

Innate immunity in breast carcinoma

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Abstract

The innate immune response, which depends on so-called pattern-recognition receptors (PRRs) is an evolutionarily old immune response able to elicit a defensive response against a vast array of pathogens. The purpose of this review is to revisit the role of innate immunity in breast carcinoma from the oldest therapeutic approach using bacillus Calmette–Guerin to the recent findings on the manipulation of the PRR pathways with unmethylated cytosine-guanosine dinucleotides (CpG motifs). Encouraging results have been obtained in prevention and local treatment of murine mammary tumors using tumor cells engineered to express stably mycobacterial antigens or directly using CpG-containing oligonucleotides. The experimental findings raise the possibility of successful anti-tumor management through stimulation of innate immunity in women at high risk of developing breast cancer and in breast cancer patients with reasonable immunological performance and low tumor load.

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Innate immunity versus adaptive immunity

Proteins, carbohydrates, lipids and nucleic acids are, for biologists, currently the ‘earth, air, fire and water’ of the ancient Greek philosophers with respect to the constituents of the natural world. All of these constituents are ‘sensed’ by cells of the innate and/or adaptive mammalian immune system. The evolutionarily older innate immune response was previously considered as a non-specific defense against foreign invaders, but recent studies have shown that this response depends on so-called pattern-recognition receptors (PRRs) to elicit a defensive response against a vast array of pathogens. A subset of these receptors, the Toll-like receptors (TLR) identify a variety of products characteristically associated with microbial species. Toll protein was originally identified in *Drosophila* as a component critical for dorsal-ventral patterning in fly embryos (Stein *et al.* 1991) and for host immunity against fungal infection (Lemaitre *et al.* 1996). The cytoplasmic domain of Toll was found to be homologous to the cytoplasmic domain of the mammalian interleukin (IL)-1 receptor, suggesting that similar receptors might be encoded in the mammalian genome. Indeed, the hunt for mammalian orthologs of Toll led to the discovery by Medzhitov and Janeway of human Toll, subsequently renamed TLR4, in 1997 (Medzhitov *et al.* 1997). More recently, at least ten additional mammalian TLRs, and agonist for some of these TLR proteins, have been identified (Akira *et al.* 2001,

Medzhitov 2001). TLR2 agonists include a variety of bacterial cell-wall products, such as peptidoglycan from Gram-positive bacteria (Schwandner *et al.* 1999, Takeuchi *et al.* 1999), bacterial lipoproteins (Aliprantis *et al.* 1999, Brightbill *et al.* 1999, Takeuchi *et al.* 2000), mycobacterial cell-wall lipoarabinomannan (Means *et al.* 1999, Underhill *et al.* 1999b) and yeast cell walls (Underhill *et al.* 1999a); TLR4 agonists include Gram-negative bacterial lipopolysaccharide (Kawasaki *et al.* 2000) and respiratory syncytial virus protein F (Kurt-Jones *et al.* 2000). Bacterial flagellin has been shown as a TLR5 agonist (Hayashi *et al.* 2001), while unmethylated cytosine-guanosine dinucleotide (CpG)-containing DNA has been identified as a TLR9 agonist (Hemmi *et al.* 2000). Therefore, through different TLRs, the innate immune system can distinguish between different classes of pathogens.

Adaptive immunity differs profoundly from the innate system in its interactions with pathogens. First, it is extremely specific, detecting subtle differences among vast numbers of proteins and responding to each of these individually. Secondly, adaptive immunity requires two signals to elicit the immune response, one for recognition of antigen and another that relies on expression of co-stimulatory molecules and cytokines by antigen-presenting cells. The mechanisms controlling the initial induction of these signals had been poorly understood until Medzhitov & Janeway (2000) proposed PRRs, in particular TLRs, as a link between

the innate and adaptive immune systems. Indeed, those investigators showed that activated TLR4 increases the expression of the co-stimulatory molecule B7.

