Novel immunotherapeutic approaches to fight metastatic breast cancer

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Nieuwe immuun therapeutische benaderingen voor het bestrijden van metastatische borst kanker

Thesis

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SCOPE OF THIS THESIS

Breast cancer is the second most common cancer in women. Breast cancer patients have a reasonable chance of becoming cured, particularly when they are diagnosed in an early phase. Eliminating primary tumors by surgery, chemotherapy, or radiation is quite successful. However, for metastases there is no cure. Thirty percent of the breast cancer cases will progress into metastatic disease, but they cannot be removed by surgery or radiation, and usually become chemoresistant. However, preclinical and clinical studies have shown that cancer vaccination has an effect on metastases but that a real breakthrough is hampered by the strong immune suppression in the tumor microenvironment (TME). Therefore, in this thesis we focused on the treatment of metastatic breast cancer using an attenuated *Listeria monocytogenes* (Listeria^{at})-based vaccine expressing tumor-associated antigen (TAA) Mage-b, combined with adjuvants such as Curcumin or alphagalactosylceramide (αGC), to reduce immune suppression in the TME in order to improve the vaccine efficacy of Listeria^{at}-Mage-b.

Chapter 1 gives an overall introduction on breast cancer, current therapeutic treatment options, cancer immunoediting, innate and adaptive immune responses against cancer, an overview of various cancer vaccines, and an introduction to the experimental work of this thesis.

Chapter 2 describes the improvement of Listeria^{at}-Mage-b vaccination by Curcumin in mice with metastatic breast cancer through the reduction of IL-6.

Chapter 3 describes the improvement of Listeria^{at}-Mage-b vaccination in mice with metastatic breast cancer by the incorporation of αGC into the Listeria^{at} cell wall.

Chapter 4 is a general discussion about the promises and problems of cancer vaccination, final conclusions about the vaccine studies performed, and future prospects of cancer vaccination.

CHAPTER 1

INTRODUCTION

Cancer

Cancer is the uncontrolled growth of abnormal cells in the body. Cancerous cells are also called malignant cells. They can develop in almost any organ or tissue, such as the lung, colon, breast, skin, bones, spleen, thymus, or in the central nervous system. Moreover, many of the cancers metastasize to other organs, depending on the type of tumors and the tumor microenvironment. Metastases are tumor cells that escape from the primary tumor, and travel via the blood stream to different organs where they can home and grow into new tumors. Breast-, lung-, kidney- and testical cancer have been described as those with the highest incidence of metastases, and 30% of the breast cancers progress into metastatic disease[1]. Numerous factors may contribute to the development of cancer including chemicals, environmental toxins, genetic problems, viruses, radiation, obesity, and excessive sunlight exposure, as well as drinking excess alcohol.

The Breast

Morphologically, the breast is a cone with the base at the chest wall, and the apex at the nipple, the center of the nipple-areola complex (NAC). The superficial tissue layer (superficial fascia) is separated from the skin by 0.5-2.5 cm of subcutaneous fat (adipose tissue). The suspensory Coopers's ligaments are fibrous-tissue prolongations that radiate from the superficial fascia to the skin envelope. The adult breast contains 14-18 irregular lactiferous lobes that converge to the nipple, to ducts 2.0-4.5 mm in diameter; the milk ducts (lactiferous ducts) are immediately surrounded with dense connective

tissue that functions as support frame work. The glandular tissue of the breast is biochemically supported with estrogen; thus, when a women reaches the menopause (cessation of menstruation) and her body estrogen levels decrease, the milk gland tissue then atrophies, withers, and disappears, resulting in a breast composed of adipose tissue, superficial fascia, suspensory ligaments, and the skin envelope. A schematic view of the breast tissue organization and mammary gland structure is shown in **Figs 1 and 2,** respectively.

Breast Cancer

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among females[2]. Most recent statistics estimated 230,480 new cases of the invasive breast cancer occurring among women during 2011 and about 2,140 new cases in men in the USA. For the year 2012, almost 39,970 deaths due to breast cancer are expected along with 226,870 new cases in the USA.

The most important risk factors that contribute to the development of breast cancer are family history (mutations in BRCA1/2 or p53 genes), prolonged exposures to endogenous or exogenous estrogens, alcohol, overweight, and physical inactivity (http://www.who.int/cancer/detection/breastcancer/en/index2.html). Other risk factors include dietary effects combined with late first childbirth, lower parity and shorter breastfeeding. Finally, the age factor is important, not only for breast but for most types of cancer.

Various types of breast cancer

Ninety five percent of all breast cancers are carcinomas, i.e. they arise from epithelial origin[3]. Breast cancer can be divided into two major types, i.e. non-invasive and invasive breast cancer.

Non-invasive breast cancer. The most prevalent type of non-invasive breast cancer is *ductal carcinoma in situ* (DCIS), and starts in the tubes (ducts) that move milk from the breast to the nipple. This type of cancer does not extend beyond the basement membrane and has a low potential for metastases. The second type of non-invasive carcinoma is *lobular carcinoma in situ* (LCIS) that arises and is confined to the milk producing glands or lobules[3]. The proportion of LCIS in benign breast disease is low, ranging between 0.5% and 4%[4], but it may progress to invasive cancer if untreated. Lobular carcinoma in situ (LCIS) is a marker for an increased risk of invasive cancer in the same or both breasts.

Invasive breast cancer. This is the type of cancer that spreads outside the basement membrane of the lobule or duct into the breast tissue. Invasive breast cancer will either remain localized to the breast or metastasize to other parts of the body, mostly lymph nodes, brain, bones, liver, or lungs and progresses into metastatic breast cancer. About 80% of all breast cancers are *invasive ductal carcinomas* (IDC), while 10–14% of all breast carcinomas are *invasive lobular carcinoma (ILC)*[5]. Furthermore, inflammatory breast carcinoma (IBC) is a form of rapidly progressing primary skin changes such as erythema, skin thickening, orange peel skin, and nipple retraction[6]. The unique appearance of IBC is due to tumor emboli that readily metastasizes into and block lymphatic vessels of the skin overlying the breast[7].

Molecular classification of breast cancer

Breast cancer has a heterogeneous phenotype. Tumors comprise of various distinct cell types with different biological features and clinical behavior. Therefore, breast cancer has been further classified based on different molecular techniques and genetic profiling into five major subtypes of breast cancers: basal-like, luminal A, luminal B, HER2+/ER–, and normal breast–like[8-10]. These five subtypes have distinct clinical outcomes. Luminal

A-type tumors have the best prognosis and are low-grade tumors[9], while luminal B type tumors are usually more aggressive demonstrating more proliferation and considered as high-grade tumors. Basal-like tumors usually have the worst prognosis. Most of the basal-like tumors poorly respond to therapy because they are often triple-negative (TN) for Estrogen Receptor (ER), progesterone receptor (PR), and HER2/neu. HER2 (human epidermal growth factor receptor) is overexpressed in 20-30% of the breast cancer cases. However, during HER2-targeted treatment its expression disappears, and therefore these patients are no longer susceptible to HER2-targeted therapies. About 20% of all breast cancers are TN, and TN breast cancer is particular increased in black women[11]. TN breast cancer is often associated with regional node metastases[12]. The molecular profiling of these subtypes of breast cancer helps to determine an optimal therapy regimen for the patients.

Treatment Options

Over the last decade, treatment of breast cancer has been dramatically improved. However, for metastatic breast cancer there is no cure. The treatment of choice depends on various factors such as the stage of cancer, the type of cancer, the genetic profile of the cancer (mutations in target genes), and whether the patient's tumor expresses ER, PR, and/or HER2/neu. The treatment options can be divided in local or systemic treatment. Local treatments involve the area of the disease only. Radiation and surgery are forms of local treatment. Systemic treatments affect the entire body. Chemotherapy is a type of systemic treatment. Most women receive a combination of treatments. For women with Stage I, II, and III breast cancer, the main goal is to treat the cancer and prevent it from returning (curing). For women with stage IV breast cancer, the goal is to improve the symptoms and help them to live longer. In most cases stage IV breast cancer cannot be cured. The various stages of breast cancer are defined as follow.

Stage I: This is the earliest stage of invasive breast cancer. At this stage, the cancer cells have spread beyond the original location and into the surrounding breast tissue. Stage II: This stage is a slightly more advanced form of stage I breast cancer. The cancer cells have spread beyond the original location and into the surrounding breast tissues, and the tumor is larger than in stage I diseases. However, stage II means the cancer has not spread to a distant part in the body. Stage III: This is a more advanced form of invasive breast cancer than stage II. The breast cancer cells have usually not spread to distant sites of the body, but they are present in several axillary (underarm) lymph nodes. The tumor may also be quite large at this stage, possibly extending to the chest wall or the skin of the breast. Stage IV: Stage IV means that the cancer has spread elsewhere in the body. The affected areas may include the bones, brain, lungs or liver, and more than one part of the body may be involved. For more detailed information see http://www.cancercenter.com/breast-cancer/breast-cancer-staging/stage-0-breast-cancer.cfm.

Local Treatment

Surgery. Surgery is advised for women with a localized breast tumor smaller than 4 cm. Surgery can be divided in lumpectomy, partial mastectomy, total mastectomy, or radical mastectomy and depends on the stage and type of breast cancer.

Lumpectomy involves removal of the tumor in the breast along with the negligible amount of surrounding tissue. Another version, *partial mastectomy* is more extensive and removes a larger amount of normal tissue surrounding the tumor. These two surgical procedures constitute 'breast conserving surgery' as removal of the complete breast is avoided, and is advised for patients with stage I or II breast cancer. Patients with a more advanced stage of breast cancer may undergo a *total mastectomy* or

complete removal of the breast and a sentinel lymph node (first node in the chain that forms the regional lymphatic system) biopsy. Sentinel lymph node biopsy may predict axillary node metastasis in breast cancer. If more advanced, tumor *radical mastectomy* is advised that includes removal of both breasts along with removal of lymph nodes in the armpits and chest. Surgery is the preferred mode of treating breast cancer when accompanied with radiotherapy or chemotherapy. For a review see Apantaku, 2002[13].

Radiation therapy. Surgical removal of a tumor is often followed by radiation therapy[13]. Clinical trials of breast conservation surgery alone or surgery plus radiation showed higher recurrence rate in women who did not receive radiation[14].

Systemic treatment

When compounds are administered through the blood vessels or orally, it is considered as systemic therapy. These compounds include chemotherapy, hormones, or more targeted therapies with small molecules, specific enzymes, or antibodies.

Chemotherapy. Chemotherapy is often advised for women with hormone receptor-negative invasive breast cancer. Although usually given through the blood stream or orally, it can also administered regionally in the area where the cancer cells are such as in the abdomen, cerebrospinal fluid, or an organ. To completely eliminate the primary tumors, standard chemotherapy regimen involves a combination of various chemotherapeutica. Most widely used combination therapies are Adriamycin (Doxyrubicin) and cyclophosphamate/Taxol, Adriamycin, and cyclophosphamide (AC/TAC), or cyclophosphamide, methotrexate, and fluorouracil/cyclophosphamde,

adriamycin and fluorouracil (CMF/CAF), or epirubicin and cyclophosphamide/cyclophosphamide, epirubicin and fluorouracil (EC/CEF), or taxotere and cyclophosphamide/taxotere, cycophosphamide and herceptin (TC/TCH). Complete recovery is usually between 60-100% of the patients receiving this combination therapies[15,16]

Hormone therapy. Hormones such as estrogens or receptors such as HER2/neu (epidermal growth factor receptor) are involved in the growth of breast cancer cells. Hormone therapy blocks the interaction between the hormone present in blood and their receptors on the tumor cells, thereby preventing the tumor cells from growing. For instance, tamoxifen is usually given to patients with early stage estrogen receptor-positive breast cancer. Another form of hormone-targeting therapy is aromatase inhibitors, that blocks the production of estrogen, and is often given to post-menopausal women with estrogen-dependent breast cancer[17].

