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Cancer-specific T helper shared and neo-epitopes uncovered by expression of the MHC class II master regulator CIITA

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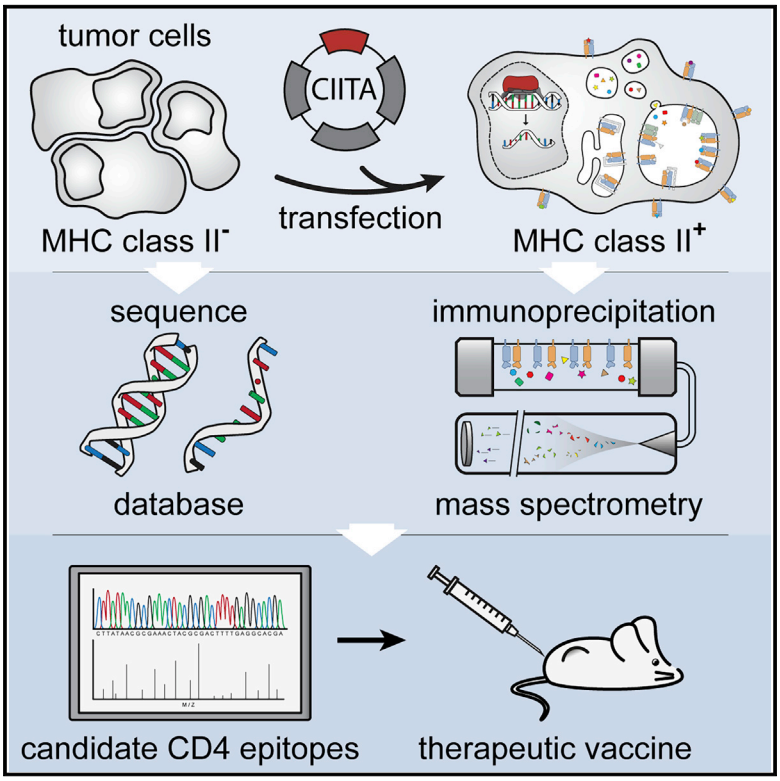
Graphical abstract



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In brief

Hos et al. show that expression of CIITA in tumor cells induces MHC class II presentation of relevant helper T cell antigens that can be identified by mass spectrometry. This method provides a platform to identify helper eptitopes and improve understanding of CD4 T cell immunity against cancer.

Highlights

- CIITA transfection of tumor cells induces cell-surface expression of MHC class II
- CIITA induces functional processing machinery for MHC class II-presented peptides
- MS analysis identifies CD4 tumor peptides of viral, shared, or mutanome origin
- Identified T helper neo-antigens are relevant targets for immunotherapy of cancer

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Cancer-specific T helper shared and neo-epitopes uncovered by expression of the MHC class II master regulator CIITA

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SUMMARY

We report an approach to identify tumor-specific CD4$^+$ T cell neo-epitopes of both mouse and human cancer cells by analysis of major histocompatibility complex (MHC) class II-eluted natural peptides. MHC class II-presented peptide sequences are identified by introducing the MHC class II transactivator (CIITA) in tumor cells that were originally MHC class II negative. CIITA expression facilitates cell-surface expression of MHC class II molecules and the appropriate peptide-loading machinery. Peptide elution of purified MHC class II molecules and subsequent mass spectrometry reveals oncoviral- and neo-epitopes as well as shared epitopes. Immunological relevance of these epitopes is shown by natural presentation by dendritic cells and immunogenicity. Synthetic peptide vaccination induced functional CD4$^+$ T cell responses, which helped tumor control *in vivo*. Thus, this CIITA transfection approach aids to identify relevant T helper epitopes presented by any MHC class II allele that would be otherwise very difficult to predict and reveals important targets for cancer immunotherapy.

INTRODUCTION

It is well documented that antigen-specific CD4$^+$ T helper responses are highly relevant in cancer immunity even when tumors are major histocompatibility complex (MHC) class II negative (Hung et al., 1998; Ossendorp et al., 1998). Their importance is attributed to CD4$^+$ T cell-dependent stimulation of effector CD8$^+$ T cell responses with improved lytic- and tumor-infiltrating capacity (Ahrends et al., 2017; Borst et al., 2018; Bos and Sherman, 2010; Hu et al., 2000; Kreiter et al., 2015; Kumai et al., 2017; Ossendorp et al., 1998; Shedlock and Shen, 2003). The relevance of neo-antigen-derived MHC class II epitopes for therapeutic immune responses in murine tumor models has been convincingly shown (Alspach et al., 2019; Kreiter et al., 2015). Therefore, the identification of tumor-specific T helper epitopes receives increased attention and has mostly centered around established prediction algorithms. However, limitations in defined anchor motifs of MHC class II-binding peptides makes available algorithms inaccurate.

In recent years, mass-spectrometric analysis of MHC-eluted peptides from the tumor cell surface has been combined with whole-exome sequencing (WES) and prediction algorithms to identify potential tumor-associated antigens (TAAs) and neo-antigens in available protein databases (Amir et al., 2011; Bassani-Sternberg, 2018; Castle et al., 2012; Hos et al., 2020; Kreiter et al., 2015; Ott et al., 2017; Sahin et al., 2017; Yadav et al., 2014). The general lack of MHC class II expression on tumor cells makes this approach less than suitable for the identification of MHC class II-presented peptides. Recent studies focused on advanced *in silico* approaches for MHC class II binding prediction algorithms, but these require validation for each MHC class II haplotype, which is difficult to apply broadly (Abelin et al., 2019; Andreatta et al., 2018). A mass-spectrometric filtering step similar to CD8$^+$ T cell epitope discovery is generally missing due to the low yield of purified MHC class II molecules isolated from cancer cells. An alternative method could be inducing MHC class II expression in tumor cells through transfection of MHC class II genes. This technique requires transfection of multiple patient-specific MHC class II genes and may also result in unwanted presentation of endoplasmic reticulum (ER)-loaded peptides, in contrast to natural loading of peptides in the endosomal route as in antigen-presenting cells (APCs) (Armstrong et al., 1997). Inclusion of MHC class II presentation machinery genes to prevent ER loading even further increases the complexity of multiple gene transfection with additional constructs like invariant chain CD74 and MHC-DM. We have circumvented these disadvantages by a simple transfection protocol with a single cDNA construct encoding the class II transactivator

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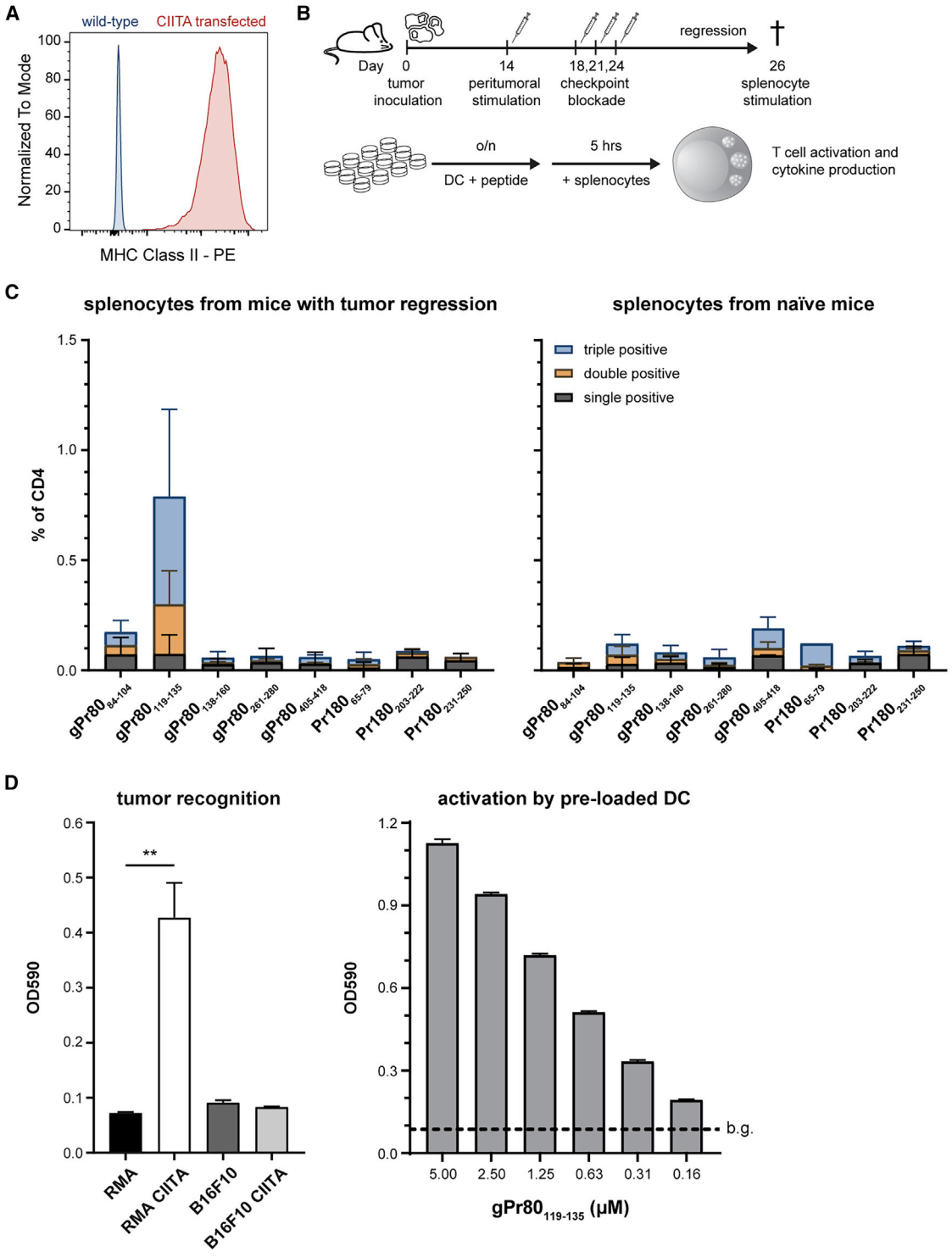

