Heteroclitic XBP1 peptides evoke tumor-specific memory cytotoxic T lymphocytes against breast cancer, colon cancer, and pancreatic cancer cells

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Introduction

High levels of proliferation characteristic of cancer cells lead to nutrient deprivation, hypoxia, and accumulation of unfolded/misfolded proteins, which in turn leads to physiologically important ER stress responses, such as the UPR. UPR is a cellular adaptive mechanism that occurs in a majority of solid tumors, which allows for tumor cell survival under stress conditions by increasing its protein folding capacity.1,2 X-box binding protein 1 (XBP1), a critical transcription activator in the UPR, regulates a subset of ER-resident chaperone genes essential for protein folding and maturation.3,4 A recent report shows that XBP1 splicing plays an important role in the regulation of glucose homeostasis, which is independent of ER stress.5 XBP1 exists as unspliced (XBP1u), a functional protein encoded by unspliced XBP1 mRNA, and spliced (XBP1s) forms via inositol requiring enzyme 1 α (IRE1 α)-mediated unconventional splicing, in which XBP1u facilitates the recruitment of XBP1 mRNA cotranslationally.6 Genome-wide profiling, along with association studies and immunohistochemistry, demonstrated that XBP1 expression was induced in a variety of primary cancers or cancer cell lines including hematological malignancies such as multiple myeloma and acute lymphoblastic leukemias,7-9 as well as breast cancers,10,11 hepatocellular carcinoma,12,13 pancreatic adenocarcinomas,14,15 and colon cancer.16,17 It has been demonstrated that XBP1 is activated in primary mammary tumors, and that its expression correlates with enhanced tumor growth.18 In support of a direct role for XBP1 in tumorigenesis, the loss of XBP1 was shown to severely inhibit tumor growth. In addition, transformed cells with XBP1 deficiency were sensitized to hypoxia and underwent apoptosis, implicating XBP1 as a survival factor.19 Thus, disrupting or targeting the XBP1 pathway is a rational approach for selective cancer cell killing, providing the basis for therapeutic strategies against multiple solid tumors.

The T-box transcription factors, T-bet and eomesodermin (Eomes), expressed in T cells have been implicated as master regulators of CD8+ T cell differentiation into Th1 type cells and the critical formation of effector and memory T cell subsets.20-22 In
the context of CD8\(^+\) memory T cells, T-bet and Eomes sustain memory phenotypes by stabilizing the expression of IL-2R \(b\), thus promoting IL-15 signaling, which is critical for continued proliferation of memory cells.\(^{23,24}\) In addition, both T-box transcription factors cooperate to promote cytotoxic T lymphocyte (CTL) formation by inducing the expression of perforin and granzyme B during early stages of CD8\(^+\) T cell activation and promote migration to inflamed tissues by inducing chemokine receptors.\(^{25-27}\) Importantly, sufficient clinical evidence demonstrates a correlation between longer survival of cancer patients and increased expression of genes representing type 1 effector T cells, in particular T-bet and Eomes.\(^{28-30}\) Therefore, T-bet and Eomes are critical for both function and homeostasis of effector and memory T cells. However, their roles in the setting of memory T cell responses in response to tumor, and their expression and function in antigen-specific CTL are not well characterized.

Our group is interested in developing a peptide-based cancer vaccine against the XBP1 antigen using engineered heteroclitic XBP1 unspliced (US)\(_{184-192}\) (YISPWILAV) and heteroclitic XBP1 spliced (SP)\(_{367-375}\) (YLFPQLISV) HLA-A2 specific peptides.\(^{31}\) Each of these selected peptides has been demonstrated to be highly immunogenic, inducing XBP1 antigen-specific CTL, which specifically target HLA-A2\(^+\) multiple myeloma (MM) cells.\(^{31,32}\) In these studies, we further evaluated the immunogenicity of these heteroclitic XBP1 peptides, and characterized the resulting XBP1 peptides-specific CTL against a variety of solid tumor cancer cell lines, which overexpress the unspliced and spliced XBP1 antigens. Our results characterized distinct phenotypic profiles for XBP1-CTL and their specific antitumor activities against HLA-A2\(^+\) breast cancer, colon cancer and pancreatic cancer cells. The immunologic antitumor activities of the CM (CD45RO\(^-\)CCR\(^+\)) and EM (CD45RO\(^-\)CCR7\(^-\)) CD3\(^+\)CD8\(^+\) cells of XBP1-CTL were shown to be driven by T-bet and Eomes transcription regulator expression within the memory subsets. These results provide the rationale for designing an immunotherapeutic approach comprised of heteroclitic XBP1 US\(_{184-192}\) and XBP1 SP\(_{367-375}\) HLA-A2 peptides as a vaccine to induce distinct XBP1-CTL memory subsets expressing critical T cell markers and transcription regulators that result in specific antitumor activities against solid tumors including breast, colon and pancreatic cancers.

Table 1. High level of XBP1 protein expression in breast, colon, and pancreatic cancer cells

| Cancer Type | Cell Line | XBP1 Unspliced | XBP1 Spliced |
|-------------|-----------|----------------|--------------|
| Breast      | MDA-MB231 | ++             | 2 ++         | 5 |
| Cancer      | MCF-7     | +++            | 3 +++        | 5 |
| Colon       | LS180     | ++++           | 4 +++++      | 5 |
| Cancer      | SW480     | ++             | 2 +++++      | 4 |
|            | WiDr      | +              | 1 +++++      | 5 |
| Pancreatic  | PATU8988T | ++++           | 4 +++++      | 5 |
| Cancer      | MiaPaCa-2 | +++            | 3 +++++      | 5 |
|            | PATU9802  | +++            | 3 ++         | 4 |
|            | PL45      | +              | 1 +++++      | 4 |
|            | MPanc96   | +              | 1 ++         | 2 |
| Prostate    | LNCaP     | –              | 0            | 0 |
| Cancer      | VCaP      | –              | 0            | 0 |