Targeted treatment. Targeted therapy is a newer type of cancer treatment. This therapy uses special anti-cancer drugs that target certain changes in a cell that can lead to cancer. These therapies are directed against pathways specifically involved in the development of cancer such as proliferative signaling, escaping immune destruction, promoting invasion and metastases, and resisting cell death. Although initial results of clinical trials with therapies targeting HER2 in metastatic breast cancer using Trastuzumab combined with chemotherapy, were highly promising, the majority of these patients become resistant[18]. Clinical trials with HER2/1 inhibitors such as Perutuzumab, and with HER2/3 or HER1/2/4 inhibitors such as Neratinib, in combination with

Trastuzumab and chemotherapy, are under way[19,20]. Lapanitib, a HER1/2 inhibitor, is more promising when combined with Trastuzumab, with less severe side effects than most conventional therapies[21]. While all these therapies are promising in patients that express the HER1/2/3/4, triple-negative patients with tumors and metastases lacking expression of receptors for estrogen, progesterone, and HER2, have the poorest prognosis because they will not responding to these therapies, and other types of therapies are under investigation but with moderate success. One such therapy is an anti-angiogenic therapy, which appeared to have less effect on overall survival than expected and data explaining this lower efficacy are not available yet. So far, the most successful anti-angiogenic therapy with tyrosine kinase inhibitor Sunitinib, targeting the vascular endothelial growth factor (VEGF) receptor, platelet-derived growth factor, c-kit, and Flt2, induce serious side effects[22], while Bevacizumab, a human antibody to VGEF, prolonged progression-free survival, but not the overall survival time. Another type of therapy is Poly (ADP-ribose) polymerase-1 (PARP) inhibitors inducing DNA repair defects in tumors but not in normal cells. Such therapy has potential[23] but is effective in BRCA1/2-mutants patients only, which makes up 0.1-0.8% of the general population[24]. The most promising targeted therapies are shown in **Fig 3.** In summary, little progress has been made over the last 25 years in the development of effective therapies against metastatic breast cancer. This underlines the urgent need for new alternative therapeutic approaches. One such alternative therapy could be cancer immunotherapy or vaccination.

Immune surveillance

In 1909, Paul Ehrlich predicted that the immune system repressed the growth of carcinomas that would otherwise occur with great frequency (Ehrlich P. Ned Tijdschr

Geneesk 5: 273-290, 1909). This statement initiated a contentious debate over immunologic control of neoplasia. Fifty years later, Burnet stated that tumor cell-specific neo-antigens could provoke an effective immunologic reaction that would eliminate developing cancers[25-27], while Thomas stated that complex long-lived organisms must posses mechanisms to protect against neoplastic disease similar to those mediating homegraft rejection (Thomas L. Discussions in cellular and humoral aspects of the hypersensitivity states, 1959). With the functional demonstration of tumor-specific antigens in the mouse, supporting the ideas of Ehrlich, Thomas, and Burnet, the immunosurveillance theory developed, which stated that sentinel thymus-dependent cells of the body constantly surveyed host tissues for nascently transformed cells. However, there has been a growing recognition that immune surveillance represents only one dimension of the complex relationship between the immune system and cancer[28]. Recent work has shown that the immune system not only protects against the development of primary non-viral cancers but also sculpts tumor immunogenicity, which developed into a new hypothesis of "cancer immunoediting". Cancer immunoediting is a process that consists of three phases, i.e. elimination (cancer immunosurveillance), equilibrium, and escape[29].

Elimination of cancer cells (cancer immunosurveillance)

Various studies have shown that deficiencies in key immunological molecules such as IFNγ and perforin enhanced hosts susceptibility to both chemically induced and spontaneous tumors, demonstrating the cancer immunosurveillance hypothesis[30-35]. Compelling data suggest that the immunosurveillance is not restricted to mouse models but also exists in humans[28,29]. These studies raised the question as to how cancer immunosurveillance functions as an extrinsic tumor suppressor and protects the immune

competent host from developing neoplasia. For instance, which cells of the immune system are involved in the protection tumor of development, what are the key molecules of the immune system in cancer immunosurveillance, and how does the immune system distinguish between a transformed cells and its normal progenitor?

Cancer immune surveillance by adaptive and innate immune cells. When tumor cells develop (by mutation of genes induced through environmental influences or hereditary), hormones as discussed at the beginning of this thesis), proteins on their membrane change which leads to their elimination by the innate and adaptive immune system during the phase of immunosurveillance. The innate immune system reacts in the early phase to danger signals and does not involve specific antigen recognition while the adaptive immune system reacts to specific antigens such tumor-associated antigens (TAA). NK cells, NKT cells, $\gamma\delta$T cells, tumor-associated macrophages (TAM), and myeloid-derived cells such as immature granulocytes and monocytes, also called myeloid-derived suppressor cells (MDSC), belong to the innate immune system. MDSC are particularly important in cancer and will be discussed later in this thesis. CD4 and CD8 T cells, as well as B cells, belong to the adaptive immune system. Below the role of these different cell types in anti-tumor reactions has been discussed. For a review see Dunn et al, 2004[29]. A schematic view of immune cells of the innate and adaptive immune system against cancer is shown **Fig 4.**

T cells. Cytotoxic T lymphocytes (CTL) are considered to be the most important players of the adaptive immune system in anti-tumor reactions. CTL are $CD3^+CD8^+$ and express the $\alpha\beta$ T cell receptor (TCR) that recognizes tumor-associated antigens (TAA) in

association with major histocompatibility complex (MHC) molecules on the tumor cells. As has become evident from *in vitro* studies, these CTL are activated when exposed simultaneously to both TAA/self-MHC complexes and co-stimulatory molecules, resulting in CTL-mediated tumor cell cytolysis. However, anti-tumor responses can also be inhibited by co-inhibitory molecules. An overview of co-stimulatory and inhibitory molecules is shown in **Fig 5AB.** Key components of CTL in killing tumor cells are IFNγ and perforins[30,35]. CD4 T cells, also called "helper T cells", recognize TAA through MHC class II molecules, and upon activation they produce large amounts of interleukin (IL)-2, which is a growth factor and activator of CD8 T cells. CD8 T cell responses to TAA will be central in this thesis and will be later discussed in more detail. In contrast to CD4 helper T cells, also regulatory CD4 T cells (T_{reg}) are involved in anti-tumor responses. However, instead of helping the CD8 T cells, they inhibit the responses of CD8 T cells[36]. T_{reg} are characterized by the expression of CD4, CD25 and Foxp3. It has been reported that T_{regs} can act antigen-dependent and -independent [36,37]

NK cells. NK cells are $TCR^-CD3^-CD4^-CD8^-CD11b^+CD16^+(CD56^+)$ and they participate in cancer immunosurveillance. They express NKG2D-activating receptors that react with ligands often over expressed on tumors such as MICA/B, and Rae-1, H60, or MULT-1[38-40], and become cytolytic in the presence of IFNγ, IL-2, TNFα[29]. In contrast to CTL, their responses are TAA-independent and they can kill tumor cells with low or no MHC expression. NK cells can also kill tumor cells through antibody-dependent cellular cytotoxicity through their Fc receptors that can interact with antibodies that recognize antigens on tumor cells. Mice depleted for NK cells were three times more sensitive for methylcholantrene (MCA)-induced cancers than wild type mice[41], demonstrating its role

in anti-tumor responses. Also in this thesis we will show that NK cells can be activated in mice with metastatic breast cancer.

NKT cells. Natural killer T (NKT) cells also participate in cancer immunosurveillance, but react as first line of defense. The most widely studied group of NKT cells expresses the invariant type of the TCR (Vα14Jα18/Vβ2, 7, or 8 in the mouse and Vα24Jα18/Vβ11 in humans)[42], CD3, CD56, NK1.1 and produce granzyme B[43]. NKT cells recognize glycolipids, presented by a MHC-like molecule CD1d[44]. They are particularly interesting because when activated they form the bridging link between the innate and adaptive immune system upon activation. This occurs through the production of a whole array of cytokines (proteins secreted by cells that regulate the immune system) including IFNγ, IL-12, IL-4, and IL-13, that can activate other cells of the innate and adaptive immune system against cancer[45]. Like NK cell depletion, it has been shown that mice depleted of NKT cells were three times more susceptible to MCA-induced cancers than wild type mice[46]. In this thesis we will show that NKT cells stimulated with a synthetic glycolipid alphagalactosylceramice (αGC), produce cytokines in correlation with improved TAA-specific T cell and NK cell responses in mice with metastatic breast cancer.

$\gamma\delta$T cells. $\gamma\delta$T cells are $CD3^+TCR\gamma^+\delta^+$, $CD8^+$ and react as a first line defense like NK and NKT cells. Upon activation they produce large amounts of IFNγ[29].

MDSC. Myeloid-derived suppressor cells (MDSC) play an important role in cancer, and particularly in metastatic cancer. They are immature myeloid cells that differentiate into

mature granulocytes, macrophages, and DC. In cancer patients, MDSC migrate from the bone marrow via the blood stream to the primary tumor. These primary tumors block their differentiation and activate these immature cells to produce immune suppressive factors such as arginase I, inducible nitric oxide synthetase (iNOS), and reactive oxygen species (ROS). Cytokines such as IL-6, IL-10, and TGFβ are also produced by MDSC, which are able to down regulate antigen-specific and non-specific T cell responses in the TME[47,48]. In mice, MDSC express both the myeloid lineage differentiation antigen Gr1 (Ly6C and Ly6G) and the α_M integrin CD11b. Two major groups of MDSC have been described: $CD11b^{+}Gr1^{high}$ ($CD11b^{+}Ly6G^{+}Ly6C^{low}$) with a granulocytic phenotype (gMDSC), and $CD11b^{+}Gr1^{low}$ ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) with a monocytic phenotype (mMDSC)[49,50]. Both mMDSC and gMDSC are immunosuppressive but may have different functions at the tumor site.

TAM. Tumor-associated macrophages (TAM) play an important role in the TME. They express M2-associated pro-tumoral functions, such as angiogenesis, matrix remodeling and suppression of the adaptive immune system[51]. TAM produce chemokines (a family of small cytokines or proteins) such as CCL2, M-CSF, and VGEF involved in the recruitment of monocytes, and differentiation into TAM, as well as CSF, IL-4, IL-10, and TGFβ[52]. These latter two cytokines are involved in the suppression of Th1 responses and naïve T cell responses through the recruitment of T_{regs}[52]. TAM are also involved in the development of angiogenesis through the production of VGEF, PDGF, TGFβ and members of the FGF family[53-55].

Key molecules of the immune system in cancer immunosurveillance. IFNγ produced by T cells, NK cells, NKT cells, γδT cells, and macrophages are relevant effector molecules in cancer immunosurveillance. For instance, endogenous production of IFNγ protected against transplanted, chemically induced and spontaneous tumors[30-32,35]. Also blocking the IFNγ receptor IFNGR1 with Abs resulted in faster tumor growth or enhanced the sensitivity for developing MCA-induced tumors with 10-120 times[56]. As mentioned above γδT cells are physiologically a relevant source of IFNγ in the cancer immunosurveillance process. IFNγ activates the innate and adaptive anti-tumor immune responses[56]. For instance, IFNγ promotes the generation of tumor-specific CD4+ Th1 T cells and CTL and activates cytocidal activity in macrophages. Moreover, IFNγ inhances the immunogenicity of tumors through the upregulation of MHC class I molecules[31].

The other important key molecule in cancer immunosurveillance is perforin. Perforin is a lytic enzyme, produced by CD8 T cells and NK cells, which when it comes in contact with tumor cells perforates the cell membrane resulting in tumor cell death[57]. Granzyme, a family of structurally related serine proteases, is secreted by NKT cells through exocytosis, and induces apoptosis of target cells[57].

Also IL-12 is important in cancer immunosurveillance. IL-12 is a heterodimer cytokine composed of two subunits, α-chain and a β-chain, both covalently linked by a disulfide bridge, and coded as IL-12p35 and IL-12 p40, respectively[58]. IL-12 is primarily produced by activated macrophages, monocytes, neutrophils, and DC[59], and strongly activates CTL and NK[60]. IL-12 activates naïve and mature T cells[61,62]. IL-12 has great anti-tumor activity, particularly against metastases[63,64]. IL-12 also possesses anti-angiogenic

activities[65-67]. Anti-tumor effects of IL-12 have been found in various mouse tumor models such as B16 melanoma, CT26 colon carcinoma, Renca renal-cell carcinoma, TSA mammary carcinoma, SCK mammary carcinoma, and many others[65,68]. While recombinant IL-12 is toxic[65,69], IL-12 produced by the immune system itself through stimulation has no side effects, as we found in our breast cancer model (see chapters 2 and 3).

Equilibrium

Originally, cancer immunosurveillance was considered to protect the host from cancer[27], by eliminating tumor cells at least at the earliest stage of cancer. This is correct for immunogenic tumor cells at a stage that immune suppression has not been induced yet. However, there is an equilibrium phase between the tumor cells and the immune system. Tumor cells may become non-immunogenic by loosing TAA expression through genetic instability[70], a well known problem in cancer, or by cytokine production such as TGFβ which reduces MHC class I expression levels on tumor cells[71]. The tumor cells that remain start producing cytokines or factors such as GM-CSF or S100[72,73] or other factors that recruit MDSC, or cytokines such as IL-10 that induce differentiation of blood monocytes into tumor-associated macrophages (TAM), or M2 macrophages[74,75]. These immune cells starts suppressing the adaptive and innate immune system, and at this point not only the non-immunogenic will enter the escape phase, but also immunogenic tumor cells since MDSC, TAM and M2 macrophages prevent the immune system from eliminating tumor cells.

