.

Direct CD4+ T cell recognition of melanoma cell lines expressing MHC-II naturally or following CIITA transduction has been reported (28, 29). However, in both of these studies a significant fraction of the TCRs tested did not recognize tumor cells directly. It is unclear whether these TCRs represent “bystander” clonotypes that do not recognize tumor antigens or tumor-specific TCRs that are unable to recognize their cognate antigen due to antigen presentation deficiencies such as those highlighted in this study. Oliveira et al demonstrate that direct recognition by CD4+ T cells was restricted to two melanomas with extremely high tumor mutational burdens, suggesting that this phenotype may confer additional necessary alterations permitting MHC-II presentation of tumor antigens (29).

While attaching an MITD to the immunogenic fragment of KRASG12V did not result in direct recognition of tumor cells by CD4+ T cells, studies have described a bias for endogenous peptides derived from membrane-bound proteins eluted from MHC-II, presumably due to membrane recycling (24, 30). The melanoma antigen Trp1 exists in the plasma membrane, which may facilitate direct presentation to CD4+ T cells by B16 tumor cells (31). Another melanoma-associated antigen, gp100, is presented by tumor cells to CD4+ T cells in a manner dependent on its transmembrane domain (32). In recent work investigating the role of neoantigen-specific CD4+ T cells in a murine sarcoma model, epitopes derived from a mutated integrin subunit, also a membrane-bound protein, were eluted from MHC-II on CIITA-transduced tumor cells (33). Our results suggest that while membrane-bound proteins may be preferentially trafficked to endosomes for direct presentation on MHC-II, the presence of an immunogenic membrane protein alone is not sufficient to promote this presentation.

Other non-classical presentation pathways have been described that may allow cytosolic or nuclear proteins access to MHC-II, including autophagy-associated presentation of intracellular proteins and TAP-dependent presentation to CD4+ T cells, whereby antigenic peptides are presumably transferred from MHC-I to MHC-II during membrane recycling (34, 35). Direct MHC-II-dependent
recognition of autologous tumor cells by CD4$^+$ T cells specific for E7 epitopes has been reported (36, 37), however, these studies both concern E7 proteins expressed by less prevalent oncogenic HPV subtypes (HPV-33 and HPV-59) presented by cervical cancer cells on HLA-DR molecules. It is therefore possible that HPV subtype, tumor histology, and restricting HLA alleles may also be relevant factors in the differential presentation of nuclear HPV antigens on MHC-II$^+$ tumor cells.

Although our data suggests that direct tumor cytotoxicity is not a likely mechanism of E6- and KRAS$_{G12V}$-specific CD4$^+$ T cells, other antitumor mechanisms of CD4$^+$ T cells have been described and adoptive transfer of tumor-specific CD4$^+$ T cells in patients with cancer has demonstrated antitumor activity (7, 38, 39). Therefore, MHC-II-restricted TCRs such as those identified in this study may be useful for ACT in human patients given their targeting of epitopes derived from driver oncoproteins restricted to HLA haplotypes estimated at 1.27% and 16.10% of the United States caucasian population, respectively, for HLA-DQA1*01:02/DQB1*05:02 and HLA-DRB5*01:01 (40). Notably, CD4$^+$ T cells aid in the priming of tumor-specific CD8$^+$ T cells by licensing antigen-bearing DCs in a CD40L-dependent manner (41–43). We demonstrate that the KRAS$_{G12V}$-specific TCR identified in this study does recognize cross-presented antigen in the context of DCs, suggesting these cells may be able to contribute to antitumor immunity via this function. CD4$^+$ T cells may also provide local help for CD8$^+$ T cells within tumors by secreting cytokines such as IL-2 and IFN-$\gamma$, supporting CD8$^+$ T cell survival and recruitment to the tumor (44). In addition, CD4$^+$ TIL may recognize antigen in the tumor microenvironment on myeloid cells such as macrophages. Indeed, preclinical studies have demonstrated that in the absence of MHC-II on tumor cells, adoptively transferred Trp1 CD4$^+$ T cells are still able to exert antitumor immunity dependent on IFN-$\gamma$ and correlating with increased cytotoxic activity of macrophages (45). However, this phenomenon appears to be dependent on tumor antigen secretion, which we would also speculate is minimal in the case of most oncogenes. Overall, our study highlights the selectivity of direct presentation of endogenous antigens by MHC-II$^+$ tumor cells and demonstrates that neither CIITA expression nor the presence of a membrane-bound antigen is sufficient alone to promote direct recognition of tumor cells by CD4$^+$ T cells. Further studies are needed to characterize which antigens may be presented directly by MHC-II$^+$ tumor cells under what conditions to fully understand the context-dependent roles of CD4$^+$ T cells in cancer.
Methods