Figure 1. MHC class II-presented T helper epitopes identified from oncoviral antigens in CIITA-transfected RMA lymphoma cells

(A) CIITA transfection resulted in stable expression of MHC class II I-A^b molecules on RMA lymphoma cells, which are of MHC class II-negative origin.

(B) C57BL/6 mice were inoculated by subcutaneous injection of 2 × 10^3 RMA cells in the right flank. After 2 weeks, established tumors were treated by an immunotherapeutic protocol consisting of peritumoral injection of CpG and agonistic OX-40 antibody, followed up by intraperitoneal injections of antagonistic PD-L1 antibody. Splenocytes from cured mice were acquired 26 days after inoculation and stimulated with synthetic-peptide preloaded DCs for the induction of antigen-specific cytokine production.

(legend continued on next page)

(CIITA). Expression of CIITA in APCs and B cells naturally functions as a recruiter of the MHC class II transcription complex, leading to the expression of all class II presentation machinery components (Reith et al., 2005; Roche and Furuta, 2015; Unanue et al., 2016). Transfection of this single gene will lead to expression of all endogenous MHC class II alleles and a natural peptide-loading machinery in the cancer cells of interest (Siegrist et al., 1995). Recent work by Alspach et al. applied the transfection of the T3 sarcoma cell line with CIITA for the confirmation of presentation by an algorithm-predicted neo-antigen (Alspach et al., 2019). In addition, Forlani et al. used the CIITA transfection to identify epitopes in known TAAs of primary glioblastoma and RA cell lines (Forlani et al., 2020). In this study, we explored this CIITA expression method as primary method for allotype-independent MHC class II-presented epitope discovery in cancer cell lines through a combined approach of exome sequencing and mass-spectrometric analysis of eluted peptides.

Here, we established the potential of this approach in identifying neo-epitopes in human colorectal cancer. We were able to determine the reliability of the approach by identification of known T helper epitopes derived from murine oncoviral antigens and disclose additional epitopes of established murine melanoma TAAs. In addition, we could identify several unique T helper neo-epitopes with single amino acid mutations in murine colorectal cancer models, which were functional as shown by tumor control upon synthetic peptide vaccination. Therefore, this single gene transfection approach is a feasible method for identification of immunologically relevant cancer-associated T helper epitopes.

RESULTS

T helper epitopes in oncoviral antigens

It is well established that $CD4^+$ T cell responses can target virally encoded peptide antigens in oncovirus-induced cancers (Boon et al., 1994). We analyzed the MHC class II-negative murine leukemia virus (MuLV)-induced lymphoma cell line RMA for virus-specific T helper epitopes. CIITA transfection of RMA cells resulted in stable expression of MHC class II (Figure 1A). The lysate of transfected cells was subsequently used for MHC class II pull-down by Y3P antibody-coated columns and was followed by elution of MHC class II-bound peptides. Mass-spectrometric analysis of eluted peptides revealed eight major sequences with several length variants derived from MuLV (Table S1). Six apparently undescribed MHC class II-binding sequences were identified, while $gPr80_{138–160}$ and $gPr80_{119–135}$ were relatively similar to previously described MuLV T helper epitopes MuLV $env_{145–158}$ (Rudensky et al., 1991) and F-MuLV $env_{122–141}$ (Iwashiro et al., 1993), respectively. Remarkably, the sequence derived from the gPr80 glycosylated envelope polyprotein, or env-$gPr80_{119–135}$, was similar but two amino acids shorter compared with a 19-mer epitope relevant in local and CD4-helped CTL responses against RMA lymphoma, as intensively studied in our group (positions 120–138) (Ossendorp et al., 1998). With the help of CIITA transfection, we could identify a naturally presented 17-mer-length variant of this relevant helper epitope by our elution approach.

To determine whether RMA tumors could raise helper T cell responses *in vivo* with specificity toward our eluted sequences, we treated wild-type RMA tumor-bearing mice with a potent immune-modulatory combination therapy consisting of CpG, antagonistic anti-PD-L1, and agonistic anti-OX40 antibodies and subsequently analyzed the induced T cell responses. (Figure 1B). Tumors fully regressed 24 days after inoculation. Two days later, splenocytes were acquired and stimulated with peptides synthesized according to the eluted sequences to determine specific T cell responses (see Figure 1C). Peptide $gPr80_{119–135}$ significantly stimulated type 1 cytokine responses in the polyclonal $CD4^+$ T cell population (Figures 1C and S1), showing that at least one of the MHC class II-eluted candidate epitopes was functionally recognized in the immunotherapeutic T cell response to this tumor.

Since wild-type RMA tumor cells are MHC class II negative, the T helper responses raised to the gPr80 MuLV sequence were likely induced by MHC class II antigen presentation of endogenous dendritic cells *in vivo*. To determine whether CIITA-transfected tumor cells present peptides similar to APCs, we used the $CD4^+$ T hybridoma reporter cell line 3A12-Z, which was originally generated from an *in-vivo*-induced $CD4^+$ T cell clone specific for the $gPr80_{120–138}$ epitope (Ossendorp et al., 1998) and can efficiently recognize MuLV-infected dendritic cells (DCs) (Figure S2). When co-cultured with tumor cell lines, the 3A12-Z cell line specifically reacts to the CIITA-transfected RMA cell line, while it does not recognize wild-type, non-transfected RMA cells or the CIITA-transfected MHC class II-expressing control cell line B16F10 (Figure 1D). Furthermore, DCs exogenously loaded with the synthetic peptide of the eluted $gPr80_{119–135}$ sequence strongly activated 3A12-Z in a concentration-dependent manner. These data support that the MHC class II-presented peptides on CIITA-transfected tumor cells are similar to antigenic peptide sequences presented in MHC class II of DCs processed from endogenous (viral protein) and exogenous (peptide) origins. The fact that this independently identified epitope induced a tumor-directed T helper immune response *in vivo* emphasizes the validity of our approach.

Melanoma-associated T helper epitopes

For both human and mouse, several melanoma-derived TAAs, as well as so-called cancer-testis antigens, have been described to elicit relevant CD8 and CD4 T cell responses (Boon et al., 1994). Using our method we found several MHC class

(C) Intracellular staining established CD40L, IFNγ, and interleukin-2 (IL-2) expression in $CD3^+CD4^+$ splenocytes and was used to determine single-, double-, and triple-positive $CD4^+$ T cells (see Figure S1 for gating strategy). Average peptide-specific responses of each mouse are shown (n = 3, error bar indicates SD).
(D) 3A12-z CD4 T hybridoma lacZ reporter cell line previously raised against the MuLV $gPr80_{120–138}$ epitope (18) was co-cultured with control RMA and CIITA-transfected RMA cells. B16F10 melanoma and B16F10-CIITA cells, stably expressing MHC class II, were used as negative controls. Recognition and activation by the reporter cell line was determined through absorption at 590 nm (left) (n = 3, p = 0.0006, two-tailed unpaired t test). 3A12-z activation by preloaded DCs with a titration of $gPr80_{119–135}$ indicated a high cross-reactivity of our reporter cell line with the identified peptide sequence presented on CIITA-transfected cells (right figure).