The vertebrate immune system has probably evolved to protect against infections that threaten survival before reproduction. Since clinically manifest tumors arise mostly after the reproductive years they may escape the attention of the immune system through various mechanisms. Most of these mechanisms (for reviews see Marincola *et al.* 2000, Khong & Restifo 2002), such as loss or downregulation of major histocompatibility complex (MHC) molecules or tumor antigens, expression of apoptosis-inducing cell surface molecules, lack of co-stimulation molecules, promote escape from tumor-specific T cell recognition. However, it is noteworthy that some mechanisms, such as secretion of immunosuppressive cytokines or expression of regulatory proteins implicated in complement resistance (Gorter & Meri 1999, Jurianz *et al.* 1999) could also affect innate immunity effectors.

Since the 1950s, when the concept was first formulated, immune surveillance against tumors has been in and out of fashion. For example, that non-virus-associated cancers such as those of breast and lung, do not arise any more frequently in people with a defective immune system than in those with a fully functional system (Groopman 1987) argues against the immune surveillance hypothesis, instead suggesting that the immune system recognizes tumors more as self than as foreign. Also, results obtained in patients receiving therapeutic immunosuppression (cyclosporine A) after kidney or heart transplantation are conflicting, since a lower incidence of breast cancer was observed, while for all other major cancers, including breast fibroadenomas (Weinstein *et al.* 2001) the incidence was higher (Stewart *et al.* 1995). Potential mechanisms to explain these effects include production by inflammatory cell infiltrates of direct or indirect modulators of breast cell growth, e.g. cytokines, peptide or steroid hormones, enzymes involved in steroid metabolism, as well as of antibodies to growth factors or their receptors.

Role of innate immunity in cancer

There is considerable historical evidence for the inverse relationship between acute bacterial infections or their toxins and cancer. Beginning in the 18th century, remarkable cases of cancer in which patients recovered after an acute bacterial infection have been reported. These cases were often called 'spontaneous regression' and some of these occurred following acute inflammation or fever (Nauts 1980). In the 1890s, William Coley, the first tumor immunologist, began to treat cancer patients with preparations derived from streptococcal cultures (Coley's toxins) to activate general systemic immunity, a portion of which might be directed against the tumor (Nauts 1989). Studies at that time also pointed to a lower incidence of cancer in patients with tuberculosis. Mycobacteria, in particular *Mycobacterium bovis* bacillus

Calmette–Guerin (BCG) have been used in experimental model systems and in humans as an approach to activate specific and/or non-specific anti-cancer immunity. The first published use of BCG as a cancer vaccine was in 1935 by Holmgren in Sweden, but it was not until the late 1950s that experimental and clinical studies generated enthusiasm for its use against a variety of cancers, including leukemia, colon cancer, lung cancer and melanoma. However, the failure of later controlled studies to support this enthusiasm, together with the advent of modern chemotherapy and radiotherapy, led to gradual abandonment of the use of BCG for cancer treatment except in the case of bladder cancer. Studies revealed that the bladder was capable of mounting a strong immune response to BCG and that close contact between BCG and the tumor was required for maximum effect in animal models (Crispen 1989, van der Meijden *et al.* 1989). In 1976, Morales *et al.* described the successful treatment of a small group of bladder cancer patients by intravesical instillation of BCG. However, wide acceptance of BCG immunotherapy for bladder cancer began after a controlled study in 1980 which demonstrated the unequivocal benefits in terms of decreased recurrence rate and increased median time to recurrence in patients who received BCG immunoprophylaxis after local surgery (Lamm *et al.* 1980).

BCG in breast carcinomas

The immune response against the intracellular pathogen mycobacteria is dominated by the fine-tuned interaction of innate and adaptive immune responses. Recent works implicate TLR proteins as key molecules in the orchestration of this immune response. Indeed, purified mycobacterial antigens, such as 19 kDa and 38 kDa lipoproteins, lipoarabinomannan and phosphatidylinositolmannan, have been reported to interact with TLR2, while infection with whole bacilli evokes a complex activation pattern involving TLR2 and TLR4 (Flynn & Ernst 2000, Flynn & Chan 2001, Thomaszyski *et al.* 2001, Heldwein & Fenton 2002, Stenger & Modlin 2002).

