The major histocompatibility complex (MHC) class II-associated Invariant chain (Ii) is present in professional antigen presenting cells where it regulates peptide loading onto MHC class II molecules and the peptidome presented to CD4+ T lymphocytes. Because Ii prevents peptide loading in neutral subcellular compartments, we reasoned that Ii− cells may present peptides not presented by Ii+. Based on the hypothesis that patients are tolerant to MHC II-restricted tumor peptides presented by Ii+ cells, but will not be tolerant to novel peptides presented by Ii− cells, we generated MHC II vaccines to activate cancer patients’ T cells. The vaccines are Ii− tumor cells expressing syngeneic HLA-DR and the costimulatory molecule CD80. We used liquid chromatography coupled with mass spectrometry to sequence MHC II-restricted peptides from Ii+ and Ii− MCF10 human breast cancer cells transfected with HLA-DR7 or the MHC Class II transactivator CIITA to determine if Ii− cells present novel peptides. Ii expression was induced in the HLA-DR7 transfectants by transfection of Ii, and inhibited in the CIITA transfectants by RNA interference. Peptides were analyzed and binding affinity predicted by artificial neural net analysis. HLA-DR7-restricted peptides from Ii− and Ii+ cells do not differ in size or in subcellular location of their source proteins; however, a subset of HLA-DR7-restricted peptides of Ii− cells are not presented by Ii+ cells, and are derived from source proteins not used by Ii+ cells. Peptides from Ii− cells with the highest predicted HLA-DR7 binding affinity were synthesized, and activated tumor-specific HLA-DR7+ human T cells from healthy donors and breast cancer patients, demonstrating that the MS-identified peptides are bonafide tumor antigens. These results demonstrate that Ii regulates the repertoire of tumor peptides presented by MHC class II breast cancer cells and identify novel immunogenic MHC II-restricted peptides that are potential therapeutic reagents for cancer patients. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.019232, 1457–1467, 2012.

Cancer vaccines are a promising tool for cancer treatment and prevention because of their potential for inducing tumor-specific responses in conjunction with minimal toxicity for healthy cells. Cancer vaccines are based on the concept that tumor cells synthesize multiple peptides that are potential immunogens, and that with the appropriate vaccine protocol, these peptides will activate an efficacious antitumor response in the patient. Much effort has been invested in identifying and testing tumor-encoded peptides, particularly peptides presented by major histocompatibility complex (MHC) class I, molecules capable of activating CD8+ T-cells that directly kill tumor cells (1, 2). Fewer studies have been devoted to identifying MHC class II-restricted peptides for the activation of tumor-reactive CD4+ T-cells despite compelling evidence that Type 1 CD4+ T helper cells facilitate the optimal activation of CD8+ T-cells and the generation of immune memory, which is likely to be essential for protection from metastatic disease.

Activation of CD4+ T cells requires delivery of a costimulatory signal plus an antigen-specific signal consisting of peptide bound to an MHC II molecule. Most cells do not express MHC II or costimulatory molecules, so CD4+ T cells are typically activated by professional antigen presenting cells (APC), which endocytose exogenously synthesized antigen and process and present it in the context of their own MHC II molecules. This processing and presentation process re-
quires Invariant chain (Ii), a molecule that is coordinately synthesized with MHC II molecules and prevents the binding and presentation of APC-encoded endogenous peptides (3, 4). As a result, tumor-reactive CD4+ T cells are activated to tumor peptides generated by the antigen processing machinery of professional APC, rather than peptides generated by the tumor cells. Because of the potential discrepancy in peptide generation between professional APC and tumor cells, and the critical role of Ii in preventing the presentation of endogenous peptides, we have generated “MHC II cancer vaccines” that consist of Ii tumor cells transfected with syngeneic MHC class II and CD80 genes. We reasoned that MHC II Ii CD80+ tumor cells may present a novel repertoire of MHC II-restricted tumor peptides that are not presented by professional APC, and therefore may be highly immunogenic. Once activated, CD4+ T cells produce IFN-γ and provide help to CD8+ T cells and do not need to react with native tumor cells. Therefore, the MHC II vaccines have the potential to activate CD4+ Th1 cells that facilitate antitumor immunity. In vitro (5) and in vivo (5–7) studies with mice support this conclusion. In vitro studies with human MHC II vaccines further demonstrate that the absence of Ii facilitates the activation of MHC II-restricted tumor-specific CD4+ T cells of HLA-DR-syngeneic healthy donors and cancer patients, and that the vaccines activate CD4+ T cells with a distinct repertoire of T cell receptors (8–12). A critical negative role for Ii is also supported by studies of human acute myelogenous leukemia (AML). High levels of class II-associated invariant chain peptide (CLIP), a degradation product of Ii, by leukemic blasts is associated with poor patient prognosis (13, 14), whereas down-modulation of CLIP on AML cells increases the activation of tumor-reactive human CD4+ T cells (14, 15).

