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The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4⁺ T lymphocytes

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Abstract

Activation of tumor-reactive T lymphocytes is a promising approach for the prevention and treatment of patients with metastatic cancers. Strategies that activate CD8⁺ T cells are particularly promising because of the cytotoxicity and specificity of CD8⁺ T cells for tumor cells. Optimal CD8⁺ T cell activity requires the co-activation of CD4⁺ T cells, which are critical for immune memory and protection against latent metastatic disease. Therefore, we are developing “MHC II” vaccines that activate tumor-reactive CD4⁺ T cells. MHC II vaccines are MHC class I⁺ tumor cells that are transduced with costimulatory molecules and MHC II alleles syngeneic to the prospective recipient. Because the vaccine cells do not express the MHC II-associated invariant chain (Ii), we hypothesized that they will present *endogenously* synthesized tumor peptides that are not presented by professional Ii⁺ antigen presenting cells (APC) and will therefore overcome tolerance to activate CD4⁺ T cells. We now report that MHC II vaccines prepared from human MCF10 mammary carcinoma cells are more efficient than Ii⁺ APC for priming and boosting Type 1 CD4⁺ T cells. MHC II vaccines consistently induce greater expansion of CD4⁺ T cells which secrete more IFN γ and they activate an overlapping, but distinct repertoire of CD4⁺ T cells as measured by T cell receptor V β usage, compared to Ii⁺ APC. Therefore, the absence of Ii facilitates a robust CD4⁺ T cell response that includes the presentation of peptides that are presented by traditional APC, as well as peptides that are uniquely presented by the Ii⁻ vaccine cells.

Keywords

Cancer vaccines; Major histocompatibility complex class II; Antigen processing and presentation

Introduction

Successful cancer vaccines must induce potent tumor-specific immunity and long term immunological memory to protect against recurrent tumors and metastatic disease. Many vaccines are aimed at boosting cell-mediated immunity, which is considered particularly promising due to its ability to destroy tumor cells. Type 1 CD4⁺ T cells, which secrete IFN γ ,

have long been recognized as critical components for the activation of CD8⁺ T cells, either through their classical role as “helper” T cells that provide cytokine support for CD8⁺ T cells [6,22], or by their induction of CD40 expression on dendritic cells (DC) (“licensing”), which in turn activate CD8⁺ T cells [5,36,40]. CD4⁺ T cells are also essential for generating CD8⁺ T memory cells, for preventing CD8⁺ T cells from being tolerized [3,19–21,41,44,45], and for recruiting cells of the innate immune system, such as macrophages. IFN γ production at the tumor site also up-regulates tumor-expressed MHC molecules to improve CTL recognition, and blocks neo-vascularization to prevent tumor proliferation [18,34,35]. Because of the central role of CD4⁺ T cells in facilitating anti-tumor immunity, we are developing vaccine strategies that specifically activate CD4⁺ T cells.

Activation of CD4⁺ T cells requires two signals: an antigen-specific signal delivered by a major histocompatibility complex class II (MHC II) molecule presenting a specific peptide, and a costimulatory signal. Expression of MHC II and costimulatory molecules is usually limited to professional antigen presenting cells (APC), such as DC. Therefore, activation of tumor-reactive CD4⁺ T cells requires that tumor antigens are endocytosed by DC and processed and presented to the appropriate CD4⁺ T cells [37]. Presentation of exogenously acquired antigen by DC is facilitated by the expression of the MHC II accessory molecule invariant chain (Ii). As newly synthesized MHC II molecules enter the endoplasmic reticulum (ER), their peptide-binding groove is occupied by Ii. From the ER, MHC II/Ii complexes traffic to endosomal compartments where Ii is degraded, and peptides derived from endocytosed, exogenously synthesized molecules are loaded with the help of HLA-DM [10]. As a result, CD4⁺ T cells that are activated by professional APC react with antigens obtained from third party (e.g. tumor) cells, but the actual peptides for which the CD4⁺ T cells are specific, are generated by DC.

Tumors synthesize antigens that are immunogenic and under some circumstances can activate an immune response that mediates tumor rejection [16,38]. However, many tumor-bearing individuals are tolerized to the tumor-derived peptides presented by their DC, and do not produce tumor-reactive CD4⁺ T cells [42,43].

