Chapter 10
Vaccination Against Breast Cancer and its Role in Prevention

Brian J. Czerniecki, Nadia Nocera, Lea Lowenfeld, Lori Showalter, and Gary Koski

Abstract The immune response against cancers, including breast cancer, are shown to play a critical role in survival. Vaccines have long been hailed as the most effective medical intervention to prevent a disease. While cancer vaccines have mostly been used therapeutically with little success in established breast cancer, their role in early breast cancer appears more promising, and primary prevention of breast cancer by vaccination is now being contemplated. The selection of vaccine targets is a critical issue, since unlike cancers with established viral etiology (e.g. cervical cancer), there is no single cause of breast cancer. Instead, there are multiple subsets of breast cancers including: Luminal A, Luminal B, HER-2, and subsets of basal-like cancer. Each of these types can be antigenically distinct, and present immune targets that may be phenotype-specific or to some degree overlapping between subsets. Three general categories of such targets are being developed as breast cancer vaccines. These include oncodrivers, breast tissue specific antigens, and cancer specific antigens. It is likely that combinations of these vaccine approaches may be best for treatment and prevention. Carriers of high-risk breast cancer mutations represent a potential target patient population for prevention. However, approximately 85 % of breast cancers occur in patients with no identified risk. Recent evidence suggests that a loss of natural immune responses against oncodrivers may identify patients at risk for early breast cancer. Devising tests to identify subjects at risk for breast cancer are needed since these will allow us to focus prevention efforts, including vaccination, on those individuals where such resources are most needed. Preventive breast cancer vaccines may be achievable with our improved understanding of breast cancer biology, and the immune response in breast cancer.

Keywords Vaccines • Breast cancer • Primary prevention • Oncodrivers • Tumor Immunity • Breast cancer stem cells • Dendritic cells

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10.1 Background: What's at Stake?

Due to the significant public health burden, breast cancer is a particularly appealing target for preventative therapy. Breast cancer is the second most common cancer and the second most common cause of cancer death among American women. The incidence and mortality rates associated with breast cancer remain stubbornly stable and high, with more than 230,000 new cases diagnosed annually and more than 40,000 breast cancer related deaths each year [1]. Worldwide about 500,000 deaths from breast cancer occur each year [2]. Furthermore, the success of standard treatment, though prolonging survival, often results in significant disfigurement. Finally, the annual cost of breast cancer treatment in the United States is estimated at $16.5 billion [3], making it one of the most expensive malignancies.

Given the complex interaction between the immune system and cancer, immunotherapy in general and vaccine prevention in particular, is an appealing option for dealing with cancer. Vaccination against specific pathogens that are known to cause cancer (e.g. EBV and lymphoma [4] or gastric cancer [5], HPV and cervical, anogenital, and oropharyngeal cancer [6], HBV/HCV and hepatocellular carcinoma [7]) prevent infection, and therefore subsequent tumorigenesis. However, only an estimated 12 % of human cancers are at this time attributable to viral infections [8] and therefore susceptible to this strategy of prevention.

The majority of cancers, including breast cancer, are not directly caused by a single pathogen, but, nonetheless, vaccines developed against over-expressed or mutated cancer associated proteins can be used to target these malignancies. The first type of anti-cancer vaccine, like their anti-microbial counterpart, is **preventative** in nature. These can be further sub-divided into two categories. Vaccines aimed at **primary prevention** are administered to patients prior to the development of disease. Ideally, these will block the development of malignancy, and the patient will never develop cancer. Vaccines aimed at **secondary prevention** are administered to patients who have a history of cancer that has been eliminated or reduced to undetectable levels through conventional therapy. These vaccines protect against later recurrence of disease. **Therapeutic vaccines**, on the other hand, are administered to patients who possess measurable tumor burdens. The aim of this approach is to generate sufficient anti-tumor immunity to favorably alter the course of existing disease, either alone or in conjunction with conventional therapy.

10.2 Breast Cancer and Immune Response

The breast, by virtue of its communication with the outside world through the nipple, is by necessity endowed with complex immune populations. Breast lobular units contain dendritic cells, CD4+ and CD8+ T cells, B cells, and NK cells [9] (Fig. 10.1). These immune cells located in breast tissue defend against microbes, but also play a role during breast involution following lactation [10] and may play a

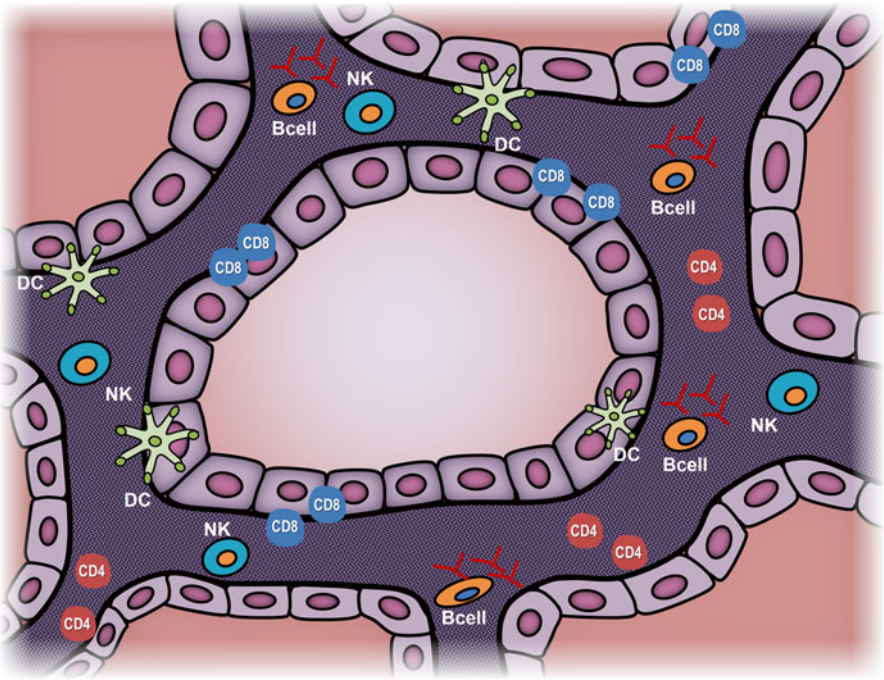

Fig. 10.1 Protective immune players in the normal breast lobule: B cells, Natural Killer cells (NK), Dendritic cells (DC), CD4+ (CD4) and CD8+ (CD8) T-cells. Myeloid and lymphoid cells are localized to the lobules with CD8s and DCs intimately integrated in the breast epithelium