The effect of BCG on experimental mammary tumors has been evaluated in different models with variable results. For example, mice implanted with the immunogenic syngeneic mammary carcinoma MC2 showed enhanced resistance to tumor development only when pretreated long term with BCG, whereas mice injected with BCG and simultaneously implanted with MC2 cells showed decreased resistance to the tumor (Vaage 1983). Results in other studies were dependent on the dose, the administration protocols and the BCG preparation used, demonstrating tumor regression in some cases but accelerated rates of tumor growth and early death in others (Likhite 1976, Yron *et al.* 1975, Yamamura *et al.* 1976).

Several studies have been conducted in breast cancer patients to compare the efficacy of BCG immunotherapy to

conventional chemotherapy and/or to evaluate the role of BCG as adjuvant in chemo-immunotherapy trials. In a randomized trial with T3 and T4 breast cancer patients following loco-regional treatment, no significant difference in the disease-free interval was observed between the group receiving BCG immunotherapy and the chemotherapy group (Serrou *et al.* 1982). However, in the same study, patients first treated with BCG responded better to chemotherapy than did patients who were not pretreated (Serrou *et al.* 1982). Subsequent studies have reported no benefit in disease-free or overall survival from the addition of BCG, administered alone or together with a tumor cell vaccine, compared with the chemotherapy regimens (Senn *et al.* 1982, Buzdar *et al.* 1986, Giuliano *et al.* 1986, Aisner *et al.* 1987, Marshall *et al.* 1987, Crowe *et al.* 1990). Moreover, toxicity in the form of painful ulcers and fevers was sometimes associated with BCG administration (Aisner *et al.* 1987).

An interesting relationship between the immunological response to BCG and the efficacy of BCG immunotherapy was observed in a study of 40 patients with advanced breast carcinoma, wherein those patients who showed good local response to BCG vaccination also had a better response to therapy (Saha *et al.* 1986). In another study, a slight negative effect was observed when BCG was used as adjunct therapy for breast cancer in women for whom BCG afforded little protection against tuberculosis (Early Breast Cancer Trialists' Group 1992). Moreover, there is some evidence for a mechanistic relationship between the variable efficacy of BCG in prophylaxis against tuberculosis and in tumor prevention and therapy (Grange *et al.* 1995). Several independent studies in different countries have shown that neonatal BCG vaccination affords some degree of protection against leukemia and other childhood cancers, but only when it also protects against tuberculosis (Grange & Stanford 1990). Thus, the variable efficacy of BCG vaccination may reflect previous imprinting of the immune system by contact with environmental mycobacteria, leading to a protective T helper 1 (Th1) response or instead to a mixed Th1/Th2 population of responsive T cells, which appears to be ineffective and results in indiscriminate tissue necrosis (Bretscher 1992).

Innate immunity and hormones

Most breast carcinomas are strongly related to estrogens. Indeed, hormonal risk factors have been clearly identified, and treatment with estrogen antagonists has recently been shown to prevent almost 50% of breast carcinomas (Fisher *et al.* 1998). However, the role of estrogens in breast carcinoma etiology is still not completely defined. Besides a direct role on the target tissue, other mechanisms might be involved, including innate immunity. In fact, numerous studies have demonstrated a role for estrogens in depressing the activity of natural killer (NK) cells (Seaman *et al.* 1978, Kalland & Forsberg 1981), one of the most important effector cell

groups of innate immunity and with an important role in tumor susceptibility. The recent finding that both estrogen receptor- α and - β are expressed on NK cells might explain the estrogen-induced immunosuppression of NK activity (Curran *et al.* 2001). Consistent with this possibility is the increase in NK activity in parallel with the decrease in estrogens after menopause.