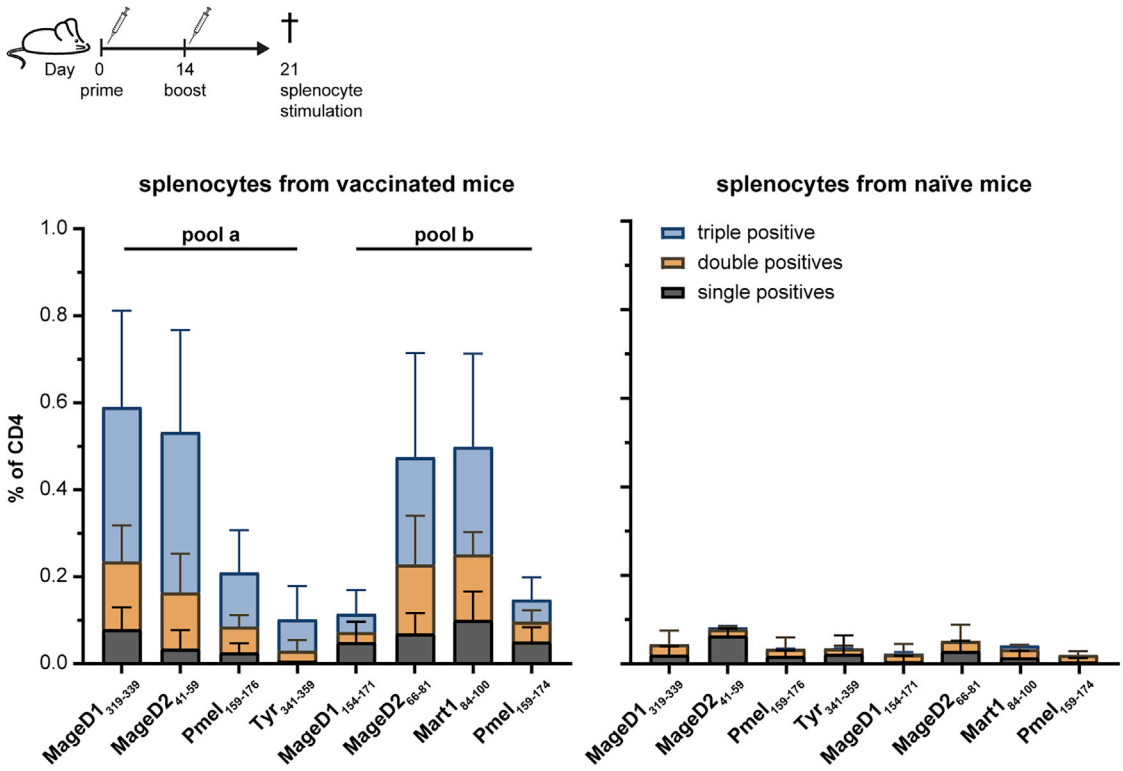

Figure 2. Emerging CD4 epitopes in well-known shared antigens in melanoma

Synthesized peptides from Table S2, with two length variants of the *Pmel*-derived sequence, were divided in two pools of four peptides for a vaccination in naive C57BL/6 mice. Each vaccination contained 20 nmol per peptide and 20 μg TLR9-ligand CpG in PBS and was subcutaneously injected at the tail base. Fourteen days after prime, mice received an identical booster vaccination accompanied by an intraperitoneal injection of 150 μg OX-40 agonistic antibody (OX-86) in PBS. Seven days after the booster vaccination, splenocytes were stimulated with peptide-loaded D1 APCs. Polyfunctional helper 1 responses are based on IL-2, CD40L, and IFNγ expressions. Single-, double-, and triple-positive populations were combined after respective backgrounds were subtracted from each single-, double-, or triple-positive population to indicate objective polyfunctionality of CD4 helper T cells upon stimulation (n = 4 mice per pool, n = 3 for mock, error bars indicate SD).

II-presented peptides different from known epitopes of various TAAs (*Tyr*, *Pmel*, *Mart1*) and cancer-testis antigens (*MageD1*, *MageD2*) in the eluate from CIITA-transfected B16F10 melanoma line (Table S2). Interestingly, several MHC class II-binding peptides were derived from antigens preferably known for CD8-specific immunity, e.g., *Pmel*, and all sequences identified appear to be unknown.

These candidate CD4 TAAs were tested for immunogenicity in naive syngeneic mice in a prime-boost vaccination setting using the synthetic peptides of the eluted sequences. Seven days after the booster vaccination, we performed a readout of established $CD4^+$ immune responses by *ex vivo* stimulation of splenocytes with individual peptides. The $CD4^+$ splenocytes of pool-vaccinated mice displayed polyfunctional type 1 helper responses upon *in vitro* stimulation with all of the identified peptides in higher or lower extent (Figure 2). These identified tumor-specific self-epitopes are currently subject for further *in vivo* studies.

Induced expression of the MHC class II presentation machinery

CIITA-transfected cancer cell lines were analyzed for protein expression of the components of MHC class II presentation machinery. Murine colorectal tumor cell lines from different origins, MC38 and CT26, were transfected with the EBO-Sfi/hCIITA plasmid. Each CIITA-transfected cell line was sorted and selected for high cell-surface expression levels of MHC class II (Figure 3A). Confocal and flow cytometric analyses of MC38 (Figure 3B) and CT26 (Figure S3A) showed cell-surface expression of MHC class II at high levels in contrast to non-transfected cells. Notably, I-A or I-E expression could not be increased by interferon γ (IFNγ) treatment of these tumor cell lines (data not shown). We analyzed the MHC class II chaperone CD74 (Ii, or invariant chain), which prevents loading of ER-originating peptides and guides MHC class II to late endosomes (Armstrong et al., 1997; Cresswell, 1996; Roche and Furuta, 2015; Suri et al., 2006; Unanue et al., 2016), and H2-DM, which assists binding of peptides in the MHC class II loading compartments (Denzin and Cresswell, 1995; Pos et al., 2012; Roche and Furuta, 2015; Rock et al., 2016; Unanue et al., 2016) Flow cytometry analysis and confocal microscopy imaging showed CIITA-induced expression of CD74 and H2-DM of transfected cell lines (Figures 3C and S3B). Moreover, the intracellular staining of both components indicates correct subcellular localization (Rock et al., 2016). Expansion of

A

Murine line	I-A/E	CD74	DM
MC38	++	+/-	++
CT26	+++	++	++

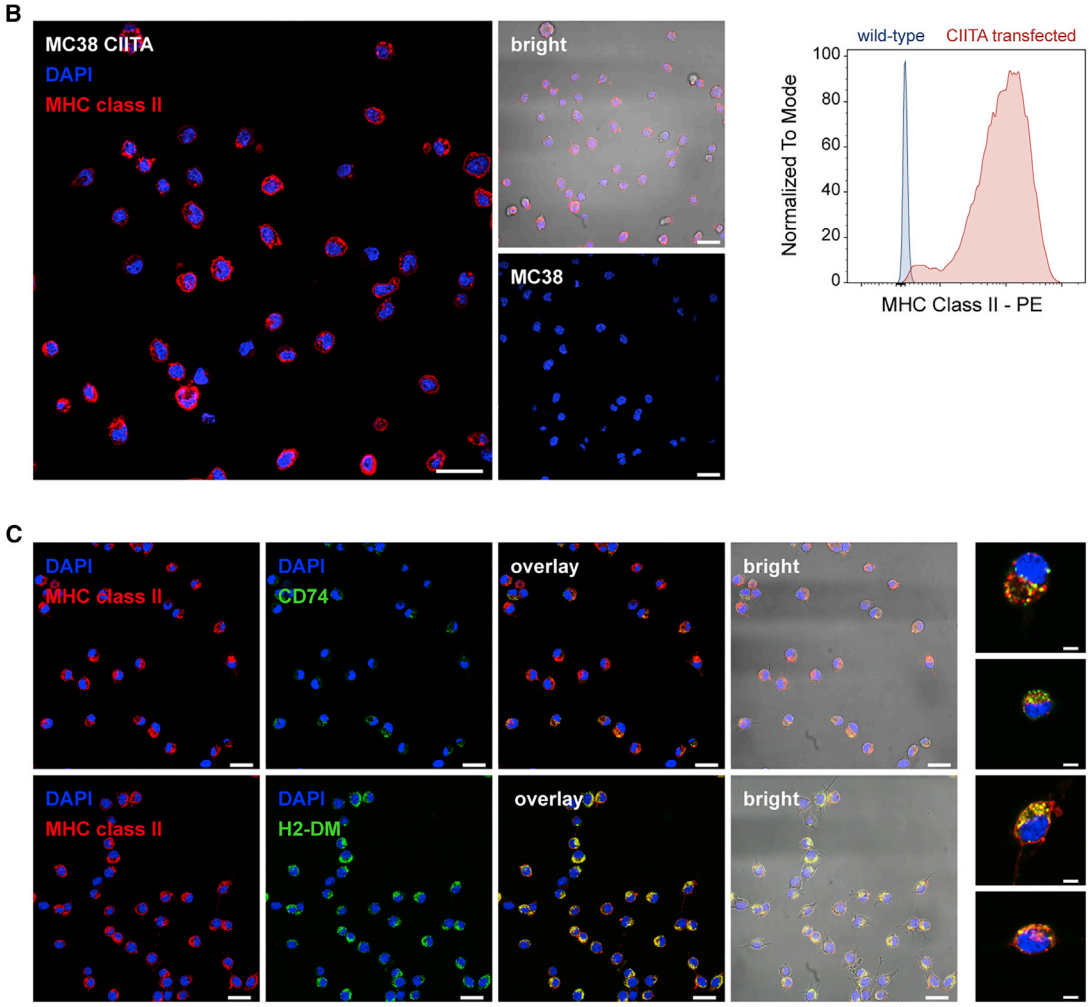

Figure 3. CIITA transfection induces stable expression and subcellular localization of the MHC class II presentation machinery

CIITA-transfected murine colorectal tumor cell lines were characterized for their expression of MHC class II presentation machinery components.