role in tumor immunosurveillance [11]. While locally regulated inflammation may control tumor proliferation, chronic inflammation has been associated with cancer development, including breast cancer [11–13]. In addition, immune suppression may increase the risk of breast cancer development, most notably in transplant patients [14] or those on immunosuppressant medications. Clearly, the immune response can play a complex role in both promoting and preventing breast cancer development. It may suppress tumor growth by destroying cancer cells, but may also select for cancer cells that are more adept to survive in an immunocompetent host [15]. This immune selection favors the development of less immunogenic tumors, allowing these tumors to escape immune surveillance—otherwise known as cancer "immunoediting" [12]. As tumor cells evade the immune system, a more aggressive phenotype is selected for and the surviving tumor cells that do not express recognized antigens will continue to evade the immune system. The complexity of the immune response to breast cancer is such that any attempts at prevention will need to be cautiously undertaken to induce only a gamma interferon (IFN) producing anti-tumor immune response. A preventative vaccine should avoid chronic inflammation and type II immune responses, which may be tumor-promoting [16]. Shifting the inflammatory response in the tumor environment can change the environment from tumor promoting to tumor eradicating [12,17] with Th1 and type

I macrophages. For example, increasing the presence and response of cytotoxic T cells (CTLs) and decreasing the presence of type II macrophages would promote a tumor eradicating environment in breast tissue.

10.3 The Problem of Developing a Preventive Breast Cancer Vaccine: Selection of target Antigens

The hallmark of the adaptive immune responses is specificity—immunity directed against individual proteins, or antigens. This allows the immune system to distinguish, based on the differential expression of proteins, between entities that should be attacked and eliminated, versus normal, healthy cells of the body, which are to be spared. In the case of vaccines against infectious agents, this is a comparatively easy task, since the evolutionary divergence between humans and microbial pathogens is so great that many of their proteins do not share significant sequence homology. These differences are easily perceived by the immune system, and vaccines directed against microbial pathogens usually elicit strong immune responses against the microbe with high specificities that do not cross-react with proteins on normal host cells. In the case of breast cancer, however, there is usually a relatively small subset of proteins that distinguish a malignant cell from its normal, healthy counterpart.

Breast cancer is a complex, multifactorial disease that develops from the normal host breast tissue. Therefore, developing preventive vaccines relies on identifying and targeting normal over-expressed, mutated, or cancer-specific targets. Three potential vaccination targets emerge including (1) oncodriver over-expressed proteins, (2) tissue specific antigens, and (3) cancer specific antigens (Fig. 10.2). We will discuss the utility of targeting each of these three groups of cancer-associated molecules in breast cancer, realizing that the best preventive vaccines may draw from a combination of these different targets.

10.4 The Case for Targeting Oncodrivers in Breast Cancer Prevention

The breast matures in distinct stages that are related to sexual development and reproduction. These stages are embryonic, prepubertal, pubertal, pregnancy, lactation and involution [19]. During early telarche, initial breast bud development occurs, however, the terminal end buds (TEB) do not complete maturation until pregnancy and lactation [20]. Following completion of lactation, a complex involution occurs causing terminal breast buds to die and the breast to return to a pre-pregnancy state.

The growth and invasion of TEB mimics cancer invasion of the breast stroma and is driven by the same oncodrivers found in many breast cancers [21]. These

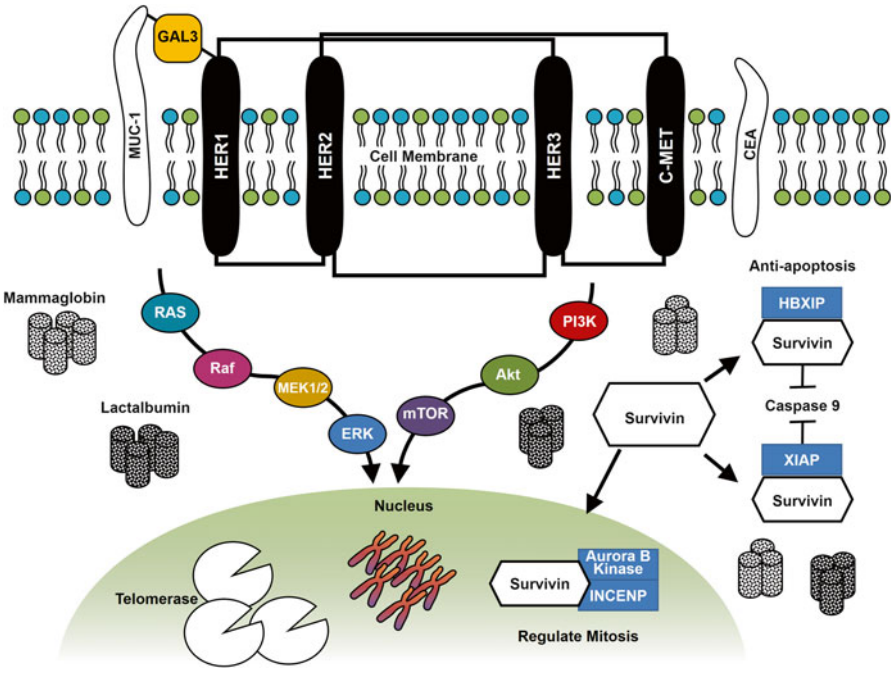

Fig. 10.2 Antigenic targets in breast cancer vaccination. Oncodrivers (HER-1, HER-2, HER-3, C-MET—*black*); Tissue Specific Breast Proteins (Mammaglobin, Lactalbumin—*grey*); Cancer Specific Proteins (Telomerase, Survivin, MUC-1, CEA—*white*)

TEBs are rapidly proliferating masses of epithelial cells that invade into stromal tissue, displaying properties associated with tumor progression-invasion, re-initiation of cell proliferation, resistance to apoptosis, and angiogenesis [20]. Carcinoma with epithelial growth factor receptor (EGFR) mutations, p53 mutations, or BRCA1 defects, such as (adeno) myoepithelial carcinoma, medullary carcinoma, metaplastic carcinoma, and ductal invasive basal carcinoma, have expression patterns similar to stem cells. In contrast, tubular, lobular, and grade 1–3 ductal invasive carcinoma have an immunophenotype similar to glandular cells. Basoluminal and ductal invasive grade 3 carcinoma with HER2 amplification fall in the intermediary cell category [21].