The effects of chemotherapy on NK activity are also worthy of consideration. Indeed, most of the drugs used for breast carcinoma therapy induce immunosuppression. However, experimental data generated in mouse models have indicated that the immunomodulatory effect of chemotherapy on NK activity is variable and largely depends on the drug itself (Gazit & Kedar 1994). Also timing of rescue of immunocompetence after treatment is variable and depends not only on the drug but also on the patient's immune system. Furthermore, in some cases, immunostimulation instead of immunosuppression has been observed, as in the case of a low dose of cyclophosphamide having been shown to increase NK activity (Tzai *et al.* 1996).

From BCG to mycobacterial antigens

The wide range of results in the previous studies conducted using the mycobacterial vaccine strain BCG might be explained, at least in part, by the variable immune status of the recipient and also by the panoply of mycobacterial components (polysaccharides, lipids, glycolipids, glycopeptidolipids) that potentially enhance or suppress the immune system (Audibert *et al.* 1977, Gaylord & Brennan 1987). The use of other mycobacterial preparations, such as the purified mycobacterial antigen, which is well characterized in terms of immunological activity, has been suggested as a means to suppress the indiscriminate necrosis while enhancing Th1-regulated selective destruction of tumor cells (Grange *et al.* 1995). In mammary experimental models, the A60 macromolecular antigen complex extracted and purified from *Mycobacterium bovis* BCG was used to prevent the growth of the murine mammary tumor EMT6. Repeated injection of mice with A60 alone prior to challenge reduced the incidence of EMT6 tumors (60–70% suppression of tumor growth vs control), and this effect was enhanced by simultaneous administration of A60 complex and irradiated isologous cancer cells (80–100% suppression) (Maes *et al.* 1995). Analysis of adoptive transfer of macrophages or T lymphocytes from A60-primed donor mice prior to EMT6 tumor challenge showed that stimulation of T lymphocytes by A60 was the key step in activating the immune cells involved in tumor rejection (Maes & Cocito 1996).

In a different approach, mammary tumor cell lines have been engineered to stably express mycobacterial antigens. Such engineered tumor cells endogenously expressing the mycobacterial protein in the same context of tumor antigens have been used as cellular vaccines. A highly immunogenic

protein (38 kDa lipoprotein) encoded by the *Mycobacterium tuberculosis* Ag38 gene has been stably expressed on the surface of N202.1A mammary tumor cells. This tumor cell line was derived from a mammary carcinoma spontaneously grown in FVB-NeuN mice transgenically expressing the rat HER2/neu proto-oncogene under the control of the mammary tissue-specific promoter of the mouse mammary tumor virus (Sfondrini *et al.* 2000). In these mice, the transgene expression starts at about 2 months of age and, by 4–5 months, the mammary glands show hyperplasia and microfoci of *in situ* carcinomas. At about 6 months, female transgenic mice begin to develop invasive carcinomas and, by 12 months, tumor incidence reaches 100% (Guy *et al.* 1992, Boggio *et al.* 1998). The spontaneous development of focal mammary tumors makes this experimental system a realistic model to evaluate immunotherapeutic strategies for cancer vaccination in individuals at risk for tumor development with defined gene mutations. Proto-neu-transgenic female mice were subcutaneously vaccinated twice at a 4-week interval starting at 2 months of age with irradiated non-transduced N202–1A tumor cells or irradiated Ag38-transduced tumor cells. Mammary glands were inspected weekly until 400 days of age. In mice vaccinated with Ag38-transduced cells, the appearance of spontaneous tumors was significantly delayed as compared with untreated control mice (at 9 months of age, only 45% of mice had tumors vs 100% of control mice), whereas no significant difference in tumor incidence was observed between mice vaccinated with non-transduced cells and controls (at 9 months of age, 82% vs 100% incidence respectively). Analysis of the anti-tumor immunity induced by Ag38-transduced cancer vaccine plus a systemic co-administration of a low dose of IL-12, which has been shown to increase natural tumor immunity in a variety of murine tumor models (Kurzawa *et al.* 1998, Taniguchi *et al.* 1998, Smyth *et al.* 2000), revealed an increased protection from spontaneous tumor development, with complete inhibition of tumor development at the end of the experiment in 2 of 12 mice, as compared with mice injected with transduced cells alone. Analysis of the cytokine secretion pattern from vaccinated mice showed that expression of the mycobacterial gene in the tumor vaccine induced a preferential Th1 response and that CD3⁺ CD8⁺ spleen cells derived from transduced cell-vaccinated mice responded to the tumor *in vitro* with interferon- γ production.

