

The Antigenic Repertoire of Premalignant and High-Risk Lesions

Juan Pablo Marquez, Sasha E. Stanton, and Mary L. Disis

Abstract

Prophylactic vaccines have been a major advance in preventing the development of infections after exposure to pathogens. When contemplating an effective approach to cancer prevention, vaccines offer unique advantages over other more standard approaches: First, once appropriately stimulated, antigen-specific T cells will travel to all sites of disease and eradicate cells bearing the proteins to which the T cells have been primed by vaccination. Second, successful immunization will further result in the development of immunologic memory, providing lifelong immunologic surveillance. There is evidence of an adaptive tumor immune infiltrate even at the

earliest stages of breast and colon cancer development. Furthermore, there is measurable immunity to lesion-associated antigens present in patients who will eventually develop malignancy even before cancer is clinically evident. Recent studies are beginning to unmask the preinvasive antigenic repertoire for these two malignancies. Preliminary experiments in transgenic mouse models of mammary and intestinal tumors suggest that immunization against antigens expressed in preinvasive and high-risk lesions may be effective in preventing the development of invasive malignancy. *Cancer Prev Res*; 8(4); 266–70. ©2015 AACR.

Introduction

Prophylactic vaccines that target pathogens, which cause significant human morbidity and mortality, has been one of the most successful interventions used in humans to prevent disease. In the United States alone, there has been a 99% decrease in the incidence of infectious diseases that are targeted by common childhood vaccinations (1). The success of vaccines in the prevention of infectious disease has resulted in exploring immune-targeting approaches for the prevention and/or treatment of a variety of non-infectious diseases, such as Alzheimer disease, atherosclerosis, cancer, and even nicotine addiction (2–4). To be effective, vaccines must arm the immune system to destroy the cause of disease. In an infection, the pathogen has been defined, but in malignancy the initiating abnormality is generally unknown. Although the specific cause of a cancer may be multifactorial, there are a limited number of genetic alterations that will stimulate the initiation and maintenance of the malignancy. If we could define the antigenic repertoire of preinvasive high-risk lesions, perhaps the immune system could be armed via vaccination to eradicate cells expressing those proteins to prevent the development of cancer.

Recent evidence indicates that type I immunity, associated with the production of IFN γ , is needed for cancer eradication. Type I immunity enhances cross-priming at the site of cancer initiation by activating local antigen-presenting cells (APC) to more effi-

ciently present immunogenic proteins (tumor antigens) to T cells. Cross-priming is the primary method by which immunity is generated against cancer (5). Immunologic memory must be generated to for surveillance and to prevent recurrence by ensuring that the destructive immune response again deploys when the cancer antigen is expressed in the future.

What is the evidence for an adaptive immune response in patients with preinvasive tumors? Are these lesions capable of being recognized by the immune system? Recent evidence discussed below demonstrates that (i) adaptive immunity, defined by the presence of lymphocytes capable of antigen recognition infiltrating the lesion, is evident in preinvasive lesions at high risk of becoming malignant; (ii) patients bearing these lesions have evidence of an immune response targeting specific tumor-associated proteins; and (iii) vaccines constructed to immunize against the preinvasive antigenic repertoire have shown promise in transgenic models of mammary and colon cancer for disease prevention. Moreover, initial human clinical trials have shown these interventions to be safe and feasible, which is essential in the otherwise healthy prevention population.

The immune environment of preinvasive tumors or high-risk lesions demonstrates an early adaptive immune response

There is clear evidence of an adaptive immune response present in lesions that are associated with a high risk of developing into invasive cancers. However, the phenotype of the adaptive immune infiltrate and the diversity of T cells that either promote tumor eradication or profoundly regulate the evolving immune response vary significantly between tissue types. Here, we compare and contrast the immune microenvironment of high-risk lesions that predispose to the development of breast or colon cancer.