We have now used mass spectrometry to identify MHC II-restricted peptides from MHC II Ii and MHC II Ii human breast cancer cells to test the concept that the absence of Ii facilitates the presentation of unique immunogenic MHC II-restricted peptides. We report here that a subset of MHC II-restricted peptides from HLA-DR7+ breast cancer cells are unique to Ii cells and are derived from source proteins not used by Ii cells. Ii peptides have high binding affinity for HLA-DR7 and activate tumor-specific T-cells from the peripheral blood of healthy donors and breast cancer patients. This is the first study to compare the human tumor cell MHC II peptidome in the absence or presence of Ii and to demonstrate that MHC II Ii tumor cells present novel immunogenic MHC II-restricted peptides that are potential therapeutic reagents for cancer patients.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Transductants, PBMC—**Human breast cancer cell line MCF10CA1 (hereafter called MCF10), its nonmalignant counterpart MCF10A (16), MCF10 transductants (MCF10/DR7/CD80, MCF10/DR7/CD80/Ii, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/Ii siRNA32; hereafter called /DR7/, /DR7/Ii, /CIITA, and /CIITA/Ii siRNA) were cultured and/or generated as described (11, 12). Cells were expanded to ~1 x 10^8 cells/line using HyFlask tissue culture flasks (Corning, Corning, NY). MCF10 cells are: HLA-DRβ1*0401, DRβ1*0701. Peripheral blood mononuclear cells (PBMC) from healthy human donors and from breast cancer patients were obtained from the University of Maryland Medical School. Healthy donor BC100206 is HLA-DRβ1*0401and *0701; healthy donors BC100306, BC061505, and BC051505 are DRβ1*0701. Breast cancer patients 3 and 10 are HLA-DRβ1*0701. Breast cancer patient 3 is stage III, ER−/PR−, HER-2/neu+; patient 10 is stage II, ER−/PR−, HER-2/neu+. Patients were bled into ACD (citrate) tubes. Within 24 h of collection PBMC were isolated on Ficoll gradients, immediately cryopreserved at a controlled freeze rate of 1 °C/min, and stored in the vapor phase of liquid nitrogen until used. PBMC were >90% viable upon thawing. Use of human materials was approved by the UMBC IRB.

**Reagents, Antibodies, and Flow Cytometry—**Chemicals were purchased from Sigma Aldrich unless otherwise noted. Monoclonal antibodies L243-FITC (HLA-DR-specific), CD80-PE, Ii, CD3-FITC, CD8-PE, CD45RO-allophycocyanin, CD25-PE-Cy7, and CD56-PE-Cy7 were purchased from BD Phamingen. HLA-A,B,C-PE-Cy5, CD4-Pacific Blue and CD4-eFluor 460 were purchased from Biolegend (San Diego, CA). Cell surface and intracellular staining for flow cytometry was performed as described (9, 12). Stained cells were analyzed using a Cyan ADP flow cytometer and Summit analysis software, v2.1 (Beckman/Coulter).

**HLA-DR Induction—**Cells were incubated at 37 °C for 48 h with 200 units IFNγ/ml. Washed cells were stained with mAbs to HLA-DR to ascertain HLA-DR expression.