Table 2. Increased XBP1 gene expression in primary cells from breast or colon cancer patients

| Series       | Tumor Type               | Total | Normal | Patient | p value | Expression in patients | Fold change |
|--------------|--------------------------|-------|--------|---------|---------|------------------------|-------------|
| Analyses by canEvolve | TCGA- Colon | Adenocarcinoma | 179 | 24 | 155 | 1.69E-12 | Upregulated | 1.60 |
| Analyses by canEvolve | TCGA- BRCA | Breast cancer | 599 | 63 | 536 | 3.70E-04 | Upregulated | 1.64 |
| Analyses by Oncomine | TCGA- Colon | Adenocarcinoma | 161 | 19 | 22 | 1.92E-06 | Upregulated | 1.91 |
| #1. Analyses by Oncomine | TCGA- BRCA | Breast cancer | 593 | 61 | 36 | 3.45E-07 | Upregulated | 2.63 |
| #2. Analyses by Oncomine | TCGA- BRCA | Invasive lobular | 61 | 36 | 3.45E-07 | Upregulated | 2.63 |
| #3. Analyses by Oncomine | TCGA- BRCA | Invasive Ductal | 61 | 392 | 6.19E-05 | Upregulated | 1.77 |
| #4. Analyses by Oncomine | TCGA- BRCA | Mixed lobular and Ductal | 61 | 7 | 1.67E-04 | Upregulated | 3.67 |
| #5. Analyses by Oncomine | TCGA- BRCA | Intraductal Cribriform | 61 | 3 | 2.86E-04 | Upregulated | 2.71 |
| #6. Analyses by Oncomine | TCGA- BRCA | Invasive Ductal & Lobular | 61 | 3 | 0.003 | Upregulated | 3.04 |
| #7. Analyses by Oncomine | TCGA- BRCA | Invasive carcinoma | 61 | 76 | 0.007 | Upregulated | 1.54 |
Figure 1. Phenotype characterization of antigen-specific CTL induced by heteroclitic unspliced XBP1<sub>165-192</sub> (YISPWILAV) and spliced XBP1 SP<sub>196-204</sub> (YLFPQLISV) peptides. XBP1-CTL were generated from HLA-A2<sup>+</sup> normal donors’ CD3<sup>+</sup> T cells by repeated stimulation with APC pulsed with a cocktail of heteroclitic XBP1 peptides. Compared to unstimulated control T cells, the XBP1-CTL (n = 3; donors A, B, C) showed enrichment of total CD3<sup>+</sup>CD8<sup>+</sup> T cells. A continuous increase in the frequency of CD3<sup>+</sup>CD8<sup>+</sup> T cells was observed from baseline (no stimulation) through four cycles of peptides stimulation (A). One week after four rounds of peptides stimulation, XBP1-CTL showed increases in both the frequency (% positive cells; B) and expression levels (MFI; C) of critical T cell markers.
Results

High level of XBP1 protein expression in breast, colon, and pancreatic cancer cells

XBP1 unspliced and spliced antigens were highly expressed at the protein level in cell lines from breast cancer (MDA-MB-231, MCF-7, BT-474), colon cancer (LS180, SW480, WiDr) and pancreatic cancer (PATU8988T, MiaPaCa-2, Panc1, PATU8902, PL45, MPanc96), but not from prostate cancer (LNCaP, VCaP) as determined by flow cytometric analyses (Table 1). The different levels of XBP1 expression (mean channel fluorescence; MFI) were classified as follows; (1) MFI < 300: −, (2) MFI 300 – 600: +, (3) MFI 600 – 1,000: ++, (4) MFI 1,000 – 1,500: ++++, (5) MFI 1,500 – 2,000: ++++, and (6) MFI > 2,000: +++++.

Increased level of XBP1 gene expression in primary cancer cells from normal cells

A significantly higher level of XBP1 gene expression was found in primary cancer cells from breast cancer patients or colon cancer patients as compared to cells from healthy donors through searching of the public databases canEvolve and Oncomine (Table 2). A significant difference \( p < 0.05 \) was detected in XBP1 gene expression using canEvolve in a series of “TCGA-colon” from colon cancer patients \( n = 155 \) with normal donors \( n = 24 \), along with a series of “TCGA-BRCA” cells from breast cancer patients \( n = 536 \) to normal donors \( n = 63 \). In addition, Oncomine database search showed significant differences in XBP1 gene expression between cells from normal donors and different types of colon cancer patients \( n = 161 \) or breast cancer patients \( n = 593 \). Pancreatic cancer patient samples were not available for the analyses.

A cocktail of heteroclitic unspliced XBP1 US184–192 (YISPWILAV) and spliced XBP1 SP196–204 (YLFPQLISV) peptides induce CD3+CD8+ cytotoxic T lymphocytes that express critical T cell markers

Repeated stimulation of purified CD3+ T cells with a cocktail of heteroclitic XBP1 US184–192 (YISPWILAV) and heteroclitic XBP1 SP367–375 (YLFPQLISV) peptides gradually increased the percentage of CD3+CD8+ T cells over a period of four weekly peptides stimulations (Fig. 1A). Baseline control CD3+CD8+ T cell percentages (donor 1: 19%, donor 2: 20%, donor 3: 26%) increased following two peptides stimulation cycles (donor 1: 59%, donor 2: 62%, donor 3: 49%) and continued to increase after a total of four peptides stimulation cycles (donor 1: 95%, donor 2: 84%, donor 3: 80%). In addition, distinct phenotypic changes were detected in the XBP1-CTL \( n = 3 \), gated CD3+CD8+ T cells) including increased frequencies (Fig. 1B) and higher MFI (Fig. 1C) of critical T cell markers CD38, CD40L, CD69, 41BB, ICOS and TCRεβ.
Heteroclitic XBP1-CTL display antigen-specific and HLA-A2-restricted antitumor activities against breast, colon, and pancreatic cancer cells

The functional immune responses of the XBP1-CTL were evaluated in response to the solid tumor cell lines. XBP1-CTL demonstrated high levels of CD107a degranulation upon recognition of HLA-A2+/XBP1+ breast cancer (MDA-MB-231; 16.8%), colon cancer (SW480; 14.3%) or pancreatic cancer cells (PL45; 13.8%) (Fig. 2A). In contrast, XBP1-CTL CD107a degranulation was not detected in response to HLA-A2-/XBP1+ tumor cells (BT-474 breast cancer cells; 4.2%, WiDr colon cancer: 4.1%, MiaPaCa-2 pancreatic cancer: 5.1%) cells, NK-sensitive K562 cells (4.1%) or HLA-A2+/XBP1− LNCaP prostate cancer cells (6.3%). The background CD107a level in XBP1-CTL alone (3.5%) was similar to those observed in the MHC mismatched or XBP1− tumor cell lines.