We are developing cell-based “MHC II vaccines” that are designed to overcome an individual’s tolerance to their tumor peptides. The MHC II vaccines are tumor cells that are genetically modified to express MHC II and costimulatory molecules. In professional APC which are Ii⁺, peptides derived from *exogenously* synthesized molecules are processed in endosomes where they bind to MHC II molecules. Because the vaccine cells do not express Ii, we have hypothesized that MHC II molecules bind peptides in the ER that are derived from *endogenously* synthesized proteins [1,32,33]. Since ER-resident peptides are likely to differ from endosomally generated peptides [9], the vaccines may present novel peptides that are not presented by professional Ii positive APC. Therefore, the vaccine cells should activate a different population of CD4⁺ T cells than that activated by Ii positive APC, thereby overcoming tolerance and stimulating tumor-specific immunity.

In previous reports MHC II vaccines were shown to have significant therapeutic efficacy in mouse models against large, established primary [2] and spontaneously metastatic [30,31] tumors. We have recently adapted the MHC II vaccine approach for human tumors and have developed several vaccines for human mammary carcinoma and ocular melanoma [13]. Similar to the mouse vaccines, the human MHC II vaccines efficiently present endogenously synthesized tumor peptides, and activate tumor-reactive CD4⁺ T cells from healthy donors [47] and from patients with primary or metastatic cancer [8]. In this report we demonstrate that human Ii negative MHC II vaccine cells are significantly better activators of tumor-reactive Type 1 CD4⁺ T cells than are Ii positive APC, and that the Ii negative vaccine cells activate an overlapping, but distinct, repertoire of CD4⁺ T cells than is activated by Ii positive APC. These findings support our hypothesis that Ii negative MHC II vaccine cells present some tumor

peptides that are not presented by professional Ii positive APC and therefore may be effective therapeutic and/or prophylactic agents for cancer patients.

Materials and methods

Cells

The human tumor cell lines Sweig and Jurkat, peripheral blood mononuclear cells (PBMC) from healthy human donors [13], MCF10CA1 human mammary carcinoma (hereafter called MCF10), and MCF10 transductants [47] were handled as described. MCF10/CIITA/CD80/Ii siRNA cells are MCF10/CIITA/CD80 cells that were rendered Ii negative by transduction with Ii siRNA 32. MHC II and Ii siRNA transductants were previously shown to express stable MHC II $\alpha\beta$ heterodimers and not express Ii as assayed by western blotting and flow cytometry [47]. PBMC were obtained by venipuncture from healthy human donors and buffy coats were stored in liquid nitrogen until used. Thawed PBMC were >90% viable. All cell lines and procedures were approved by the institutional review boards of the participating institutions.

Retroviral constructs, transductions and drug selection

The human major histocompatibility complex class II associated invariant chain (Ii) cDNA (Genbank accession no. NID X00497), containing both start codons for p33 and p35 isoforms of Ii, was cloned from sp64/p33 (kindly provided by Eric Long, NIH) [23], into the pLPCX vector (Clontech) containing a puromycin resistance gene. To construct the pLPCX/Ii vector containing the Ii gene, Ii was excised from sp64/p33 with *SalI* and *EcoRI* (New England Biolabs; Beverly, MA, USA) and inserted into the pLPCX vector digested with *XhoI* (New England Biolabs) and *EcoRI* (*XhoI* having compatible cohesive ends with *SalI*). Proper insertion of the Ii gene was confirmed by sequence analysis. Sequencing reactions were carried out using the "ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1" (Perkin Elmer Applied Biosystems Division, Foster City, CA, USA) according to the manufacturer's protocol using a PTC-200 Thermal Cycler (MJ Research, BioRad, Hercules, CA, USA) and an ABI PRISM 3700 DNA Analyzer (Applied Biosystem). Retroviruses were prepared, and tumor cells were transduced with the retroviruses as described [13]. Transduced cells were selected in 0.3 $\mu\text{g/ml}$ puromycin for 2–3 weeks after transduction, and thereafter maintained in MCF10 medium without puromycin. Transduced cells were periodically monitored by flow cytometry for expression of the transduced genes, which were stably expressed for >12 months.