Escape of tumor cells

Once in the escape phase, there is a strong interaction between tumor cells and MDSC, TAM and M2 macrophages, resulting in the production of high levels of cytokines and factors that suppresses the immune system such as IL-6, IL-10, TGFβ, iNos, arginase[51,72,76,77], and finally leading to escape of the tumor cells from the immune system. All those cells that normally can eliminate the tumor cells directly or indirectly such as CD8 and CD4 T cells, as well as NK cells, NKT cells, γδT cells are now functionally inhibited. This is the moment that tumors develop, and in a later phase depending on the tumor microenvironment and the type of tumor, metastases will develop. For a schematic view of the elimination of tumor cells, equilibrium and escape of tumor cells see **Fig 6.** For a review about the immunoediting theory see Dunn et al, 2004[29]

Cancer vaccination

While immunosurveillance is able to eliminate tumor cells in an early phase, there is a moment as discussed above that the immune system fails. This is the moment were vaccines or immunotherapy can help the immune system to eliminate the tumor cells by reducing immune suppression, by the generation of cytokines that can stimulate the immune system, and by the delivery of TAA molecules at large numbers into antigen-presenting cells in vivo, which is the central theme of this thesis. While standard therapies such as chemotherapy, radiation, surgery or targeted therapies fail to eliminate metastases, cancer vaccination may be the best and most benign option for preventing or curing metastatic cancer.

Various cancer vaccines

Developing bacterial or viral vaccines is different from developing cancer vaccines. Bacteria or viruses express membrane antigens that are foreign to the human immune system, and are therefore able to activate the immune system against these antigens. Tumors do not express foreign membrane antigens in the same way as bacteria or viruses because tumors are derived from normal human cells, expressing primarily the same antigens as normal cells do. However, several decades of research into the presence of tumor-specific antigens have shown that many tumors express antigens that are not expressed in normal adult tissues except testis and placenta. Examples are MAGE, GAGE, BAGE, LAGE, NY-ESO-1[78-81]; overexpress antigens that are present at low levels in normal tissues, such as CEA, HER/neu-2, MUC1[82-84]; or show altered antigen expression by mutation in cellular genes, such as MUM1, cdk4, ß-catenin[85-87]. These TAA are able to activate specific T cells if optimally presented (see below), and are therefore suitable targets for the development of cancer vaccines. For a schematic overview of more various TAA see **Table 1**.

The first attempts to develop cancer vaccines on the basis of irradiated tumor cells were unsuccessful[88]. Tumor cells are poor antigen-presenting cells (APCs) due to the low expression of MHC and co-stimulatory molecules and poor processing of antigens and presentation of TAA on the membrane. Over the last decade, new generations of TAA-based cancer vaccines have become available that are much more powerful in activation of the immune system than irradiated tumor cells. This new generation of vaccines is able to activate different T cells depending on processing of exogenous or endogenous proteins produced by the vaccine, and subsequent presentation of the antigen (TAA peptides) by APC to the immune system. Endogenous proteins, for instance delivered

into the cytoplasm of an APC by a DNA vaccine, are processed by cytoplasmic enzymes resulting in small peptides, then transported to the endoplasmic reticulum, where peptides can associate with newly synthesized MHC class I molecules. These peptide/MHC class I complexes migrate to the membrane of the APC for presentation to the immune system and for subsequent activation of naive CTL. Exogenous proteins, for instance from purified protein or conjugate vaccines, are internalized by APC via endocytosis to an endosomal compartment, where they are digested into peptides and associated with MHC class II molecules. These peptide/MHC class II complexes migrate to the membrane of the APC for presentation to the immune system and for subsequent activation of naïve T helper cells. However, exogenously produced proteins can also be taken up by APC and then presented in the context of MHC class I molecules (epitope spreading)(for a review see Cohen et al., 1998)[89]. Below, several cancer vaccines will be discussed that are particularly powerful in the induction of CTL responses.

Peptide-based vaccines

Use of peptide-based vaccines is an approach to initiate TAA-specific CTL responses. Such vaccines obviate the need to digest proteins into peptides, a process that is often impaired in tumor cells. Peptide-based vaccines consist of dendritic cells (DC) (isolated from the cancer patients themselves) loaded with synthetic peptides derived from TAA that are expressed, but inadequately presented by the tumor. These peptides assemble with MHC molecules that are highly expressed at the cell membrane of DC. Injection of these peptide-loaded DC into cancer patients leads to presentation of TAA-peptide/self-MHC complex to the immune system, activating TAA-specific CTL, resulting in the destruction of TAA-expressing tumor cells. Clinical trials in patients with melanomas, prostate cancer, B-lymphomas or multiple myelomas using DC/peptide vaccines have been promising[90]. However, a major disadvantage of their use is that the production

procedures are difficult, expensive and time-consuming. Indeed, the DC need to be isolated from the cancer patient, expanded *in vitro*, then loaded with peptide and then re-injected into the patient, all under sterile conditions. It is difficult to obtain sufficient viable DC with this approach. To circumvent these difficulties, several clinical trials in patients with melanomas, AIDS, breast, ovarian, or colorectal carcinomas, have been performed with some success using TAA-peptides without DC but in the presence of cytokines such as IL-2 and GM-CSF (for a review see Gravekamp)[91]. It is difficult to load DC with peptides *in vivo*, because the injected peptides need to compete with existing peptides associated with the MHC molecules at the membrane of DC.

Tumor cell-dendritic cell hybrid vaccines

Generation of hybrids between autologous tumor cells and allogeneic (monocyte-derived) DC, presenting antigens expressed by the tumor in concert with co-stimulating capacities of DC is another approach to activate TAA-specific CTL[92]. An advantage is that unknown tumor antigens are included in this type of vaccine. Caution must be taken to avoid activating autoimmunity against normal cells[93,94]. These hybrids demonstrated the induction of MHC class I-restricted CTL reactive with MUC1 TAA. A human clinical vaccine trial with this tumor cell-dendritic cell hybrid vaccine showed promising results in metastatic breast cancer (for a review see Gravekamp)[91], but the procedures are as difficult as with peptide-based vaccines.

Delivery through DNA vectors

Use of DNA vaccines allows activation of TAA-specific CTL. Like the above described DC-based vaccines, DNA-based vaccines circumvent the poor APC function of the tumor cells since the antigens delivered by the DNA vaccines will be presented by professional APC that do express high levels of MHC and co-stimulatory molecules. A

conventional DNA vaccine is a bacterial plasmid (for instance pCDNA3.1) containing an eukaryotic promoter (required for transcription), a Kozak sequence (required for translation) and the gene of interest, followed by a polyadenylation signal (to prevent degradation of mRNA). The gene of interest can be any DNA sequence that may activate tumor-specific T cell responses.

Intramuscular or epidermal immunization with a DNA vaccine leads to DNA uptake into APC such as bone marrow-derived DC, macrophages, or Langerhans cells[95]. CpG rich motifs (high frequency of unmethylated CG sequences) present in bacterial DNA, enable stimulation and maturation of APC[96]. APC express pattern recognition receptors (PRR) that bind CpG rich motifs[97]. It might be speculated that interaction between CpG motifs and PRR may lead to internalization of the DNA vaccine like internalization of proteins, but unlike proteins might escape degradation. Circular DNA is less prone to degradation than linear DNA. In addition, it has been shown that cell types other than APC are involved in DNA uptake as well[89]. For instance, intramuscular injection of a DNA vaccine leads to the uptake and expression of the DNA vaccine in myocytes. However, myocytes do not function as APC, because they lack the important co-stimulatory molecules required for priming of naïve T cells. Epidermal DNA immunization allows the uptake of the DNA vaccine in keratinocytes. Keratinocytes, like myocytes, do not function as APC. It has been suggested that myocytes and keratinocytes deliver soluble antigen (exogenous) to APC, resulting in antigen presentation in context with MHC Class I or II molecules, resulting in activation of naïve CTL, or T helper cells, respectively. Cutaneous bombardment with DNA, using the gene gun is different from epidermal or intramuscular immunization[98]. It results in direct delivery (physically) of the DNA into the cytoplasm of Langerhans cells. These DC migrate to regional lymph nodes in order to present antigens delivered by the DNA vaccine to naïve CTL.

mRNA-based vaccines

mRNA-based vaccines can code for all types of proteins, are easy and cost efficient to produce, have a favorable safety profile and enables the induction of combined immune responses[99]. Over the last few years major developments have been made in the field. Clinical approaches use mRNA either for direct administration or for engineering of adoptive transferred dendritic cells. However, there are still challenges to overcome such as the short half-life of extracellular mRNA, and relatively weak target specific immune responses[100,101].

Delivery through live attenuated viruses or bacteria

An efficient delivery of TAA in vivo is a key issue for developing strong immune responses against cancer. In this regard, live vaccination strategies including various attenuated live bacterial and viral vectors have attracted great attention. Several attenuated bacterial strains such as *Salmonella, Lactococcus lactis*, *and Listeria monocytogenes* have been tested for the delivery of antigens in vivo[102-105]. Also viruses as vaccine vectors have been used such as Vaccinia, Adenovirus, Herpes Simplex virus, Paramyxovirus, and Retroviruses[102,106,107]. Even non-pathogenic variants of parasites such as Leishmania tarentolae, Toxoplasma gondii, and Trypanosoma cruzi has been used for gene delivery in vivo[108,109]. These bacteria, viruses and parasites have great potential to deliver target genes in vivo. However, safety aspects are still an issue, particularly for viruses and much less for bacteria and parasites. An overview of the different cancer vaccines in human clinical trials is shown in **Table 2.**

Listeria monocytogenes

In our laboratory, we used a highly attenuated *Listeria monocytogenes* (Listeria^{at}), for the delivery of TAA into APC. This Listeria^{at} was originally developed in the laboratory of Dr.

Yvonne Paterson, University of Pennsylvania, PA. She attenuated the Listeria bacteria to reduce the pathogenicity as described below. Listeria^{at} consists of Listeria plasmid pGG34 and a Listeria background strain XFL-7. The Listeria plasmid pGG34, expresses the positive regulatory factor (prfA) and Listeriolysin O (LLO)[110]. prfA regulates the expression of other virulence genes, and is required for survival in vivo and in vitro[110]. The coding region for the C-terminal part of the LLO (cytolytic domain that binds cholesterol in the membranes) protein in the plasmid has been deleted, but Listeria^{at} is still able to escape the host vacuole[111]. Mutations have been introduced into the prfA gene and the remaining LLO (expressed by the pGG34 vector), which further reduced the pathogenicity of Listeria^{at} [111].

Initially, we used the Listeria^{at} because it naturally infects professional APC (monocytes), and targets antigen delivery to both the class I MHC pathway of endogenous antigen presentation and the class II MHC pathway of exogenous antigen presentation. This DNA delivery system (containing the same antigen as expressed by the tumors) successfully protected mice from renal or colorectal tumors[112]. Advantages of Listeria^{at} are the higher efficiency of DNA uptake into APC and subsequent processing and antigen presentation compared to the conventional DNA immunizations described above, and the possibility of oral administration. We found that preventive vaccinations with Listeria^{at} expressing TAA Mage-b protected mice from metastatic breast cancer[113].

Later our lab discovered that Listeria^{at} also infects tumor cells in vitro and in vivo, and kills tumor cells through the generation of high levels of reactive oxygen species (ROS) without side effects[114]. This was possible because Listeria^{at} survives and multiplies in the tumor microenvironment because of the strong immune suppression[114], but is efficiently cleared in normal tissues that lack immune suppressions by

macrophages, NK cells and T cells[115,116]. These various pathways of Listeria^{at} are highly attractive for the development of cancer therapies.

Most dramatically were the semi-therapeutic immunizations (one before and two after tumor development) with Listeria^{at} expressing TAA Mage-b[114]. This resulted in the complete elimination of all metastases and the primary tumor was reduced by 95%. This high efficacy of Listeria^{at}-Mage-b was obtained by the generation of excellent T cell and NK cell responses, and the direct kill of tumor cells by Listeria^{at}-induced ROS. The strong T cell and NK cell responses, even after tumor development, could be induced because the first immunization was administered before tumor development in the absence of immune suppression. However, exclusive therapeutic immunizations with Listeria^{at}-Mage-b were much less effective due to the strong immune suppression in the tumor microenvironment[114]. Cancer vaccination is highly promising, particularly against metastases, but a real breakthrough is hampered by the strong immune suppression in the tumor microenvironment. Therefore, reduction of immune suppression is of great value for the development of effective vaccines against cancer. This thesis is about the improvement of vaccine efficacy by the development of combination therapies with Listeria^{at}-Mage-b and curcumin or alphagalactosylceramide (αGC) against metastatic breast cancer. Both compounds reduce immune suppression and/or convert immune suppression into immune stimulation in tumor-bearing mice.