**MHC II Peptide Isolation, LC-MS/MS Analysis—**Approximately 1 x 10^6 cultured cells per cell line were harvested and lysed on ice for 1 h in lysis buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1% 3-[[(3-cholamidopropyl)dimethylammonio]propanesulfonate; (17) containing a Complete Mini Protease Inhibitor Mixture tablet (Roche). Lysates were ultra centrifuged at 129,888 g for 1 h at 4 °C in a SW40 Ti swinging bucket rotor and the supernatants harvested and stored at −80 °C. MHC II peptides were obtained by HPLC (Biologic HR, BioRad, Hercules, CA) as follows: Thawed supernatants were pre-cleared on a 5 ml Protein G Sepharose column equilibrated with lysis buffer and then applied to a 2 ml in-house generated L243 mAb (pan HLA-DR) Sepharose column (9) equilibrated with two column volumes of lysis buffer. The loaded column was sequentially washed with 20 column volumes of 0.2 N acetic acid, and eluates were lyophilized and stored at −80 °C. MHC II peptides were obtained by FACET biocompatible strong cation exchange (SCX) trapping column and separated on Acquity UPLC (Waters) using a 50 cm, 15 μm, C18 column (Waters). Mobile phases were: 0.1% formic acid in water (A), 80% acetonitrile, 20% water with addition of 0.1% formic acid (B), and 2 mM ammonium bicarbonate in water (C). Peptide samples were loaded onto the SCX trapping column and washed with 40% B, 96% A for 0.75 min, then eluted onto an analytical column over 10 min at 15% C, 4% B. Peptides were separated by a gradient of mobile phase B, which was 15, 45, 100, and 4% at min. 20, 40, 50, and 55, respectively. Eluted peptides were ionized on a Thermo Scientific (nanospray ionization) LTQ XL mass spectrometer interfaced with a LTQ XL (ThermoFisher) electrospray ionization source. The electrospray capillary was at 200 °C temperature and under 1.7 kV voltage. For each cell line two affinity purifications were performed, and for each...
affinity purification two LC-MS/MS runs were conducted. Mass spectrometric analysis was performed using unattended data-dependent acquisition mode, in which the mass spectrometer automatically switched between acquiring a survey mass spectrum (full MS) and consecutive CID of up to four most abundant ions (MS/MS). To facilitate identification of a broad range of peptides, dynamic exclusion for MS/MS was used. Individual precursor ions were selected no more than twice over the duration of 20 s, and were then placed in the exclusion list for 120 s. The m/z tolerance window for dynamic exclusion was 1.5 Da.

Peptide Identification—Spectra were searched against the International Protein Index (IPI) human database, (version 3.26, 67665 entries) on BioWorks 3.3.1 SP1 platform (ThermoFisher), using SEQUEST and the following search parameters: peptide mass tolerance: 2 Da; fragment mass tolerance: 1 Da, no enzyme specificity. Results of the SEQUEST searches (.out files) were converted into mzXML files and analyzed on Peptide Prophet using Trans-Proteomic Pipeline version 4.3 revision 1 (18). Peptides that were identified in both affinity purifications and both LC-MS/MS runs were considered as reliable identifications. These peptides had a minimal Xcorr score of 1.5 for the charge state of 1, a minimal Xcorr score of 2.0 for the charge state of 2, a minimal Xcorr score of 2.5 for the charge state of 3, and a minimal Peptide Prophet probability of 0.07. Peptides with Peptide Prophet probability greater than 0.05 were subjected to Artificial Neural Network (ANN) analysis. We purposefully included all peptides identified with a more relaxed filtering criterion to facilitate MHC II peptide binding prediction accuracy. Subcellular localization of source proteins for peptides was determined using LOCATE subcellular localization database (http://locate.imb.uq.edu.au/) and WOLF Psort (http://wolfpsort.org/).

Artificial Neural Network Analysis—Two hundred and sixty seven published HLA-DR7-restricted peptides of 9–25 amino acids in length (MHCBN database, version 4.0; http://www.imtech.res.in/raghava/mhcbn/index.html) were used to train an ANN. The network was a three layer back propagation network, trained to produce four output classes, representing highest to lowest binding affinities. The amino acid strings were initially coded by amino acid names. A “1” in a specific position of the 20 node input corresponded to the particular amino acid in the ordered list of 20 amino acids; the other positions being set to “0.” The binding region was assumed to be nine amino acids in length. Therefore, larger peptides were broken into (n-9) by 9-mers prior to coding. This produced 1318 9-mer sequences. The resultant 9-mer sequences in the larger peptide sequence. The resultant 9-mer sequences were then coded in the same manner as those used for training the network. Five peptides unique to li- and two peptides present in both li- and lii- cells scored >0.92 (H binding class).

PBMC Activation—Seven MS-identified peptides and Her2/neu peptide 776 (20, 21) were synthesized in the University of Maryland, Baltimore biopolymer facility. Peptides were assessed for their ability to activate T cells as previously described (9, 11, 12). Briefly, PBMC from HLA-DR7+ donors were primed with peptide (2 μg/ml), expanded with IL-15, and boosted with either MCF10 vaccine cells, MHC II- lii-transductants, or nonmalignant MCF10A cells. T-cell activation was assessed by measuring IFNγ production by ELISA. Percent T-cell activation = (100% × (pg IFNγ for experimental peptide/pg IFNγ for Her2 p776)).