Cytokine production (IFNγ, IL-2) was measured within the CD3+/CD8+ T cells of XBP1-CTL in response to the respective solid tumor cell line. The XBP1-CTL had increased IFNγ production (Fig. 2B) as well as IL-2 production (Fig. 2C) in response to HLA-A2+/XBP1+ MDA-MB-231 breast cancer cells (IFNγ+ cells: 15.8%, IL-2+ cells: 4.1%), SW480 colon cancer (IFNγ+ cells: 16.1%, IL-2+ cells: 4.4%) or PL45 pancreatic cancer (IFNγ+ cells: 10.2%, IL-2+ cells: 1.6%) as compared to the control XBP1-CTL alone (IFNγ+ cells: 0.8%, IL-2+ cells: 0.4%). In addition, the XBP1-CTL displayed low or no cytokine production in response to MHC mismatched (HLA-A2+/XBP1−) tumor cells (BT-474 breast cancer – IFNγ+ cells: 3.4%, IL-2+ cells: 1.7%, WiDr colon cancer – IFNγ+ cells: 4.3%, IL-2+ cells: 1.9%, MiaPaCa-2 pancreatic cancer – IFNγ+ cells: 1.2%, IL-2+ cells: 0.6%) or XBP1 antigen-mismatched HLA-A2+ tumor cells (LNCaP prostate cancer – IFNγ+ cells: 1.3%).

We further evaluated the poly-functional antitumor activities of the XBP1-CTL by measuring IFNγ production/CD69 upregulation or IFNγ production/CD107a degranulation against the respective solid tumor cell line. XBP1-CTL IFNγ production/cell activation (IFNγ+/CD69+ cells: Q2) was detected against HLA-A2+/XBP1+ breast cancer cells (MDA-MB-231: 15.7%, MCF-7: 7.2%), but not to MHC mismatched breast cancer cells (BT-434: 2.2%), HLA-A2+/XBP1− prostate cancer cells (LNCaP: 2.9%), nor HLA-A2+/XBP1+ prostate cancer cells (VCaP: 2.9%) (Fig. 2D). The XBP1-CTL also exhibited IFNγ production/degranulation (IFNγ+/CD107a+ cells: Q2) against HLA-A2+/XBP1+ pancreatic cancer cells (Panc1: 11.7%, PL45: 15.7%) and HLA-A2+/XBP1+ colon cancer cells (LS180: 18.2%, SW480: 25.7%), but not against MHC mismatched pancreatic cancer (MiaPaCa-2: 1.5%), nor colon cancer cells (WiDr: 2.3%) (Fig. 2E). Taken together, these results

Figure 2. For figure legend, see page 6.
Importantly, XBP1-CTL recognized and responded to both native unspliced XBP1184–192 (NISPWILAV), native spliced XBP1 SP367–375 (ELFPQILSV), heteroclitic XBP1184–192 (YLFPQLISV) and spliced XBP1 SP196–204 (YLFPQLISV) peptides. Specifically, CD107a degranulation and IFNγ production were evaluated in response to K562-A*0201 cells presenting either native unspliced XBP1184–192 (NISPWILAV), native spliced XBP1 SP367–375 (ELFPQILSV), heteroclitic XBP1184–192 (YLFPQLISV), or heteroclitic XBP1 SP367–375 (YLFPQLISV) peptide. K562-A*0201 cells alone or K562-A*0201 cells presenting an irrelevant HLA-A2-specific influenza virus matrix protein58–66 (IMP58–66: GILGFVFTL) peptide were used as controls. XBP1-CTL (n = 3) induced CD107a degranulation and IFNγ production in response to each heteroclitic XBP1 peptide. These functional immune responses were higher than those against K562-A*0201 cells alone or the cells presenting irrelevant IMP58–66 peptide. Importantly, XBP1-CTL recognized and responded to both native and heteroclitic XBP1 unspliced and native XBP1 spliced peptides as seen by comparable levels of CD107a degranulation (6–8%; Fig. 3A), IFNγ production (1–1.2%; Fig. 3B) and poly-functional CD107a degranulation/IFNγ production (0.4–0.7%; Fig. 3C). Therefore, these results indicate that XBP1-CTL are able to respond to their specific heteroclitic peptides and more importantly their respective native peptides, which can be presented as epitopes on the tumor cells.

A cocktail of heteroclitic unspliced XBP1184–192 (YISPWILAV) and spliced XBP1 SP367–375 (YLFPQILSV) peptides evoke CTL with a memory CD3+CD8+ T cell phenotype

We further evaluated XBP1-CTL for their memory: naïve phenotypic profile by flow cytometry. Representative analyses demonstrate differentiation of CD3+CD8+ T cells from naïve (CD45RO–CCR7+ into CM (CD45RO+CCR7+), EM (CD45RO+CCR7+) and finally TE (CD45RO–CCR7−) cell subsets (Fig. 4A). Distinct changes were seen in the CM [day 2: 60%, day 4: 62%, day 6: 38%] and EM [day 2: 7%, day 4: 19%, day 6: 40%] populations beginning after three cycles of XBP1 peptides stimulation. Further analysis of the XBP1-CTL (n = 3) demonstrated continued differentiation of CD3+CD8+ T cells between baseline (no peptides stimulated; naïve cells: ~60%, EM: <10%) (Fig. 4B) and four cycles of XBP1 peptides stimulation (naïve cells: <5%, EM: 50–70%) (Fig. 4C). Based on these results, we chose to evaluate XBP1-CTL after four rounds of peptides stimulation for their function and antitumor activities within the different CD3+CD8+ memory: naïve T cell subsets.