Retrospective analyses of samples derived from a variety of preinvasive breast lesions have suggested that the adaptive immune system is responding to abnormalities arising in the

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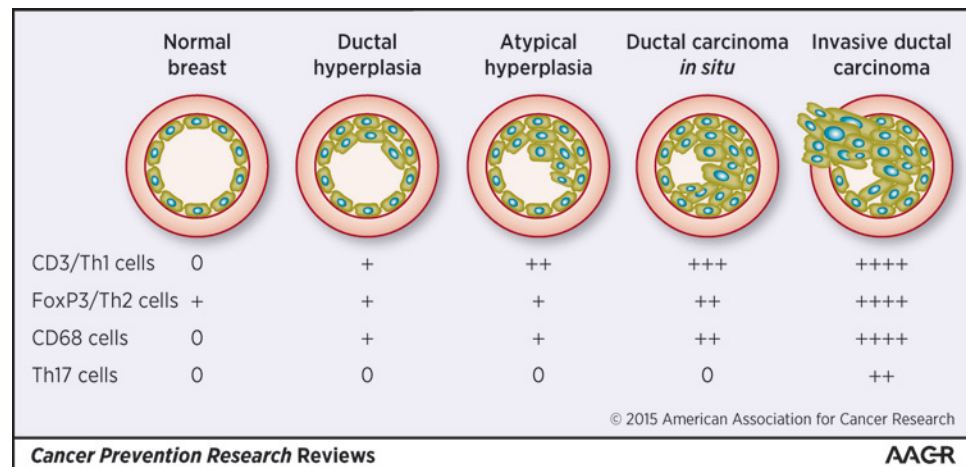
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Figure 1.

Evolution of adaptive immunity during breast cancer progression. As breast cells develop progressive atypia and then invasion of the basement membrane (pink circle) in invasive carcinoma, the adaptive immune response also increases. Immune infiltrate is graded from 0 (no infiltrate), + (low infiltrate), ++ (intermediate infiltrate), +++ (high infiltrate), to +++++ (very high infiltrate).



breast tissue before the histologic diagnosis of cancer. In one study of 53 mastectomy samples, investigators evaluated the levels of CD3⁺ and CD20⁺ lymphocytes as well as the presence of CD68⁺ macrophages in collected tissues (6). Specimens included a continuum from normal breast tissue to benign proliferative disease, ductal carcinoma *in situ* (DCIS), and invasive carcinomas. All immune cell levels in normal breast tissue were low, but significant increases in cellular infiltrates were already becoming evident in proliferative lesions. While the mean CD3⁺ T-cell infiltrate in normal breast tissue was 2.8 cells/high-power field (hpf), the mean level of CD3⁺ cells had already increased significantly in benign proliferative breast tissue to 81.5 cells/hpf ($P < 0.01$). The level of T cells in DCIS was similar, 84.0 cells/hpf however, levels of T cells further increased in invasive disease to a median of 103.7 cells/hpf ($P < 0.01$). The innate immune infiltrate, as evidenced by CD68⁺ cells, a marker for macrophages, was minimal in normal breast tissue (mean of 1.3 cells/hpf), increased to 3.8 cells for benign proliferative disease, 12.7 for DCIS, and 22.1 for invasive cancers with similar trends in the surrounding stroma ($P < 0.01$; ref. 6).

Cellular infiltrates associated with immune suppression increase as breast cancer becomes invasive. Investigators exploring the role of FOXP3⁺ regulatory T cells in breast cancer evaluated 237 invasive breast cancers and 62 DCIS-archived tissues and demonstrated increased FOXP3⁺ infiltrate in DCIS (mean 4/hpf; $P = 0.01$) and invasive breast cancers (mean 15/hpf; $P \leq 0.001$), as compared with normal breast (mean 0.5/hpf). Multivariate analysis indicated that levels of tumor-infiltrating FOXP3⁺ cells greater than the median predicted a poor prognosis both in invasive breast cancer and in DCIS. DCIS patients with "high" FOXP3⁺-infiltrating cells (defined as ≥ 4 cells/hpf) had a shorter relapse-free survival (HR, 2.81; 95% CI, 0.99–7.99; $P = 0.05$) than those with lower levels of FOXP3⁺ cells (7). A subsequent study has demonstrated that increases in the CD3⁺FOXP3⁺ T-cell/CD3⁺ T-cell ratio favor a regulatory immunosuppressive response as preinvasive lesions progress to invasive disease (Fig. 1; ref. 8). In an investigation of 32 invasive breast cancers with elements of both normal breast epithelium and DCIS present in the surgical specimen, there was a significantly increased intratumoral CD3⁺FOXP3⁺ T-cell to CD3⁺ T-cell ratio by immunohis-