Cell culture
TIL were harvested and cultured as previously described (46). Briefly, primary tumor tissue was dissected into 2-3 mm fragments which were placed in a 24 well plate and cultured in RPMI-1640 supplemented with 10 percent human serum AB, penicillin-streptomycin, HEPES, gentamicin, and 6000 IU/ml IL-2. Extravasated TIL were cultured by half media replacements every 2-3 days. Primary human T cells from peripheral blood were cultured in 50/50 media consisting of 50 percent AIM V plus 50 percent RPMI-1640 supplemented with 10% human serum AB, penicillin-streptomycin (100 units/ml each, Gibco), 300 IU/ml IL-2, 5 ng/ml IL-7, and 5 ng/ml IL-15. Patient-derived tumor cell lines were generated and cultured as previously described (17). Briefly, primary tumor tissue was cut into <2mm fragments and dissociated using a GentleMACS Octo Dissociator (Miltenyi). Tumor cells were resuspended in F-Media containing 5 µM Rho Kinase inhibitor and plated over a bed of irradiated 3T3 fibroblasts (30 Gy). After initial culture, tumor cells were propagated in a 3:1 mix of irradiated 3T3 conditioned media and F-Media containing 5 µM Rho Kinase inhibitor. CaSki (ATCC), NCI-H2444 (ATCC), DAN-G (ATCC), J76 Jurkat (ATCC), 3T3-CD40L, and B lymphoblastoid cell lines (B-LCLs) were maintained in RPMI media supplemented with L-glutamine and HEPES (10mM, Gibco), 10% fetal bovine serum (FBS), 1mM sodium pyruvate (Gibco), 1x MEM Non-Essential Amino Acids (Gibco), and penicillin-streptomycin (100 units/ml each, Gibco). 293GP (ATCC) were maintained in DMEM media supplement with 10% FBS and penicillin-streptomycin (100 units/ml each, Gibco). IHW03304, GM3107, KAS011, D8, and D66 cells were gifts from Alessandro Sette (La Jolla Institute for Immunology, La Jolla, CA).

ELISpot assays
TIL or PBMC were plated at 100,000 cells per well in ELISpot plates coated with anti-interferon-γ (IFN-γ) antibodies (1-D1K, Mabtech). Cells were restimulated with HPV-16 E6 and E7 PepMix (JPT) peptide pools, peptide pool representing selected oncogenic mutations, or indicated individual peptides at 5 µg/ml for pools or 10 µg/ml for peptides for 22 hours before developing ELISpot plates according to the manufacturer’s instructions (Mabtech). 5 µg/ml PHA-L was used as a positive control.

Ex vivo expansion culture
PBMC were plated with peptide pool representing selected oncogenic mutations at 5 µg/ml. Fresh media containing 10 IU/ml IL-2 was added on days 4, 7, and 10. On Day 14, PBMC were restimulated for use in either ELISpot or cytokine secretion assays.

**TCR sequencing**

TIL or PBMC were restimulated with 5 µg/ml HPV-16 E6 PepMix (JPT) or KRAS<sup>G12V</sup> peptide and IFN-γ-secreting cells were fluorescently tagged using the Human IFN-γ Secretion Assay kit according to manufacturer instructions (Miltenyi). Single IFN-γ positive cells were sorted into a 96 well plate using a FACS Aria (BD) and RNA sequencing was performed using an Illumina Miseq to identify paired TCR alpha and beta chains. For identification of the E6-specific CD4<sup>+</sup> TCR clone, IFN-γ-secreting cells were sorted as above and TCR alpha and beta sequences were amplified by nested, multiplexed PCR as previously described (47). PCR products were submitted for Sanger sequencing (ETON Biosciences). For TCRβ frequency analysis, PBMCs pre- and post-14 day ex vivo expansion with indicated peptides were sent to Adaptive Biotechnologies.

**HPV expression analysis**

RNA was isolated from PT-56 primary tumor cells and tumor cell line and submitted for bulk RNA sequencing. Paired-end sequencing reads were mapped using BWA (v0.7.12) (48) to the HPV complete genome (Genbank accession NC_001526.2) and its corresponding transcriptome. The resulting SAM file was converted to a sorted BAM and indexed, followed by counting mapped reads using SAMtools (v1.2) (48).