Curcumin

Curcumin (diferulolylmethane), a polyphenol derived from the plant *Curcunina longa*, commonly called turmeric, has a broad anti-cancer effect through down regulating transcription factor NFkB thereby affecting down stream genes such as c-myc, Bcl-2, COX-2, NOS, Cyclin D1, TNFα and MMP9 [40]. Various studies have shown that curcumin kills breast tumor cells in vitro[117-121]. Also it has been shown that curcumin improves

therapeutic efficacy of doxorubicin or of B16-R lysate against B16-R melanoma in mice[122], and that curcumin prevents tumor-induced T cell apoptosis in mice[123]. Many clinical trials with curcumin are ongoing or have been completed **(Table 3).** Curcumin is also known for reducing immune suppressive cytokines such as IL-6 through the NFkB pathway[124]. IL-6 is strongly produced in mice and human with metastatic breast cancer, particularly in TNBC[125,126]. IL-6 is a potent regulator of dendritic cell (DC) differentiation in vivo[127], and is able to turn on the expression of signal transducer and activator of transcription (STAT)3 in DC. However, high levels of STAT3 can prevent DC from maturation[127] and subsequent presentation of antigens[128]. This in turn may lead to T cell unresponsiveness. In a previous study we found evidence that IL-6 strongly inhibited T cell responses in the TME and that anti-IL-6 antibodies restored the T cell responses[113]. We have also shown that IL-6 strongly inhibited T cell responses to Mage-b and that elimination of IL-6 with anti-IL-6 antibodies restored T cell responses to Mage-b[113]. Based on these results, we hypothesized that curcumin could improve the vaccine efficacy of Listeria^{at}-Mage-b by improving T cell responses through the reduced IL-6 production. This hypothesis has been tested in Chapter 2.

α-Galactosylceramide (αGC)

αGC is a glycolipid and represents one potentially useful class of adjuvants that have shown promise in preclinical studies for immunotherapy of cancers[129]. These glycolipids mediate their effects on the immune system by binding to an MHC class I-like molecule called CD1d, creating a complex that is recognized by a population of conserved effector lymphocytes known as natural killer T cells (NKT cells)[130]. Several subsets of NKT cells have been defined, with the most abundant being the so-called type 1 or invariant NKT cell (iNKT) subset which is highly responsive to αGC and highly conserved between

primates and mice[44,131]. It has been shown that formation of complexes of CD1d with αGC intracellularly in antigen presenting cells initiates rapid NKT cell activation[132]. This cascade of immune reactions that is initiated by NKT cells in response to αGC has been shown in mouse models to generate innate and adaptive immunity against a wide range of cancers and infections[132-136]. Based on these observations we hypothesized that addition of αGC to the LM-Mb vaccine could improve the vaccine efficacy, in part through enhancement of specific T cell responses to Mage-b. This hypothesis has been tested in Chapter 3.

Introduction to the experimental work

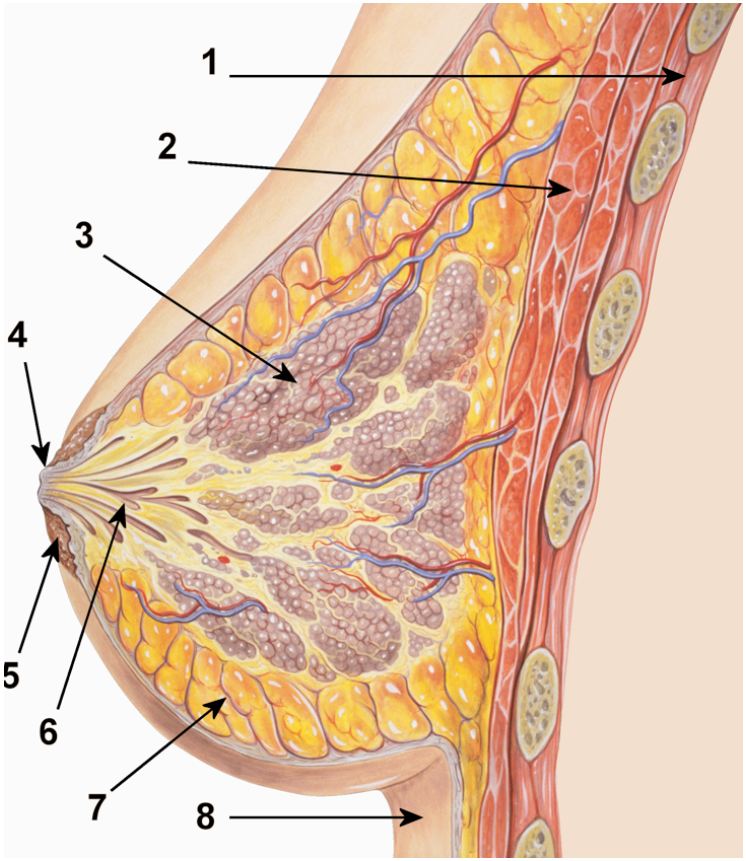
Cancer vaccination is particularly promising to combat metastatic cancer. However, a real breakthrough is hampered by immune suppression in the TME. Therefore, the main goal of this thesis project is focused on the reduction or conversion of immune suppression in the TME. To reach this goal we have used two different approaches. The first approach involved reduction of immune suppressive cytokine IL-6. IL-6 strongly inhibits T cell responses in the TME, is a growth promoter for tumor cells, and contributes to the development of metastases. IL-6 is produced by at least 50% of all breast cancers (particularly stem-like breast cancer cells)[137], and various immune cells in the TME (this thesis). We hypothesized that agents that reduce IL-6 will improve the efficacy of the vaccination through improved T cell responses. To test this hypothesis we developed a combination therapy of Listeria^{at}-Mage-b and Curcumin in a metastatic breast cancer model 4T1, in collaboration with Bharat Aggarwal, MD Anderson, Houston, Texas. This study has been described in Chapter 2.

The second approach involved the conversion of immune suppression into immune stimulation by generation of a cascade of immune-stimulating cytokines,

through the activation of NKT cells with αGC. We hypothesized that the generation of immune-stimulating cytokines by αGC, will improve the efficacy of Listeria^{at}-Mage-b vaccinations through improved T cell responses to Mage-b. This hypothesis was tested, in collaboration with Dr. Steven Porcelli of Microbiology and Immunology of Albert Einstein College of Medicine, in chapter 3.

In summary, the various approaches to eliminate or convert immune suppression are of crucial importance for successful cancer vaccination. Another important issue was to develop non-toxic combination therapies. We have shown earlier that Listeria^{at}-Mage-b is non-pathogenic and is naturally cleared by the immune system within 3-5 days[114], and curcumin and αGC are both non-toxic compounds. Our ultimate goal is to apply our combination therapies in human clinical trials, with higher efficacy but lower side effects compared to current treatment regimens of metastatic breast cancer.

Figure 1: Schematic view of breast tissue organization



1. **Chest wall**
2. **Pectoralis muscle**
3. **Lobules**
4. **Nipple**
5. **Areola**
6. **Milk duct**
7. **Fatty tissue**
8. **Skin**

Source: http://www.who.int/cancer/detection/breastcancer/en/index2.html

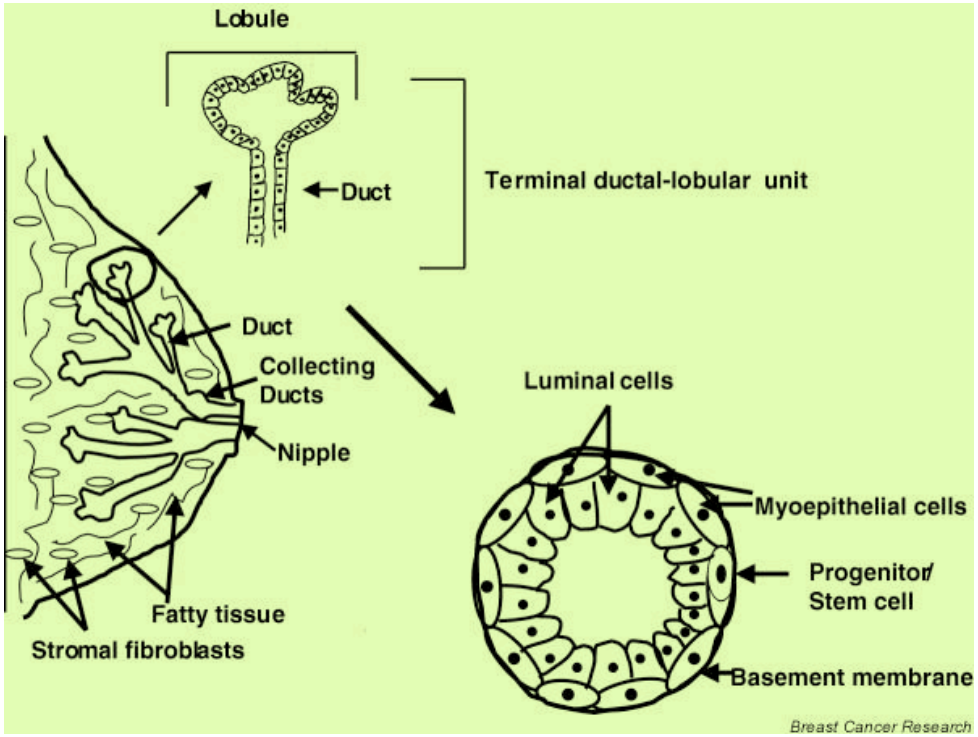


Figure 2: Structure of the mammary gland. The mammary gland consists of a branching ductal system that ends in terminal ducts with their associated acinar structures, termed the terminal ductal-lobular units (TDLUs), together with interlobular fat and fibrous tissue. Terminal ductal–lobular unit (TDLU), composed of ductal cells, is the unit thought to be the origin of most breast cancers. The stroma is composed of fatty tissue (adipocytes) and fibroblasts. Also shown are the two primary types of cells in normal ducts: outer contractile myoepithelial and inner columnar luminal cells. A putative progenitor/stem cell is also indicated.
Source: Dimri et al. Breast Cancer Research 2005 ***7****:171 doi:10.1186/bcr1275*

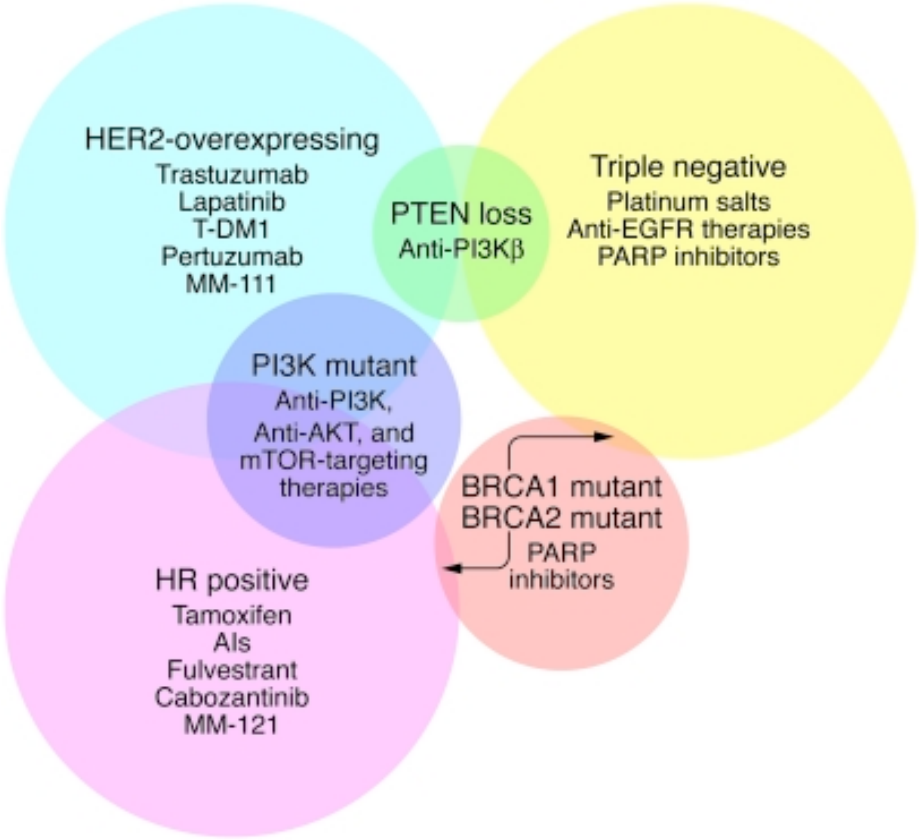


Figure 3: Venn diagram of breast cancer subtypes and their overlapping molecular targets. This figure shows some of the most promising targeted agents in development and discuss considerations for the optimal design of clinical trials of targeted therapies in breast cancer. The sub-classification nomenclature currently used in the clinic.
Source: Higgins and Baselga, 2011, J Clin Inv 121: 3797-3803[199]