tochemistry between normal breast (ratio, 0.005), DCIS (ratio, 0.01; $P < 0.0001$, as compared with normal breast), and invasive disease (ratio, 0.030; $P = 0.004$, as compared with DCIS), with similar significant trends observed in the surrounding stroma. The CD68⁺ tumor-associated macrophage infiltrate was also increased when comparing normal breast with DCIS (mean infiltrate 116 in normal and 139 in DCIS; $P = 0.007$), validating previously reported analyses (8). Recent studies have suggested that elevated levels of infiltrating CD4⁺ T cells that secrete IL17 (Th17) accompany the increase in regulatory T cells in invasive breast cancers (9). The presence of both these cell types is associated with a more aggressive phenotype. Th17 cells are the type of adaptive immune cell associated with the development of autoimmunity and tissue destruction, and cytokines secreted by Th17 cells have potent proliferative effects that may stimulate cancer progression (10). In an analysis of over 200 invasive breast cancers, the increased number of Th17 cells, as assessed by histopathology, was associated with high histologic grade and lack of expression of hormone receptors on tumors (11). Patients with increased Th17 cells infiltrating their tumors had a shorter disease-free survival than individuals with lower levels of these cells ($P < 0.01$). These data suggest that preinvasive breast tumors stimulate an adaptive immune response. However, early in that response, immune regulation occurs and, if significant, confers a poor prognosis (Fig. 1).

In contrast with breast cancers, colon adenomas develop in a highly inflammatory microenvironment where bacteria present in the gut constantly stimulate the immune system. For this reason, self-regulation is significant in normal colonic epithelium with regulatory T cells and Th2 cells present in the tissues (Fig. 2; ref. 12). The development of inflammatory bowel disease may represent a failure of immune regulatory cells keeping the bacterial induced inflammatory response in check. Patients with ulcerative colitis, a condition known to be associated with the development of colon cancer, have increased numbers of Th17 cells in the intestinal mucosa (13). Increased Th17 cells have been found in the invasive cancers of patients with preexisting chronic inflammatory syndromes and most likely contribute to tumor development.

Few studies have been conducted evaluating the immune infiltrate in human polyps or adenomas. One recent analysis of

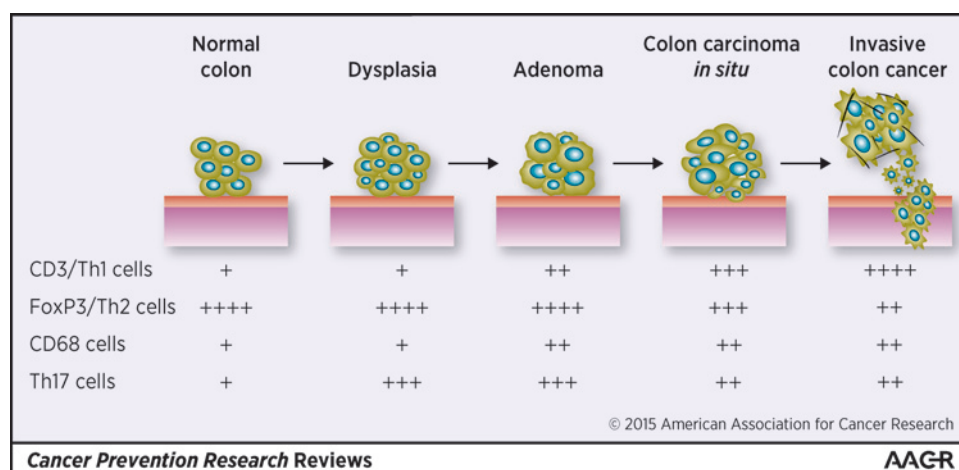


Figure 2. Evolution of adaptive immunity during colon cancer progression. As the epithelial cells of the colonic lumen develop atypia and then invade the basement membrane (pink bar), adaptive immunity is regulated. Immune infiltrate is graded from 0 (no infiltrate), + (low infiltrate), ++ (intermediate infiltrate), +++ (high infiltrate), to ++++ (very high infiltrate).

36 colon adenomas, including both low-grade and high-grade dysplasia, noted that the lesions demonstrated diverse immune infiltrates as compared with normal surrounding tissues (14). While there was a significant increase in CD4⁺ T cells in the adenomas as compared with benign lesions ($P = 0.009$), there was association of increased T-cell infiltration with increasing dysplasia ($P = 0.06$). The role of adaptive immunity in invasive colon cancers is well established. Several studies have demonstrated that intratumoral infiltration of type I T cells, Th1 and CD8⁺ T cells as well as memory T cells, predicts favorable outcome from early-stage invasive disease (15–17). In multivariate analysis, densities of CD45RO and CD8⁺ T cells in early-stage colon cancers independently predicted rates of complete remission and improved overall survival (15). The presence of an adaptive immune response, either Th17 or Th1, infers that T cells are responding to immunogenic proteins expressed in the tumor environment (Fig. 2).