**Whole exome sequencing**

Whole exome sequencing and RNA-seq were performed by Tempus Labs (Chicago IL) using their XE (whole exome + RNA) products. FFPE biospecimens were distributed by the Moores Cancer Center to Tempus for next generation sequencing along with matched reference DNA using peripheral blood specimens. Sequence reads from Exome-Seq of the tumor and normal samples were aligned to the reference genome GRCh38 using SpeedSeq Align (v0.1.0) (49). Exome variants were identified using SpeedSeq Somatic and variants were annotated using SNPeff (v4.3i) (50).

**T cell retroviral transduction**
TCR nucleotide sequences were synthesized and cloned in the MSGV1 retrovirus backbone using a BioXP 3200 (Codex). The sequence was codon optimized for expression in human cells. The human TCR constant regions were exchanged for mouse TCR constant regions with substitutions to enhance surface expression and promote preferential pairing (51, 52). Human PBMC were isolated from buffy coats. CD4+ or CD8+ T cells were removed by magnetic selection and the negative fraction containing all remaining lymphocytes was cultured in T cell media with 50 ng/ml anti-CD3 antibody (OKT3) for 2 days. TCR retroviral supernatants were generated by cotransfection of 293GP cells with the MSGV1 vector containing the relevant TCR sequence and RD113 plasmid. Two days after transfection the retroviral supernatants were harvested and either used fresh or frozen at -80ºC. Transductions were performed on RetroNectin-coated plates (Takara) as previously described. Murine TCRβ constant region expression was assessed by flow cytometry to determine transduction efficiency. TCR-engineered T cells were used no sooner than day 7 following initial stimulation.

**T cell functional assays**

5 x 10⁴ TIL, transduced T cells, or J76 Jurkat cells were cocultured with 1 x 10⁵ B-LCLs pulsed overnight with indicated concentrations of peptide or 5 x 10³ tumor cells seeded the previous day in 96 well U-bottom or flat-bottom plates respectively. Where indicated, anti-CD28 agonist (clone CD28.2, Biolegend) or anti-PD-L1 blocking (clone 29E.2A3, Biolegend) antibodies were added to T cell and tumor cell cocultures at 5 and 10 µg/ml respectively. For cytokine and activation marker assays, 1x Cell Activation Cocktail (Biolegend) containing PMA and Ionomycin was used as a positive control. For HLA blocking studies, 20 µg/ml of the following antibodies were added to peptide pulsed B-LCLs at least 2 hours prior to addition of T cells: anti-HLA-DQ (Tü169, Biolegend), anti-HLA-DR (L243, Biolegend), or Mouse IgG2a isotype control (MOPC-173, Biolegend). Where indicated, tumor cells were cultured for 72 hours with 10 ng/mL recombinant IFN-γ prior to addition of T cells. Supernatants were harvested after 18-24 hours and IFN-γ was measured by ELISA (Thermo Scientific) and cell pellets were stained for flow cytometry. Cytotoxicity assays were performed by coculture of T cells with tumor cells at indicated effector-to-target ratios. Briefly, 5 x 10³ tumor cells were seeded and cultured overnight before adding T cells at the indicated ratios. Target cell lysis was determined using the xCELLigence Real-Time Cell Analyzer (ACEA Biosciences) which assessed electrical impedance due to cell adherence every 30 minutes until the end of the experiment. The data was analyzed using the xCELLigence RTCA
software package and results were reported as cell index normalized to 1 at the time when T cells were added.

**MHC-II presentation assay**

Synthetic DNA constructs encoding the following were generated and cloned into MSGV1 using a BioXP 3200 (Codex): The N-terminal 25 amino acids of the human HLA-B gene, followed by the first 50 amino acids of HPV-16 E6 or 25 amino acids of KRAS\(_{G12V}\) fused to the C terminal 55 amino acids of human HLA-B, a T2A self-cleaving element, and the coding sequence of eGFP. Viral supernatants were generated as described above and autologous B-LCLs were transduced on retronectin coated plates after overnight stimulation on a bed of irradiated (50 rad) 3T3 cells expressing human CD40L (3T3-CD40L). Transduction efficiency was assessed by flow cytometric quantification of eGFP expression. Transduced B-LCLs were stimulated with 3T3-CD40L in the presence of 200 IU/ml recombinant human IL-4 (Peprotech) for two successive rounds of 2-3 days prior to coculture with autologous TIL.