Antibodies, immunofluorescence, western blots

HLA-DR-FITC, CD80-PE, FITC-isotype and PE-isotype controls were from BD Biosciences. CD4-FITC and CD8-FITC were from Miltenyi Biotec Inc. (Auburn, CA, USA); HLA-DM-PE was from BDBiosciences (San Diego, CA, USA). Culture supernatants of hybridomas W6/32 (pan HLA-A, B, C), L243 (pan anti-HLA-DR), and PIN1.1 (anti-Ii) were prepared, and tumor cells and PBMC were stained for cell surface markers, or fixed and stained for Ii as described [13]. T cell receptor (TCR) $V\beta$ repertoire of CD4^+ T cells was determined by three color flow cytometry analysis using the IO Test Beta Mark TCR $V\beta$ Repertoire kit (Beckman Coulter, San Diego, CA, USA), which consists of monoclonal antibodies (mAbs) to 24 distinct TCR $V\beta$ families (about 70% coverage of normal human TCR $V\beta$ repertoire). Each test includes three directly coupled TCR $V\beta$ mAbs, conjugated to PE, FITC or both PE and FITC. PBMC were stained and analyzed for TCR $V\beta$ usage according to the manufacturer's instructions, with the following modification: prior to staining with $V\beta$ mAbs, 1×10^5 PBMC were stained with 1 μg of biotin conjugated anti-human CD4 (eBioscience, San Diego, CA, USA), then washed and stained with streptavidin-PerCP (BD). Viable CD4^+ T cells were gated according to CD4 staining and side scatter, and this population was analyzed for $V\beta$ TCR usage. Western blots were done as described [47].

T cell priming and T cell depletions

Transduced MCF10, (MCF10/DR7/CD80, MCF10/DR7/CD80/Ii, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/Ii siRNA) cells were plated on day 0 in MCF10 medium at $2-4 \times 10^5$ cells/4 ml in 6 cm dishes and the cells allowed to attach before irradiating with 25 Gy. The next morning MCF10 medium was removed and $1-2 \times 10^7$ PBMC in 4 ml of T cell medium (Isocoves Modified Dulbeccos Medium, 1% gentamicin, 1% penicillin, 2 mM Glutamax, 0.01 M HEPES, 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 0.05 mM mercaptoethanol (VWR West Chester, PA, USA), 5% human AB serum (Genini Bio-Products Woodland, CA, USA) were added to each 6 cm dish, and the cells were incubated at 37°C for 3–5 days. At the end of this incubation period, non-adherent cells (PBMC) were harvested, washed twice, and resuspended at 1×10^6 PBMC/2 ml T cell medium supplemented with human recombinant IL-15 (40 ng/2 ml) (Peprotech, Rocky Hill, NJ, USA). PBMC were then plated in 24 well plates at 1×10^6 cells/2 ml/well. After a 7 day culture period, the non-adherent cells (“primed PBMC”) were removed, washed twice, and then rested in T cell medium for 1–3 days before being used in antigen presentation assays. In some experiments, PBMC were depleted for CD4⁺ or CD8⁺ T cells before priming, using LD columns and CD4 or CD8 microbeads according to the manufacturers’ directions (Miltenyi, Auburn, CA, USA).

Antigen presentation assays

MHC II vaccine cells were plated at 2.5×10^4 cells/200μ MCF10 medium/well in 96 well plates and allowed to adhere for 2–4 h. MCF10 medium was then removed and primed PBMC in T cell medium were added at 5×10^4 PBMC/200 μl/well. After 48 h of incubation, the supernatants were analyzed for IFNγ by ELISA [13], and the non-adherent cells were analyzed for TcR Vβ repertoire by flow cytometry. Multiplex cytokine analyses were done in the Cytokine Core Facility at the University of Maryland, Baltimore using the Luminex 100 System (Luminex, Austin, TX, USA) according to the manufacturer’s directions.

HLA haplotyping

PBMC from healthy donors and MCF10 cell lines were HLA typed using SSP-ABDR DNA typing trays (OneLambda, Canoga Park, CA, USA) according to the manufacturer’s recommendations. MCF10 are A33, B55, DR7. PBMC 100704 are A11, A29; B44, B51; DR4, DR7, DR53. PBMC 111504 are A1, A68; B35, B57; DR1, DR7, DR53; PBMC 123104 are A33, A36; B-, B44; DR1, DR7, DR53.