Next, we investigated the expression levels of critical antigen-specific T cell markers on XBP1-CTL. Higher expression levels (MFI) were observed for CD28, ICOS (Inducible T-cell costimulator; CD278), CD69 and CD40L within the CD45RO+ memory as compared to CD45RO− non-memory CD3+CD8+ XBP1-CTL (n = 3) (Fig. 4D). Further analyses also demonstrated increased frequencies of CD28, ICOS, CD69, and CD40L expression within the specific CM and EM subsets as compared to the naïve and terminal effector XBP1-CTL subsets (Fig. 4E). In addition, the XBP1-CTL CM cells had higher levels of CD28, ICOS, CD69, and CD40L expression as compared to the EM subset, while not much difference was detected between the naïve and terminal effector cells of XBP1-CTL subsets.

A high level of cell proliferation was included by memory subsets of XBP-CTL in response to breast, colon or pancreatic cancer cells in an antigen-specific and HLA-A2-restricted manner

The antitumor activities of the XBP1-CTL were further analyzed using a 6 d CFSE-proliferation assay. The proliferation of XBP1-CTL was evidenced by a decrease in the fluorescence intensity of CFSE-labeled cells following stimulation with irradiated tumor cells. Flow cytometric analyses showed a high level of XBP1-CTL proliferation in response to HLA-A2+ breast cancer.
cells (MDA-MB-231; 70.0%) (Fig. 5A), HLA-A2+ pancreatic cancer cells (PATU8902; 70.3%, Panc1; 68.5%) (Fig. 5B), and HLA-A2+ colon cancer cells (LS180; 55.4%, SW480; 51.2%) (Fig. 5B). In contrast, the XBP1-CTL did not proliferate in response to HLA-A2+ breast cancer (BT-434), HLA-A2+ pancreatic cancer (MiaPaCa-2), HLA-A2+ colon cancer (WiDr) cells or the NK-sensitive K562 cells (Figs. 5A, B). Therefore, these results demonstrate XBP1-CTL proliferation in an antigen-specific and HLA-A2-restricted manner against breast cancer, colon cancer, and pancreatic cancer cells.

We further analyzed cell proliferation within the specific XBP1-CTL memory cell subsets in response to HLA-A2+/XBP1+ breast (MDA-MB-231), colon (LS180) or pancreatic (Panc1) cancer cells. The memory cell subset (CD45RO+/CD3+CD8+) of XBP1-CTL displayed a higher level of cell proliferation as compared to the non-memory cells subset (CD45RO−CD3−CD8−) in response to breast cancer (MDA-MB-231; 92% memory CTL vs. 5% non-memory CTL) (Fig. 5C), colon cancer (LS180; 88% memory CTL vs. 12% non-memory CTL) (Fig. 5D) or pancreatic cancer (Panc1; 87% memory CTL vs. 18% non-memory CTL) (Fig. 5E) cells. Furthermore, a higher level of cell proliferation was detected within the EM subset as compared to the CM subset of XBP1-CTL in response to breast cancer MDA-MB-231 (97% EM vs. 34% CM, Fig. 5C), colon cancer LS180 (95% EM vs. 62% CM, Fig. 5D) or pancreatic cancer Panc1 (95% EM vs. 39% CM, Fig. 5E) cells. Therefore, these results demonstrate a strong XBP1-CTL proliferative response within specific memory cell subsets in response to each of the solid tumor cell lines.

The central memory subset of XBP1-CTL possesses a high level of antitumor activities against breast, colon or pancreatic cancer cells

Finally, we investigated the functional antitumor activities within the specific CM (CCR7+/CD45RO+/CD3+CD8+) and EM (CCR7+/CD45RO+/CD3+CD8+) subsets from the XBP1-CTL. In contrast to the proliferation results, the CM subset of XBP1-CTL had greater cytokine production and CD107a degranulation as compared to the EM subset in response to the respective HLA-A2+/XBP1+ solid tumor cell lines. Specifically, IFNγ production was higher by the CM CTL population in response to breast cancer (MDA-MB-231: 76.8% CM vs. 17.6% EM; MCF-7: 58.8% CM vs. 7.0% EM), colon cancer (LS180: 18.8% CM vs. 2.1% EM; SW480: 13.2% CM vs. 2.8% EM), and pancreatic cancer (Panc1: 40.4% CM vs. 2.1% EM; PL45: 35.5% CM vs. 8.5% EM) cells (Fig. 6A). IL-2 production was also higher by the CM than EM CTL against breast cancer (MDA-MB-231: 90.4% CM vs. 19.6% EM; MCF-7: 58.3% CM vs. 6.0% EM), colon cancer (LS180: 13.2% CM vs. 1.7% EM; SW480: 18.6% CM vs. 2.3% EM), and pancreatic cancer (Panc1: 40.4% CM vs. 2.1% EM; PL45: 35.5% CM vs. 8.5% EM) cells (Fig. 6B). In addition, CD107a degranulation (associated with cytotoxicity) was higher in the CM than EM subset of XBP1-CTL against breast cancer (MDA-MB-231: 90.2% CM vs. 18.0% EM; MCF-7: 65.2% CM vs. 13.3% EM), colon cancer (LS180: 33.1% CM vs. 8.2% EM; SW480: 65.7% CM vs. 8.0% EM), and pancreatic cancer (Panc1: 47.4% CM vs. 10.3% EM).
cells of XBP1-CTL (n = 3) (donor 1: 12% memory vs. 8% non-memory, donor 2: 13% memory vs. 5% non-memory, donor 3: 18% memory vs. 1% non-memory) (Fig. 7B). Overall, the frequency of cells expressing T-bet was higher than Eomes in memory cells of XBP1-CTL (n = 3) (Mean: T-bet 63% vs. Eomes 11%). Consistent with T-bet and Eomes expression, the level of granzyme B expression was higher in memory as compared to non-memory CD3⁺CD8⁺ XBP1-CTL (n = 3) in response to HLA-A2⁺ breast cancer (MDA-MB231), pancreatic cancer (Panc1) or colon cancer (SW480) cells (Fig. 7C). Among XBP1-CTL generated from the three different HLA-A2⁺ donors, CTL from Donor 3 showed the most memory cells, which correlated with increased expression of T-bet (Fig. 7A), Eomes (Fig. 7C) and granzyme B (Fig. 7C) within the cell subset. These results indicate that XBP1-CTL upregulate both critical transcription factors (T-bet, Eomes) required for type 1 immune responses and demonstrate increased levels of the cytolytic effector molecule granzyme B in support of their functional antitumor activities.