In a subsequent study, the mycobacterial gene encoding the cell wall-associated 19 kDa lipoprotein was stably expressed in the TS/A mammary tumor cell line, and the engineered cells were used to vaccinate mice previously immunized with BCG (Martino *et al.* 2001). Mice that received the two-step vaccination protocol developed a strong anti-TS/A delayed-type hypersensitivity reaction. Efficacy of this vaccination, evaluated as protection against challenge with parental TS/A tumor cells, was demonstrated by the significantly increased survival of vaccinated mice as

compared with controls and by the complete rejection of the tumor in some mice.

From mycobacterium antigen to CpG oligonucleotides

In a study to identify the component of BCG responsible for its anti-tumor activity, Tokunaga *et al.* (1984) purified a fraction from BCG, designated MY-1, which contained 70% DNA and 28% RNA; injection of this fraction into CDF1 mice and strain 2 guinea pigs induced regression of a carcinoma and line 10 tumor respectively. RNase-digested MY-1 (the DNA-rich fraction) was more effective than undigested MY-1 against tumors, while DNase-digested MY-1 had reduced activity, suggesting that the BCG DNA was the relevant component for strong anti-tumor activity. Subsequent studies demonstrated that the potent immunostimulatory activity of bacterial DNA was due to the presence of unmethylated CpG dinucleotides within a specific pattern of flanking bases, i.e. CpG motifs (Krieg *et al.* 1995, Lipford *et al.* 1997). Prokaryotic and vertebrate DNAs differ in the relative abundance of CpG dinucleotides and the degree of cytosine methylation, raising the possibility that these structural differences are used by immune cells to discriminate pathogen-derived ‘dangerous’ from ‘self’ DNA. Recent studies indicate that innate immunity recognition of these motifs is mediated by TLR9 (Hemmi *et al.* 2000). Theoretically then, anti-tumour responses might be augmented by bacterial DNA- and CpG-containing oligonucleotide (ODN)-induced activation of B cells (Krieg *et al.* 1995), NK cells (Ballas *et al.* 1996), macrophages, and dendritic cells (Jakob *et al.* 1998, Sparwasser *et al.* 1998, Hartmann *et al.* 1999). Furthermore, CpG-ODNs reportedly act as an adjuvant for Th1 polarization of cellular responses (Chu *et al.* 1997). Promising results have been obtained using CpG-ODNs as an adjuvant for immunization against tumor antigens (Weiner *et al.* 1997, Kim *et al.* 1999, 2000, Davila & Celis 2000, Miconnet *et al.* 2002), and an anti-tumor effect of CpG-ODNs has been recently evaluated in different experimental models (Wickstrom 1997, Smith & Wickstrom 1998, Blazar *et al.* 2001, Dow *et al.* 1999, Carpentier *et al.*, 1999, 2000, Lanuti *et al.* 2000, Warren *et al.* 2000, Ballas *et al.* 2001, Hafner *et al.* 2001, Kawarada *et al.* 2001, Sfondrini *et al.* 2002b).