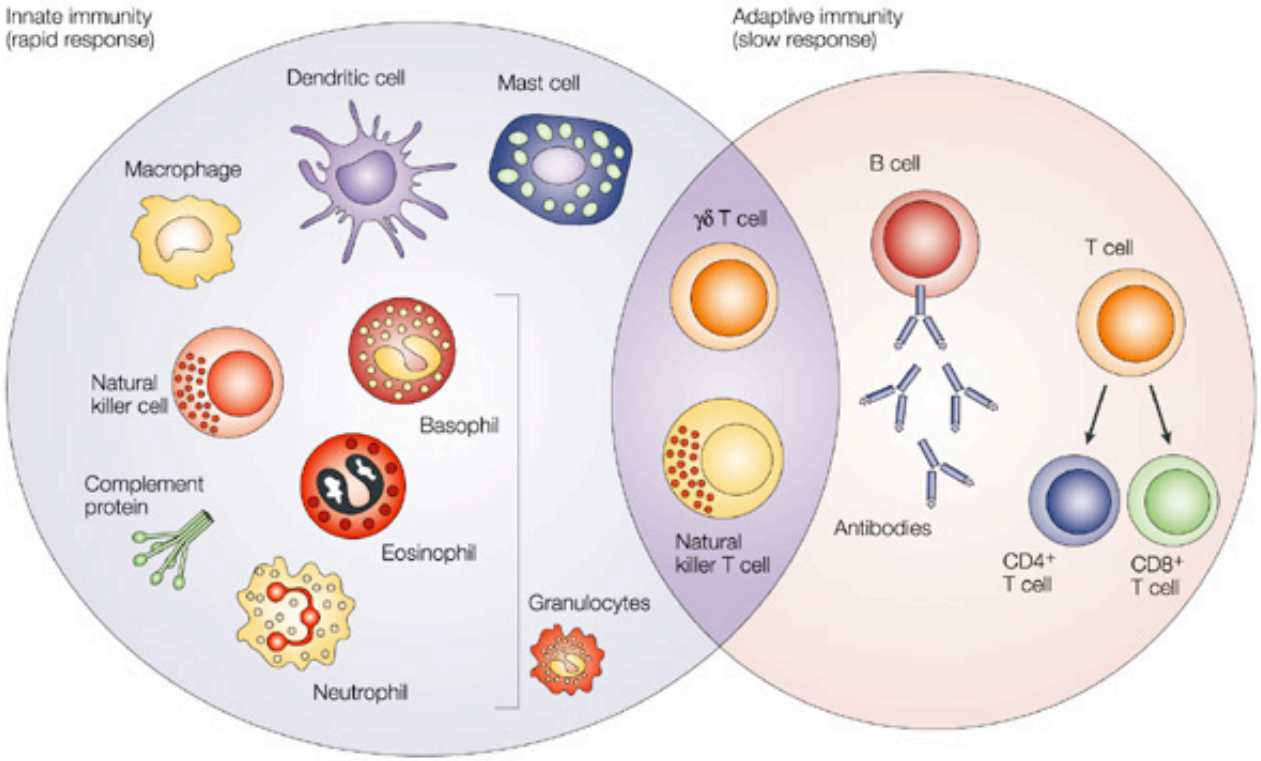


Figure 4: Immune recognition of tumours. The immune system can be broadly divided into innate and adaptive components, with extensive crosstalk between the two. The **innate immune** response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The **adaptive immune** response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδT cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.
Source: Dranoff. Cytokines in cancer pathogenesis and cancer therapy, Nature Reviews Cancer 4: 11-22, 2004[200]

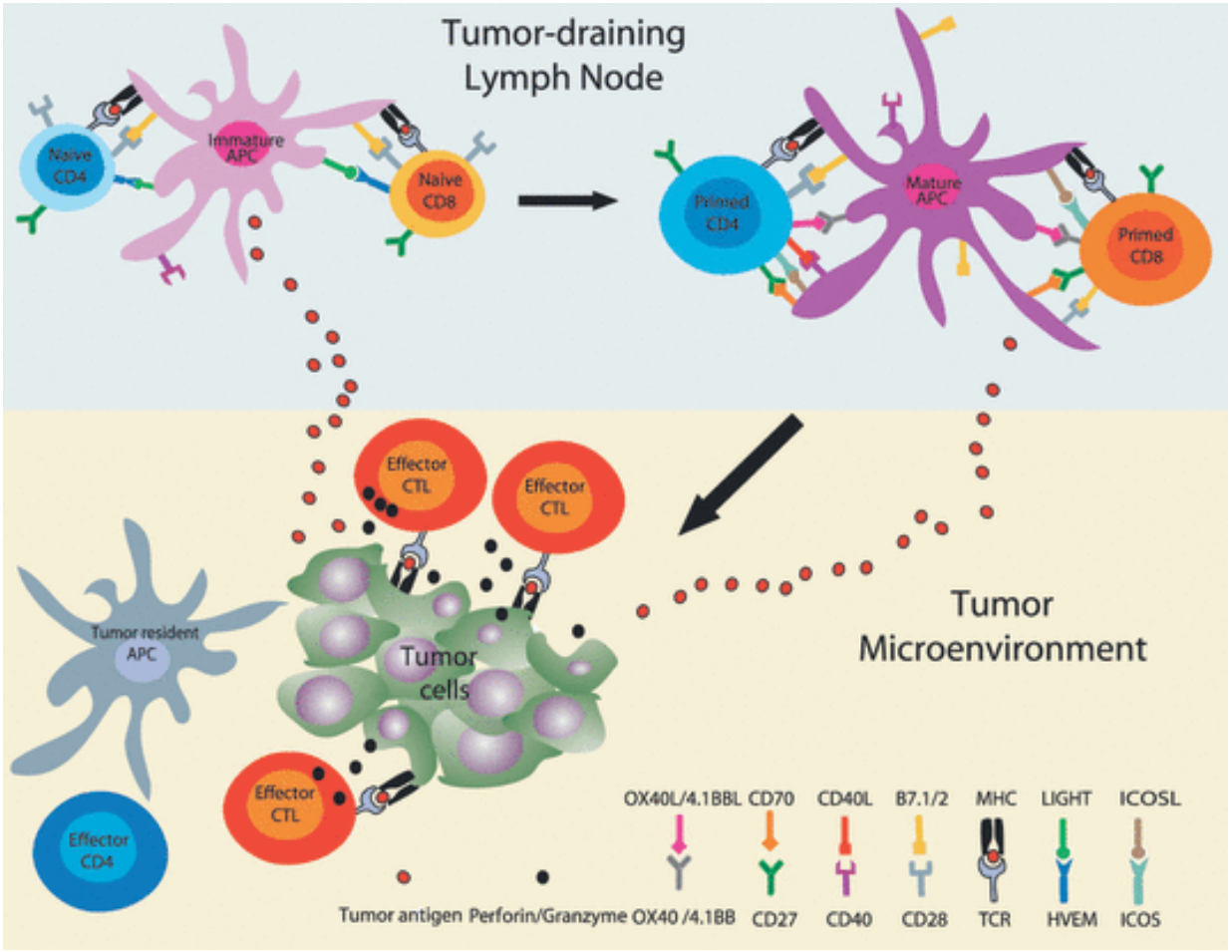


Figure 5A: Schematic of positive costimulatory pathways that could regulate specific stages in an anti-tumor immune response. Depicted are representations of the priming phase in secondary lymphoid organs, and the effector phase within the tumor microenvironment. Most costimulatory signals can be envisioned to improve aspects of the immune response in both compartments, to improve productive cross-priming by APCs, and to help maintain the desired functional properties of effector cell subsets. Specific receptor/ligand interactions are defined in the lower right section. APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte.
Source: Driessens et al. Costimulatory and coinhibitory receptors in anti-tumor immunity. Immunonol Rev, 229: 126-144, 2009[202]

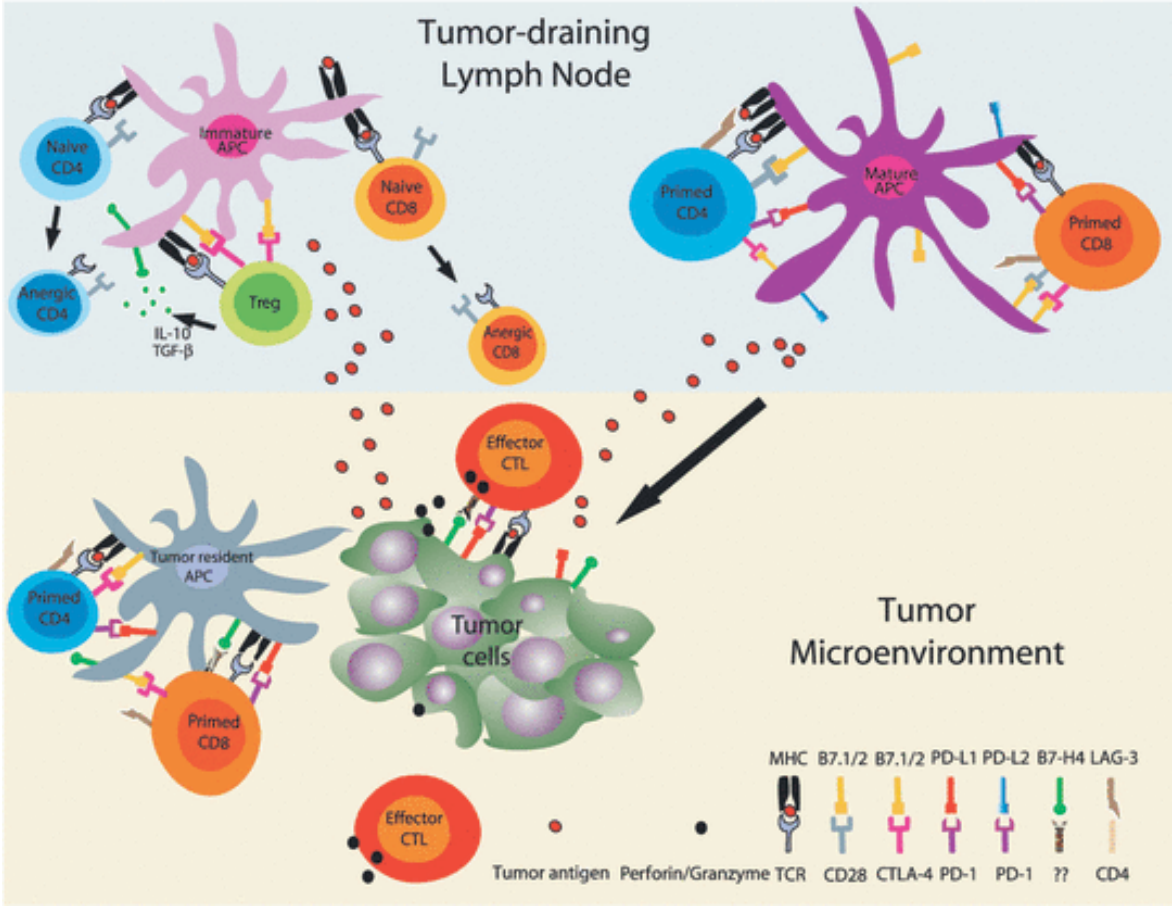


Figure 5B: Schematic of coinhibitory pathways that can be active within tumor-draining lymph nodes and the tumor microenvironment. Depicted are representations of the priming phase in secondary lymphoid organs, and the effector phase within the tumor microenvironment, along with key coinhibitory pathways that could dampen anti-tumor T-cell responses at each level. Several inhibitory signals, such as PD-L1/PD-1 interactions, can regulate both the effectiveness of tumor antigen cross-priming and the function of effector cells at tumor sites. Not all pathways are necessarily relevant to each subtype of cancer, nor do they necessarily coexist in all individual tumors. Specific receptor/ligand interactions are defined in the lower right section. PD, programmed cell death.