T-bet⁺ CM and Eomes⁺ CM subsets of XBP1-CTL are enhanced for IFNγ production in response to breast cancer, colon cancer or pancreatic cancer cells

Next, we evaluated T-bet expression and IFNγ production within the specific memory:naive subsets of XBP1-CTL (n = 3). The highest cell frequency of T-bet expression was detected within the EM subset (39–47%) followed by the CM (10–26%), effector (2–22%), and naive (<1%) CD3⁺CD8⁺ T cells (Fig. 8A). However, the highest frequency of T-bet⁺/IFNγ⁺ cells was detected within the CM subset of XBP1-CTL, consistently in response to the respective tumor cells including breast cancer (Fig. 8B), pancreatic cancer (Fig. 8C) or colon cancer (Fig. 8D) cells. Compared to the CM subset, EM subset induced a lower level of IFNγ production within their T-bet⁺ cell population upon the stimulation. Either naive cell (data not shown) or effector cell subset (Figs. 8B, C, D) of XBP1-CTL displayed no significant level of T-bet⁺/IFNγ⁺ cells to the tumor cells. Furthermore, all IFNγ producing cells expressed T-bet, but not all the T-bet expressing cells produce IFNγ in stimulation with the tumor cells (Figs. 8B, C, D), suggesting that maintaining a CM maturation state for antigen-specific CTL might be an important strategy in vaccine development.

XBP1-CTL (n = 3) were analyzed further for Eomes expression and cytokine production. Consistent with T-bet expression, the EM subset of XBP1-CTL (n = 3) had the highest level of

EM; PL45: 52.0% CM vs. 4% EM) cells (Fig. 6C). XBP1-CTL generated from two different individuals showed the same pattern of antitumor responses, higher IFNγ production (Fig. 6D), IL-2 production (Fig. 6E), and CD107a degranulation (Fig. 6F), by the CM subset as compared to EM cell population. Therefore, these results demonstrate that the CM subset of XBP1-CTL have superior cytokine production and cytolytic activity (CD107a expression and IFNγ production) as compared to EM cells against breast cancer, CD107a degranulation (as compared to EM cells against breast cancer, colon cancer or pancreatic cancer cells.

Memory cells within XBP1-CTL have enhanced expression of T-bet and Eomes

T-bet and Eomes transcription factors are master regulators of Th1 development and are critical for optimal development of type 1 immune responses (18–20). In these studies, we evaluated T-bet, Eomes and granzyme B expression within the XBP1-CTL. Overall, memory subset (CD45RO⁺/CD3⁺CD8⁺) of XBP1-CTL (n = 3) displayed a higher frequency of T-bet⁺ cells as compared to non-memory subset (CD45RO⁻/CD3⁺CD8⁺) (donor 1: 58% memory vs. 22% non-memory, donor 2: 71% memory vs. 15% non-memory, donor 3: 62% memory vs. 2% non-memory) (Fig. 7A). In parallel, a higher frequency of Eomes⁺ cells was also detected in memory (CD45RO⁺/CD3⁺CD8⁺) than non-memory (CD45RO⁻/CD3⁺CD8⁺) of XBP1-CTL are enhanced for IFNγ production in response to breast cancer, colon cancer or pancreatic cancer cells.

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Eomes expression (Fig. 9A). Additionally, IFNγ+ production was detected exclusively in Eomes+ fraction of memory CD3+CD8+ T cells of XBP1-CTL, in response to the various HLA-A2+ tumor cells. The highest level of IFNγ+ production was observed within the CM subset expressing Eomes in response to breast cancer (MB231, 12–19%; Fig. 9B), pancreatic cancer (Panc1, 25–34%; Fig. 9C) or colon cancer cells (SW480, 10–16%; Fig. 9D). Overall, IFNγ production was higher in CD3+CD8+ T cells expressing Tbet than Eomes in response to the various solid tumor cells. Therefore, these results provide additional evidence that heteroclitic XBP1 peptides induce different subsets of memory CD3+CD8+ T cells, which express the critical transcription factors which regulate memory cell development and Th1 development.

**Discussion**

Peptide-based vaccines have distinct advantages over individualized vaccines in regards to cost of production, manufacture of vaccine products, and straightforward measurements to detect antigen-specific immune responses in patients. They have been shown to mount a safe and effective immune response to treat different types of cancer; for instance, in a randomized phase 3 trial in patients with advanced melanoma, patients receiving a vaccine consisting of the HLA-A2-restricted gp100 peptide plus incomplete Freund’s adjuvant, followed by IL-2 administration, showed a higher response rate and longer progression-free survival than the patient cohort who received IL-2 alone. In addition, a vaccine for renal cell cancer patients consisting of multiple tumor-associated HLA-A2-restricted peptides demonstrated improved disease control in early stage clinical studies, evidenced by prolonged survival in patients who mounted a specific immune response to the vaccine combined with a single-dose cyclophosphamide. Thus, application of promising immunogenic HLA-A2-specific peptides targeting tumor-associated antigens can offer new vaccine strategies to enhance antitumor efficacy under optimal conditions.