Statistical analysis and TCR Vβ usage calculations

Mean, SD, and statistical significance as measured by Student’s *t* test were calculated using Excel v2002. Percent increase in TCR Vβ usage = $100\% \times [(\% \text{ cells with specific TCR V}\beta \text{ after activation with Ii}^- \text{ or Ii}^+ \text{ APC})/(\% \text{ cells with specific TCR V}\beta \text{ before activation})]$.

Results

MCF10 human mammary carcinoma cells transduced with an Ii (p33) expression vector express Ii

To study the effects of Ii on the repertoire of peptides presented by MHC II-transduced human breast cancer cells, we have constructed an expression vector that contains p33, which is the dominant isoform of Ii [23] (Fig. 1a). MCF10 cells previously transduced with retroviruses encoding MHC II and CD80 molecules (MCF10/DR7/CD80) were further transduced with Ii retroviruses. The resulting cells (MCF10/DR7/CD80/Ii) were stained with mAbs for cell surface expression of HLA-DR, HLA-DM, and CD80 (live cells), or for internal Ii expression (fixed cells), and analyzed by flow cytometry (Fig. 1b). MCF10/DR7/CD80/Ii cells express Ii protein, and their levels of HLA-DR and CD80 are similar to the levels expressed by Ii[−]

MCF10/DR7/CD80 cells. Neither population expresses HLA-DM. Expression of the Ii p33 isoform by MCF10/DR7/CD80/Ii cells was further confirmed by western blotting using the human B cell lymphoma cell line Swei as a positive control for p33 (Fig. 1c). Therefore, Ii is expressed in MCF10/DR7/CD80/Ii breast cancer cells, and its expression does not alter expression of HLA-DR7, HLA-DM, or CD80.

Ii⁻ transductants prime CD4⁺ T cells more efficiently than Ii⁺ transductants

Previous studies have established that Ii⁻ MCF10/DR7/CD80 transductants bind MHC II-restricted peptides and activate CD4⁺ T cells that were primed by peptide pulsing; however, these studies did not determine if the Ii⁻ vaccines prime CD4⁺ T cells [47]. To test whether Ii⁻ vaccine cells efficiently prime CD4⁺ T cells, HLA-DR7⁺ PBMC from healthy human donors were depleted for CD8⁺ T cells and co-cultured (primed) with irradiated MCF10/DR7/CD80 vaccine cells. Following 3 days of culture, non-adherent cells (lymphocytes) were removed, expanded in IL-15 for 6 days, re-cultured (boosted) with MCF10/CD80 or MCF10/DR7/CD80 cells, and the supernatants analyzed for IFN γ (Fig. 2a). Boosting with MCF10/DR7/CD80, but not with the MCF10/CD80, cells induces IFN γ release, indicating that the transduced MHC II allele (DR7) is essential for priming of CD4⁺ T cells and that allogeneic MHC I differences between the vaccine cells and the responding PBMC do not result in IFN γ release. Therefore, the vaccine cells prime naïve CD4⁺ T cells and the priming is MHC II-dependent.

To determine if Ii⁺ cells are as effective as the Ii⁻ vaccines in priming, DR7⁺ PBMC from healthy donor 100704 were primed with Ii⁻ (MCF10/DR7/CD80) or with Ii⁺ (MCF10/DR7/CD80/Ii) cells, expanded in IL-15, rested, and boosted with the transductants used for priming. PBMC were either not depleted, or depleted for CD4⁺ or CD8⁺ T cells prior to priming (Fig. 2b). Ii⁻ vaccine cells induced more than twice as much IFN γ production as Ii⁺ cells and the response is virtually eliminated by depletion of CD4⁺ T cells prior to priming, indicating that CD4⁺ T cells are the only cells activated to produce IFN γ . Priming and boosting with Ii⁻ cells also gave 1.5–2 fold more CD4⁺ T cells compared to priming and boosting with Ii⁺ cells (data not shown).

To ascertain the type of response induced by the Ii⁻ vaccine cells, the supernatants from the boosted cultures of Fig. 2b were assayed by multiplex analysis for IL-2, IL-4, IL-10, IL-15, and IFN γ (Fig. 2c). MCF10/DR7/CD80 vaccine cells induce high levels of the type 1 cytokine IFN γ , intermediate levels of IL-2, and very low levels of the type 2 cytokines IL-4 and IL-10. Therefore, MCF10/DR7/CD80 vaccine cells are significantly more efficient than Ii⁺ cells for priming type 1 tumor-reactive CD4⁺ T lymphocytes.