(A) Expression levels of MHC class II presentation machinery components after transfection with CIITA, as determined by flow cytometry (+/−, intermediate; ++, high; +++, very high).

(B) Confocal fluorescence microscopy of CIITA-transfected MC38 (MC38 CIITA) stained for MHC class II (I-A^b) expression and DAPI, compared with non-transfected MC38 (MC38). On the right, flow cytometric analysis of MHC class II expression of CIITA-transfected (red) and control (blue) MC38 cells.

(C) Chaperone proteins CD74 and H2-DM were separately co-stained with MHC class II in the top and bottom row, respectively. Two single-cell zooms were added on the right for detailed visualization of invariant chain staining and H2-DM localization combined with MHC class II. Scale bars indicate 25 μm (wide images) or 5 μm (single-cell images in C).

transfected cells allowed specific MHC class II pull-down from cell lysates, followed by acid elution and mass spectrometry. This resulted in the identification of 3,000–5,000 different peptides per cell line within a false discovery rate of 1% and a mascot score ≥35 (see Figure S4). Gibbs clustering of the identified sequences was performed, and binding groove position-specific enrichments of amino acids for the MHC class II molecules could be shown (Figure S4A). For the C57BL/6 cell lines MC38 and RMA, appropriate patterns could be identified specific for I-A^b, although another grouped pattern was observed in the RMA eluate. Likewise, near-identical I-A^d patterns were observed in the BALB/c-derived CT26 cell line eluate, while I-E^d patterns could not be identified. Peptide lengths were in accordance with expected MHC class II-binding peptide distributions, which peak around 16- and 17-mer (Figure S4B).

Table 1. Exome sequencing of MC38 cells and analysis of MHC class II-presented peptides by CIITA-transfected MC38 cells resulted in the identification of seven sequences containing amino acid variants

Protein source	Gene symbol	Position	Sequence (X = variant)	WT	Length variants
Bone morphogenetic protein receptor type-2	*Bmprll*	317–335	TELP**Q**GDHYKPAISHRDLN	R	6
Discoidin domain-containing receptor 2	*Ddr2*	308–330	SEASEWEP**H**AVYFPLVLDDVNPS	T	5
E3 ubiquitin-protein ligase MYCBP2	*Mycbp2*	79–94	RIYTTALSDRD**L**AGSS	Q	1
Protocadherin 18	*Pcdh18*	479–494	SP**W**AYITTVTATDPDL	G	1
Structural maintenance of chromosomes protein 2	*Smc2*	766–780	Q**N**KAEEKYEALENKM	K	1
Transmembrane protein 131	*Tmem131*	1591–1603	KQR**H**TSPTPASPS	Q	1
Zinc finger MIZ domain-containing protein 1	*Zmiz1*	271–287	RPPADFTQPAA**S**AAAAA	A	1

Amino acid variation, gene symbol, amino acid positions in the source protein, WT amino acid sequence, and the number of identified length variants are indicated.

Identification of tumor-specific MHC class II-presented neo-antigens

WES information combined with mass spectrometric analysis of MHC class II I-A^b-eluted peptides from the CIITA-transfected MC38 colorectal tumor resulted in the identification of seven candidate neo-antigens (Table 1). Eluted neo-antigen peptides varied in length from 13 to 23 amino acids, of which mutated Bmpr2 and Ddr2 gene products were presented with several peptide length variants. All mutated peptide sequences within the presented peptides contained a single amino acid change, which significantly modified these residues in terms of polarity, charge, or hydrophobicity. To determine whether the identified eluted peptides were immunogenic *in vivo*, naive syngeneic mice were prime-boost vaccinated to raise $CD4^+$ T cell responses to the synthetic peptides of these sequences. Seven days post booster vaccination, $CD4^+$ T cell responses were analyzed in the spleen. Out of seven candidates, three neo-antigens (point-mutated sequences of the Ddr2, Pcdh18, and Zmiz1 gene products) induced significant type 1 helper $CD4^+$ responses (Figure 4A). In addition, no cross-reactivity to the wild-type (non-mutated) sequence of two neo-peptides was observed, as determined with *in-vitro*-established T cell lines directed to the Pcdh18 and Zmiz1 mutated peptides. In contrast, T cells to Ddr2 were cross-reactive to both wild-type and mutated peptides (Figure S5A), and no $CD8^+$ T cell responses were observed after vaccination (Figure S5B).

Next, immunotherapeutic efficacy of these immunogenic T helper peptides was explored. The candidate neo-epitopes were divided in two pools of "immunogenic" and "non-immunogenic" neo-antigens. These pools were used to prophylactically vaccinate naive mice and compared with a vaccination group with non-tumor-related antigenic T helper peptides. After a prime-boost vaccination, the animals were challenged with wild-type MC38, and tumor growth was monitored. Average tumor size in the group that received immunogenic neo-antigens Ddr2, Pcdh18, and Zmiz1 was significantly smaller 21 days after challenge compared with mice vaccinated with non-tumor-specific help (MuLV-env, PADRE, OVA), while tumors in mice vaccinated with the non-immunogenic peptide pool (Bmpr2, Mycbp2, Smc2, and Tmem131) were only slightly smaller than the tumor non-specific peptide group (Figures 4B and S5C).

CD8-specific neo-epitopes of the MC38 tumor model have been reported previously, and two neo-antigens were identified as prime targets in MC38-specific anti-tumor immunity: Adpgk and Rpl18 (Hos et al., 2020; Yadav et al., 2014). To test the capacity of our candidate helper peptides to improve tumor-specific cytotoxic T lymphocyte (CTL)-mediated protection, the previously tested helper vaccination groups were combined with both CTL synthetic long peptide antigens in a prophylactic vaccination experiment. This resulted in reduced average tumor growth compared with a non-specific help vaccination and extended survival of mice vaccinated with a combination of CTL and immunogenic helper peptides (Figure S5D). In contrast, the non-immunogenic helper peptides did not improve tumor control compared with the CTL-antigens only.

To determine the therapeutic efficacy of the immunogenic helper neoantigens, tumor-bearing mice were vaccinated with CpG adjuvanted synthetic peptides 6 and 12 days after tumor inoculation. In this setting, the CTL peptides alone could not prevent tumor outgrowth, but we observed a significant tumor control in mice vaccinated with a combination of CTL and helper epitopes and improved survival (Figure 4C). Splenocyte analysis of therapeutically cured mice from a separate experiment indicated that a combinatorial vaccination with CTL and helper peptides improved the induction of polyfunctional neoepitope-specific $CD8^+$ and $CD4^+$ T cell responses (Figure S5D). These results show the immunotherapeutic relevance of the T helper neo-antigen-derived peptides identified with the CIITA method.

T helper neo-epitope in CT26 tumor cell line

The approach was also used for another MHC class II haplotype using the well-known CT26 cell line: a colorectal cancer model established from BALB/c mice. An identical analysis of WES and RNA sequencing data of this cell line combined with CIITA transfection (see Figure S3 for induced expression of MHC class II, DM, and CD74) and purification of MHC class II molecules followed by mass-spectrometric analysis of MHC class II eluted peptides was applied. We could identify a single MHC class II I-A^d-presented neo-epitope with a mutation in the *Magi1* gene, which was detected in five length variants varying from 15 to 19 amino acids in length (within positions [pos.] 370–388) with a centrally located