During tumorigenesis, the high proliferation rate of cancer cells requires increased activities within the ER machinery to facilitate protein folding, assembly, and transport. This high demand leads to ER stress and accumulation of unfolded/misfolded proteins, ER calcium depletion, altered glycosylation, nutrient deprivation, oxidative stress, DNA damage, and/or energy perturbation/fluctuations. In addition, the high level of ER involvement triggers a group of signal transduction pathways, collectively termed as UPR, to modify transcriptional and translational programs and maintain ER homeostasis. The UPR pathways are activated in a great variety of tumor types, and protect tumor cells from stressful conditions as well as immune surveillance. Based on these observations, extensive research and clinical trials have been focused on targeting UPR pathways to treat different types of cancer patients including those with MM, mantle-cell or non-Hodgkin’s lymphoma, acute myeloma leukemia, breast cancer, colorectal cancer, ovarian cancer, lung cancer, sarcoma, renal cancer or melanoma. A few examples of UPR-targeted cancer drugs in development and their mechanisms include Bortezomib (proteasome inhibitor), Tanespimycin (HSP90 inhibitor), Retaspimycin (HSP90 inhibitor), Versipelostatin (GRP78 inhibitor), and Irestatins (IRE1α inhibitor).

The purpose of these studies was to evaluate an immunotherapeutic approach for targeting the XBP1, an important transcription activator of UPR, in a variety of solid tumor. Previously, we
identified highly immunogenic heteroclitic XBP1 US\textsubscript{184–192} (YISPWILAV) and XBP1 SP\textsubscript{367–375} (YLFPQLISV) peptides with higher HLA-A2 binding affinities and stabilities than their native counterparts. In these studies, XBP1-CTL were generated using a cocktail of HLA-A2 unspliced and spliced heteroclitic XBP1 peptides and were analyzed for their functional immune activities against solid tumors including breast, colon, and pancreatic cancer cells that highly over-express XBP1 unspliced and spliced proteins. Our results confirmed our previous findings on the immunogenicity of the heteroclitic XBP1 peptides to induce CTL with distinct phenotypic profiles including highly activated memory CD8\textsuperscript{T} cells. In addition, these studies demonstrate distinctive upregulation of critical molecules on the expanded CD3\textsuperscript{+}CD8\textsuperscript{+} T cells including CD28, ICOS, CD69, and CD40L cell surface antigens as compared to non-memory CD3\textsuperscript{+}CD8\textsuperscript{+} T cells.

**Figure 4.** Differentiation of memory: naive CD3\textsuperscript{+}CD8\textsuperscript{+} (T) cell subsets upon the stimulation with heteroclitic XBP1 peptides. XBP1-CTL differentiation into specific memory: naive CD3\textsuperscript{+}CD8\textsuperscript{+} T cell subsets was evaluated by flow cytometry. Representative analyses after 0, 2, 3 or 5 cycles of heteroclitic XBP1 peptides stimulation show the differentiation of CD3\textsuperscript{+}CD8\textsuperscript{+} T cells from naive cells (Q1) into CM (Q2), EM (Q4) and TE (Q3) cells (A). A summary of memory: naive CD3\textsuperscript{+}CD8\textsuperscript{+} T cell frequency is demonstrated between baseline T cells (B) and XBP1-CTL induced with four cycles of peptides stimulation (C). The memory CD3\textsuperscript{+}CD8\textsuperscript{+} T cells of XBP1-CTL (n = 3) had increased level (D) and higher cell frequency (E) expressing CD28, ICOS, CD69, and CD40L cell surface antigens as compared to non-memory CD3\textsuperscript{+}CD8\textsuperscript{+} T cells.
Figure 5. For figure legend, see page 13.
Figure 5. For figure legend, see page 13.
CM (CD45RO⁺CCR7⁺) CD3⁺CD8⁺ T cells retain a greater capacity for secondary re-expansion and long-term persistence and are enabled by efficient homeostatic self-renewal, whereas EM (CD45RO⁻CCR7⁻) CD3⁺CD8⁺ T cells rapidly exert effector functions upon antigen encounter. However, there remain controversies in defining the immune response of the specific memory T subsets within antigen-specific CTL against tumor cells. In these studies, we characterized the antitumor activities of the two different memory cell subsets, CM and EM, from XBP1 antigen-specific CTL against a variety of solid tumor cells. We chose to evaluate the XBP1-CTL after the fourth round of peptide stimulation, due to high levels of antigen-specific cells within both memory cell subsets of the CD3⁺CD8⁺ T cell population. Our results demonstrate a higher level of XBP1-CTL proliferation within the EM as compared to the CM population in response to HLA-A2⁺/XBP1⁺ MDA-MB-231 breast cancer cells, but not HLA-A2⁺/XBP1⁺ BT-434 breast cancer cells, HLA-A2⁺/XBP1⁻ LNCaP, nor HLA-A2⁺/XBP1⁻ VCaP prostate cancer cells. In addition, XBP1-CTL proliferated in response to HLA-A2⁺/XBP1⁺ pancreatic cancer cells (PATU8902, Panc1) and colon cancer cells (LS180, SW480), but not to HLA-A2 mismatched pancreatic cancer (MiaPaCa-2), nor colon cancer cells (WiDr). Specific XBP1-CTL proliferation was detected within the memory subsets (EM > CM) in response to HLA-A2⁺/XBP1⁺ MDA-MB-231 breast cancer (C), LS180 colon cancer (D) or Panc1 pancreatic cancer cells (E). XBP1-CTL in media alone was used to determine background cell proliferation.