**Generation of dendritic cells for antigen presentation assays**

Dendritic cells were generated using the plastic adherence method as previously described. Briefly, frozen PBMCs were thawed and plated in DC media (RPMI 1640 supplemented with L-glutamine, 5% heat-inactivated human AB serum, and 100 units/ml each penicillin-streptomycin) for 2 hours at 37°C. Nonadherent cells were removed by washing with warm PBS and adherent cells were cultured in DC media supplemented with 800 U/ml GM-CSF (Tonbo) and 500 U/ml IL-4 (Peprotech) for 6 days. 1 µg/ml KRAS\(_{G12V}\) peptide or 20 µg/ml KRAS\(_{G12V}\) or wildtype KRAS protein (Abcam) was added directly to immature DCs overnight. The next day, DCs were harvested and cocultured with TCR-engineered T cells at a 1:1 ratio overnight before assessing CD137 upregulation by flow cytometry.

**Retroviral transduction of tumor cells**

The coding sequence of human CIITA was cloned into MSGV1 by Gibson Assembly. The coding sequences of HLA-DRB5*01:01 alpha and beta chains separated by a P2A sequence were cloned into a modified pHAGE lentiviral vector upstream of a T2A sequence followed by a truncated CD34 reporter sequence by Gibson Assembly. Retroviral supernatants were generated with MSGV1 as described above. Lentiviral supernatants were generated by cotransfection of 293T cells with pHAGE, tat, rev,
gag/pol, and vsv-g plasmids. Viral supernatants were collected 24 hours later and concentrated by centrifugation in Amicon Ultra 5 tubes (Sigma Aldrich). Tumor cells were plated and one day later the media was replaced with retroviral or lentiviral supernatant supplemented with 10 µg/mL Polybrene. Lentiviral 4 hours later, RV supernatant was aspirated and replaced with culture media. MHC-II+ and CD34+ tumor cells were sorted using a FACS Fusion cell sorter (BD Biosciences).

Flow Cytometry

Cells were labeled with fluorochrome-conjugated monoclonal antibodies to the following antigens: anti-human CD3-PE (OKT3, Tonbo), CD4-PE-Cyanine7 (SK3, Tonbo), CD8-APC (Hit8a, Tonbo), CD34-Alexa Fluor 647 (581, Biolegend), CD69-PerCP-Cyanine5.5 (FN50, Biolegend), CD137-PE (4B4-1, Miltenyi), PD-1-BV650 (EH12.2H7, Biolegend), HLA-II-APC (Tü39, Biolegend), and anti-mouse TCRβ-APC (H57-597, Tonbo). Dead cells were stained with either DAPI or LIVE/DEAD Fixable Aqua (Invitrogen). E6_{29-38}-HLA-A*02:01-PE tetramer was assembled and labeled by the NIH Tetramer Core Facility. Cells were stained with 1 µg/mL tetramer for 1 hour on ice. Antibody staining for surface antigens was performed for 15 minutes on ice. For intracellular cytokine staining (ICS), T cells were cocultured with peptide pulsed APCs for 4-6 hours in the presence of Brefeldin A. Cells were permeabilized and stained for intracellular cytokines with the Cytofix/Cytoperm Kit (BD Biosciences) after surface staining according to manufacturer's instructions. The following fluorochrome-conjugated antibodies were used for cytokine staining: IFN-γ-APC (4S.B3, Biolegend), IL-2-FITC (MQ1-17H12, Biolegend), TNF-α-BV785 (Mab11, Biolegend), and Granzyme B-PE (QA16A02, Biolegend). Data were acquired with a BD FACSCelesta flow cytometer (BD Biosciences) and analyzed with FlowJo v10.7 software (FlowJo).

Statistics

Statistical analyses were performed with Prism 8 (GraphPad Software). For ICS comparisons, an unpaired T test was used. For comparing HLA blocking antibody-mediated suppression of T cell signaling, a two-way ANOVA was used. P values of <0.05 were considered statistically significant.

Study Approval
The de-identified human samples used in this study were obtained under informed consent through University of California San Diego (UCSD) IRB protocol 101391CX “Tumor Environment Phenotyping and Cell Isolation.” Written informed consent was provided by the individuals in the study.