HER family members—HER-2, HER-3 and HER-1 (EGFR) as well as hepatocyte growth factor (c-MET), are expressed during breast development and growth [22]. HER-2 is also expressed during TEB growth during pregnancy. These same drivers have also been shown to be overexpressed in many breast cancers, suggesting their potential role in breast tumorigenesis. HER-2 is the classic example of a tumorigenic protein, and is overexpressed in both DCIS lesions and 20–30 % of invasive breast cancers (IBC) [23]. HER-3 has been found to promote HER-2-induced changes in breast epithelium before, during, and after tumor formation [24], and is expressed in numerous triple-negative cancers. HER-3 overexpression

is associated with worse outcomes and increased recurrence in several cancers, including breast cancer [25]. EGFR or HER-1 is also overexpressed in half of triple negative or basal breast cancers [26]. C-Met has been found to be expressed in triple-negative and some estrogen-expressing cell lines. The targeting of HER-3 and HER-1 is beginning to be explored using antibodies and kinase inhibitors to block the down-stream signaling pathways [26].

We have shown many of these oncodrivers to be expressed in early breast cancers, such as DCIS. DCIS is a proliferation of malignant epithelial cells confined within the basement membrane of mammary ducts, and appears to be a precursor lesion to IBC. These oncodrivers expressed in early DCIS lesions may be ideal targets for breast cancer prevention because of their key role in driving growth, invasion, and metastatic spread. Since these proteins are normally expressed in mammary development, innate immune responses may exist for controlling these oncodrivers. If these immune responses exist, then these oncodriver proteins may be suitable targets for breast cancer vaccination.

10.5 Evidence for Immune Responses Against Oncodrivers

HER-2 has been the focus of numerous immune interventions. Peptides derived from this molecule can be recognized by CD8+ T cells in MHC class I molecules. One of the most studied immunogenic peptides derived from HER-2 is E75 or (369–377). E75 is a peptide that binds HLA-A2 and has been administered as a vaccine in numerous clinical studies [27–29]. It has generated CD8+ T cell responses when administered to patients with HER-2 expressing breast cancers [29]. HER-2 derived peptides have also been identified that bind MHC class II molecules and activate anti-HER-2 CD4+ cells [30–32]. These peptides have been used to successfully prevent recurrence in patients with HER-2 positive breast cancer [30–32] and cause regression of DCIS lesions [33–36]. Other forms of anti-HER-2 vaccination are also being tested in trials including DNA vaccines, protein and RNA vaccines to drive anti-HER-2 immunity for treatment [30, 37, 38].

We observed that healthy individuals have surprisingly high frequencies of circulating anti-HER-2 CD4+ Th1 cells that secrete INF-γ and TNF-α [39]. This anti-HER-2 CD4+ Th1 response is lost during HER-2 breast tumorigenesis [40] beginning very early in the process during DCIS and more profoundly at the time of invasion [39] (Fig. 10.3). Furthermore, in patients with HER-2+ IBC, low anti-HER-2 immune responses are associated with increased risk of recurrence and lack of achieving complete responses to neoadjuvant chemotherapy [39]. Additional situations where the anti-HER-2 immune response is lowered may increase susceptibility to breast cancer development. For example, nulliparous women who have higher HER-2 gene expression and lower anti-HER-2 immune responses compared with parous women also have an increased risk of breast cancer [41]. Post-partum, when it is known that pre-menopausal women

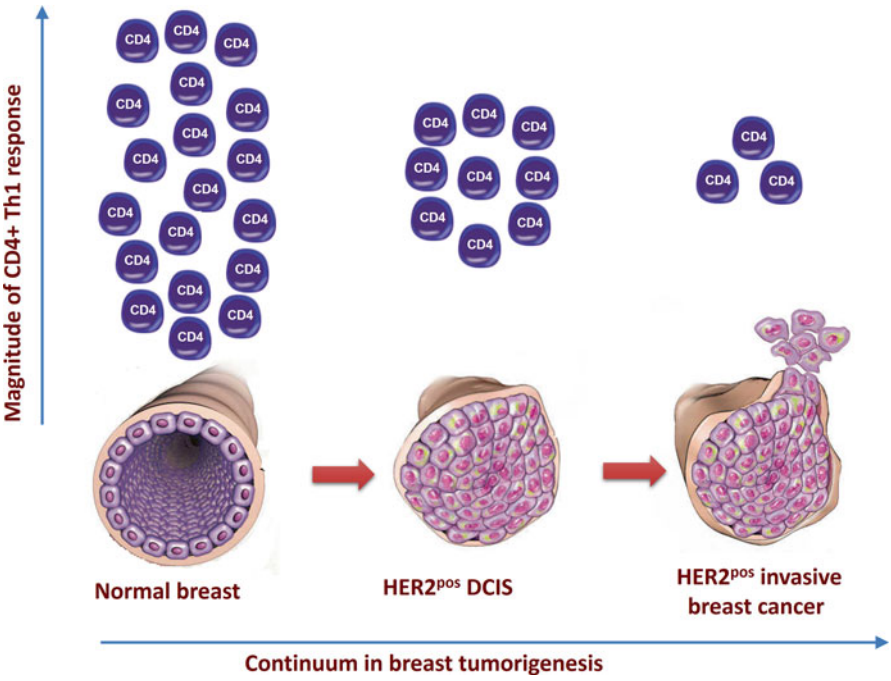

Fig. 10.3 Progressive loss of the anti-HER2 Th1 immune response along the breast cancer continuum

have some increased risk of breast cancer development in the 5-year window following pregnancies [42,43], women may display similarly high HER-2 gene expression and low anti-HER-2 immunity. We have developed a simple blood test that can measure anti-HER-2 CD4+ Th1 responses. Identifying patients with depressed anti-HER-2 CD4+ Th1 immune responses may be particularly useful in capturing those patients with HER-2 DCIS or IBC that are not detected with screening mammograms. Finally, although this deficient immune response is not corrected by surgery, radiation, or chemotherapy, we have shown that HER-2 peptide pulsed dendritic cells (DC1) activated to secrete high levels of IL-12 can be used in vaccination to augment and restore the anti-HER-2 immune response [39].

There are similar losses in CD4+ Th1 immunity being identified in HER-3 and other oncodrivers, suggesting this may be a common theme in breast cancer development. The ability to identify patients with suppressed immune responses against oncodrivers and correct the defective response prior to the development of breast cancer may be a feasible approach to breast cancer prevention. The benefits of targeting oncodrivers using vaccines are that these drivers are over-expressed in cancer cells, so only the aberrant cells would be targeted. This process may be a natural surveillance mechanism that the immune response uses to control proliferating cells during growth and development.