Various transcription factors, repressors, and other regulators are known to be critical for generation of specific T cell subsets. Among them, T-bet and Eomes have been demonstrated as important transcription regulators involved in the differentiation of CD8⁺ T cells into Th1 type cells. Both T-
Figure 6. For figure legend, see page 16.
**bet** and **Eomes** have a critical role in the generation and promotion of central and EM CD8$^+$ T cells and in elimination of established tumors by the T cells. However, the role of these transcription factors in shaping the memory T cell pool and eliciting tumor-specific memory T cell subsets needs to be further elucidated. In current studies, we describe the association of **T-bet** and **Eomes** in antitumor activities within the memory T cell subsets of antigen-specific CTL in response to a variety of solid tumor cancers. Of particular interests were to define and characterize the expression patterns of **T-bet** and **Eomes** within the XBP1 antigen-specific effector or memory CD3$^+$CD8$^+$ T cell subsets, which demonstrated specific functional activities against breast cancer, colon cancer, and pancreatic cancer. These studies showed that the XBP1-specific CTL express higher frequencies of T-bet$^+$ and Eomes$^+$ within the memory populations as compared to their non-memory counterparts. Specifically, XBP1-CTL EM subset had the highest frequency of T-bet$^+$ and Eomes$^+$ CD8$^+$ T cells followed by the CM CTL subset. However, upon recognition of HLA-A2$^+$ XBP1 antigen expressing cancer cells, the CM subset of XBP1-CTL expressing T-bet or Eomes had the greatest functional antitumor activities including IFNγ production IL-2 production, granzyme B expression, and CD107a degranulation. Thus, out in vitro analyses provide the evidence of the critical role of **T-bet** and **Eomes** transcription factors regulating the antitumor activities within antigen-specific CTL and support results from other studies demonstrating that both transcription regulators cooperate to promote CTL formation during CD8$^+$ T cell activation.$^{25-27}$

Recent studies have indicated that the administration of long peptides encompassing Th1 and CTL epitopes has the potential to elicit both CD4$^+$ and CD8$^+$ T-cell responses through cross-presentation, and more favorable clinical outcomes are associated with immune responses toward many MHC class I and class II epitopes derived from multiple tumor antigens.$^{63,64}$ Therefore, both CD4$^+$ and CD8$^+$ T cells play an important role in inducing and maintaining antitumor immunity, and we are interested in identifying long peptides specific to XBP1, CD138 and CS1 antigens and developing cancer vaccines based on respective single polypeptide encompassing epitopes, which can elicit both Th1 and CTL responses.

In summary, we demonstrate ex vivo that a cocktail of highly immunogenic HLA-A2 specific peptides, heteroclitic XBP1 US184–192 and heteroclitic XBP1 SP367–375, can evoke XBP1-specific CTL with antitumor activities against a variety of solid tumor cancers that express high levels of unspliced and spliced XBP1 proteins. These data provide evidence for induction of a complete repertoire of XBP1-specific CM and EM CD3$^+$CD8$^+$ T cells, which have a high proliferative capacity and strong antitumor activity against breast, colon and, pancreatic cancer cells.
Therefore, we provide the framework for a cancer vaccine trial with the cocktail of heteroclitic XBP1 peptides to evoke CTL targeting both the unspliced and spliced XBP1 protein in order to delay or prevent disease progression in a variety of suitable cancer patient populations.

**Materials and Methods**

**Cell lines**

Breast cancer (MDA-MB-231, MCF-7, BT-474), colon cancer (LS180, SW480, WiDr), and prostate cancer (LNCaP, VCaP) cell lines were obtained from ATCC. The pancreatic cancer cell lines (PATU8988T, MiaPaCa-2, Panc1, PATU8902, PL45, MPanc96) were kindly provided by Dr. A. Kimmelman (Harvard Medical School, Boston, MA). The T2 cell line was provided by Dr. J. Mlojdrem at the University of Texas M. D. Anderson Cancer Center. MDA-MB-231 and SW480 cell lines were cultured in Leibovitz’s L-15 Medium (ATCC); MCF-7, BT-474, LS180 and WiDr cell lines were cultured in EMEM (Gibco-Life Technologies); LNCaP cells was cultured in RPMI-1640 Medium (Gibco-Life Technologies); and VCaP, PATU8988T, MiaPaCa-2, Panc1, PATU8902, PL45, MPanc96 and T2 cell lines were cultured in DMEM (Gibco-Life Technologies) media supplemented with 10% fetal calf serum (FCS; BioWhittaker), 100 IU/mL
penicillin, and 100 μg/mL streptomycin (Gibco-Life Technologies).

**Reagents**

Fluorochrome conjugated mouse or rabbit anti-human HLA-A2, CD3, CD8⁺, CD38, CD40L, CD69, 41BB, ICOS, TCRαβ, CCR7, CD45RO, CD107a, IFNγ, IL-2, T-bet, and granzyme B monoclonal antibodies (mAbs) were purchased from Becton Dickinson. Fluorochrome conjugated mouse anti-human Eomes mAb was purchased from eBioscience. Rabbit anti-human XBP1 unspliced isoform antibody and donkey anti-rabbit IgG-PE were purchased from Novus Biologicals. Mouse anti-human XBP1 spliced isoform antibody and goat anti-mouse IgG-PE were purchased from R&D Systems. Recombinant human IL-2, IL-4, IFNα and TNF-α were purchased from R&D Systems, and human GM-CSF was obtained from Immunex.

**Synthetic peptides**

HLA-A2-specific native XBP1 184–192 (NISPWILAV), native XBP1 SP367–375 (ELFPQLISV), heteroclitic XBP1 184–192 (YISPWILAV), heteroclitic XBP1 SP367–375 (YLFPQLISV), and influenza virus matrix protein 58–66 (GILGFVFTL) peptides were synthesized by standard Fmoc (9-fluorenylmethyl-oxycarbonyl) chemistry, purified to >95% using reverse-phase chromatography, and validated by mass-spectrometry for molecular weight (Biosynthesis). Lyophilized peptides were dissolved in DMSO (Sigma), diluted in AIM-V medium (Gibco-Life Technologies), and stored at −140°C.