**Author contributions:** S.E.B., M.S.N., and S.P.S. designed the experiments. S.E.B., M.S.N., R.R.T., A.B., L.M., and M.B. performed experiments. A.L.R.P. and J.A.G. performed bioinformatic analyses under supervision of B.P. S.E.B. and M.S.N. analyzed data and performed statistical analyses. E.E.W.C. and A.M.M. provided clinical samples and data. S.E.B. and M.S.N. prepared the figures and wrote the manuscript with S.P.S. Order of co-first authors was determined by an objective assessment of the relative amount of the work attributable to each author by the last author.

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References


**Figure Legends**

**Figure 1: Identification of E6 reactive CD4⁺ and CD8⁺ TIL from HPV-16 positive HNSCC.**

A IFN-γ ELISpot assay results from tumor-infiltrating lymphocytes (TIL) derived from different tumor fragment cultures stimulated with indicated peptide pools at 5 µg/ml or 5 µg/ml PHA-L as a positive control. Data represent the average of 3 technical replicates in a single experiment. B Cytokine secretion assay results from TIL fragments with ELISpot reactivity demonstrating IFN-γ and IL-5 production from both CD8⁺ and CD4⁺ T cell subsets after stimulation with 1 µg/ml of indicated peptide pools. TIL subsets with E6-specific IFN-γ production greater than DMSO controls (designated with bolded gates) were sorted for T cell receptor (TCR) sequencing.
Figure 2: Functional characteristics of an HLA-A*02:01-restricted E6-specific TCR from Hu-56 TIL. 

A Histogram demonstrating 1 µg/ml HLA-A*02:01/E629-38 tetramer binding to Jurkat cells expressing an irrelevant TCR, a previously described TCR with known E6 specificity, or the TCR identified among the dominant CD8+ clonotype in patient TIL. B Representative FACS plots demonstrating specific upregulation of CD69 on Jurkat cells when stimulated with 1 µg/ml E629-38 peptide. C Functional avidity curves comparing activation of TCR-expressing Jurkat cells stimulated with a titration of peptide concentrations. Data represents the mean +/- SEM for 3 independent experiments. D Cellular impedance measurements to determine the kinetics of tumor cell death after recognition of CaSki cells by primary human T cells expressing the F29 TCR compared to mock transduced donor T cells. Normalized cell index values over time for E:T ratios 10:1, 3:1, and 1:1 are
shown compared to tumor cells cultured without effectors. Data are represented as mean values of 3 technical replicates +/- SEM. Data are representative of 2 independent experiments. E Cellular impedance measurements to determine the kinetics of tumor cell death after recognition of autologous HNSCC-56 tumor cell line by primary human T cells expressing the F29 TCR compared to mock transduced donor T cells. Normalized cell index values over time for E:T ratios 10:1, 3:1, and 1:1 are shown compared to tumor cells cultured without effectors. Data are represented as mean values of 3 technical replicates +/- SEM. Data are representative of 2 independent experiments.
Figure 3: Functional characteristics of clonally expanded HLA-DQ-restricted E6-specific CD4+ TIL. **A** Representative FACS plots (left) and quantification (right) of intracellular cytokine staining for IFN-γ from TIL fragment 12 post rapid expansion protocol after stimulation with 1 µg/ml E6 pepmix. Data represents the mean +/- SEM from 3 independent experimental replicates. **** p<0.0001 unpaired T test. **B** IFN-γ production by CD4+ TIL stimulated with a titration of E6,1-15 peptide pulsed onto autologous B-LCLs. Data represent mean +/- SEM from 2 independent experimental replicates. **C** Normalized IFN-γ production by CD4+ TIL cultured with 1 µg/ml E6,1-15 peptide pulsed B-LCLs preincubated with indicated HLA blocking antibodies. Data represents mean +/- SEM from 3 independent experimental replicates. ** p<0.01 two-way ANOVA. **D** IFN-γ production by CD4+ TIL stimulated with indicated B-LCL lines pulsed with 1 µg/ml E6,1-15 expressing mismatched HLA-DQ alleles. **E** Representative FACS plots (left) showing upregulation of activation markers PD-1 and CD69 after culturing TCR-transduced primary CD4+ T cells with autologous B-LCLs alone or pulsed with 1 µg/ml E6,1-15. Quantification of CD69 expression by TCR-transduced primary CD4+ T cells (right). Data represent 2 independent experiments.
Figure 4: Autologous tumor cells fail to present endogenous E6\textsubscript{1-15} on MHC-II to CD4\textsuperscript{+} T cells. 

A Representative histograms of FACS staining for HLA-II surface expression on HNSCC-56 tumor cells left unstimulated, cultured with IFN-\textgreek{g} for 72 hours, or transduced with CIITA. 