10.6 The Case for Targeting Tissue Specific Breast Proteins

The breast contains several tissue-specific proteins that are found in very few other organs, making these proteins breast-specific. Mammaglobin and lactalbumin are two important examples [44, 45]. Mammaglobin (MAM) is a member of the uteroglobin gene family that is highly expressed in the mammary epithelium and is over-expressed in up to 80 % of breast cancers [44]. Lactalbumin is conditionally produced only during lactation, but is expressed in over 60 % of breast cancers [46]. Immune responses have been generated against both of these proteins [45, 47, 48]. Both CD4+ and CD8+ T cell as well as antibody responses develop as a consequence of vaccination [46, 47]. Clinical trials have already shown that these immune responses are reproducible in human patients, with an increase in CD8+ T cells capable of lysing MAM+ breast cancer cells [48]. Murine models vaccinated against lactalbumin have been shown to prevent breast cancer development along with increased CD4+ and CD8+ T cell response [45, 49]. Mammaglobin may be useful in preventing a broad range of breast cancers as it is expressed in up to 80 % of estrogen receptor (ER) positive cancers and up to 40 % of triple-negative cancers [48]. Lactalbumin appears to be more highly expressed in triple-negative breast cancer [50], suggesting that vaccinating against lactalbumin may be most useful in preventing triple negative breast cancers.

The benefit of tissue-specific antigen vaccination is the low likelihood of life-threatening autoimmune pathologies, since expression of these antigens is limited to the breast. These antigens are also only minimally expressed on healthy and non-lactating tissue; therefore, when cells over-expressing these antigens arise, they are easily recognized. In this setting, vaccination acts as an immunologic mastectomy, eliminating duct cells that express lactalbumin or mammaglobin. Vaccination would be restricted to women who do not wish to lactate, have completed lactating, or are post-menopausal, since inducing these responses in lactating breasts can cause tremendous mastitis as seen in mouse models [51]. Nonetheless, there is continued progress in developing these vaccines against lactalbumin and mammaglobin in triple negative cancer.

10.7 The Case for Targeting Cancer Specific Proteins for Breast Cancer Prevention

In addition to oncodriver targets and breast tissue-specific targets, cancer-specific proteins that are abundantly expressed in transformed cells represent a third potential class of vaccine target. Examples of these proteins include telomerase, survivin, MUC-1 and differentiation antigens, such as carcinoembryonic antigen (CEA). All of these antigens are over-expressed in different types of breast cancers and are essential in transformation of normal cells to tumor cells. Telomerase is expressed in most tumors and prevents loss of telomeric DNA during the rapid cell division

characteristic of tumor growth [52]. Survivin is nearly undetectable in most normal adult tissues, but is highly expressed in some breast cancers, participating in the control of apoptosis, angiogenesis and proliferation [53]. MUC-1 is a mucoglycoprotein that is upregulated and hypoglycosylated in breast cancer. CEA is also a glycoprotein molecule that is overexpressed in many cancers, mainly gastrointestinal cancers, but has also been found in up to 50 % of breast carcinomas [54]. Targeting these antigens early, when they are initially over-expressed, is important in preventing IBC.

Immune responses have been induced against each of these cancer-specific molecules. In fact, clinical studies have already shown that vaccination against telomerase induces a peptide specific CD8+ immune response, increases progression free, and increases overall survival [55, 56]. Survivin has been shown to produce a CD8+ T cell response in vitro [57]. Clinical trials with survivin vaccination against prostate cancer have shown disease remission and regression [58], but clinical trials with survivin vaccination against breast cancer have yet to yield significant results [59]. Because MUC-1 is a glycoprotein, it tends to be weakly immunogenic. To ameliorate its weak immunogenicity, clinical trials in patients with breast cancer have coupled MUC-1 with Bacillus Calmette-Guerin (BCG) and tetanus toxoid in vaccines, which have been shown to have a selective immune response against MUC-1 [60–62]. Like MUC-1, CEA is also a glycoprotein and has been used in a recombinant vaccine with vaccinia. The MUC-1-vaccinia combination has been tested against many cancers, including breast cancer, with a good immune response and even showing a pathologic complete response [54].

Cancer-specific antigens are good targets for vaccination, thus group of antigens may be best utilized in secondary prevention of early lesions for primary prevention of invasive cancer. An example would be vaccination of DCIS, which is considered a precursor to IBC, this although secondary prevention would truly be primary prevention of invasive breast cancer. Eradicating DCIS at a pre-invasive stage with a continued immune response to the causal antigen would prevent future breast cancer. Telomerase, survivin, and MUC-1 are all expressed in DCIS [63–65], making these antigens good candidates for primary prevention and treatment against breast cancer. In fact, clinical studies have applied a MUC-1 vaccine in the setting of patient with a history of advanced colon adenoma—a precursor lesion to colon cancer—and found that these patients were able to exhibit long lasting immunity to the MUC-1 antigen [66].

10.8 The Special Case for Targeting Breast Cancer Stem Cells in Prevention

Breast malignancies may arise from specialized breast cancer stem cells (BCSC), or cancer initiating cells [67]. BCSC have been associated with late recurrences, and may very well be early precursor cancer initiating cells. Numerous groups are now focusing efforts to grow out stem cells that are CD44 high and CD24 low or ALDH1 positive as a means to develop strategies to target these cells. While oncodrivers,

such as HER-2, have been shown to be expressed on BCSC [68], there may be additional unique BCSC antigens [69]. As described below, we have developed vaccines against HER-2, clearly a BCSC associated molecule [68]. Once identified, additional unique BCSC antigens could be similarly targeted by vaccination.

10.9 The Lack of Evidence to Target Viral antigens

Some malignancies have been shown beyond doubt to have a strong viral component in their etiology. The best examples include the association of human papilloma virus types 16 and 18 with cervical, anogenital, and head and neck cancers, hepatitis B virus with hepatocellular carcinoma, EBV with Burkitt's lymphoma, Hodgkin's disease, and undifferentiated nasopharyngeal carcinoma, and Human Herpesvirus 8 (HHV8) with Kaposi's sarcoma. Vaccines have been developed against both HPV and HBV. Gardasil and Cervarix target the major capsid protein L1 of HPV and the hepatitis B vaccine is based on the major surface antigen of the virus (HBsAg). Targeting viral antigens to protect against breast cancer is dependent upon the extent to which viruses play a role in breast carcinogenesis. The causal relationship between viruses and human breast cancers remains controversial. Nonetheless, there are several suspect viruses that are being actively investigated. Three of the most prominent are briefly discussed here.