HLA-DM expression does not affect CD4⁺ T cell activation by MHC II vaccine cells

In professional APC such as DC, HLA-DM facilitates the binding of peptides derived from exogenously synthesized molecules by displacing the class II-associated Ii peptide (CLIP) from the peptide binding groove of MHC II molecules [10]. Since MCF10/DR7/CD80/Ii vaccine cells contain Ii, but do not contain DM (Fig. 1b), their reduced antigen presentation activity may be due to an inability to replace CLIP with other peptides. If MCF10/DR7/CD80/Ii cells have this defect and are unable to replace CLIP, then their cell surface HLA-DR molecules will have bound CLIP. To assess this possibility, live MCF10/DR7/CD80/Ii cells were stained with the CerCLIP mAb which detects MHC II/CLIP complexes (Fig. 3a). MCF10/DR7/CD80/Ii cells express approximately the same amount of HLA-DR/CLIP complexes at the cell surface as the B cell lymphoma Swei, which constitutively expresses HLA-DR, Ii, and DM. As expected, MCF10/DR7/CD80 cells do not express HLA-DR/CLIP complexes at the cell surface since they do not contain Ii. Therefore, MCF10/DR7/CD80/Ii cells do not express significantly higher levels of CLIP than Swei cells, making it unlikely that their reduced

antigen presentation activity is due to excessive levels of CLIP interfering with MHC II-restricted peptide presentation.

Because professional APC such as DC express DM, we have prepared MHC II vaccines using the MHC class II transactivator (CIITA), which coordinately up-regulates MHC II, Ii, and DM [15], and an siRNA to down-regulate Ii (MCF10/CIITA/CD80/Ii siRNA cells) [47]. As measured by flow cytometry, these vaccine cells express high levels of HLA-DR, very low levels of HLA-DR/CLIP complexes at the cell surface (Fig. 3a), and levels of HLA-DM similar to Sweig cells (Fig. 3b). Since PBMC that are syngeneic to the MCF10 vaccines are not available, and CIITA up-regulates all HLA-DR, -DP, and DQ alleles, the CIITA transductants cannot be used as priming agents because they will induce MHC II allogeneic responses. However, allogeneic T cell activation should not occur during the short duration of the boosting response, so the CIITA transductants can be used as boosting agents. To determine if DM expression impacts the ability of Ii⁻ vaccine cells to boost CD4⁺ T cells, DR7⁺ PBMC from healthy donor 111504 were depleted of CD8⁺ T cells, primed with Ii⁻ MCF10/DR7/CD80 cells, and boosted with Ii⁻DM⁻ (MCF10/DR7/CD80), Ii⁻DM⁺ (MCF10/CIITA/CD80/Ii siRNA), or Ii⁺DM⁺ (MCF10/CIITA/CD80) cells (Fig. 4a). Boosting with Ii⁻DM⁻ or Ii⁻DM⁺ cells induces a strong response, while boosting with Ii⁺DM⁺ cells gives a much poorer response. As expected, allogeneic MHC I differences between the boosting cells and responding PBMC do not activate CD4⁺ T cells because MCF10/CIITA/CD80 cells do not cause significant IFN γ release. Therefore, DM expression does not impact the boosting of CD4⁺ T cells that are primed with Ii⁻ vaccine cells.

Although DM expression is irrelevant for the boosting of CD4⁺ T cells that are primed with Ii⁻ cells, it may affect the boosting of CD4⁺ T cells that are primed with Ii⁺ cells. To test this possibility, DR7⁺ PBMC from healthy donor 123104 were depleted for CD8⁺ T cells and primed with Ii⁻ MCF10/DR7/CD80 or Ii⁺ MCF10/DR7/CD80/Ii cells and boosted with Ii⁻DM⁻ (MCF10/DR7/CD80), Ii⁺DM⁻ (MCF10/DR7/CD80/Ii), Ii⁻DM⁺ (MCF10/CIITA/CD80/Ii siRNA), or Ii⁺DM⁺ (MCF10/CIITA/CD80) cells (Fig. 4b). Ii⁻ cells are significantly more efficient than Ii⁺ cells for boosting CD4⁺ T cells, regardless of DM expression. Therefore, Ii⁻ MHC II vaccine cells are more effective activators of CD4⁺ T cells than Ii⁺ cells, and the decreased efficiency of the Ii⁺ cells is unlinked to HLA-DM expression.