B IFN-\textgreek{g} production by CD4\textsuperscript{+} TIL after culture with autologous tumor cells with or without addition of 1 \( \mu \)g/ml E6\textsubscript{1-15} peptide. Data represent mean +/- SEM of 2 independent experimental replicates. 

C IFN-\textgreek{g} production by CD4\textsuperscript{+} TIL after culture with autologous B-LCLs, -LCLs stably expressing E6\textsubscript{1-50} with an endosomal targeting sequence, or PMA + Ionomycin. Data represent mean of 2 independent experimental replicates. 

D Cellular impedance measurements to determine the kinetics of tumor cell death after recognition of autologous HNSCC-56 tumor cell line by E6-specific CD4\textsuperscript{+} TIL compared to HNSCC-56 CIITA or HNSCC-56 CIITA pulsed with 1 \( \mu \)g/ml E6\textsubscript{1-15}. Normalized cell index values over time for E:T
ratios 10:1, 3:1, and 1:1 are shown compared to tumor cells cultured without effectors. Data are represented as mean values of 3 technical replicates +/- SEM. Data are representative of 2 independent experiments.
Figure 5: Identification of an HLA-DRB5*01:01-restricted KRAS$^{G12V}$-specific TCR from blood of a PDAC patient. A IFN-γ ELISpot assay results from ex vivo expanded PBMC restimulated with 5 µg/ml indicated peptides or pools. Data represent the average of 3 technical replicates in a single experiment. B Cytokine secretion assay results from ex vivo expanded PBMC restimulated with 5 µg/ml indicated peptides. C Correlation of TCRβ frequencies among PBMC pre- and post- ex vivo peptide expansion. D IFN-γ secretion by TCR-engineered primary CD4$^+$ T cells stimulated with autologous B-LCLs pulsed or electroporated with 1 µg/ml indicated peptides or in vitro transcribed RNA, respectively. Data representative of 4 independent experiments. E IFN-γ secretion by TCR-engineered primary CD4$^+$ T cells stimulated with indicated B-LCL lines pulsed with 1 µg/ml KRAS$^{G12V}$ peptide. Data representative of 2 independent experiments. F PD-1 upregulation by TCR-engineered primary CD4$^+$ T cells stimulated with a DRB5*01:01 transfected murine fibroblast cell line pulsed with 1 µg/ml indicated peptides. Data representative of 2 independent experiments.
Figure 6: MHC-II+ KRAS\textsuperscript{G12V+} tumor cells do not present KRAS\textsuperscript{G12V}-derived epitopes to CD4\textsuperscript{+} T cells. A Expression of CD34 reporter gene and HLA-DQ/DR/DP by indicated human tumor cell lines transduced with HLA-DRB5*01:01 and CIITA. B Representative FACS plots (left) and quantification (right) of CD137 upregulation after coculture of TCR-engineered CD4\textsuperscript{+} T cells with designated CIITA/HLA-DRB5*01:01-expressing tumor lines with or without 1 \mu g/ml KRAS\textsuperscript{G12V} peptide. Data represent two independent experiments. C Cellular impedance measurements to determine the kinetics of tumor cell death after recognition of NCI-H2444 CIITA tumor cell lines by KRAS\textsuperscript{G12V}-specific TCR-engineered CD4\textsuperscript{+} T cells compared to cells pulsed with 1 \mu g/ml KRAS\textsuperscript{G12V} or wildtype KRAS peptide. Normalized cell index values over time for E:T ratio 10:1 is shown compared to tumor cells cultured without effectors. Data are represented as mean values of 3 technical replicates +/-
SEM. Data are representative of 2 independent experiments. **D** Quantification of CD137 upregulation by TCR-engineered CD8\(^+\) T cells expressing an HLA-A\(^*\)03:01-restricted TCR specific for KRAS\(^{G12V}\) cocultured with CaSki or NCI-H2444 tumor cells. Data represented as the mean of 2 independent experimental replicates. **E** Quantification of CD137 upregulation by TCR-engineered CD4\(^+\) T cells cocultured with HLA-matched DCs pulsed with 1 \(\mu\)g/ml KRAS\(^{G12V}\) peptide or fed 20 \(\mu\)g/ml KRAS\(^{G12V}\) or KRAS whole protein overnight. CD137 upregulation was normalized to the peptide-pulsed control sample. Data represented as the mean of 2 independent experimental replicates.