In experimental mammary tumors, the therapeutic effect of CpG-ODNs was evaluated in the proto-neu transgenic model (see above). In these mice, systemic repeated administration of CpG-ODNs to maintain the innate immune system on continuous alert was found to prevent spontaneous mammary tumor development (Sfondrini *et al.* 2002a). In that study, transgenic female mice were treated intraperitoneally with CpG-ODNs every 10 days starting at 10 weeks until 54 weeks of age; spontaneous tumor appearance was significantly delayed as compared with untreated control mice (at 10 months of age, tumor incidence was 54% vs 100%

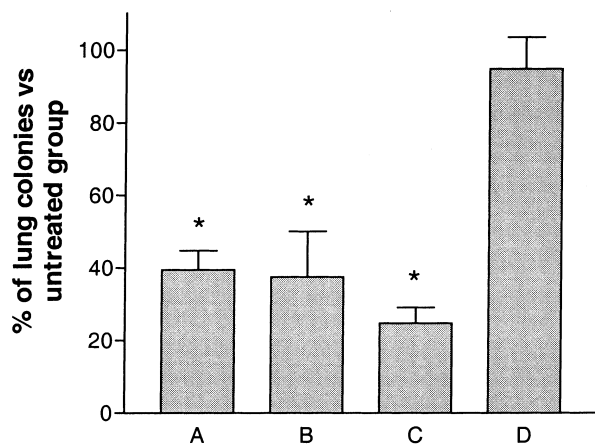


Figure 1 Superficial lung metastases (\pm s.d.) in the pericardiac lobe at 4 weeks after i.v. injection of 3×10^5 N202.1A tumor cells. Mice were inoculated i.p. with 20 μ g CpG-ODNs 4 h before (A) or 2 h (B) or 48 h (D) after tumor cell injection, or with 40 μ g CpG-ODNs 4 h before tumor injection and for the following 4 days (C). Untreated mice received tumor cells only. * $P < 0.05$ (unpaired *t*-test).

respectively). Moreover, at the end of the experiment, four of 11 mice were completely cured of spontaneous tumor development. Immunohistochemical analysis of mammary tissue glands from CpG-ODN-treated mice revealed foci of macrophage infiltration in small nodules of *in situ* carcinomas, and *in vitro* assay using splenic lymphocytes obtained from the treated mice revealed NK cell cytotoxic activation induced by CpG-ODN. Significant inhibition of tumor growth was also observed in mice bearing small spontaneous mammary tumors and injected with CpG-ODNs directly at the tumor site for 5 days (at day 50 from treatment, mean tumor volume was reduced by 36% as compared with controls). Moreover, significant inhibition of experimental lung metastases was observed in mice of the same strain inoculated i.v. with N202.1A mammary carcinoma cells and treated with CpG-ODNs, even at a single low dose, 4 h before or 2 h after tumor cell inoculation (61% and 63% reduction of lung tumor colony number respectively, vs controls) (Fig. 1).

Conclusions

Extremely encouraging results have been obtained in prevention and local treatment of murine mammary tumors. The findings raise the possibility of successful anti-tumor management through CpG-ODN stimulation of innate immunity in women at high risk of developing breast cancer and in breast cancer patients with reasonable immunological performance and low tumor load. Of course, several points must be considered before CpG-ODN treatment finds its way into the clinical setting. First, some effective immune adjuvants

that are well tolerated in mice are toxic in humans, and other adjuvants that are highly effective in rodents are much less effective in humans. With respect to toxicity, preliminary results from an initial CpG-ODN clinical trial (Heckelsmiller *et al.* 2002) suggest a low systemic toxicity, a finding supported by studies in primates (Heckelsmiller *et al.* 2002). Moreover, several antisense ODNs used in human clinical trials have contained CpG motifs and, to date, there are no reports of an association with autoimmunity and no evidence of anti-DNA antibody formation (Glover *et al.* 1997). Regarding the potential differing activity of adjuvants in human and rodents, studies have already indicated that ODNs, able to activate human immune cells, differ from the ODNs that activate the murine system (Krieg 2002), indicating the need to identify the appropriate CpG-ODNs for use in humans.

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