Peptide Binding Assay—The procedures of (22) and (23) were modified as follows: DR7/CD80 cells (6 × 105 cells/well of 96 well plates) were washed twice with excess ice-cold PBS and cells were resuspended in 100 μl of 0.1315 M citric acid in PBS (pH—3) for 60 s. Solution was neutralized by addition of 150 μl Iscoves Modified Dulbecco’s medium with 2% FCS (IMDM-2% fetal calf serum), cells washed twice with PBS-2% fetal calf serum, and 0 to 350 μg/ml of peptide in PBS-2% fetal calf serum (250 μg/well) or PBS-2% fetal calf serum added to each well. Following 16–24 h incubation at 4 °C, cells were washed, stained with L243-FITC mAb and analyzed by flow cytometry. Delta mean channel fluorescence (ΔMCF) = (MCF of cells + peptide) – (MCF of cells + PBS-2% fetal calf serum).

Statistical and Bioinformatics Analysis—Statistical analyses of IFNγ production and comparison of T-cell activation by tumor-derived peptides versus Her2 p776 peptide was determined by two-tailed equal and/or unequal variance t test (Microsoft Excel 2007). p values < 0.05 were considered significant. Error bars represent the standard error of the mean.

RESULTS

MCF10 Transductants Express MHC II and Do Not Express li—We have used human mammary adenocarcinoma MCF10 cells (16) (genotype HLA-DR4, -DR7) that constitutively express MHC I and do not constitutively express MHC II, li, or CD80 (Fig. 1A, 1B). Two types of transfectants have been generated: (1) Stable transfectants that are HLA-DR7+ CD80+ and differentially express li (MCF10/DR7/CD80 and MCF10/DR7/CD80/li; hereafter called /DR7) and /DR7/li) were generated by transfection with a bicistronic vector encoding the α and β chains of HLA-DR7, a vector encoding the CD80 co-stimulatory molecule, ± a vector encoding li. These transfectants were used for analyzing peptides bound to the HLA-DR7 allele. (2) MCF10 cells stably expressing multiple MHC II alleles were generated by transfection with the MHC class II transactivator (CIITA), a transcription factor that coordinately up-regulates all MHC II alleles, li, and MHC I-associated...
proteins (24). Ii expression in CIITA transfectants was extinguished by cotransfection with siRNA for Ii. Ii^+/H11001 and Ii^-/H11002 CIITA transfectants (MCF10/CIITA/CD80 and MCF10/CIITA/CD80/Ii siRNA; hereafter called /CIITA and /CIITA/Ii siRNA) were used to obtain a broader pool of MHC II-restricted peptides because these cells express multiple HLA-DR alleles. All transfectants were generated as described (11, 12), and stably expressed their transgenes as assessed by flow cytometry (Fig. 1B).

**MHC II^-/li^- Tumor Cells Present MHC II-restricted Peptides Not Presented by MHC II^+/li^- Cells**—To determine if the presence of li impacts the repertoire of MHC II-restricted peptides, two independent batches of li^- and li^+ CIITA transfectants (MCF10/CIITA/CD80 and MCF10/CIITA/CD80/li siRNA; hereafter called /CIITA and /CIITA/li siRNA) were used to obtain a broader pool of MHC II-restricted peptides because these cells express multiple HLA-DR alleles. All transfectants were generated as described (11, 12), and stably expressed their transgenes as assessed by flow cytometry (Fig. 1B).

**Peptides from li^-/H11002 (/DR7 and /CIITA/Ii siRNA) and li^-/H11001 (/DR7/li peptides)** and **supplemental Table S2 (/CIITA and /CIITA/li siRNA peptides)**. For the HLA-DR7 transduced lines, 22.6% of peptides were unique to /DR7, 59.4% of peptides were unique to /DR7/li cells, and 17.9% of peptides were shared by /DR7 and /DR7/li cells. For the CIITA transductants, 18.6% of peptides were unique to /CIITA/li siRNA cells, 77.6% of peptides were unique to /CIITA cells, and 3.7% of peptides were shared by /CIITA and /CIITA/li siRNA cells. These results demonstrate that the absence of li enables the presentation of MHC II-restricted peptides that are not presented by li^+ cells.

Peptides from li^-/DR7 and /CIITA/li siRNA and li^+ (/DR7/li and /CIITA) cells ranged in length from 9 to 36 amino acids, with an average length of 19.48, 20.17, 21.67, and 19.59, respectively (Fig. 2C). Many of the peptides were derived from the same protein cleaved at different locations, giving rise to sets of nested peptides. /DR7/li unique peptides contained eight nested sets, whereas there were no nested sets among /DR7 unique peptides. Seven and four nested sets were identified for /CIITA and /CIITA/li siRNA cells, respec-
HLA-DR bound peptides from two independent preparations of DR\(^{-}\)Ii\(^{-}\) MCF10 vaccine cells and from DR\(^{+}\)Ii\(^{+}\) MCF10 transfectants were purified using affinity chromatography, sequenced using LC-MS/MS, assigned to proteins using SEQUEST, and analyzed using ANN. Peptides with the highest predicted HLA-DR7 binding affinity were selected for further study.