Immunity to cancer-associated antigens can be detected before the diagnosis of invasive malignancy

Patients with preinvasive high-risk lesions that predispose them to the development of breast or colon cancer have measurable immunity to specific proteins expressed in their tumors. Presumably, the humoral immune response detected is reflective of immune surveillance recognizing aberrant cells and mounting an adaptive immune response.

Individuals with preinvasive breast cancers have detectable antibody immunity to mutated or overexpressed tumor-associated proteins that are not found in age-matched controls. In a study of 94 individuals with no cancer, 40 DCIS patients, and 97 patients with invasive breast cancers, the presence of autoantibodies to one of six tumor antigens—p53, c-MYC, HER2, NY-ESO-1, BRCA2, and MUC1—was found in 45% of patients with DCIS and 64% of patients with invasive breast cancer, giving a sensitivity of 85% to detect women who have DCIS or invasive disease as compared with women with no breast malignancy (18). Investigators evaluating 235 serum samples (93 controls, 82 DCIS patients, and 60 patients with invasive breast cancer) identified DCIS-associated antigens by proteomics. A panel of tumor-associated autoantibodies PPIA, PRDX2, FKBP52, HSP60, and MUC1 could discriminate patients with DCIS from controls with area under the curve

(AUC) 0.85 (95% CI, 0.61–0.92; ref. 19). A second five-antigen autoantibody signature has been demonstrated in patients with DCIS consisting of antigens RBP-J κ , HMGN1, PRSC1, CIRBP, and ECHDC1. Autoantibodies directed against these proteins could differentiate the sera of 20 DCIS patients from 20 patients with early-stage invasive breast cancers (AUC, 0.794; 95% CI, 0.674–0.877). Furthermore, this signature was associated with a higher risk of recurrence and could split DCIS patients into a low risk and high risk of recurrence population ($n = 44$ DCIS patients, $P = 0.011$; ref. 20).

Tumor-associated autoantibodies are also found in women who will subsequently develop breast cancer, raising the question of whether detected autoantibodies are reflecting the antigenic repertoire of preinvasive disease. Two studies have evaluated the autoantibody repertoire using sera derived from the Women's Health Initiative study. The first study focused on evaluating for the presence of known tumor-associated antigens in the sera of women who subsequently developed breast cancer. Four antigens—HER2, p53, CEA, and Cyclin B1—had the ability to differentiate between women with breast cancer from case-matched controls with an AUC of 0.79 (95% CI, 0.72–0.85; ref. 21). The second study used proteomics to identify autoantibody signatures associated with breast cancer risk and identified autoantibodies in the glycosylation and spliceosome pathways that could discriminate women who will develop breast cancer from controls that do not. Autoantibodies to nine proteins in the glycolysis pathway (including GAPDH, ENO1, PKM2, and ALDOA) were found more commonly in women who would develop breast cancer, as compared with case-matched controls with an AUC of 0.68 (95% CI, 0.59–0.78) and 14 autoantibodies to components of the spliceosome pathway (including SRSF1, U2AF1, HISTHID, and HSPA8) predicted women who would develop breast cancer, as compared with case-matched controls with an AUC of 0.73 (95% CI, 0.63–0.82). These data suggest that even in preinvasive disease, the immune system recognizes specific tumor-associated antigens and mounts an adaptive immune response.

Cancer-associated immune responses are also present in patients before the clinical diagnosis of colon cancer; however, a restricted number of antigens have been studied. A blinded case-control study was performed on 97 postmenopausal women who developed colon cancer after they were recruited to the UK Collaborative Trial of Ovarian Cancer Screening and 97 women

with no history of cancer (22). Sera from these women were analyzed for the presence of MUC1, MUC4, and p53 antibodies. The presence of MUC1 and MUC4 IgG antibodies could discriminate between patients who would develop colon cancer from controls with 8% to 13% sensitivity and 95% specificity, and the addition of p53 to the panel increased the sensitivity to over 30%. The immunogenicity of MUC was further validated in the preinvasive setting via the evaluation of MUC5AC antibodies in patients with colorectal polyps versus invasive cancers (23). While MUC5AC antibodies could be detected in about 30% of controls, incidence increased to 45% of patients with polyps and 60% of colorectal cancer patients. Furthermore, antibody levels were significantly correlated with the expression of MUC5AC in polyp sections.