**Induction of XBP1 peptides-specific CTL**

XBP1 peptides-specific CTL (XBP1-CTL) were generated ex vivo by repeated stimulation of CD3⁺ T lymphocytes obtained from HLA-A2⁺ normal donors with a cocktail of heteroclitic XBP1 peptides-pulsed APC, either mature DC or T2 cells. In brief, APC in serum-free AIM-V medium were pulsed overnight at 37°C and 5% CO2 in humidified air with a cocktail of heteroclitic XBP1 184–192 and heteroclitic XBP1 SP367–375 peptides (50 μg/mL). Peptides pulsed APC were washed, irradiated, and used to prime CD3⁺ T cells at a 1:20 APC/peptides (stimulator)-to-CD3⁺T cell (responder) ratio in AIM-V medium supplemented with IL-2 (50 U/mL) and 10% human AB serum (BioWhittaker). The CTL cultures were restimulated with peptide-pulsed-APC every 7 d for a total of 5 cycles.

**Analyses of XBP1 gene expression in cancer patients**

Expression analysis of the XBP1 gene was performed using canEvolve (www.canevolve.org)⁶⁵ and Oncomine (www.oncomine.org)⁶⁶ databases. The Oncomine database covers two subtypes for colon adenocarcinoma and six subtypes of breast cancer, as well as the appropriate control sample data. A differential analysis in Oncomine was used to compare expression in cancer versus healthy donors, with the student’s t-test to calculate p values.⁶⁵ The canEvolve database uses linear models to calculate differential gene expression between groups and calculates the p values for given dataset.

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Figure 7. Enhanced levels of T-bet, Eomes and granzyme B expression in memory CD3⁺CD8⁺ T cell subsets of heteroclitic XBP1-CTL. The expression level of T-bet, Eomes and granzyme B was analyzed within the CD45RO⁺ memory and CD45RO⁻ non-memory subsets of XBP1-CTL (n = 3). A higher frequency of cells expressing T-bet (A) and Eomes (B) was detected within the CD45RO⁺ memory cells as compared to CD45RO⁻ non-memory CD3⁺CD8⁺ T cells. In addition, the frequency of granzyme B⁺ cells was increased in CD45RO⁺ memory cells as compared to CD45RO⁻ non-memory CD3⁺CD8⁺ T cells in response to the respective tumor cells (C).
Evaluation of XBP1 expression in tumor cell lines

The cancer cell lines were evaluated for intracellular expression of XBP1 unspliced or XBP1 spliced protein by flow cytometry. In brief, cells were permeabilized using the Cytotox/Cytoperm solution (BD) and stained with rabbit anti-human XBP1 unspliced isoform or mouse anti-human XBP1 spliced isoform mAbs for 30 min at room temperature, washed with Perm/Wash solution (BD) and stained with donkey anti-rabbit IgG-PE or goat antimouse IgG-PE, respectively, for 30 min at 4°C. The cells were washed with Perm/Wash solution and fixed in 2% formaldehyde-PBS. The cells were analyzed using a FACS Canto II™ flow cytometer and DIVA™ v6.1 software (Becton Dickinson) after gating on the tumor cells population.

Phenotypic analysis of XBP1-CTL and evaluation of critical T cell markers

XBP1-CTL were evaluated for the frequency of CD3⁺ CD8⁺ T cells and expression levels (% positive cells, mean fluorescence intensity (MFI)) of critical T cell surface markers including CD45RO, CCR7, CD38, CD40L, CD69, 41BB, ICOS, and TCRβ. After staining with each specific antibody, the cells were washed and fixed in 2% paraformaldehyde. The cells were

Figure 8. For figure legend, see page 19.
Figure 8. T-bet expression and IFN-γ production by memory XBP1-CTL subsets in response to solid tumor cells. The memory-naïve cell subsets of XBP1-CTL (n = 3) were evaluated for T-bet expression by flow cytometry. A higher frequency of T-bet expressing cells was observed within the EM subset of XBP1-CTL as compared to the CM and TE subsets (A). However, the highest level of T-bet+ /IFN-γ+ cells was observed within the CM subset of XBP1-CTL against HLA-A2+ MDA-MB231 breast cancer (B), HLA-A2+ Panc1 pancreatic cancer (C) or HLA-A2+ SW480 colon cancer cells (D).
analyzed using a LSRII Fortessa™ flow cytometer and DIVA™ v7.1 software (BD) after gating on non-memory or memory CD3⁺CD8⁺ T cell populations.

**IFNγ/IL-2 production, CD107a degranulation, granzyme B, and T-bet/Eomes expression in XBP1-CTL**

The functional antitumor activities of XBP1-CTL were examined against a variety of solid tumor cell lines for IFNγ/IL-2 production, CD107a degranulation, granzyme B, and T-bet/Eomes expression. XBP1-CTL were co-incubated with various stimulator cells in the presence of CD107a-FITC mAb, Brefeldin A and Monensin (BD) were added after 1 h incubation. After an additional 5 h incubation, cells were stained with mAbs specific to surface antigens including CD3, CD8⁺, CD45RO, CCR7, and/or CD69. The cells were fixed/permeabilized, and stained with mAb specific to IFNγ, IL-2, granzyme B, T-bet or Eomes. The cells were analyzed using a LSRII Fortessa™ flow cytometer and DIVA™ v7.1 software after gating on non-memory or memory CD3⁺CD8⁺ T cell populations.

**XBP1-CTL proliferation in response to tumor cell lines**

To evaluate tumor-specific cell proliferation, CFSE (Molecular Probes) labeled XBP1-CTL were co-incubated with irradiated (20 Gy) HLA-A2⁺ or HLA-A2⁰ cancer cell lines. On day 6, the cultures were harvested, stained with anti-CD3, CD8⁺, CD45RO and CCR7 mAbs, and analyzed by flow cytometry to determine their specific cell proliferation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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