The first indication that breast cancer could have an infectious etiology came from studies initiated by Bittner in the 1930s [70]. The apparent filterable agent was later identified as a retrovirus designated mouse mammary tumor virus (MMTV). This virus could integrate into the genome of adult mice and be transmitted vertically through the endogenous route, or alternatively be transmitted to offspring through milk during nursing. The discovery of this virus, which was found to cause breast tumors in both captive-bred and wild mice, spurred vigorous investigations into the possible viral causes of breast cancer in humans.

Subsequently, MMTV, or a closely related virus (about 95+% sequence homology with MMTV) [71] has been discovered in some human breast cancers and designated human mammary tumor virus (HMTV) [72]. Viral gene sequences have been reported in 38 % of breast cancers, but only 1 % of normal breast tissues [73]. Interestingly, correlations have been reported between geographical regions of low breast cancer incidence and prevalence of detectable viral sequences [74], as well as more frequently detected viral genes in certain breast cancer subsets like gestational and inflammatory breast cancer [75, 76].

Also implicated in breast cancer etiology are the human papilloma viruses (HPV). High-risk human papilloma viruses type 16, 18 and 33 cause cellular transformation through early gene products (particularly E6 and E7), which act as oncoproteins that inhibit apoptosis and dysregulate cell cycle. HPV infection also induces a particular cytopathic effect in squamous epithelial cells that leads to the formation of a koilocyte, which is characterized by an enlarged, darkly-staining nucleus with pronounced cytoplasmic perinuclear clearing (e.g. "halo"). A number of studies

have used standard PCR to detect high-risk HPV strains in breast tumors, but not in the surrounding normal breast tissues [77–81]. More recently, Heng and co-workers performed in situ, as well as standard, PCR (with sequencing) and histopathology to assess presence of koilocytes in breast cancer. The in situ assay was designed to minimize the possibility of contamination by localizing the viral DNA to the nucleus. The investigators found evidence of high-risk HPV in breast cancer lesions, but also detected it in surrounding normal tissues and in the tissues of some healthy breasts (although frequency was higher in cancerous tissue). This was explained by the fact that even in the well-established relationship with cervical cancer, HPV infection precedes the development of malignancy, but does not guarantee eventual cancer development. Interestingly, koilocytes were observed in 18 of 28 (66 %) breast cancer specimens, and all of these were shown HPV-positive by in situ PCR [82, 83]. Taken together, these data suggest a possible link between HPV and breast cancer.

There is also a possible link between Epstein Barr virus (EBV) and breast cancer. EBV is a γ-Herpesvirus that has a strong tropism for B lymphocytes and epithelial cells. EBV principally manifests as infectious mononucleosis. The virus infects most individuals by young adulthood, and establishes a state of latency that lasts for the lifetime of the individual. During latency, only a subset of EBV genes is expressed. Certain triggers can reactivate the virus leading to re-establishment of lytic infection. Studies to determine an association between EBV and breast cancer have sought to detect viral genetic material via qPCR [84], PCR plus tissue microarray [85], and in situ hybridization [86], and to detect expressed viral proteins, such as Epstein-Barr nuclear antigens (EBNAs) and Latent membrane protein-1 (LMP-1), via immunohistochemistry [87]. These studies detected evidence of EBV genes or gene products in a subset of breast cancers.

In summary, it should be reiterated that a viral etiology for breast cancer remains highly controversial, and whereas we have cited a number of studies purporting to demonstrate the presence of viral products in breast tumors, a considerable body of work from numerous laboratories have reported either failure to find any association of these viruses with human breast cancer [88–90] or have attributed detection to contaminating, virally-infected but non-cancerous cells [87] or cross-reaction of detecting reagents with non-viral products [91]. Further studies are clearly necessary to settle this issue, and if a consensus is reached that certain viruses promote breast carcinogenesis, the associated viral antigens should be included in breast cancer vaccines.

10.10 Making Immunization More Effective: Vaccine Adjuvants

Immune response evolved primarily to deal with microbial infection. Therefore, elements of the innate immune system (such as dendritic cells) sense pathogen associated molecular patterns (PAMPs), become activated, and present pathogen-derived protein antigens complexed with MHC molecules to T lymphocytes, which are the agents of adaptive immunity. Since neither tumors nor pure protein antigens (such

as synthetic peptides) derived from tumors contain PAMPs, vaccine preparations including only tumor-derived proteins are unlikely to be strongly immunogenic and will poorly activate innate immunity. Adjuvants are compounds that amplify the immunogenicity of vaccines. Such adjuvants were originally developed to enhance vaccines against infectious diseases, but they are likely to be necessary for generating effective anti-tumor immunity. Adjuvants are thought to act by two general mechanisms. The first is the "depot" effect, which conserves the antigen at the site of injection for an extended period of time, where it is released slowly to provide long-term stimulation to the immune system. The second is through direct or indirect activation of components of the innate immune system. The natural adjuvant is an activated dendritic cell (DC), which can be prepared from peripheral blood DC precursors including monocytes [92]. For secondary prevention, as discussed later, this may be an ideal vaccine adjuvant; however, for primary prevention the harvesting of personalized DC is cumbersome and not cost-effective. An alternative simplified vaccine adjuvant must be selected.

The first adjuvant to gain wide use was aluminum salt (alum). Precipitation of vaccine immunogens with alum, and the attendant enhancement of immunity using this mixture, was first observed with diphtheria toxoid [93]. Alum has subsequently been employed in a variety of vaccines against infectious agents licensed for use in the United States. Despite its extensive use, the mechanisms by which alum amplifies immune responses are still uncertain. Alum has an excellent safety record spanning decades, but, unfortunately, it is probably unsuitable for generating powerful anti-cancer immunity. Alum largely induces Th2-dominated immunity [94]. Th2-responses are characterized by strong antibody production, and IL-4 and IL-5 producing T cells. Effective anti-tumor immunity, on the other hand, requires robust cell-mediated immunity characterized by IFN-gamma secreting "Th1"-polarized T cells and cytotoxic T cells.

Freund's adjuvant consists of paraffin oil that is mixed with an aqueous solution of the vaccine antigens to form an emulsion. Freund's "complete" adjuvant adds a killed preparation of bacteria (e.g. Mycobacterium) to enhance immunity, while the "incomplete" adjuvant contains only oil. It is likely that some of the adjuvant effect of Freund's complete adjuvant is derived from the PAMP molecules provided by the Mycobacteria. This adjuvant has been used for decades to induce powerful immunity in experimental animals; however, it is not suitable for humans, largely due to toxicity—i.e. severe inflammatory responses at the site of injection.