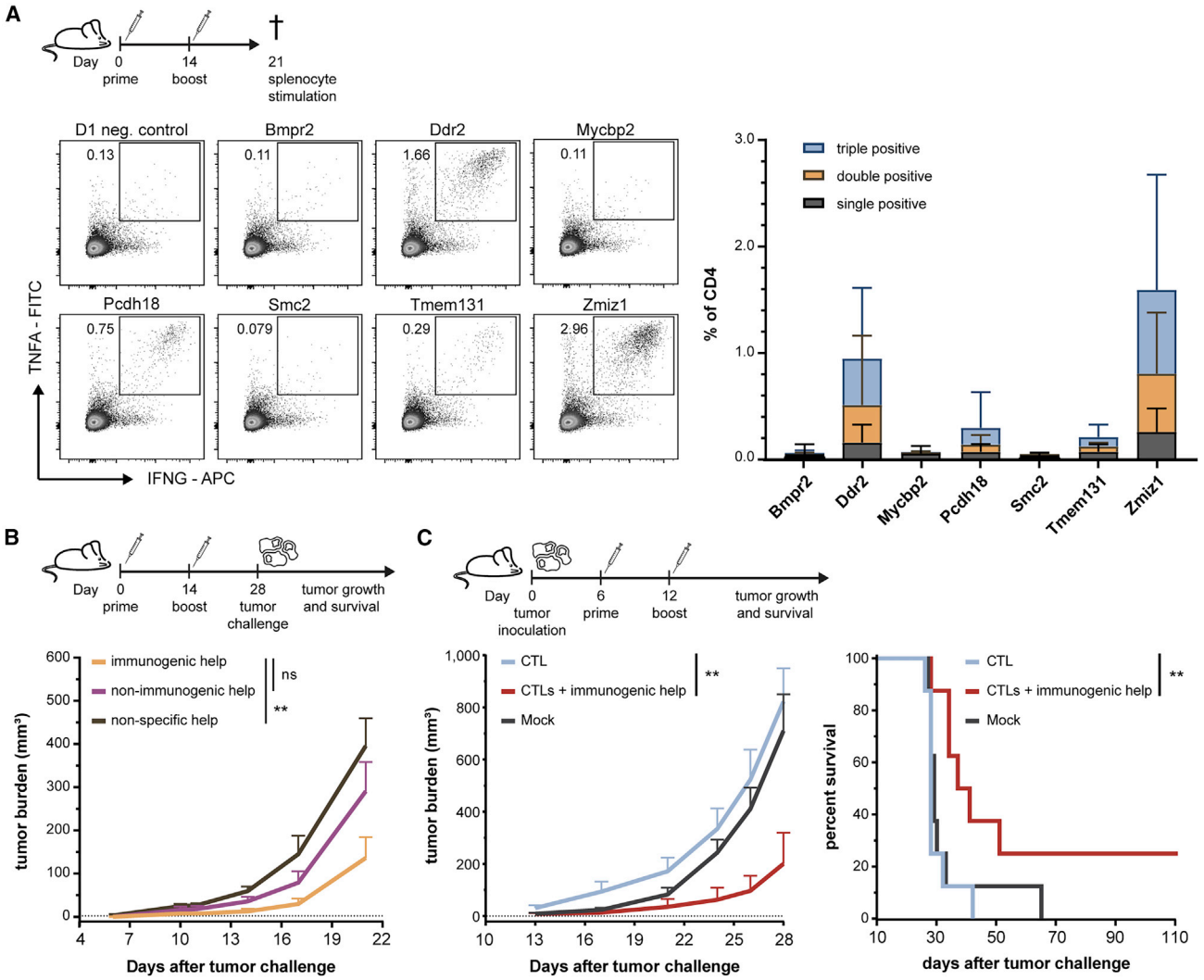

Figure 4. Identification of MHC class II-presented neo-epitopes in colorectal cancer

(A) In three separate experiments, naive C57BL/6 mice were vaccinated in a prime-boost setting with synthetic peptides of the longest identified mutated sequence of each potential neo-antigen. Seven days after boost, splenocytes were *ex vivo* analyzed for specificity by stimulation with synthetic peptide-loaded D1 DCs and subsequent cytokine production of IFNγ, tumor necrosis factor α (TNF-α), and IL-2. (Left) Concatenated cytokine-producing $CD4^+$ populations from a single experiment after stimulation are shown (n = 3). (Right) Combined results are presented from three independent vaccination experiments (n = 39 for Ddr2, Pcdh18, and Zmiz1 peptide pool vaccination; n = 24 for Bmpr2, Mycbp2, Smc2, and Tmem131 peptide pool vaccination). Polyfunctionality was determined similarly to Figure 1C.

(B) Three neo-antigen-specific peptides with "immunogenic" help (Ddr2, Zmiz1, and Pcdh18) were used for prophylactic vaccination and compared with "non-immunogenic" help (Bmpr2, Mycbp2, Smc2, and Tmem131; p = 0.2808) or non-specific help (MuLV-env, PADRE, and OVA; p = 0.0077). Fourteen days after booster vaccination, mice were challenged with 5×10^5 MC38 tumor cells by subcutaneous injection in the right flank. Tumor growth is indicated (n = 7 per group, error bars indicate SEM, p values were determined by two-way ANOVA statistical tests).

(C) Combination of identified immunogenic helper neo-epitopes with previously identified (Hos et al., 2020) CTL neo-epitopes in Rpl18 and Adpgk for therapeutic vaccination setting. Tumors were inoculated by subcutaneous injection of live MC38 cells in the right flank. Six and twelve days after inoculation, mice were vaccinated subcutaneously in the tail-base area with a mix of synthetic peptides and CpG. Tumor burden was followed and limited to a max of 1,000 mm^3. Average tumor growth shows effective control of the CTL+ immunogenic help therapeutic vaccine versus CTL peptide alone (p = 0.0031, n = 8, error bars indicate SEM and statistical significance was determined by a two-way ANOVA statistical test) and long-term improved survival (p = 0.0116, log rank test).

amino acid variant (D > N, pos. 379). We synthesized the shortest (SP) and longest (LP) versions of the discovered sequences and tested immunogenicity *in vivo*. Both peptides were immunogenic and elicited polyfunctional type 1 helper immunity in the $CD4^+$ population (Figure S6). No cross-reactivity with the non-variant wild-type peptide sequences was observed.

Discovery of human MHC class II-presented neoantigens

To determine whether our approach induces class II presentation on MHC class II-negative human tumor cells, we applied CIITA transfection to three human colorectal carcinoma cell lines, RKO, HCT15, and HCT-116. CIITA-induced expression

A

Human line	DP	DQ	DR	CD74	DM
RKO	+++	++	+++	+++	+++
HCT15	++	++	++	++	++
HCT116	++	-	++	++	+

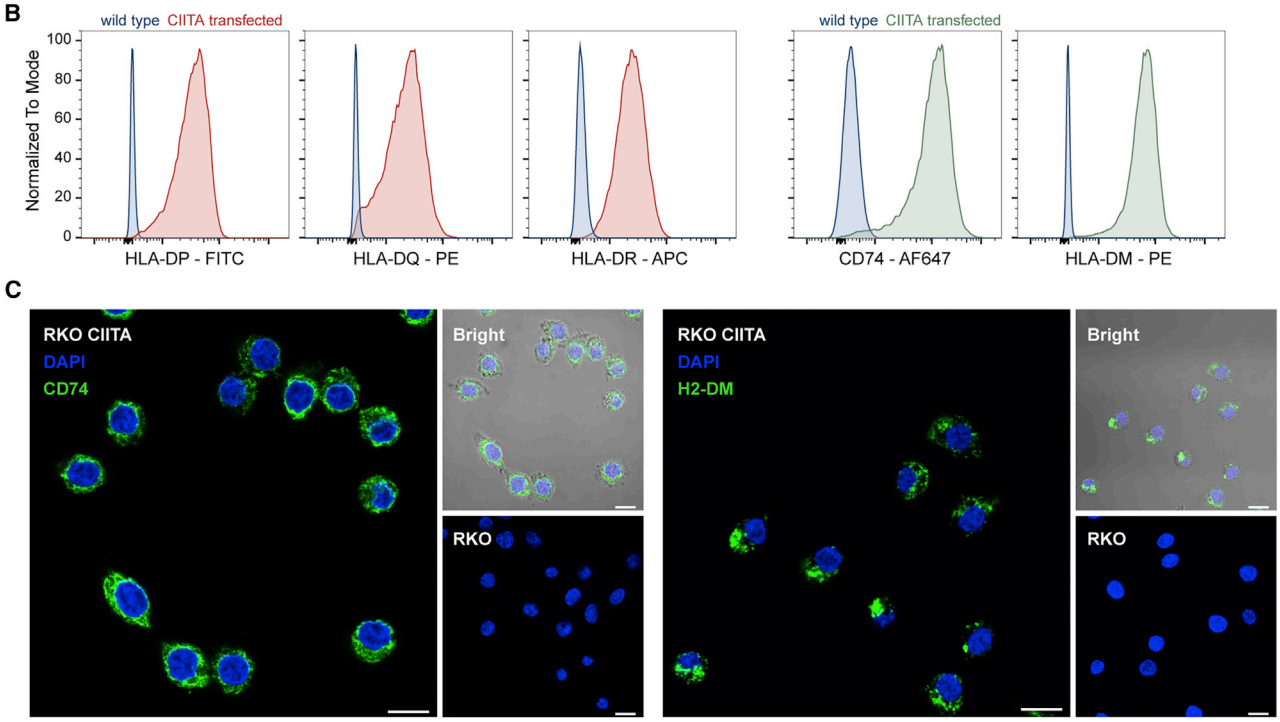

Figure 5. Identification of potential MHC class II neo-antigens in CIITA-transfected human cancer cell lines

(A) Summary of the CIITA-transfection-induced expression levels of MHC class II and chaperone proteins in human colorectal cell lines, as determined by intracellular staining.