Ii⁻ and Ii⁺ vaccine cells activate overlapping, but distinct, repertoires of CD4⁺ T cells

If Ii⁻ vaccine cells present novel tumor antigen epitopes that are not presented by Ii⁺ cells, then Ii⁻ and Ii⁺ cells should activate different repertoires of CD4⁺ T cells. This hypothesis was tested by assessing the TCR V β repertoires of the CD4⁺ T cells activated by Ii⁺ vs. Ii⁻ cells. PBMC from DR7⁺ healthy human donors were primed and boosted with Ii⁻ (MCF10/DR7/CD80) or Ii⁺ (MCF10/DR7/CD80/Ii) cells, and the non-adherent cells were harvested and triple-stained for CD4 and 24 different TcR V β s. The CD4⁺ T cells were gated (Fig. 5a) and analyzed for V β expression. Figure 5b shows the TCR V β family usage by CD4⁺ T cells from healthy donors 123104 and 100704 before and after activation with either the Ii⁻ or Ii⁺ cells. Table 1 shows the number of TCR V β families that are increased at least 20% in response to Ii⁻ or Ii⁺ cells in these two donors plus a third healthy donor (111504). Some TCR V β families are equally activated by both Ii⁻ and Ii⁺ cells, while other families are activated exclusively by Ii⁻ or Ii⁺ APC. Therefore, Ii⁻ and Ii⁺ cells activate some of the same TCR V β families, but they also activate distinct families, demonstrating that Ii⁻ vaccine cells activate CD4⁺ T cells that are not activated by standard APC that are Ii⁺.

Discussion

The human MHC II vaccines described in this report are generated by transducing Ii⁻MHC I⁺ mammary carcinoma cells with CD80 and MHC II alleles syngeneic to the prospective

recipient. Previous studies demonstrated that such vaccines induce tumor-specific immunity that results in rejection of established primary and metastatic disease in mouse models [2,12, 29,31]. This report extends the mouse studies to humans and demonstrates that the absence of Ii in the vaccine cells is a critical element for vaccine efficacy. We find that MHC II vaccines prime and boost type 1 CD4⁺ T cells in vitro to release high levels of IFN γ . MHC II vaccines consistently induced greater expansion of CD4⁺ T cells that secreted more IFN γ and expressed a different repertoire of T cell receptor gene families than CD4⁺ T cells primed and/or boosted by the same cells expressing Ii. Collectively, these observations are consistent with the hypothesis that the absence of Ii facilitates a more robust CD4⁺ T cell response that includes the presentation of tumor peptides that are presented by Ii⁺ APC, as well as distinct peptides that are uniquely presented by Ii⁻ APC.

There are several possible explanations why the absence of Ii promotes greater expansion of T cells and increased IFN γ secretion by activated CD4⁺ T cells. In contrast to professional APC, the peptide binding groove of newly synthesized MHC II molecules of MHC II vaccines is not occupied by Ii or CLIP, and may bind peptides derived from endogenously synthesized molecules either in the ER or as they traffic from the ER to endosomes. If the ER contains peptides that are not present in endosomes, then the genetically modified tumor cells may be presenting peptides that are not presented by professional APC. Such antigen presentation would result in the activation of CD4⁺ T cells that are not typically activated by professional Ii⁺ APC. Other studies using different experimental approaches support this possibility. For example, Ii⁻ MHC II vaccine cells activate CD4⁺ T cells to peptides derived from diverse subcellular compartments, whereas Ii⁺ APC do not [33], consistent with the concept that Ii⁻ MHC II vaccine cells present a different repertoire of tumor peptides than the repertoire presented by Ii⁺ APC. Studies with Ii knockout mice similarly show that the absence of Ii facilitates the presentation of novel endogenous epitopes, while most endogenous antigens that are presented in the presence of Ii are also presented in the absence of Ii [7]. These findings are also corroborated by biochemical studies in which mass spectroscopy analysis of MHC II-bound peptides demonstrated that Ii⁻ APC display peptides that are not presented by Ii⁺ APC [28].