HLA-DR\(^{-}\) MCF10 vaccines and Ii\(^{-}\) transfectants present distinct and partially overlapping repertoires of peptides. Values are the number of peptides that are unique or shared between DR\(^{-}\)Ii\(^{-}\) MCF10 vaccine cells and DR\(^{+}\)Ii\(^{+}\) MCF10 transfectants.

Peptides isolated from HLA-DR7-transfected and CIITA-transfected Ii\(^{-}\) and Ii\(^{+}\) MCF10 cells are similar in length. D, Peptides isolated from HLA-DR7-transfected Ii\(^{-}\) and Ii\(^{+}\) MCF10 cells contain nested sequences. Data are from two independent analysis of each preparation of cells.

**Fig. 2.** A, Experimental design for isolation and identification of HLA-DR-bound peptides. HLA-DR bound peptides from two independent preparations of DR\(^{-}\)Ii\(^{-}\) MCF10 vaccine cells and from DR\(^{+}\)Ii\(^{+}\) MCF10 transfectants were purified using affinity chromatography, sequenced using LC-MS/MS, assigned to proteins using SEQUEST, and analyzed using ANN. Peptides with the highest predicted HLA-DR7 binding affinity were selected for further study. B, HLA-DR\(^{-}\) MCF10 vaccines and Ii\(^{-}\) transfectants present distinct and partially overlapping repertoires of peptides. Values are the number of peptides that are unique or shared between DR\(^{-}\)Ii\(^{-}\) MCF10 vaccine cells and DR\(^{+}\)Ii\(^{+}\) MCF10 transfectants. C, Peptides isolated from HLA-DR7-transfected and CIITA-transfected Ii\(^{-}\) and Ii\(^{+}\) MCF10 cells are similar in length. D, Peptides isolated from HLA-DR7-transfected Ii\(^{-}\) and Ii\(^{+}\) MCF10 cells contain nested sequences. Data are from two independent analysis of each preparation of cells.
Fig. 2D shows examples of nested sets. Nested sets of peptides ranging in length from 13–23 are characteristic of MHC II-restricted peptides (25, 26) and demonstrate that the peptides presented by tumor cells share physical characteristics with MHC II-restricted peptides presented by professional APC.

**Ii***/H11002 Cells Present Peptides Derived from Source Proteins Not Used by **Ii***/H11001 Cells—Protein Prophet was used to identify the source proteins for the peptides, which are shown in supplemental Tables S3 and S4. Annotated spectra for the proteins identified by a single peptide per protein are shown in supplemental Figs. S1–S3. The source proteins were analyzed using the LOCATE subcellular localization database and WOLF Psort to identify the cellular source and determine if the peptides presented by **Ii**– and **Ii**+ cells were derived from proteins residing in similar locations (Fig. 3A). The source proteins for all three categories of peptides (unique to **Ii**– or **Ii**+ cells; shared by **Ii**– and **Ii**+ cells) and for both DR7 and CIITA transductants originated in similar compartments, with most proteins residing in the cytoplasm, nuclei, and mitochondria, and fewer in the endoplasmic reticulum, cytoskeleton, peroxisome, plasma membrane, lysosome, and Golgi.

Although source proteins for peptides from **Ii**– and **Ii**+ cells originated in the same subcellular compartments, a subset of peptides in **Ii**+ cells were derived from proteins not used by **Ii**– cells (Fig. 3B). Specifically, 29 and 19% of source proteins used by **Ii**+ /DR7 and CIITA/ **Ii** siRNA cells were not represented in the protein pool from /DR7/ **Ii** and /CIITA **Ii**+ cells.
respectively. Likewise, 53.5% and 74.6% of source proteins used by Ii^+/H11002/DR7 and Ii^+/H11001/DR7 cells were not used by Ii^+/H11002 and Ii^+/CIITA/Ii siRNA cells, respectively. These results suggest that a subset of peptides presented by Ii^+/H11002 and Ii^+/H11001 cells are derived from a different pool of source proteins.