Source: Driessens et al. Costimulatory and coinhibitory receptors in anti-tumor immunity. Immunonol Rev, 229: 126-144, 2009[202]

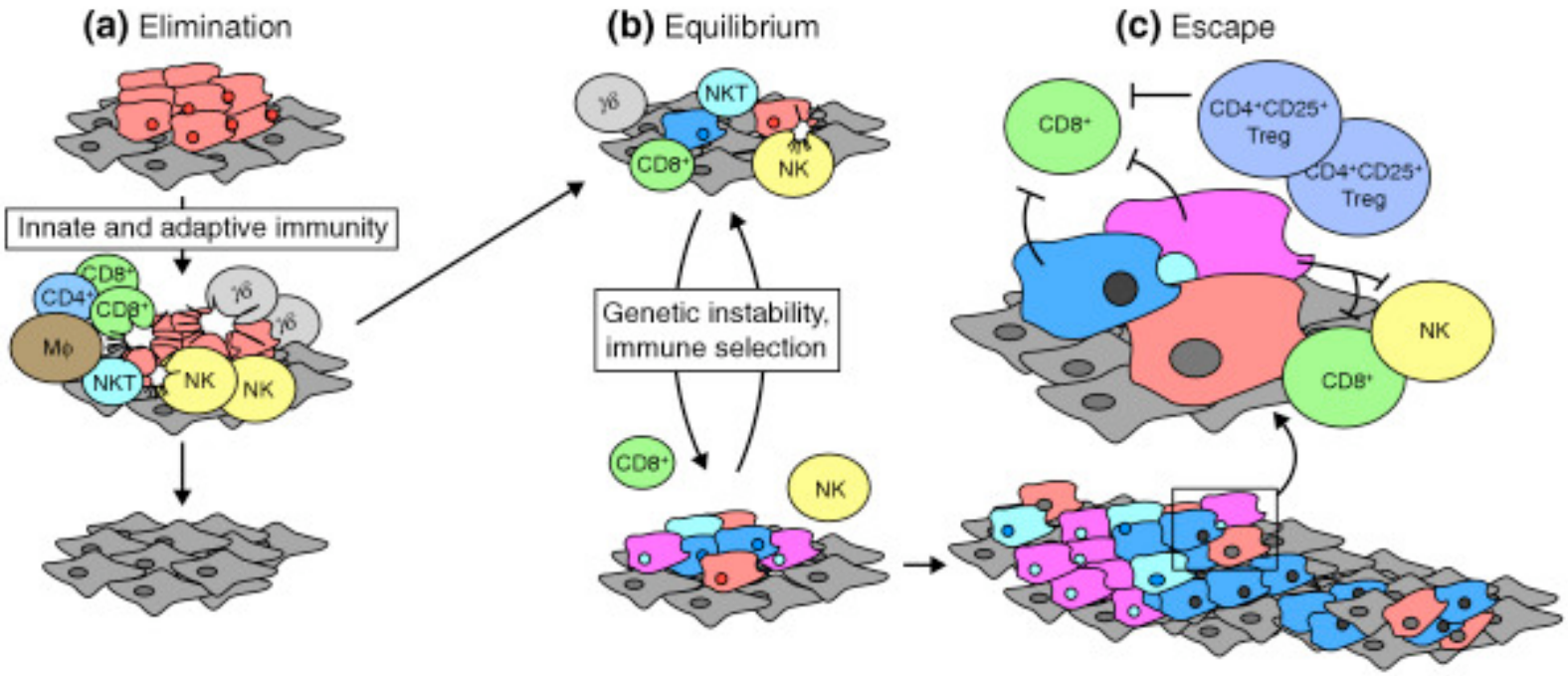


Figure 6: The three Es of cancer immunoediting: elimination, equilibrium, and escape. (a) After transformation of cells in a normal layer (diamond-shaped cells) into cancerous cells (with irregular shapes), attack by various different cell types of the immune system (indicated by round cells) may lead to elimination of the cancerous cells. **(b)** If elimination is unsuccessful, the immune system and the cancer can reach an equilibrium in which immune cells keep the cancer in check but cannot remove it completely. During the elimination phase, there is selection on the cancer cells, whose genomes are also unstable. This can lead to escape **(c)**, in which mutated cancer cells become able to inhibit the immune system. The cancer can then grow unchecked. Figure modified from [2]. $CD4^+$, $CD8^+$, $CD4^+CD25^+$ Treg, γδ and NKT cells are all types of T cell; Mφ cells are macrophages and NK cells are natural killer cells.
Strausberg. Tumor microenvironments, the immune system and cancer survival. Genome Biology 2005 ***6****:211*[201]

Table 1: List of most relevant TAAs recognized by T cells

Shared Antigens	Type of Tumor	Normal tissue distribution	Ref
Cancer Testis Antigens			
BAGE GAGE MAGE-A/B NY-ESO-1 SSX	melanoma, lymphoma, lung, bladder, colon, and breast carcinomas	spermatocytes, spermatogonia, testis, placenta, ovary cells	160-163
Differentiation Antigens			
Gp100 Melan-A/Mart-1 Tyrosinase PSA CEA Mammaglobin-A	melanoma, prostate cancer, colon and breast carcinomas	melanocytes, epithelial tissues, prostate, colon	164-166, 167-171, 185
Overexpressed Antigens			
P53 HER2/neu Livin Survivin	esophagus, liver, pancreas, colon breast, ovary, bladder, and prostate carcinomas	ubiquitous (low level)	172-175
Unique Antigens			
Unique Antigens			
β-catenin-m β-actin/1/m Myosin/m HSP70-2/m HLA-A2-R170J	melanoma, non-small lung cancer, renal cancer	N/A	176-179
Unique Shared Antigens			
Tumor-associated Carbohydrate Antigens			
GM2 GD2 GD3 MUC-1 sTn globo-H	melanoma, neuroblastoma, colorectal, lung, breast, ovarian and prostate cancer	epithelial tissues (e.g. renal, intestinal, colorectal)	180-184

Adapted from Buonaguro et al, 2011. Clin and Vacc Immunol 18: 23-24[203]

Table 2: Overview of different vaccination strategies employed in clinical trials

VACCINE	PHASE	TUMOR	NOTE	REF
Vaccines with vectors				
PSA-TRICOM	II	Prostate	8.5 mos OS improvement vs Placebo	186
PSA-TRICOM	II	Prostate	16.4 mos OS improvement in HPS>18 mos	187
PANVAC-VF	III	Pancreatic	Failed>OS. Pts with life expectancy < 3mos	188
Vaccines with peptides				
Provenge	III	Prostate	4.1 mos improvement vs placebo	189,200
Oncophage	III	Melanoma	Prolonged OS in M1a or M1b	191
Gp100: 209-217 (210M)	III	Renal	No difference in DFS and OS	192
Stimuvax	IIb	Lung	Improvement versus BSC in locoregional stage IIIB	193
Vaccines with tumor cells or tumor cell lysates				
OncoVAX	III	Colon	Significant improvement in DFS and OS in stage II	194-196
Reniale	III	Renal	Significant improvement in DFS and OS	197, 186
GVAX	III	Prostate	Failed to improve OS vs docetaxel	188
GVAX	III	Prostate	Failed. Higher death rate in vacc+doc vs doc alone	188
Vaccines with RNA				
mRNA from PCa cell lines	I/II	Prostate	Immunological response	198
Vaccines with attenuated live bacteria[1]				
Listeria-E7	I/II	Cervix (invasive)	Flu-like symptoms; Detection of E7-specific T cell responses	103

OS=overall survial; PFS= Progression-free survival; HPS=Halabi-predicted survival; DFS=disease-free survival
*Adapted from: from Vergati et al. J of Biomed Biotechnol **2010**, doi:10.1155/2010/596432 (2010)*[186]

Table 3: A list of most important completed, ongoing, and suspended clinical trials with curcumin in patients with different types of cancer

Type of Cancer	Trial condition	Trial	Identifier No./Ref*
Colon Cancer	Completed	The effects of curcuminoids on aberrant crypt foci	NCT00176618
Colon Cancer	Completed	Curcumin in colon cancer	NCT00027495
Colon Cancer	Ongoing (Phase II)	Curcumin in lower GI tract in FAP	NCT00248053
Colon Cancer	Ongoing (Phase II)	Curcumin for chemoprevention colon cancer	NCT00118989
Colon Cancer	Ongoing (Phase II)	Curcumin in colon cancer in smokers with aberrabant crypt foci	NCT00365209
Colon Cancer	Suspended	Curcumin effect on biomarkers of colon cancer cell turnover	NCT00003365
Colon Cancer	Not yet open (Phase III)	Curcumin, gemcitabine, and celebrex in metastatic colon cancer	NCT00542711
Colon cancer	Suspended	Curcumin for intestinal FAP	NCT00641147
Pancreatic Cancer	Ongoing (Phase II)	Curcumin in advanced pancreatic cancer	NCT00094445
Pancreatic Cancer	Ongoing (Phase III)	Curcumin, gemcitabine, and celebrex in pancreatic cancer	NCT00486460
Pancreatic Cancer	Ongoing (Phase II)	Curcumin with gemcitabine	
Oral Cancer	Ongoing (Phase II/III)	Oral premalignant lesions	See$
Oral Cancer	Ongoing (Phase II/III)	Oral premalignant lesions	See$
Cervical Cancer	Ongoing (Phase II/III)	Cervical cancer (stage IIb, IIIb)	See$
Gall bladder Cancer	Ongoing (Phase II)	Gall bladder Cancer	See$
Osteosarcoma	Ongoing	Curcumin and ashwaganda root powder extract	NCT00689195

www.clinical trials.gov; $www.charakinternational.com/pdfs/clinic_trial.pdf. FAP= familial adenomatous polyposis
Source: Shehzad et al, 2010, Arch Pharm Chem Life SCi 2010, 9, 489-499[204]

CHAPTER 2

Manuscript under revision with Cancer Medicine

Title: *Curcumin Improves the Therapeutic Efficacy of Listeria^{at}-Mage-b Vaccine in Correlation with Improved T Cell Responses in Blood of a Triple Negative Breast Cancer model 4T1*

Running Title: Curcumin improves therapeutic efficacy of Listeria^{at}-Mage-b

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Keywords: Cancer vaccines, triple-negative breast cancer, Curcumin, Metastases, T cells

Abstract

Success of cancer vaccination is strongly hampered by immune suppression in the tumor microenvironment (TME). Interleukin (IL)-6 is particularly and highly produced by triple negative breast cancer (TNBC) cells, and has been considered as an important contributor to immune suppression in the TME. Therefore, we hypothesized that IL-6 reduction may improve efficacy of vaccination against TNBC cancer through improved T cell responses. To prove this hypothesis, we investigated the effect of curcumin, an inhibitor of IL-6 production, on vaccination of a highly attenuated *Listeria monocytogenes* (Listeria^{at}), encoding tumor-associated antigens (TAA) Mage-b in a TNBC model 4T1. Two therapeutic vaccination strategies with Listeria^{at}-Mage-b and curcumin were tested. The first immunization strategy involved all Listeria^{at}-Mage-b vaccinations and curcumin after tumor development. Since curcumin has been consumed all over the world, the second immunization strategy involved curcumin before and all therapeutic vaccinations with Listeria^{at}-Mage-b after tumor development. Here we demonstrate that curcumin significantly improves therapeutic efficacy of Listeria^{at}-Mage-b with both immunization strategies particularly against metastases in a TNBC model (4T1). The combination therapy was slightly but significantly more effective against the metastases when curcumin was administered before compared to after tumor development. With curcumin before tumor development in the combination therapy, the production of IL-6 was significantly decreased and IL-12 increased by myeloid-derived suppressor cells (MDSC), in correlation with improved CD4 and CD8 T cell responses in blood. Our study suggests that curcumin improves the efficacy of Listeria^{at}-Mage-b vaccine against metastases in TNBC model 4T1 through reversal of tumor-induced immune suppression.

Introduction

Triple negative breast cancer (TNBC), defined as tumors lacking estrogen receptor (ER), progesterone receptor (PR), and HER2/neu accounts for about 20% of all breast cancers, and

is particularly increased in black women[1]. Women with TNBC represent high-grade tumors that are large and commonly associated with regional node metastases, and recur at distant sites, especially within the first 5 years of diagnosis[2] . The absence of any specific targeted therapy for TNBC or basal subtype limits the therapeutic options to cytotoxic therapy[3,4], indicating the need for new therapeutic approaches. Immunotherapy may be our best and most benign option for preventing or curing TNBC. However, immune suppression in the tumor microenvironment (TME) remains as a potential limitation to immunotherapy. Myeloid-derived suppressor cells (MDSC) are one of the most important players in mediating TME-associated immune suppression, with tumor-associated macrophages (TAM), Tregs, and M2 macrophages also playing a role[5-8]. Interleukin (IL)-6 is one of such immune suppressive cytokines that is frequently and highly produced by metastatic breast cancers in humans and mice, and particularly by TNBC[9-11]. TNBC are enriched for stem-like breast cancer cells (CD44+/CD24-/low), which are typically aggressive and highly resistant to current therapies[12-15]. These stem-like breast cancer cells produce high levels of IL-6, and have the capacity to metastasize[16]. Moreover, IL-6 is capable of converting dormant breast cancer cells into an actively growing tumor.

IL-6 is a potent regulator of dendritic cell (DC) differentiation in vivo, and is able to turn on the expression of signal transducer and activator of transcription (STAT)3 in DC[17]. However, high levels of STAT3 can prevent DC from maturation and subsequent presentation of antigens[18]. This in turn may lead to T cell unresponsiveness. In a previous study we found high levels of IL-6 produced by breast cancer cells and by immune cells in their TME in an aggressive TNBC mouse model 4T1[19]. This IL-6 strongly reduced T cell responses to Mage-b, but elimination of IL-6 using anti-IL-6 antibodies restored T cell responses to Mage-b in vitro[20].