The success of Freund's in animal models has led to the search of other, less toxic, oil/water emulsion adjuvants that might be useful for humans. These include MF59 and AS03, manufactured by Novartis and Glaxo Smith Klein, respectively. Both adjuvant preparations are based on squalene, a 30-carbon lipid molecule originally derived from shark liver oil, but also obtained from a number of plant sources. Although not yet licensed in the United States, both of these adjuvants are utilized in Influenza vaccine preparations in Europe.

Synthetic or chemically-modified Toll-like receptor (TLR) agonists represent another highly promising avenue of investigation. TLR agonists directly activate dendritic and other antigen-presenting cells of the innate immune system through

their associated PAMP receptors. There are approximately ten known TLRs in humans, each identifying a different restricted set of possible ligands common to broad classes of potential pathogens. Ligation of TLR receptors induces enhanced antigen-presenting function of dendritic cells, and stimulates the secretion of cytokines and chemokines, which enhances the adaptive immune responses. Several of these receptors are being targeted by candidate adjuvants.

For example, Monophosphoryl lipid As (MPL) is a chemically altered, detoxified form of cell wall lipopolysaccharide from Salmonella Minnesota strain R595. Despite its chemically altered nature, it retains recognition by TLR4 and TLR2 and activates a MyD88-dependent signaling pathway that triggers secretion of pro-inflammatory cytokines and chemokines [95]. It is also associated with the generation of Th1 immunity [96]. A licensed HPV vaccine (Cervarix; GSK) contains MPL as an adjuvant, and a similar adjuvant formulation has been tested in vaccines in clinical trials against other viruses—including Herpes Simplex and Norovirus [97, 98], and cancers—including melanoma and colorectal cancer [99, 100]. Other TLR ligands being investigated in clinical trials as vaccine adjuvants include the double-stranded RNA mimic and TLR3 agonist, poly-ICLC for ovarian cancer and glioma [101, 102], and CpG DNA oligonucleotides (TLR9 agonists) and imiquimod (TLR7 agonist) for melanoma [103].

10.11 Enhancing Effector Function: Checkpoint Inhibitors

Ideally, cancer vaccines would work as stand-alone prevention for cancer as they so effectively do for a variety of infectious diseases. While this may be achieved in primary prevention, in the case of pre-existing disease, where the goal is either therapy or secondary prevention, vaccination will almost certainly have to be combined with other interventions to achieve maximal effect. This is largely because the presence of pre-existing disease either presents the immune system with too large a disease burden to eliminate without additional help, or because the tumors themselves exert regulatory influences on the immune system that may partially blunt or attenuate anti-tumor immunity.

One highly promising field of investigation is “checkpoint inhibitors”. T lymphocytes, which are largely responsible for dealing with both infection and cancer, are able to receive a variety of input signals that regulate their functional activity. Some of these signals activate the lymphocytes. Such signals are necessary to set immune responses in motion against microbial or malignant threats. Other signals are inhibitory, and are often referred to as “checkpoint” signals [104, 105]. Checkpoint signals are also important for maintaining homeostasis, because immune responses should not continue after the challenge has been eliminated, and normal, healthy tissues need to be spared from off-target immune attack. Receptors that receive these inhibitory signals represent the checkpoints that govern the limits of immune responses. These checkpoints become highly relevant for anti-tumor immunity because cells comprising malignant tumors often subvert these systems

of inhibitory control as a means of escaping the immune response. Checkpoint inhibitor drugs interfere with this strategy of subversion.

There are many possible receptor/ligand interactions forming checkpoints that tumors may use to evade immunity, but the two that are most studied, and that are being developed as therapeutic targets are the CTLA-4/B7 interaction and the Programmed death receptor (PD-1)/Programmed death receptor ligand (PD-L1 and PD-L2) interaction [106, 107].

CTLA-4 (i.e.CD152) is a surface receptor on T lymphocytes. CTLA-4 competes with another receptor, CD28, for interaction with B7-family co-stimulatory molecules (CD80 and CD86), which are expressed by dendritic cells and other antigen-presenting cells. One of the earliest steps in T cell activation occurs when dendritic cells present processed peptide antigen to T cells in the context of self MHC molecules. This antigenic signal is sensed by T cells through the T cell receptor. A second signal provided by the dendritic cell is through expression of surface co-stimulatory molecules, including CD80 and CD86. Resting T cells express high levels of CD28 (relative to CTLA-4), the counter-receptor for these co-stimulatory molecules. This interaction supplies an important second signal to the T cells that allows them to proceed to an activated state and avoid a state of chronic inactivation (anergy). Following T cell activation, levels of CTLA-4 begin to rise [108]. In contrast to CD28, ligated CTLA-4 supplies an inhibitory signal to the T cells that limits the scope of their effector function [109]. Monoclonal antibody-based drugs such as Ipilimumab have been developed that block signaling through CTLA-4 [110], preventing activated T cells from receiving feedback signals that will limit their activity. These drugs maintain T cells in a prolonged state of high effector activity, thereby improving the anti-tumor immune response. Ipilimumab has been tested in a number of clinical trials alone [111], in conjunction with chemotherapy [112], or in combination with vaccination [113]. Improved clinical responses have been observed in a subset of patients, but a relatively high rate of adverse effects has been reported, including diarrhea, colitis and dermatitis, and occasional more serious off-target toxicities to the liver and thyroid gland. These side-effects have limited the use of CTLA-4-blocking therapy, but the cases of improved clinical responses have spurred the search for other checkpoint inhibitors.

Programmed death receptor-1 (PD-1) is a transmembrane protein that is expressed on T lymphocytes. The ligands are PD-L1 and PD-L2. PD-L1 is expressed by activated dendritic cells, macrophages, B cells, and a variety of normal tissues. PD-L2 was initially thought to be found only on antigen-presenting cells, but it has now been identified in a number of immune and non-immune cell types, depending on a certain environmental factors [114]. When effector T lymphocytes are signaled through PD-1 by PD-L1 or PD-L2, they are negatively regulated in their activation, proliferation and expression of effector function. Consequently, transgenic mice lacking PD-1 suffer from several chronic inflammatory pathologies, indicating that this molecular interaction is critical for avoiding autoimmunity [115–117]. Also of significance, most tumor lines express PD-L1 or PD-L2, suggesting that tumors are subverting this system of autoimmune avoid-

ance to escape anti-tumor immunity. Consequently, PD-1 has come under scrutiny as a target for improving anti-tumor immune responses, and several monoclonal antibody-based therapeutics that interfere with PD-1 signaling (e.g. Pembrolizumab; Merck, Nivolumab; Bristol-Myers Squibb) are being developed and tested for treatment of solid tumors, including malignant melanoma and breast cancer [118–120]. Many of these studies provide evidence of objective responses and improvements in progression-free survival. The toxicity profile of these agents appears to be more promising than anti-CTLA therapy.