Peptides Presented by Ii^+ and Ii^- Cells Have Similar Binding Affinity for MHC II and Contain Redundant Binding Motifs—To determine if Ii expression affects the binding affinity of MHC II-restricted peptides, all of the peptides identified by LC-MS/MS from /DR7 and /DR7/Ii cells (1385 and 2219 peptides, respectively) were subjected to Artificial Neural Net (ANN) analysis. The peptides of /CIITA transductants were not included in this analysis because we could not determine which of these peptides were restricted to HLA-DR7 versus HLA-DR4. The ANN was trained on the MHCBN database of 267 peptides with known high, medium, or low binding affinity for HLA-DR7 (27, 28). Of the peptides in Ii^+/DR7 cells, 5.1% scored >0.92, placing them at the top of the high affinity class, and 8.7% scored >0.62 placing them lower in the high affinity class. The remaining peptides scored <0.62 and were in medium and low affinity classes. Approximately the same percentage of peptides from Ii^+/H11001/DR7/Ii cells were in each class (5.4% highest affinity, 8.2% next highest affinity). Surprisingly, ANN analysis identified a previously unpublished binding motif in the peptides isolated from /DR7 and /DR7/Ii cells (Fig. 4A). Most of the peptides from both Ii^- and Ii^+ cells

![Fig. 4. MS-identified peptides isolated from MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii cells bind peptides in multiple registers and have predicted high affinity binding to HLA-DR7. A, Consensus HLA-DR7 binding motif for MS-identified peptides as determined by ANN, and published HLA-DR7 binding motif from SYFPEITHI database. B, MS-identified peptides contain multiple overlapping binding motifs for HLA-DR7. Example of one peptide is shown. C, Seven MS-identified peptides with high predicted binding affinity used in the subsequent PBMC activation studies of Fig. 5 and supplemental Fig. S4.](image-url)
in the high and medium binding affinity classes contained multiple binding cores indicating that these peptides could bind to HLA-DR7 in multiple registers. Fig. 4B shows a representative peptide that contains four potentially high or medium binding regions. These data demonstrate that Ii does not impact the binding affinity of MHC II-restricted peptides, and suggest that strong binding could be because of multiple binding sites within a given peptide.

**Peptides From Ii Cells Bind to HLA-DR7 and Activate Tumor-specific T Cells from Healthy Donors and Breast Cancer Patients**—To determine if the peptides identified by MS were bona fide tumor antigens, five peptides unique to Ii/H11002 vaccines and two peptides shared by Ii/H11002 and Ii/H11001 cells from the highest predicted binding class and isolated from HLA-DR7-transfectants were selected for further study (Fig. 4C). To ascertain the relative immunogenicity of the MS-identified peptides, T cell activation by these peptides was compared with T-cell activation by the immunogenic Her2/neu peptide p776–790 (Her2 p776) (21). HLA-DR7+ healthy donor PBMC were primed with peptides, rested, expanded in IL-15, and boosted with /DR7 or /DR7/Ii cells (Fig. 5A). Priming with peptides derived from Ii- vaccine cells (peptides 46 and 43) and boosting with the corresponding Ii- vaccine cells gave maximum activation as measured by IFNγ production (Fig 5B). Similar results were obtained for the three other peptides unique to Ii- cells (peptides 45, 47, and 48; supplemental Fig. S4A) and for the two peptides shared by Ii- and Ii+ cells (peptides 42 and 44; supplemental Fig. S4B). An irrelevant peptide (HEL 46–61) gave only background levels of IFNγ (supplemental Fig. S4C). Tumor-derived peptides activated T cells because 90% of the cells after priming and before boosting were CD3+ and depletion of CD3+ cells before boosting eliminated IFNγ production (75.5 ± 17.6 pg/ml undepleted versus 0 pg/ml CD3-depleted). Table I compares the relative activation by the MS-identified peptides as compared with activation by Her2 p776. Peptides 43, 47, and 48 (present only in Ii- cells) and peptides 42 and 44 (shared by Ii- and Ii+ cells) were as effective as Her2 p776 peptide in activating PBMC, and Ii- peptide 45 was significantly more effective. These results demonstrate that the peptides identified by
null
MHC II ligands can reach many thousands of peptides (32), so only a subset of the HLA-DR7 peptidome has been identified in the present study. However, the novel peptides we have identified are functional, immunogenic and tumor-specific, making them potential agents for therapeutic cancer vaccines.

Surprisingly, ANN, the bioinformatics approach we used to identify MHC II-restricted peptides and predict their binding affinity, yielded a binding motif that was not identified by previous analyses (33, 34). The validity of ANN was confirmed in that six of the seven peptides that were identified by ANN as being high affinity binders were strongly immunogenic for four healthy donors and two breast cancer patients. ANN analysis has the advantage of not only identifying key anchor residues, but also evaluates the contributions of neighboring residues and therefore may give a more accurate assessment of affinity.