Agents that are able to inhibit IL-6 are of great value for immunotherapies against TNBC and other IL-6-producing cancers. One such agent could be curcumin. Curcumin (diferulolylmethane), a polyphenol derived from the plant *Curcunina longa*, commonly called

turmeric, has a broad anti-cancer effect through down regulating transcription factor NFkB thereby affecting down stream genes such as c-myc, Bcl-2, COX-2, NOS, Cyclin D1, TNFα and MMP9 [21]. Curcumin is also known for reducing immune suppressive cytokines such as IL-6 through the NFkB pathway[22]. It has been shown that curcumin improves therapeutic efficacy of doxorubicin or of B16-R lysate against B16-R melanoma in mice, and that curcumin prevents tumor-induced T cell apoptosis in mice[23]. In a previous study we developed a Listeria^{at}-based vaccine expressing tumor-associated antigen (TAA) Mage-b[20]. Mage-b is homologous to Mage-a[24], and its human homologue MAGE-A is expressed in 26% of the TNBC[25]. Vaccination with Listeria^{at}-Mage-b showed to be highly effective against metastatic breast cancer in a TNBC model 4T1 in a preventive setting[20]. However, Listeria^{at}-Mage-b was less effective in a therapeutic setting because of immune suppression in the TME. Here, we demonstrate that curcumin improved therapeutic efficacy of Listeria^{at}-Mage-b by reducing the production of IL-6 and increasing the production of IL-12, in correlation with improved T cell responses in blood of the TNBC 4T1 model. Most important, we found a dramatic effect of the combination therapy on the metastases without having side effects. The results of this study may provide new opportunities to improve efficacy of other types of vaccines and/or against other IL-6-producing cancers.

Materials and Methods

Mice.

Normal female BALB/c mice (3 months old) were obtained from Charles River and maintained in the animal husbandry facility Albert Einstein College of Medicine according to the Association and Accreditation of Laboratory Animal Care (AACAC) guidelines. All mice were kept under Bsl-2 condition as required for Listeria vaccinations.

Cells and cell culture

The TNBC 4T1 cell line, derived from a spontaneous mammary carcinoma in a BALB/c mouse[26], was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM mixed nonessential amino acids, 2 mM L-glutamine, insulin (0.5 HSP units/ml) penicillin (100 units/ml) and streptomycin (100 μg/ml).

Listeria^{at}-based vaccine

In this study, a highly attenuated *Listeria monocytogenes* (Listeria^{at}) has been used for the delivery of TAA Mage-b in vivo, as described previously[20]. The Listeria^{at} plasmid pGG-34, expresses the positive regulatory factor A (prfA) and one of the virulence genes Listeriolysin O (LLO)[27]. The coding region for the C-terminal part of the LLO (cytolytic domain that binds cholesterol in the membranes) protein in the plasmid has been deleted, but Listeria^{at} is still able to escape the vacuole[28]. Mutations have been introduced into the prfA gene and in the LLO, which further reduced the pathogenicity of the Listeria^{at} [27]. The background strain XFL-7 lacks the prFA gene, and retains the plasmid in vitro and in vivo[28]. Listeria^{at}-Mage-b, expressing nucleotide fragment 311-660 of mouse Mage-b, was developed earlier in our laboratory[20].

Curcumin

As indicated in the text below, a dose of curcumin (95% curcuminoid)(Alfa Aesar, Ward Hill, MA) of 0.8 or 2 gram/kg (20 or 50 mg/mouse), in olive oil was administered orally. Piperine (black pepper) of 20 mg/kg (0.48 mg/mouse) was added to the olive oil in all studies with curcumin. Piperine improves the bioavailability with 2000%, and has been successfully used in humans and animals[29]. Piperine is a known inhibitor of hepatic and intestinal glucuronidation, a process that breaks down curcumin in vivo[30,31].

Immunization and tumor challenge

In this study, two different immunization protocols were tested. The first immunization protocol consisted of three therapeutic immunizations with Listeria^{at}-Mage-b and curcumin. Briefly, mice received 0.5x10^5 4T1 tumor cells in the mammary fat pad on day 0, then 0.5x10^7 CFU of Listeria^{at}-Mage-b, or Listeria^{at} or saline intraperitoneally (ip) on days 2, 9, and 16, and finally curcumin orally (50 mg curcumin + 0.48 mg black pepper in olive oil/mouse) on days 4, 5, 6, 11,12, and 13 (Immunization protocol A). All mice were euthanized on day 17, and analyzed for the number of metastases and tumor growth. All untreated 4T1 mice developed a primary tumor that extended to the chest cavity lining and metastasized predominantly to the mesenteric lymph nodes (MLN), and less frequently to the diaphragm, portal liver, spleen, and kidneys within 14 days (metastases were visible as nodules and counted by eye) as described previously[20]

The second immunization protocol consisted of three therapeutic immunizations with Listeria^{at}-Mage-b, but curcumin was administered before tumor development. Briefly, mice received curcumin orally (50 mg curcumin + 0.48 mg black pepper in olive oil/mouse) on days 0, 1 and 2, then 0.5x10^5 4T1 tumor cells in the mammary fat pad on day 5, and finally three therapeutic immunizations (ip) with 1x10^4 CFU Listeria^{at}-Mage-b, Listeria^{at} or saline on days 8, 11 and 14 (Immunization protocol B). All mice were euthanized on day 16 and analyzed for metastases and tumor growth as described above.

Flow cytometry analysis

Cells were isolated from spleen and blood as described previously[32]. Briefly, red blood or spleen cells were lysed according standard protocols, and the remaining leukocyte population was used for analysis. Single cell suspensions were also obtained from primary tumors using GentleMacs combined with a mild treatment of the cells using Collagenase, Dispase, and DNAse I, according to the manufacturer's instructions (Miltenyi, Biotec Inc, Auburn, CA).

Cells were first incubated with an Fc blocker (anti-CD16), and subsequently with the antibodies for the identification of different cell types. For MDSC, anti CD11b and Gr1 antibodies were used. $CD11b^{+}Gr1^{low}$ represents monocytic MDSC (mMDSC), and $CD11b^{+}Gr1^{high}$ granulocytic MDSC (gMDSC). Anti-CD8 antibodies were used to identify CD8 T cells and anti-CD4 to identify CD4 T cells. Anti-CD45 antibodies were used to identify the leukocyte population in the primary tumors. To detect the production of intracellular lymphokines the cytofix/cytoperm kit from Pharmingen according to the manufacturer's instructions, and antibodies to IL-6, IL-12, and IFNγ, were used. Appropriate isotype controls were used for each sample. Depending on the sample size, 10,000-500,000 cells were acquired by scanning using a Fluorescence Activated Cell sorter (flow cytometry)(Beckton and Disckinson; Excalibur), and analyzed using Flojo software as described previously[32]. Cell debris and dead cells were excluded from the analysis based on scatter signals and use of Fixable Blue or Green Live/Dead Cell Stain Kit (Invitrogen). In blood and spleens, MDSC were analyzed in the total live gated leukocyte population, and T cells in the total live gated lymphocyte population. In the tumor cell suspension, MDSC and T cells were analyzed in the total live gated $CD45^{+}$ (leukocyte) population. All antibodies were purchased from BD Biosciences Pharmingen.

Cell proliferation, Mitotic Index, and Apoptosis

Cell proliferation: 4T1 cells (2000 cells in 0.1 ml) were cultured with different doses of curcumin in dimethyl sulfoxide (DMSO) for 72 h, then cell viability was analyzed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) method using a microtiter plate reader at a wave length of 570 nm. *Mitotic Index:* Sections of 1 mm thick of primary tumors of mice treated with Listeria^{at}-Mage-b and curcumin or with saline were stained with Hematoxylin and Eosin (H and E) and subsequently analyzed for the number of cells in mitosis by light microscopy. *Apoptosis:* Early and late apoptosis was analyzed by Annexin-V and TUNEL

assay, respectively. For the Annexin-V assay, 4T1 tumor cells were cultured with or without 100 μM of curcumin for 24h, and subsequently incubated with Annexin-V antibodies (BD Biosciences), for the detection of apoptosis. For the TUNEL Assay, the ApoTag® *In Situ* Apoptosis detection (Millipore) was used. Briefly, slides were deparaffinized through graded alcohols to PBS. TUNEL staining was performed using ApopTag® In Situ Apoptosis Detection Kit (Millipore). Briefly, samples were Proteinase K digested (20 μg/mL) for 15 minutes at room temperature. Endogenous peroxidases were blocked using 3% H_2O_2 for 5 minutes at RT. Samples were washed and placed in Equilibration Buffer for 10 seconds followed by TdT enzyme incubation in reaction buffer for 1 hr at 37°C. Samples were incubated in the Anti-Digoxigenin, washed and developed using DAB (3,3' diaminobenzidine). Slides were briefly counterstained in hematoxylin and mounted using Permount (Fisher Scientific). From each tissue, two sections were analyzed, and from each section the number of apoptotic cells in 10 fields were counted by light microscopy. The TUNEL assay and Mitotic Index analyses were performed in the Laboratory of Dr. Rani Sellers, Director of Histology and Comparative Pathology Core Facility, Albert Einstein College of Medicine.

Pathological examination

All pathological analyses were performed by Dr. Rani Sellers, Director of Histology and Comparative Pathology Core Facility, Albert Einstein College of Medicine. Briefly, normal tissues such as kidneys, heart, lungs, liver and spleen were fixed in 10% formaldehyde for 48 hrs, and then kept in 70% ethanol until use. Sections of 1 mm thick were stained with H and E, and analyzed for pathological damage by light microscopy.

Results

Curcumin administered after tumor development significantly improved therapeutic effect of Listeria^{at}-Mage-b in the 4T1 model

Here we tested whether curcumin could improve the efficacy of Listeria^{at}-Mage-b vaccination in the model 4T1. Listeria^{at}-Mage-b and curcumin were alternately administered after tumor development (Immunization protocol A). As shown in **Fig 1A,** the number of metastases in the mice that received Listeria^{at}-Mage-b and curcumin was significantly lower compared to all control groups. Also the tumor weight in the mice that received Listeria^{at}-Mage-b and curcumin was significantly lower than in the mice that received Listeria^{at} or curcumin alone, but not compared to the mice that received Listeria^{at}-Mage-b alone **(Fig 1B).** Curcumin alone had no significant effect on the tumor weight compared to the saline group.

Curcumin administered before tumor development also significantly improved therapeutic effect of Listeria^{at}-Mage-b in the 4T1 model

Since curcumin is frequently used in food all over the world we tested whether curcumin could improve therapeutic vaccine efficacy of Listeria^{at}-Mage-b when consumed before tumor development (Immunization protocol B). Here we used a low dose of Listeria^{at}-Mage-b (10^4 CFU) at a high frequency (every 3 days; 4 times totally) in order to obtain a continuous delivery of Listeria^{at}-Mage-b in vivo without having side effects. Using this immunization protocol, the number of metastases in the mice that received Listeria^{at}-Mage-b and curcumin was significantly decreased compared to all control groups **(Fig 2A).** Also the tumor weight in the mice that received Listeria^{at}-Mage-b and curcumin was significantly lower compared to all control groups (**Fig 2B).** Curcumin alone had also a significant effect on the metastases and primary tumors compared to the saline group **(Fig 2B)**. The growth kinetics of the primary tumors was analyzed as well in mice that received Listeria^{at}-Mage-b and curcumin, and confirmed the results shown in **Fig 2B**, i.e. on day 14 the tumor size in mice that received Listeria^{at}-Mage-b and curcumin was significantly lower compared to all other control groups **(Supplementary Information Fig S1).**

The combination therapy with curcumin before and Listeria^{at}-Mage-b after tumor development was slightly but significantly more effective against the metastases than curcumin and Listeria^{at}-Mage-b both after tumor development **(Figs 2AB and 3AB),** i.e. the number of metastases in the combination therapy with curcumin before tumor development was 4±1, and after tumor development 31±12 (Mann-Whitney p=0.0017).