The presentation of different and/or more immunogenic epitopes by Ii⁻ MHC II vaccines may also be due to the presentation of “cryptic” peptides, a term coined by investigators studying autoimmunity. Cryptic peptides are peptides that are not presented in the thymus during central tolerance induction, but are present in the periphery and which bind with high affinity to MHC molecules. Such peptides have been identified and shown to induce T cell responses to self antigens [4,17,24,39]. If the absence of Ii in MHC II vaccine cells results in altered antigen processing and presentation, as we have hypothesized, then the vaccine cells may present cryptic tumor peptides which potentially induce a more robust CD4⁺ T cell response.

The MCF10/DR7/CD80 and MCF10/CIITA/CD80/siRNA vaccines both activate tumor-reactive CD4⁺ T cells in the absence of Ii and have the potential to be clinically useful. We have proposed using the vaccines in non-autologous patients who are HLA-DR-matched to the vaccine cells, to avoid customized vaccine preparation for individual patients [8]. Each vaccine has its pros and cons. Advantages of the multiple allele CIITA-based vaccines include (a) their presentation of tumor peptides on multiple MHC II alleles, (b) their ease of production via a universal construct encoding the CIITA, Ii siRNA, and CD80, (c) the potential to use tumor cells that constitutively express MHC II, and (d) increased expression of MHC class I molecules due to up-regulation of MHC I by the CIITA. A drawback of the CIITA vaccines is their potential to activate allo-MHC II responses, which may overwhelm the tumor-specific MHC II-restricted response in non-fully MHC II-matched recipients. A major advantage of the single allele vaccines is the ease of HLA-DR-matching of the vaccine to an individual patient. A potential disadvantage of the single allele vaccines is that an IFN γ -inducible tumor cell line

could be up-regulated for Ii if the vaccine enters or induces an inflammatory environment. However, this disadvantage can be overcome if non-IFN γ -inducible cells are used or if the cells are co-transduced with Ii siRNA. Both the single allele and multiple allele vaccines could be stored as frozen cells, thereby minimizing reagent preparation time for individual patients.

Several clinical observations support our hypothesis that tumor cell co-expression of Ii with MHC II blocks tumor cell immunogenicity and favors tumor progression by impeding T cell activation to endogenously synthesized tumor antigens. Chamuleau and colleagues have observed that acute myelogenous leukemia patients whose myeloid leukemic blasts are HLA-DR⁺CLIP⁻ or low have a significantly better clinical prognosis than patients whose blasts are DR⁺CLIP^{high} [11]. Since the failure to remove CLIP from MHC II molecules reduces endogenous antigen presentation [25], this observation is consistent with the concept that HLA-DR expression in the absence of Ii favors the activation of tumor-reactive CD4⁺ T cells. Similarly, the co-expression of Ii by HLA-DR⁺ hepatocellular carcinoma cells is associated with a very poor prognosis [46]. Likewise, tumor cell expression of an isoform of Ii that blocks endogenous antigen presentation is associated with poor prognosis in chronic lymphocytic leukemia patients [48]. If the vaccine cells are the actual APC in vivo, then they may be particularly useful for treating cancer patients whose DC are dysfunctional due to tumor burden [26,27]. If the vaccine cells are not the relevant APC in vivo, and CD4⁺ T cells are activated via cross-dressing [14] or by conventional antigen presentation via DC, then the vaccines are useful reagents for producing novel peptide-MHC complexes. Regardless of the precise mechanism by which the MHC II vaccines activate tumor-reactive CD4⁺ T cells, they efficiently prime and boost CD4⁺ T cells, and the absence of Ii is critical for their activity.

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Fig. 1.

MHC II breast cancer vaccines prepared from MCF10 cells express HLA-DR and CD80 and do not express invariant chain unless transduced with a p33 Ii construct. **a** Invariant chain retroviral vector. **b** MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii transductants stained for Ii (mAb Pin1.1), CD80 (mAb CD80), HLA-DR (mAb L243), or HLA-DM (mAb DM), and analyzed by flow cytometry. **c** Western blot of MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii cells and control Sweig and Jurkat cells probed for Ii with the PIN1.1 mAb. Data of panels **b** and **c** are representative of three independent experiments

Fig. 2.