(B) Flow cytometric analysis of MHC class II presentation components in CIITA-transfected RKO cells (HLA-DP, -DQ, and -DR: red; HLA-DM and CD74: green) versus non-transfected (wild-type) RKO cells (blue).

(C) Confocal imaging after staining of CD74 (left) and HLA-DM (right) in CIITA-transfected and non-transfected RKO cells.

of all three human MHC class II molecules, HLA-DR, -DQ, and -DP, was established in the cell lines after transfection, and each CIITA-transfected cell line was sorted and selected for high cell-surface expression levels of MHC class II (Figures 5A and S7A). We used RKO for further analysis of all three HLA class II alleles (Figure 5B). CIITA transfection induced CD74 and HLADM expression as shown by flow cytometry and confocal imaging (Figures 5B and 5C). Subsequently, HLA class II eluates of -DR, -DQ, and -DP molecules were collected separately and analyzed by mass-spectrometric analysis. We characterized the peptide patterns by Gibbs clustering and length distribution of the three eluates (Figure S7B). Gibbs clustering of HLADR resulted in the elucidation of known enrichments at the anchor positions 1 and 9, while the HLADP and -DQ elutions were unique. Length distributions were normal in all elutions, with the majority of the sequences between 13 and 19 amino acids long. Detailed analysis of individual peptides revealed 10 independent gene products that contained single amino acid variants, as shown in Table 2. These results show the potential of this approach to detect MHC class II-presented neo-antigens in human cancer cells.

DISCUSSION

The search for reliable methods to identify T cell neo-epitopes is central in personalized approaches for immunotherapeutic treatment of cancer. Technological progress in WES and mass-spectrometric analysis of MHC-eluted peptides improved confidence and allows physiologically relevant peptide identification presented by MHC on the tumor cell surface. A lack of expressed MHC class II on most tumor cells prevents such a wet molecular approach for the identification of $CD4^+$ T helper epitopes. Several studies indicated that these epitopes are essential for the activation of $CD8^+$ T cells in the priming phase as well as (recall) effector phases (Ahrends et al., 2017; Borst et al., 2018; Ossendorp et al., 1998; Schepers et al., 2002). In this study, we have independently developed a straightforward and versatile method to uncover T helper (neo-)epitopes from cancer cells: a single gene transfection

Table 2. MHC class II-eluted peptide sequences from CIITA-transfected RKO cells containing amino acid variants

Protein source	Gene symbol	Position	Sequence (X = variant)	WT	HLA precipitate	Length variants
V-type proton ATPase 116 kDa subunit a isoform 2	*Atp6v0a2*	184–200	KVEAFEKMLWRVCKGY**A**	T	DP	1
Insulin-like growth factor 2 receptor	*Igf2r*	2149–2164	SLGDIYFKLFRASG**V**M	D	DP	3
Frizzled 6	*Fzd6*	633–650	VDGKGQAGSVSESA**Q**SEG	R	DQ	5
Solute carrier family 4, anion exchanger, member 2	*Slc4a2*	181–195	E**T**TPRASKGAQAGTQ	A	DQ	1
Neurosecretory protein VGF (precursor)	*Vgf*	380–394	**G**EDEEAAEAEAEAEE	E	DQ	1
Dihydrolipoamide dehydrogenase (precursor)	*Dld*	105–120	NSHYYHMAHGKDF**V**SR	A	DR	2
N-ethylmaleimide-sensitive factor	*Nsf*	562–575	FPFI**N**ICSPDKMIG	K	DR	1
PDZ and LIM domain protein 7 isoform 1	*Pdlim7*	133–147	GQ**S**LRPLVPDASKQR	P	DR	1
Ubiquitin chain A (or B)	*Uba52*	36–55	IPPDQQRLIFA**S**KQLEDGRT	G	DR	1
Ubiquitin-conjugated enzyme E2 D3	*Ube2d3*	12–28	ALKRINKELSDL**V**RDPP	A	DR	1

Amino acid variants were identified by comparing our mass spectrometry-identified peptides against the publicly available COSMIC database with known mutations in RKO. Ten potential neo-epitopes with identified length variants are shown with the mutated variants indicated in bold/underlined text.

method with a cDNA expression construct of CIITA, the master regulator for MHC class II expression and presentation. This epitope discovery method is based on the induction of stable MHC class II expression on tumor cells, which are normally MHC class II negative, and expansion of the cells, followed by mass-spectrometric analysis of peptides eluted from purified MHC class II molecules. The approach by mass-spectrometric analysis of MHC eluate has been effective in the identification of relevant neoepitopes for MHC class I molecules in combination with sequencing of the tumor exome (Castle et al., 2012; Hos et al., 2020; Kreiter et al., 2015; Ott et al., 2017; Sahin et al., 2017; Yadav et al., 2014). Prediction algorithms are deemed faster but less reliable for MHC class II-binding peptides (Abelin et al., 2019), while the wet approach in this study identifies relevant MHC class II-presented antigenic peptides with high efficiency. An alternative method would be *in vitro* loading of primary tumor material on APCs, but this is technically more challenging. The full-length human CIITA cDNA appeared to be applicable for all tumor cell lines we used and was compatible with material of murine and human origin. The expression of all MHC class II presentation machinery components was highly stable in most cases.

Expression of only MHC class II molecules in tumor cells does not suffice, since physiological MHC class II peptide loading requires invariant chain CD74 and accessory molecules like DM. This was illustrated two decades ago when Ostrand-Rosenberg and colleagues showed aberrant peptide loading in the ER in the absence of CD74 (Armstrong et al., 1997). Also, there are three different MHC class II molecules, each with an alpha and beta chain and inherent polymorphism, thus complicating the off-the-shelf approach by such an MHC class II-only approach.

Using the RMA lymphoma model, we showed that our CIITA-induced MHC class II expression-elution approach could identify the well-known dominant MuLV envelope T helper epitope. The exact length of the naturally presented peptide epitope from tumor cells was only now established. Importantly, we confirmed that transfection of RMA equaled the presentation of the relevant T helper epitope by DCs of both endogenous gPr80 protein and exogenous peptide. Therefore, we immunologically analyzed promising antigen sequences from all murine cancer cell lines for inducing adequate $CD4^+$ type 1 helper responses. For the MC38 model, we could confirm the relevance of several immunogenic helper epitopes in anti-tumor immunity. For the other murine and human tumor models, we report on the identification of CD4-specific oncoviral antigens, TAAs, and tumor-specific neo-epitopes. Our findings are in line with two recent reports on CIITA transfection as an identification tool for $CD4^+$ epitopes on human cell lines, which resulted in the identification of presented peptide sequences from established TAAs relevant in glioblastoma (Forlani et al., 2020) and $CD4^+$ T cell responses to TAAs in 10 out of 19 patients with hepatocellular carcinoma during a clinical phase I/II trial (Löffler et al., 2022).

The consistent identification of immunogenic $CD4^+$ T cell epitopes by the CIITA method underscores its translational advantage independent of MHC haplotype. How well this approach can be applied in the clinic remains to be established since the CIITA method is relatively time consuming due to transfection, selection, and expansion of tumor cells. *In silico* prediction methods have the benefit of rapid utilization, however with lower epitope identification confidence. The required algorithms are currently underdeveloped, as was exemplified by our Gibbs clustering of the RKO eluted peptides, where only one of three binding motifs overlapped with the known patterns for RKO-specific HLA genes. Therefore, the CIITA-transfection approach has several advantages. Firstly, this method may contribute to better characterization of MHC class II-binding motifs and improve algorithm definition for individual MHC alleles. Secondly, *in silico* prediction is inherently probabilistic and requires validation of MHC binding, which is not necessary for the CIITA method. Strikingly, we have observed that the immunogenicity rate of our identified epitopes upon synthetic peptide vaccination of CD4

T cells in mice is relatively high (13 out of 24 tested peptides), including both neo- and shared cancer epitopes. Therefore, the CIITA method will significantly increase the "hit rate" of immunogenic epitopes for cancer-specific CD4 responses. Lastly, this comprehensive method can contribute directly to our understanding of CD4 immunity *in vivo*, including the shaping of the T cell repertoire to neo- and shared cancer antigens, thus improving options for immunotherapy.