The presence of specific autoantibodies in patients with preinvasive or high-risk lesions but not controls may be a reflection of the aberrant expression of these proteins very early in the malignant transformation. Autoantibodies may represent an attempt to mount an immune response to eliminate cells expressing these alterations. Could vaccination against immunogenic proteins associated with preinvasive disease create high magnitude adaptive immunity of the correct T-cell phenotype to result in destruction of the lesion before progression to invasive cancer?

Vaccinating to prevent cancer by targeting proteins included in the preinvasive antigenic repertoire

Transgenic mouse models of cancer have been essential for evaluating cancer prevention strategies, and immunotherapeutic approaches are no exception. Initial studies exploring breast cancer prevention used the BalbC ERBB2-expressing transgenic mouse and showed that vaccinating the mice with tumor cell lysates that overexpressed HER2 with IL12 as an adjuvant prevented development of hypertrophy and tumors in 90% of the mice ($P < 0.005$) as compared with control mice, where 100% developed atypical hyperplasia by 3 weeks and invasive carcinoma by 15 weeks (24). A DNA vaccine expressing the entire HER2 open-reading frame in another HER2-neu transgenic mouse model demonstrated that vaccinating mice starting at 3 months of age delayed tumor development compared with empty vector ($P < 0.0001$), but vaccinating mice at 6 months of age did not delay tumor development, suggesting that mice at this later age already had established malignant disease (25). More recent investigation has demonstrated the superiority of using multiantigen vaccines over approaches focusing on a single protein. Vaccinating against three breast-specific antigens that are commonly overexpressed in DCIS and invasive breast cancer (HER2, IGFBP2, and IGFIR) increased both disease-free (HR, 3.959; 95% CI, 2.018–7.768; $P < 0.0001$) and overall survival (HR, 4.335; 95% CI, 1.886–9.962; $P < 0.0002$) in TgMMTV-neu mice. The tumors that did develop in vaccinated mice were smaller, had a significantly slower growth rate, and had an increased CD8⁺ T-cell infiltrate compared with controls ($P = 0.0007$; ref. 26).

Transgenic mouse models have also been used to show the efficacy of a MUC1-targeting vaccine to prevent colon cancer in a MUC-1 transgenic mouse model of inflammatory bowel disease (27). Mice in the MUC1-immunized group had a significantly longer time to rectal prolapse, an indicator of disease, than control mice ($P = 0.043$). The vaccine was also associated with the elimination of MUC1-expressing cells as

well as a decrease in immunosuppressive elements in the immune microenvironment (neutrophils and myeloid derived suppressor cells, MDSC). Based on these promising results, MUC1 vaccination has been translated to the clinical setting. Thirty-nine patients with advanced colon adenomas (1 cm or more with villous or high-grade histology) were immunized with a MUC1 peptide-based vaccine. All adverse events collected were grade 1, with 80% of subjects developing erythema at the vaccine site. Almost 50% of patients developed high-titer MUC-1-specific antibodies after vaccination. Those patients that developed immunity with immunization demonstrated amnestic responses after a booster vaccine at 52 weeks, indicating the development of immunologic memory. The lack of immunity in the remainder of the patients was associated with high levels of circulating MDSCs, providing a defined target to modulate for vaccine failures (28). Strategies to reduce the level of MDSC will, most likely, enhance vaccine efficacy in all patients.

Conclusions

The importance of the preexisting immune response has emerged in both colon and breast cancers, and there is further evidence that the immune response to the atypical cells begins in preinvasive disease (16, 29). Autoantibodies present in patients that will develop colon or breast cancer but not in normal controls suggest that the immune system is able to recognize the preinvasive lesions. However, evidence of immunosuppressive infiltrate predicting worse outcome in preinvasive lesions suggests that, similar to invasive lesions, preinvasive tumors are able to escape the immune system. Priming the immune system to recognize and destroy cancer as it develops has distinct advantages over more classic forms of chemoprevention. One advantage is that exposure to antigen to stimulate T cells can occur over a short period of time, yet immunologic memory generated from this exposure can last a lifetime. Also, T cells will travel to any site in the body where cancer occurs and eliminate cells that have begun to express the aberrant protein against which the T cell was primed. Finally, immune therapies have been shown to be generally well tolerated with minimal side effects, which is critical in preventative vaccines that will be given to otherwise healthy high-risk patients. One key to effective vaccines for cancer prevention is the identification of the preinvasive antigenic repertoire that will provide the targets for active immunization.

Disclosure of Potential Conflicts of Interest

M.L. Disis has ownership interest (including patents) in Epithany and University of Washington. No potential conflicts of interest were disclosed by the other authors.

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Cancer Prevention Research

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