In professional APC MHC class II molecules are loaded with peptides in MHC endosomal compartments when the acidic pH causes the release of Ii from the MHC II molecule. The peptides are derived from either exogenous antigens that are endocytosed and degraded in the endocytic vesicles, or from endogenously synthesized nuclear and cytosolic material that accesses endosomal compartments by autophagy (35). Because the vaccine cells lack Ii, their MHC II molecules can potentially be loaded with peptide in any of the subcellular compartments preceding the MIIC. Therefore, newly synthesized and recycling MHC II molecules in the vaccines are likely to be exposed to a broader repertoire of peptides than MHC II molecules of Ii (i.e., professional) APC. The finding that Ii peptides consist of fewer nested sets relative to Ii peptides further supports the concept that peptides are either loaded onto MHC class II molecules, or that MHC II molecules traffic through different subcellular compartments in Ii- and Ii cells, resulting in the novel peptides identified in this report. Surprisingly, most of the peptides identified from Ii cells and Ii cells are derived from endogenous proteins, consistent with the concept that autophagy is a prominent source of MHC II-restricted peptides in Ii cells. The absence of Ii may facilitate MHC II trafficking through the secretory pathway rather than the endosomal pathway where they encounter a different peptide repertoire. The absence of Ii and its intrinsic endosomal trafficking sequence (36) may or may not affect MHC II trafficking because the MHC II β chain also contains an endosomal trafficking signal (37). Our previous antigen presentation studies using endosomal and proteasomal inhibitors support peptide loading in the endosomal route (38, 39); however, confocal microscopy studies indicate that vaccine cell MHC II molecules also traffic via the secretory pathway (40).

The different peptidomes of Ii- and Ii cells combined with the observation that some of the peptides of Ii cells are derived from source proteins not present in the repertoire of Ii cells, confirm our original hypothesis that the absence of Ii facilitates the presentation of novel peptides. Whether the differences in intracellular trafficking patterns or differential use of endocytosis or autophagy are responsible for the different peptidomes requires further study. Regardless of which pathway and process are used, the absence of Ii enables the presentation of immunogenic, bonafide nonconventional tumor peptides.

Acknowledgments—We thank Lydia Grmaj for help with culturing MCF10 transductants for MHC II peptide purification, Christopher Ecker for help with peptide subcellular localization analysis, Virginia Clements for technical support, Dr. Amy Fulton for the MCF10A cells, Drs. Saranya Chumsri and Dean Mann for healthy donor and breast cancer patients’ PBMC, and Drs. Elizabeth Jaffe and Sheherazade Sadegh-Nasseri for advice on assessing peptide binding affinity.

* This work was supported by National Institutes of Health (NIH) (NIH RO1CA84232 and RO1CA115880). OC is the recipient of DOD breast cancer program pre-doctoral fellowship W81XWH-10-1-0027 and was partially supported by NIH CBI T32GM 066706.

This article contains supplemental Figs. S1 to S6 and Tables S1 to S4.

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REFERENCES

1. Fang, L., Lonsdorf, A. S., and Hwang, S. T. (2008) Immunotherapy for advanced melanoma. J. Invest. Dermatol. 128, 2596–2605
2. Mellman, I., Coukos, G., and Dranoff, G. (2011) Cancer immunotherapy comes of age. Nature 480, 480–489
3. Neefjes, J., Jongsma, M. L., Paul, P. A., and Bakke, O. (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat. Rev. Immunol. 11, 823–836
4. Germain, R. N. (2011) Uncovering the role of invariant chain in controlling MHC class II antigen capture. J. Immunol. 187, 1073–1075
5. Armstrong, T. D., Clements, V. K., Martin, B. K., Ting, J. P., and Ostrand-Rosenberg, S. (1997) Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc. Natl. Acad. Sci. U.S.A. 94, 6886–6891
6. Humphreys, R. E., Hillman, G. G., von Hofe, E., and Xu, M. (2004) Forcing tumor cells to present their own tumor antigens to the immune system: a necessary design for an efficient tumor immunotherapy. Cell. Mol. Immunol. 1, 180–185
7. Baskar, S., Glilcher, L., Nabavi, N., Jones, R. T., and Ostrand-Rosenberg, S. (1995) Major histocompatibility complex class II+T lymphocytes by major histocompatibility complex class I+ cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. J. Exp. Med. 181, 619–629
8. Bosch, J. J., Thompson, J. A., Srivastava, M. K., Iheagwara, U. K., Murray, T. G., Lotem, M., Ksander, B. R., and Ostrand-Rosenberg, S. (2007) MHC class II-transduced tumor cells originating in the immune-privileged eye prime and boost CD4(+) T lymphocytes that cross-react with primary and metastatic uveal melanoma cells. Cancer Res. 67, 4499–4506
9. Dissanayake, S. K., Thompson, J. A., Bosch, J. J., Clements, V. K., Chen, P. W., Ksander, B. R., and Ostrand-Rosenberg, S. (2004) Activation of tumor-specific CD4(+) T lymphocytes by major histocompatibility complex class II tumor cell vaccines: a novel cell-based immunotherapy. Cancer Res. 64, 1867–1874
10. Srivastava, M. K., Bosch, J. J., Thompson, J. A., Ksander, B. R., Edelman, M. J., and Ostrand-Rosenberg, S. (2006) Lung cancer patients’ CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells. Cancer Immunol. Immunother. 57, 1493–1504
11. Thompson, J. A., Dissanayake, S. K., Ksander, B. R., Knutson, K. L., Disis, M. L., and Ostrand-Rosenberg, S. (2006) Tumor cells transduced with the MHC class II Transactivator and CD80 activate tumor-specific CD4+...
12. Thompson, J. A., Srivastava, M. K., Bosch, J. J., Clements, V. K., Ksander, B. R., and Ostrand-Rogersen, S. (2008) The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4+ T lymphocytes. *Cancer Immunol. Immunother.* 57, 389–398