The elucidation of therapy-relevant MHC class II-binding epitopes remains a challenge due to the sensitivity of the mass-spectrometric analysis pipeline. We normally aim to culture 10^8–10^9 cells to reliably obtain a deep ligandome, although lower numbers can be used too. Recent work by Ramarathinam et al. (2020) takes advantage of the use of carrier channels, based on the work of Budnik et al. (2018) and adapted for the MHC class I ligandome analysis of low cell numbers. This resulted in a decrease of cell numbers down to 10^3–10^5, and, although with limited depth, it still identified a number of TAAs (Budnik et al., 2018; Ramarathinam et al., 2020). This approach could similarly be applied to the identification of MHC class II-derived tumor epitopes and opens up new avenues for peptide elutions from small tumor samples. For clinical application, however, available tumor material may still be limited, and making cell lines or organoid cultures of patient material may prove essential. Due to the implementation of three-dimensional (3D) scaffolding, success rates for the establishment of cultured primary material have reached up to 90% for some tumor origins (Dijkstra et al., 2018; Schütte et al., 2017; van de Wetering et al., 2015; Weeber et al., 2015). Likewise, the formation of spherical bodies of cancer cells improves the potential for this transfection method.

Our work shows that the induction of the MHC class II presentation machinery in cancer cells expressing shared cancer antigens, oncoviral antigens, and neo-antigens enables the identification of proven and essential peptide epitopes for specific vaccination against cancer. This method presents an important step forward to specific immunotherapeutic strategies in the clinic and in the interplay of tumor-specific $CD4^+$ and $CD8^+$ immune responses in cancer.

Limitations of this study

This study has some limitations. We have applied CIITA transfection on several tumor cell lines to discover CD4 T cell epitopes with potential for specific immunotherapy against cancer. The method is well suited for established cell lines but has not been developed for clinical translation transfection of primary tumor material followed by patient-specific analysis. Secondly, immunogenicity of the human MHC class II epitopes, derived from human tumor cell lines, has not been analyzed *in vivo*. Furthermore, additional experiments are needed to establish antigen presentation of the identified cancer-derived epitopes by DCs. This requires extensive loading of fresh tumor material to professional APCs, peptide elution, and mass spectrometry. Finally, the unavailability of MHC class II tetramers folded with the identified epitopes prevented us from addressing fundamental questions on immune functions of tumor-specific CD4 T cells at tumor site and draining lymphoid organs.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

B.J.H. was the primary designer and performer of experiments, interpreter of data, and writer of this manuscript. E.T., M.G.M.C., and W.R. provided help with the design, execution, and data interpretation of experiments. J.v.d.B., D.R., and N.F.C.C.d.M. contributed to this manuscript through the analysis and processing of tumor and mouse sequencing data and prediction analysis. P.J.v.d.E. provided the hCIITA vector for transfection. G.M.C.J., A.H.d.R., and P.A.v.V. were vital in the elution, mass spectrometry, and identification of MHC class II-binding peptides. F.O. was the principal investigator responsible for supervision during each step of this project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-I-Ab, clone Y-3P	BioXCell	BE0178; RRID: AB_10949066
anti-I-Ab/d/I-Ed, clone M5/114	BioXCell	BE0108; RRID: AB_10950841
anti-HLA-DR, clone B8.11.2	In-house	N/A
anti-HLA-DQ, clone SPV-L3	In-house	N/A
anti-HLA-DP, clone B7/21	In-house	N/A
anti-I-A/I-E PE, clone M5/114.15.2	eBioscience	12-5321-82; RRID: AB_465928
anti-H2-DM, clone 2E5A (primary)	BD Pharmingen	552405; RRID: AB_394380
anti-CD74, clone 829706 (primary)	R&D systems	MAB7478
donkey-anti-rat IgG(H + L) Alexa Fluor 488 (secondary)	ThermoFisher Scientific	A-21208; RRID: AB_141709
anti-HLA-DR APC, clone G46-6	BD Pharmingen	560896; RRID: AB_10563218
anti-HLA-DQ PE, clone 1a3	Leinco Technologies	H138; RRID: AB_2892856
anti-HLA-DP FITC, clone B7/21	Leinco Technologies	H129; RRID: AB_2892854
anti-CD74, clone MB741 (primary)	BD Pharmingen	555,538; RRID: AB_395922
goat-anti-mouse IgG (H + L) Alexa Fluor 647	ThermoFisher Scientific	A28181; RRID: AB_2536165
anti-HLA-DM PE, clone MaP.DM1	Biolegend	358003; RRID: AB_2562027
agonistic OX40, clone OX-86	In-house	N/A
antagonistic PD-L1, clone MIH-5	In-house	N/A
CD3ε EF450, clone 17A2	eBioscience	48-0032-82; RRID: AB_1272193
CD4/L3T4 FITC, clone RM4-5	ThermoFisher	11-0042-82; RRID: AB_464896
CD4/L3T4 BV605, clone RM4-5	BioLegend	100547; RRID: AB_11125962
CD8α FITC, clone 53-6.7	BD Biosciences	553031; RRID: AB_394569
CD8α APC-R700, clone 53-6.7	BD Biosciences	564983; RRID: AB_2739032
TNFα FITC, clone MP6-XT22	eBiosciences	11-7321-41; RRID: AB_10670212
CD40L PE, clone MR1	ThermoFisher	12-1541-82; RRID: AB_465887
IFNγ PE-Cy7, clone XMG1.2	BD Biosciences	557649; RRID: AB_396766
IFNγ APC, clone XMG1.2	Invitrogen	17-7311-82; RRID: AB_469504
IL-2 APC, clone JES6-5H4	BD Biosciences	554429; RRID: AB_398555
Chemicals, peptides, and recombinant proteins		
Peptides synthesized as noted in tables	In-house synthesis	N/A
CpG (ODN, 1826) - TLR9 ligand	InvivoGen	tlrl-1826-1
SAINT-DNA	Synvolux Products	SD-2001-XX
Deposited data		
MC38 sequence database	(Hos et al., 2020) Sequence Read Archive	BioProject ID: PRJNA564288
CT26 sequence database	This publication Sequence Read Archive	BioProject ID: PRJNA880469
RKO somatic mutation database	COSMIC database https://cancer.sanger.ac.uk/cosmic	COSS2302003
Experimental models: Cell lines		
RMA	Ljunggren et al., 1990	N/A
MC38	Mario P. Colombo	N/A
CT26	Mario P. Colombo	N/A
B16F10	ATCC	CRL-6475

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RKO	ATCC	CRL-2577
HCT-15	ATCC	CCL-225
HCT116	ATCC	CCL-247
Experimental models: Organisms/strains		
C57BL/6JRj	Janvier Labs, France	N/A
BALB/cByJ	Charles River Laboratories	N/A
Recombinant DNA		
EBO-Sfi/CIITA	Steimle et al. 1993	N/A
Software and algorithms		
FACSDiva		N/A
FlowJo vX		N/A
Graphpad prism v8		N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ferry Ossendorp (F.A.Ossendorp@lumc.nl).

Materials availability

Requests for reagents can be directed to the lead contact author. Generated cell lines will be made available upon request after completion of a materials transfer agreement.

Data and code availability

- The sequence databases used for the determination of expressed mutations in MC38 is previously published and available on the Sequence Read Archive (SRA) under Bioproject ID PRJNA564288. The sequence databases for the determination of expressed mutations in CT26 is available upon publication on the SRA under Bioproject ID PRJNA880469. Somatic mutations of RKO are publicly available on the COSMIC database under identifier COSS2302003.
- No original code was reported in this report.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

Murine colorectal cell lines MC38 or CT26 present in Leiden were originally a gift from Mario P. Colombo, and human colorectal carcinoma cell lines HCT-15, HCT116, RKO and B16F10 were purchased from ATCC. HLA-typing of the RKO cell line was performed in-house. All cell lines were grown in IMDM medium (Lonza) supplemented with 8% Fetal Calf Serum (Greiner), 100 IU/mL penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 25 μM 2-mercaptoethanol (culture medium) and at 37°C, 95% relative humidity, 5% CO_2. Cell lines were mycoplasma tested before the start of experiments. STR typing is performed on human cell lines for tracking of cell line 'identity'.