MCF10/DR7/CD80 vaccine cells prime and boost healthy donor PBMC and are better activators of CD4⁺ T cells than MCF10/DR7/CD80/Ii cells. **a** PBMC from healthy donor 100704 were depleted for CD8⁺ T cells, primed with MCF10/DR7/CD80 vaccine cells, boosted with MHC II negative control (MCF10/CD80) or vaccine cells (MCF10/DR7/CD80), and T cell activation quantified by measuring IFN γ release by ELISA. CD8-depleted PBMC contained <2% CD8⁺ T cells and 88.6% CD4⁺ T cells at the start of priming. Data are representative of three independent experiments from three different healthy donors. **b** PBMC from healthy donor 100704 were either not depleted or depleted for CD4⁺ or CD8⁺ T cells prior to priming and boosting with Ii⁻ (MCF10/DR7/CD80) or Ii⁺ (MCF10/DR7/CD80/Ii)

cells. T cell activation was quantified by measuring IFN γ release. After depletion and before priming, CD4-depleted PBMC were <2% CD4 $^{+}$ and 74.3% CD8 $^{+}$; CD8-depleted PBMC were 78.2% CD4 $^{+}$ and <1.5% CD8 $^{+}$; not depleted PBMC were 44.2% CD4 $^{+}$ and 38.3% CD8 $^{+}$. Data are representative of four independent experiments using PBMC from three different healthy donors. **c** Supernatants from primed and boosted PMBC from panel B were tested for multiple cytokines by multiplex analysis

Fig. 3.

MHC II vaccine cells do not express HLA-DR/CLIP complexes at the cell surface. Live MCF10 transductants were stained for cell surface HLA-DR/CLIP complexes (CerCLIP mAb), HLA-DR (L243) (**a**), or HLA-DM (**b**) and analyzed by flow cytometry

Fig. 4.

HLA-DM expression does not affect CD4⁺ T cell activation by MHC II breast cancer vaccine cells. **a** PBMC from healthy donor 111504 were depleted for CD8⁺ T cells, primed with Ii⁻ (MCF10/DR7/CD80) vaccine cells, and boosted with Ii⁻ (MCF10/DR7/CD80, MCF10/CIITA/CD80/li siRNA), Ii⁺ (MCF10/CIITA/CD80), or control (CD80) transductants. T cell activation was quantified by measuring IFN γ secretion. After depletion and before priming PBMC were 87.5% CD4⁺ and <2% CD8⁺. **b** PBMC from DR7⁺ healthy donor 123104 were depleted for CD8⁺ T cells, primed with Ii⁻ (MCF10/DR7/CD80) or Ii⁺ (MCF10/DR7/CD80/li) cells and boosted with Ii⁻ or Ii⁺ transductants as in **a**. After depletion and before priming PBMC were 80.7% CD4⁺ and 4.9% CD8⁺. Data of panels **a** and **b** are representative of 2–4

independent experiments with 2 healthy donors and 3 experiments with 1 healthy donor, respectively

Fig. 5.

Ii⁻ and Ii⁺ MHC II breast cancer cells activate different repertoires of CD4⁺ T cells. PBMC from healthy donor 123104 or 100704 were depleted for CD8⁺ T cells, primed and boosted with Ii⁻ (MCF10/DR7/CD80) or Ii⁺ (MCF10/DR7/CD80/Ii) cells and stained with mAb to CD4, mAb to TCR Vβ subtypes, or isotype control mAb. Gated CD4⁺ T cells were analyzed for the percent of CD4⁺ T cells expressing a given TCR Vβ. P values are indicated. Data are the average of three independent experiments for each PBMC donor

Table 1

Ii^- and Ii^+ breast cancer cells activate over-lapping and distinct repertoires of CD4^+ T cells

PBMC donor	Number of TCR $\text{V}\beta$ families expanded after activation ^a		
	Ii^+ APC	Ii^- APC	Ii^+ and Ii^- APC ^b
123104	1	2	6
100704	1	3	10
111504	1	6	5

^a Expansion of a given TCR $\text{V}\beta$ family is defined as a statistically significant increase ($P < 0.5$) of at least 20% in the percent of CD4^+ T cells after priming and boosting compared to unprimed CD4^+ T cells

^b Number of common TCR $\text{V}\beta$ families expanded by both Ii^- and Ii^+ APC