10.12 DCIS as a Model for Prevention

The immunogenicity of breast cancer that has been described above makes breast cancer a particularly promising candidate for vaccination designed to generate "secondary cancer prevention". Specifically, breast cancer tumor antigens have been observed to initiate a tumor-specific adaptive immune response. [121, 122] and lymphocytic infiltration is associated with improved survival [123, 124].

Early vaccine trials have focused on later stages of disease when standard treatments have failed. Under these conditions, cancer vaccines have had limited success—even vaccines that were able to stimulate an immune response did not demonstrate corresponding clinical improvement [125].

With the introduction of screening mammography, pre-invasive lesions are increasingly diagnosed. Ductal carcinoma in situ (DCIS) represents greater than 20 % of breast cancer cases diagnosed. Pre-invasive or early stage disease may be a better suited target for vaccination and cancer prevention for a variety of reasons [126]. These include:

- Patients with pre-invasive or early stage breast cancer may be more adept at responding to vaccination as they are usually otherwise healthy.
- Patients with pre-invasive or early stage breast cancer do not require adjuvant cytotoxic treatment which may induce immunosuppression via immunosuppressive cytokines, anergy, lymphopenia, impaired antibody production, inhibition of immune effector function, reduction of MHC expression, or inhibition of co-stimulatory proteins [127–132] (There are some chemotherapies, like cyclophosphamide and 5-Fluorouracil, that may induce immunogenic cell death and eliminate regulatory immune subsets, which would actually enhances the immune response [133]).
- The slow progression from DCIS to invasive breast cancer gives time for the patient to receive neoadjuvant booster vaccinations and develop a robust immune response,
- The smaller tumor burden of early disease may be more amenable to penetration and destruction by the immune effector cells
- Both immune and clinicopathological responses to neoadjuvant treatment can be assessed rapidly at the time of surgical resection.

Treatment of DCIS may (1) prevent the progression to invasive disease, (2) decrease the extent of surgical resection or the need for radiation therapy, thereby reducing the associated morbidity resulting from current treatments, and (3) lower the risk of subsequent recurrence and the associated psychological fear.

DCIS is a non-obligate precursor to invasive breast cancer—this means not all patients with DCIS will progress to invasive breast cancer. DCIS is frequently present on routine autopsy, suggesting that up to 15 % of DCIS lesions may be clinically insignificant [134, 135]. Therefore, ideal treatment of DCIS should be provided preferentially to higher risk patients. High-risk patients have an increased risk of invasive disease, subsequent recurrence, and require more aggressive treatment (e.g. mastectomy or lumpectomy with radiation).

Conventional predictors of high risk DCIS include patient, tumor, and treatment factors, including: younger age, family history of breast cancer, tumor size, tumor grade, and resection margin [136]. More recently, molecular markers that are prognostic in invasive breast cancer have also been shown to be expressed in DCIS [137]. In fact, HER-2/neu is overexpressed in DCIS (56 %) as compared to invasive breast cancer (11 %) [138], and HER-2 positivity is significantly associated with a higher rate of invasive disease [139, 140] and increased risk of recurrence [141] in patients with DCIS. This association suggests that HER-2 may have a critical role in cancer progression, or at least represent as a biomarker for increased risk of invasive disease. Therefore, HER-2-targeted therapy in DCIS may be of particular benefit in preventing the development of invasive breast cancer, or alternatively eliminate HER-2 expressing cancer stem cells. The latter would leave behind less harmful non-cancer stem cells with favorably less malignant phenotypes.

We have taken this approach in patients with HER-2-expressing DCIS in two neoadjuvant studies using HER-2 pulsed type I activated dendritic cell (DCI) vaccines. The advantages of this approach are that the DC are activated ex vivo where they cannot be further influenced by tumor factors, and that there is no adjuvants including aluminum compounds as the DC1 are the adjuvant themselves. The drawback to this personalized approach is that DC precursors must be obtained from each individual subject. In our first clinical trial of our anti-HER2 dendritic cell vaccine, we vaccinated patients who were diagnosed with $HER2^{pos}$ DCIS (either HER-2 2+ or 3+). Patients underwent leukapheresis with elutriation of blood product to provide monocytes (DC precursors) for vaccine preparation. Monocytes were cultured overnight in GM-CSF and IL-4-containing culture medium (to induce DC differentiation), pulsed with six HER-2/neu MHC class II binding peptides, and rapidly matured using IFN-γ and LPS. If the patient was HLA-$A2^{pos}$, the monocyte pool was divided in half and pulsed with either MHC class I binding peptide 369–377 or 689–697. Four to six weekly injections were administered into bilateral groin lymph nodes. In the second study, we randomized patients to injections in the groin nodes, the breast in the region of DCIS, or both sites.

The vaccine was well tolerated with only grade 1 and 2 toxicities observed and no cases of unacceptable toxicity. Vaccination with HER-2/neu peptide pulsed DC1s induced both $CD4^{pos}$ and $CD8^{pos}$ HER-2/neu-reactive T-cells, infiltration of

lymphocytes into the breast around the DCIS tumor (Fig. 10.4), and durability of the response >48 months. Additional complement-dependent, tumor lytic antibodies were induced in some subjects, suggesting an additional effector role. Clinical response (i.e. no evidence of disease found in the breast at the time of surgical resection) occurred in about 30–35 % of ER^{neg} $HER\text{-}2^{pos}$ subjects, but only 4 % of ER^{pos} $HER\text{-}2^{pos}$ patients experienced no residual disease [34,36]. Combining anti-estrogen therapy with vaccines in this latter group resulted in complete response rates of 30 %, similar to the ER^{neg} $HER\text{-}2^{pos}$ subjects (*submitted for publication*). Further studies combining HER-2 pulsed DC1 vaccines with HER-2 targeted blockade are underway in an effort to further increase pathologic complete response rates, decrease the extent of surgical and cytotoxic therapy used to treat for high risk DCIS lesions, and prevent subsequent breast cancer events.