Animal models

Naïve C57BL/6JRj (Janvier Labs, France) and BALB/cByJIco mice (Charles River Laboratories) female mice were purchased at an age of 8–10 weeks and housed under specified pathogen-free conditions in animal facilities of the Leiden University Medical Center. Female only was decided for ethical reasons. Long term housing of males causes excessive discomfort due to fighting, while females can be swapped between nests to prevent grooming. We expect no confounding effects on the outcome of experiments from this decision. All animal experiments were according to ethical guidelines and compliant to the requirements on expertise, animal welfare and licensing as determined by the Experiments on Animals Acts issued by the Dutch government and enforced by the Netherlands Central Animal Experiment Committee, and according to guideline of the Federation of European Laboratory Animal Science Associations (FELASA). Full sequences spanning all length variants of eluted peptides as shown in the figures were synthesized in-house. Animals involved in RMA endogenous anti-viral antigen specific immunity were S.C. injected in the right flank at day 0 with 2 x 10^3 live RMA cells in 200 μL PBS. Tumors were established after 14 days and mice were treated by peri-tumoral injection of 25 μg CpG

(ODN 1826 – TLR9 ligand, InvivoGen tlrl-1826-1) and 150 μg agonistic OX-40 antibody (clone OX-86, BioXCell) in PBS. Next, mice were treated with intraperitoneal injection of 200 μg antagonistic PD-L1 antibody (MIH-5, in-house production) in PBS at 18, 21, and 24 days after tumor challenge. Tumors were fully regressed 26 days after tumor challenge and spleens were resected for *ex vivo* splenocyte stimulation. For immunogenicity tests, naïve mice were immunized by subcutaneous injections in the base of the tail with 50 μL PBS containing single or pools of peptides, 20 nmol each (peptide stocks dissolved in DMSO at 10 mM), supplemented with 20 μg CpG. Primed mice were subsequently boosted fourteen days later with the same peptides, but co-treated by intraperitoneal injection of 150 μg agonistic OX-40 antibody (clone OX-86, in-house production) in 200 μL PBS. Animals involved in MC38 challenge were prophylactically vaccinated identical to the immunogenicity test and by indicated peptide pools. Tumor cells were S.C. injected in the right flank with 5×10^5 live MC38 cells in 200 μL PBS. Animals involved in therapeutic vaccination against MC38 were inoculated with 3×10^5 live MC38 cells. Six and twelve days after inoculation, mice were vaccinated by subcutaneous injections in the tail-base with synthetic peptides mixed with CpG as indicated. Therapeutic antibodies were used when indicated. Tumor sizes where measured 2–3 times a week and 500 mm^3 (length x width x depth) was maintained as humane endpoint for neoantigen helper peptide pools, 1000 mm^3 for CTL ± helper pools, and 1500 mm^3 for therapeutic challenge. Animals in the same cage/nest were allocated to different groups to minimize the effect of nests on the outcome of experimental group.

METHOD DETAILS

CIITA transfection of tumor cell lines

Tumor cells were seeded in 24-well plates in 0.5 mL complete culture medium. The next day, cells were transfected with EBO-Sfi/hCIITA (Steimle et al., 1993) using SAINT-DNA (Synvolux, Leiden, The Netherlands). Briefly, complexes at a 1:20 ratio (μg DNA:μl SAINT-DNA) were prepared by diluting 1 μg EBO-Sfi/CIITA DNA in 199 μL PBS followed by the addition of 20 μL SAINT-DNA. The mixture was incubated for 5–10 min at room temperature. Complexes were added directly on top of the cells in the following quantities: 250 ng, 100 ng and 50 ng DNA per well. Approximately 24h post-transfection, cells were trypsinized, pooled and transferred to a T75 culture flask. The following day, Hygromycin B (AG Scientific, San Diego, CA) was added. RMA, MC38, RKO and HCT116 cells were selected with 500 μg/mL Hygromycin B. B16F10 cells were selected with 600 μg/mL Hygromycin B. CT26 and HCT-15 cells were selected with 700 μg/mL Hygromycin B. After 7 days of selection, cells were harvested and stained with antibodies directed against MHC Class II and sorted. The EBO-Sfi/hCIITA plasmid encoding the full length hCIITA cDNA.

Analysis of MHC class II presented peptides

Identification of MHC class II-associated neoantigens was performed with a similar strategy as previously reported for MHC class I (Hos et al., 2020). In short, DNA and RNA were isolated from the MC38 and CT26 cell lines in culture to determine the expression of neo- and tumor-associated antigens. Tails of inbred C57BL/6Jico and BALB/cByJIco mice (Charles River Laboratories) were used as source of DNA to serve as reference for the identification of somatic mutations in the respective mouse cancer cell lines. Viral sequences were acquired through database searches for discovery of presented viral antigens in CIITA transfected RMA, and somatic mutations in RKO were collected from the COSMIC database. Tumor cell lysate immunoprecipitation of MHC class II was conducted by passage through columns coated with antibodies to I-A^b Y-3P (BioXCell, cat. BE0178) or I-$A^{b/d}$/I-E^d M5/114 (BioXCell, cat. BE0108) in case of murine lysates, and in-house produced HLA-DR (clone B8.11.2), -DQ (clone SPV-L3), and -DP (clone B7/21) antibodies in case of human RKO-CIITA lysate, essentially as previously described (van Balen et al., 2020). Column eluates where subsequently analyzed by on-line C18 nanoHPLC MS/MS. In a post-analysis process, raw data was converted to peak lists and submitted to the Uniprot *Mus musculus* database (UP000000589.fasta; June 12 2020; 55398 entries) or *Homo sapiens* database (UP000005640.fasta; June 12 2020; 20596 entries), respectively for protein identification. The aforementioned databases were supplemented with the appropriate sequences obtained from WES.

Analysis of expressed MHC class II components

Flow cytometric analysis: CIITA-transfected mouse cell lines were stained with antibodies to MHC class II I-A/I-E (PE, clone M5/114.15.2, eBioscience), H2-DM (primary: rat anti mouse H2-DM, clone 2E5A, BD Pharmingen; secondary: donkey anti rat IgG(H+L), alexa fluor 488, ThermoFisher Scientific), and CD74 (rat anti mouse, clone 829706, R&D systems; secondary: donkey anti rat IgG (H+L), alexa fluor 488, ThermoFisher Scientific) antibodies. CIITA-transfected human cell lines were stained with fluorescent-labeled anti-HLA-DR (APC, clone G46-6, BD Pharmingen), -DQ (PE, clone 1a3, Leinco Technologies), -DP (FITC, clone B7/21, Leinco Technologies), CD74 (primary: clone MB741, BD Pharmingen; secondary: alexa fluor 647 goat anti mouse IgG (H+L), ThermoFisher Scientific), and HLA-DM (PE, clone MaP.DM1, Biolegend) antibodies. The sorting of MHC Class II positive cells was performed on BD FACSAria II SORP (BD Biosciences).

Confocal microscopy

75.000 cells were seeded on the glass part of 35 mm poly-d-lysine glass bottom culture dishes (MakTeK) plates and cultured overnight at 37°C. On the following day, the medium was aspirated and the cells were washed once with PBS. Subsequently, the cells were fixed by adding 150 ul of 4% paraformaldehyde and incubating for 15 min at room temperature. The fixative was washed twice by adding and aspirating PBS. Cells were permeabilized for 5 min with 0.1% PBS/Tween. The solution was removed and a solution of

1% BSA in 0,1% PBS/Tween was added for blocking and incubated for 5 min at 37°C. Next, the blocking solution was removed and a solution containing primary antibodies in 1% BSA/PBST was added and incubated for 30 min at 37°C. After incubation, the antibody solution was washed with 1% BSA/PBST and the secondary antibodies were added in case of unlabeled primary antibody in 1% BSA/PBST and incubated for 30 min at 37°C. The antibody solution was washed first with 1% BSA/PBST and then with PBS. Once the PBS was aspirated, a drop of ProLong™ Gold Antifade Mountant with DAPI (Invitrogen) was added and left to cure overnight at room temperature. The next days the cells were imaged using a confocal scanning laser microscope SP5 (Leica).

T cell activation assays

The read-out of peptide specific T cell responses was performed through o/n pre-culture of 5×10^4 DC's of the growth factor dependent cross-presenting dendritic cell-line D1 (Winzler et al., 1997) with 5 μM synthetic peptide as described in the text, whereupon T cells and 2 μg/mL brefeldin A (Sigma-Aldrich) were added. Analysis of activation markers and intracellular cytokine production was started through extracellular staining of CD3ε (EF450, clone 17A2, eBioscience), CD4 (FITC or BV605, clone RM4-5, Thermo Fisher or BioLegend), and CD8α (APC-R700 or FITC, clone 53–6.7, BDBiosciences) in FACS buffer (0,5% Bovine Serum Albumin, 0,02% Sodium Azide). Afterward, cells were washed and o/n treated with 0.5% paraformaldehyde to subsequently be stained for TNFα (FITC, clone MP6-XT22, eBioscience), CD40L (PE, clone MR1, Thermo Fisher), IL-2 (APC, clone JES6-5H4, BD Biosciences), and IFNγ (PE-Cy7 or APC, clone XMG1.2, BD Biosciences or Invitrogen) through the use of perm/wash (BioLegend). Intracellular cytokine and activation marker gates were used to determine single, double, triple or quadruple positive events. All samples were analyzed on a BD Bioscience LSR-II with FACSDiva for data-capture software. FlowJo vX (FlowJo, LLC) was used for sample analysis and processing.

QUANTIFICATION AND STATISTICAL ANALYSIS

Graphpad prism v8 was used for statistical analysis. Respective statistical details and tests are mentioned in the legends of the figures. Significance was determined when p-values are below 0.05 and specific p values per test are mentioned in the legends. In general, statistical analysis on animal experiments on average tumor outgrowth comparisons were statistically tested by two-way ANOVA, and survival with log-rank tests. Incidentally, mice were preliminarily excluded from tumor outgrowth and survival experiments for the prevention of unethical suffering.