13. Chamuleau, M. E., Souwer, Y., Van Ham, S. M., Zevenbergen, A., Westers, T. M., Berkhof, J., Meijer, C. J., van de Loosdrecht, A. A., and Ossenkoppele, G. J. (2004) Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. *Cancer Res.* 64, 5548–5550

14. van Luijning, M. M., Chamuleau, M. E., Thompson, J. A., Ostrand-Rogersen, S., Westers, T. M., Souwer, Y., Ossenkoppele, G. J., van Ham, S. M., and van de Loosdrecht, A. A. (2010) Class II-associated invariant chain peptide down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased CD4+ T-cell responses. *Haematologica* 95, 485–493

15. van Luijning, M. M., van den Ancker, W., Chamuleau, M. E., Zevenbergen, A., Westers, T. M., Ossenkoppele, G. J., van Ham, S. M., and van de Loosdrecht, A. A. (2011) Absence of class II-associated invariant chain peptide on leukemic blasts of patients promotes activation of autologous leukemia-reactive CD4+ T cells. *Cancer Res.* 71, 2507–2517

16. Pauley, R. J., Soule, H. D., Tait, L., Miller, F. R., Wolman, S. R., Dawson, P. J., and Heppner, G. H. (1993) The MCF10 family of spontaneously immortalized human breast epithelial cell lines: models of neoplastic progression. *Eur. J. Cancer Prev.* 2 67–76

17. Deponie, F. R., Olan, A. T., Zarling, A. L., McMiller, T. L., Salay, T. M., Norris, A., English, A. M., Shabanowitz, J., Engelhard, V. H., Hunt, D. F., and Topalian, S. L. (2009) Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12073–12078

18. Deutsch, E. W., Mendoza, L., Shiteynberg, D., Farrah, T., Lam, H., Tasman, N., Sun, Z., Nilsson, E., Pratt, B., Prazen, B., Eng, J. K., Martin, D. B., Nesvizhskii, A. I., and Aebersold, R. A guided tour of the Trans-Proteomic Pipeline. *Proteomics* 10, 1150–1159

19. Borstnik, B., and Hofacker, G. L. (1985) Functional aspects of the neutral patterns in protein evolution, Guiderland: Academic Press

20. Sotiriadou, R., Perez, S. A., Gritzapis, A. D., Sotropoulou, P. A., Echner, H., Heinzl, S., Mamakaki, A., Pavlec, G., Voelter, W., Baxevanis, C. N., and Papamichail, M. (2001) Peptide HER2(776–788) represents a naturally processed broad MHC class II-restricted T cell epitope. *Br. J. Cancer* 85, 1527–1534

21. Salazar, L. G., Fikes, J., Southwood, S., Ishioka, G., Knutson, K. L., Gooley, T. A., Schiffman, K., and Disis, M. L. (2003) Immunization of cancer patients with HER-2/neu-derived peptides demonstrating high-affinity binding to multiple class II alleles. *Clin. Cancer Res.* 9, 5559–5565

22. Thomas, A. M., Santarius, L. M., Lutz, E. R., Armstrong, T. D., Chen, Y. C., Huang, L. Q., Laheru, D. A., Goggins, M., Hruban, R. H., and Jaffee, E. M. (2004) Mesothelin-specific CD8+ T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J. Exp. Med.* 200, 297–306

23. Kessler, J. H., Mommaas, B., Mutia, T., Huijbiers, I., Vissers, D., Benchkhijsen, W. E., Schreuder, G. M., Ofringa, R., Goumyl, E., Meleif, C. J., van der Burg, S. H., and Drijfhout, J. W. (2003) Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using flou-