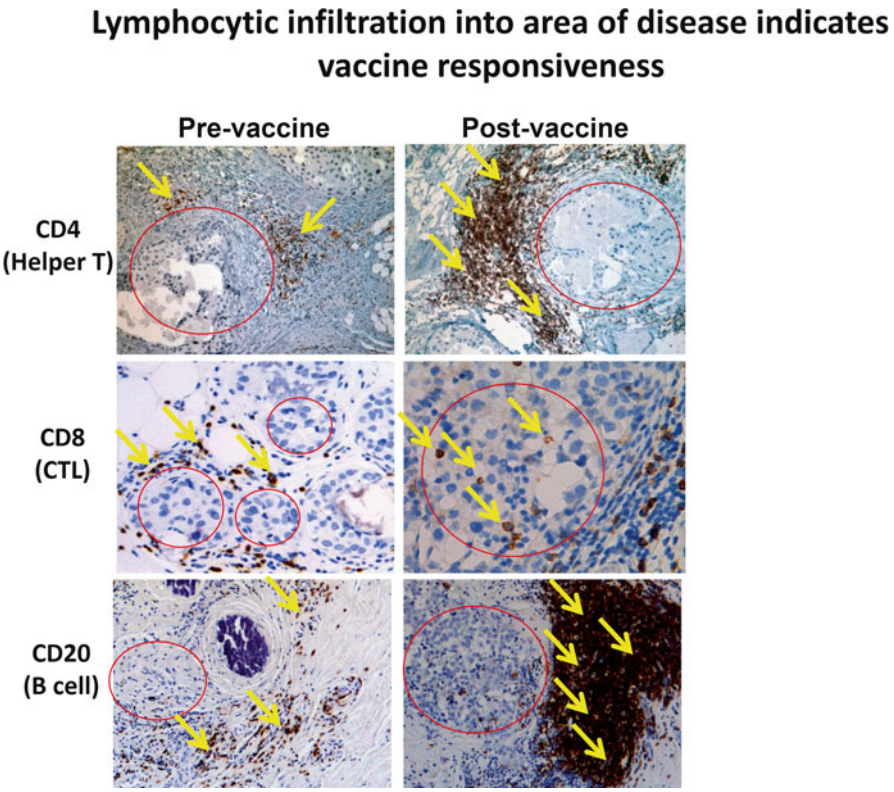

Fig. 10.4 Pre-vaccine biopsies were compared to post-vaccine surgical specimens by staining thin tissue sections for $CD4^{pos}$ "helper" T cells, $CD8^{pos}$ cytotoxic T cells, and $CD20^{pos}$ B lymphocytes. Areas of DCIS are subtended by *red circles*, lymphocytic infiltrates are stained *dark brown* and highlighted with *yellow arrows*. Note large increase in CD4 T cells in periductal areas after vaccination. $CD8^{pos}$ cells typically did not increase as dramatically, but often were observed entering the diseased duct. Somewhat unexpectedly, $CD20^{pos}$ B cells dramatically increased for some subjects in periductal regions after vaccination

10.13 Identifying High Risk Groups

10.13.1 Genetic Predisposition

Patients that are at high risk for developing breast cancer can be divided into those with genetic predisposition and those with acquired risk. While we can identify those with genetic predisposition quite readily, these patients account for only 10–15 % of all breast cancer patients. Despite the development of whole genome sequencing, genetic mutation identification has outstripped our ability to offer numerous treatment alternatives/ for prevention. Surgery remains the most effective treatment, but does so at an enormous cost to the patient. Other prevention techniques, such as anti-estrogens or surgical oophorectomy, are modestly risk reducing, but also with substantial side effects. Vaccination of women who have a genetic predisposition to develop breast cancer is a particularly appealing strategy for prevention.

For example, BRCA1 carriers are at increased risk of developing triple-negative breast cancers [142]. These patients present with a highly aggressive tumor at a young age, can be offered only limited available treatment options, including highly toxic chemotherapy, and still often succumb to recurrent disease. Oncodrivers, including HER-3, EGFR, and c-MET are overexpressed on triple negative tumors, and therefore represent potential targets for vaccination. Similarly, the lactalbumin protein is also over-expressed in triple negative tumors, and presents another potential target for vaccination. Cancer-specific targets, including MUC-1, telomerase, and survivin, could also be targets of vaccination therapy in this setting. These high-risk patients and their potential vaccine targets are both readily identifiable and are therefore well-suited to be treated with preventative vaccination. Because of the high lifetime risk of developing invasive breast cancer (60–80 %). This group is particularly well-suited for breast cancer preventive vaccines.

10.13.2 Acquired Risk

Identifying the patients with acquired risk is more difficult, but not impossible. For example, pre-menopausal women have been shown to be at increased risk for developing breast cancer in the 5 years following pregnancies [143–145]. Some of these patients have diminished immune response gene expression related to dendritic cells and T cell function [146]. With the rapid blood immune tests that we have developed, we can identify a diminished anti-HER-2 immune response or a transient loss of immune responses against oncodrivers, such as HER-2, HER-3 and c-MET. Women with a decreased immune response may be at increased risk of developing post-partum breast cancer. Additionally, this deficit can be corrected by vaccination.

Many of the acquired risk-associated breast cancers have HER-2, HER-3, and c-MET oncodrivers in early DCIS since these are the main oncodrivers involved in

breast elongation. In contrast, HER-2-expressing breast cancer rarely are associated with patients with BRCA1 or BRCA2 mutations. Vaccinations targeting HER-2 may be a very effective way to prevent non-hereditary breast cancer, and clinical trials are in progress [34–36] and being developed in large scale Phase III trials in patients with DCIS.

Finally, there is an increased risk of breast cancer development in patients taking immunosuppressive medications, particularly following organ transplantation [14]. Vaccination against oncodrivers, tissue specific antigens, or cancer specific antigens may be able to augment the immune responses and reduce breast cancer risk in these populations where immune suppression needs to be maintained.

10.14 Realizing the Potential

Clearly the immune response can determine the outcome and influence survival in invasive breast cancer [39, 95]. The loss of immune responses against oncodrivers early during tumorigenesis further suggests a crucial role of the immune response against protection for the development of breast cancer [39]. Vaccinations against oncodrivers to restore immunity may help to prevent breast cancer. Blood tests to measure the immune responses may be used to identify individuals at risk of developing breast cancer, and allow for vaccination prior to developing invasive disease. Developing vaccines against oncodrivers, breast tissue specific antigens, and cancer specific antigens will be useful to develop in the armamentarium for breast cancer prevention in those with identified risk including those with genetic predisposition. Since many acquired breast cancers have HER-2 involved even vaccinations to correct the anti-HER-2 immunity may be a good starting point for prevention in these patients. The time is nearing that we can now begin to realize the potential of using vaccines to prevent breast cancer and in the next decade will begin to realize this potential.

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