The effects of Listeria^{at}-Mage-b and curcumin on MDSC in vivo

Since MDSC strongly contributes to immune suppression in the TME, we analyzed the effect of the combination therapy on MDSC in blood and primary tumors of mice immunized according to immunization protocol B. In total blood, the percentage of MDSC was extremely high (~80%)**(Fig 3A).** This percentage was strongly reduced to ~20% by the combination of Listeria^{at}-Mage-b and curcumin compared to the saline group, but was also significantly lower compared to all other control groups **(Fig 3A).** More detailed analysis showed that granulocytic (g)MDSC was predominantly responsible for the strong decrease in percentage of MDSC **(Fig 3BC).** In the primary tumors, the percentage of MDSC (in CD45^{+} population) was much lower than in blood (~12%), and the effect of Listeria^{at}-Mage-b and curcumin on MDSC was much less robust than in blood. The combination therapy slightly but significantly reduced the percentage of MDSC and gMDSC (but not of monocytic (m)MDSC) compared to the saline or curcumin groups only **(Fig 3DEF).**

Curcumin reduced the production of IL-6 in primary tumors and in MDSC

Here, we analyzed the effect of curcumin on the production of IL-6 in total tumor cell lysates, in MDSC of primary tumors and blood, and in serum of the 4T1 model. In the tumor cell lysates **(Fig 4A)** and in mMDSC and gMDSC of the primary tumors **(Fig 4BC)** we found that curcumin significantly reduced IL-6 levels compared to the control groups**.** In blood, IL-6 was significantly reduced by curcumin in mMDSC compared to the Listeria^{at}-Mage-b group **(Fig**

4E), but IL-6 was not produced by gMDSC. In serum, IL-6 was undetectable and therefore not shown

Also Listeria^{at}-Mage-b reduced IL-6 levels in the primary tumors (tumor cell lysates) **(Fig 4A),** but not in MDSC in blood and primary tumors **(Fig 4B-C).** Moreover, Listeria^{at}-Mage-b significantly increased the production of IL-6 in sub populations of the MDSC (with an exception of gMDSC in tumors), probably to protect them selves from immune clearance, but as mentioned above curcumin strongly reduced the IL-6 production in both types of MDSC in blood **(Fig 4DE).**

Curcumin administered before and Listeria^{at}-Mage-b after tumor development improved the IL-12 production by MDSC and T cell responses to Mage-b

Here we analyzed the IL-12 production in subpopulations of gMDSC and mMDSC in blood of mice that received the combination of curcumin before and Listeria^{at}-Mage-b after tumor development. A significant increase was found in the percentage of IL-12-producing gMDSC and mMDSC in the combination group compared to all other groups **(Fig 5AB),** but not in the primary tumor (data not shown). These results raised the question whether the lower number of MDSC **(Fig 3),** the decreased IL-6 levels **(Fig 4)** and increased IL-12 production **(Fig 5AB)** induced by Listeria^{at}-Mage-b and curcumin**,** could improve T cell responses in vivo. For this purpose, we analyzed the production of IFNγ by CD4 and CD8 T cells in blood and primary tumors in vaccinated and control mice by flow cytometry. IFNγ is a marker for T cell activation. The cells were analyzed in all groups without re-stimulation in order to determine whether the T cells were activated in vivo by the combination therapy compared to the control groups. It appeared that the combination of Listeria^{at}-Mage-b and curcumin significantly improved the percentage of CD4 and CD8 T cells producing intracellular IFNγ compared to all control groups in blood **(Fig 5CD),** but not in tumors (data not shown). We also analyzed T cells responses in the spleen upon re-stimulation with Mage-b in vitro. As shown in **Fig 5E**,

Listeria^{at}-Mage-b and curcumin strongly improved the number of CD8 T cells to Mage-b, secreting extracellular IFNγ.

Curcumin inhibited proliferation of tumor cells and killed tumor cells through apoptosis

Several reports describe that curcumin inhibits proliferation and kills tumor cells through apoptosis, including breast tumor cells[33-35]. We found that curcumin inhibited the growth of 4T1 tumor cells in vitro **(Fig 6A),** and mitosis of the tumor cells in vivo **(Fig 6B).** In addition, we found that curcumin killed tumor cells through apoptosis in vitro as shown by Annexin-V (early apoptosis) **(Fig 6C),** and in the primary tumors in vivo as shown by the TUNEL assay (late apoptosis) **(Fig 6D).** A representative example of apoptotic cells by the TUNEL assay is shown by light microscopy in **Fig 6E.**

Listeria^{at}-Mage-b is non-pathogenic and curcumin is non-toxic

In a previous study we have shown that Listeria^{at}-Mage-b is non-pathogenic[20], while curcumin, consumed through food all over the world, is non-toxic[31]. However, the combination of Listeria^{at}-Mage-b and curcumin has never been tested. Here, we demonstrate by pathological examination of various normal tissues (as kidney, heart, lungs, liver, and spleen) in tumor-bearing mice that the combination of Listeria^{at}-Mage-b and curcumin is non-pathogenic and non-toxic**,** but primarily activated the innate immune system. Most obvious was the increased extramedullary myeloid hematopoiesis in the spleen and liver of mice that received Listeria^{at}-Mage-b and curcumin compared to the saline group. An example of extramedullary myeloid hematopoiesis in the liver is shown in the **Supplementary Information Fig S2.** An overview of pathological analysis of normal tissues of tumor-bearing mice that received Listeria^{at}-Mage-b and curcumin is shown in **Table S1 of the Supplementary Information**.

Discussion

Patients with TNBC have the poorest prognosis. One of the main problems of current therapies against TNBC is their inability to target metastases and their high toxicity. They do not respond to therapies that target ER, PR, and HER2/neu because their tumors lack the expression of these receptors/molecules, and other types of therapies such as tyrosine kinase inhibitor Sunitinib, targeting vascular endothelial growth factor (VEGF), or therapies targeting c-kit or Flt2, or Bevacizumab, a human antibody to VGEF[36-40], are under investigation but with moderate success. In the study presented here, we developed two non-toxic vaccination strategies in a preclinical TNBC mouse model 4T1. We demonstrated that three therapeutic vaccinations with a highly attenuated non-pathogenic Listeria^{at}-based vaccine, expressing TAA Mage-b, and non-toxic curcumin significantly reduced the number of metastases compared to Listeria^{at}-Mage-b or curcumin alone. However, curcumin alone had no significant effect on the primary tumors and metastases. Others described that curcumin killed tumor cells[41-45]. These studies were all performed in vitro, while we analyzed the effect of curcumin on the primary tumor and metastases in vivo, which may be an explanation for the different results. Different types of tumor cells, concentrations and time points of curcumin administration may lead to different results as well[46].

We also tested three administrations of curcumin before tumor development followed by three immunizations with Listeria^{at}-Mage-b after tumor development. This immunization protocol was slightly but significantly more effective against the metastases compared to Listeria^{at}-Mage-b and curcumin both after tumor development. Most interestingly, curcumin alone significantly reduced the number of metastases and tumor growth, in contrast to administering curcumin after tumor development. These results suggest that consuming curcumin before cancer develops may provide an advantage over consuming curcumin after cancer develops in the battle against metastatic breast cancer.

Curcumin is known for reducing the production of IL-6[47,48]. Here we demonstrate that curcumin significantly reduced the production of IL-6 in vivo in the primary tumors (tumor cell lysates), and in MDSC of blood and primary tumors. Also Listeria^{at}-Mage-b reduced the production of IL-6 significantly, but this reduction was stronger by the combination of Listeria^{at}-Mage-b and curcumin.

MDSC are important regulators of the immune system in the TME[5,6], and therefore became one of our most important targets in this study. As mentioned above, curcumin reduced the production of IL-6 significantly in MDSC in blood and primary tumors. To our surprise, the combination of Listeria^{at}-Mage-b and curcumin significantly increased the production of IL-12 in gMDSC and mMDSC in blood (but not in tumors). It has been reported that IL-12 activates naïve and mature CD4 and CD8 T cells[49,50], which may have happened in this study as well. An interesting observation was that the combination of Listeria^{at}-Mage-b and curcumin significantly reduced the number of MDSC (predominantly gMDSC) in blood of the TNBC model 4T1. It is possible that MDCS infected with Listeria^{at}-Mage-b became a target for Listeria^{at}- and Mage-b-specific T cell and perhaps NK cell responses because the combination therapy improved these immune responses to Listeria^{at} and Mage-b by reducing IL-6, and increasing IL-12 production. Since Listeria^{at}[51] and curcumin kill 4T1 tumor cells directly (this study), it is also possible that the combination therapy prevented the tumor cells from growing in the early phase of treatment, and consequently prevented migration of the MDSC to the TME. We found that curcumin alone decreased the percentage of MDSC in blood (although this effect was much stronger when Listeria^{at}-Mage-b was combined with curcumin). Reduction in the percentage of MDSC by curcumin was also found by others in a xenograft model of colon cancer[52]. They concluded that reduction in IL-6 production by curcumin reduced the mobilization of MDSC to the primary tumors. Others found that activated T cells might express Fas ligand and induce apoptosis of Fas^{+} MDSC[53]. In

conclusion, various pathways may lead to the reduction in MDSC and more analysis is required.

The decrease in IL-6 and increase in IL-12 production, the improved CD4 and CD8 T cell responses in blood and spleen, and the dramatic reduction in the number of metastases by the combination therapy strongly suggest that T cell responses contributed to the effect on the metastases. However, this strong reduction by the combination therapy is not only an effect of Mage-b-specific immune responses. As shown previously, Listeria^{at} exhibits several pathways to kill tumor cells, i.e. Listeria^{at} infects tumor cells in vivo and in vitro, and kills tumor cells directly through high levels of reactive oxygen species (ROS)[51]. Moreover, we have shown that Listeria^{at}-activated CD8 T cells eliminated Listeria^{at}-infected tumor cells in vivo[51]. In addition, we have shown that curcumin kills 4T1 tumor cells through apoptosis (this study). Therefore, it is most likely that the synergistic effects of the multiple pathways of Listeria^{at}-Mage-b and curcumin as described above, are responsible for the overall strong therapeutic effect on the metastases in this TNBC model 4T1.

The therapeutic effect of the combination therapy was strong but less pronounced on the primary tumors compared to the metastases. It is possible that the production of IL-6 was not sufficiently reduced in the primary tumors (IL-6 was reduced in the tumor cell lysates by ~65% by Listeria^{at}-Mage-b and curcumin treatment), and another inhibitory cytokine such as TGFβ, which is highly produced by 4T1 tumor cells[54], may play a role as well. However, most primary tumors can be removed by surgery, radiation or chemotherapy, while metastases are unresectable, and usually chemoresistant despite aggressive and toxic follow-up[55].

The highly attenuated Listeria^{at} of this study is non-pathogenic, and naturally cleared by the immune system within 3-5 days[51], which is different from wild type Listeria^{at} that multiplies in hepatocytes in the liver or epithelial cells of the gastrointestinal tract[56,57]. Moreover, the side effects of the combination therapy of Listeria^{at}-Mage-b and curcumin in the 4T1 model were minimal, i.e. primarily induction of inflammatory responses in the liver and spleen and no

significant findings were observed in other normal tissues such as heart, lungs and kidneys. Therefore, Listeria^{at}-Mage-b and curcumin may be of value as a non-toxic adjuvant therapy, to prevent the development of metastases in TNBC patients that produce IL-6 and express MAGE. This study may be a platform for improvement of other cancer vaccines by curcumin and against other IL-6-producing cancers.

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Legends

Figure 1: Significant reduction in the number of metastases by therapeutic immunizations with Listeria^{at}-Mage-b and curcumin in 4T1 tumor-bearing mice. BALB/c mice were immunized with Listeria^{at}-Mage-b and treated with curcumin after tumor development (Immunization protocol A), and analyzed for the frequency of metastases (**A)** and tumor weight **(B).** This experiment was performed two times with 5 mice per group. Average of two experiments. Mann-Whitney $p<0.05$ is significant. * $p<0.05$, **<0.01, ***<0.001, ****<0.0001. ns=not significant. All groups were compared to LM-Mb+Curc. In addition, curcumin alone was compared to the saline group.

Figure 2: Significant reduction in the number of metastases by preventive administration of curcumin followed by therapeutic immunization with Listeria^{at}-Mage-b

in 4T1 tumor-bearing mice. BALB/c mice were treated with curcumin before tumor development and immunized with Listeria^{at}-Mage-b after tumor development (Immunization protocol B), and analyzed for the frequency of metastases **(A),** tumor weight **(B).** This experiment was performed three times with 5 mice per group. Average of three experiments. Mann-Whitney $p<0.05$ is significant. * $p<0.05$, **<0.01, ***<0.001, ****<0.0001. ns=not significant. All groups were compared to LM-Mb+Curc. In addition, curcumin alone was compared to the saline group.

Figure 3: The effect of Listeria^{at}-Mage-b and curcumin on MDSC in 4T1 tumor-bearing mice. BALB/c mice were treated with curcumin before tumor development and immunized with Listeria^{at}-Mage-b after tumor development (Immunization protocol B), and analyzed for MDSC ($CD11b^{+}Gr1^{+}$)**(A),** gMDSC ($CD11b^{+}Gr1^{high}$)**(B),** and mMDSC ($CD11b^{+}Gr1^{low}$) **(C)** in blood and for MDSC **(D),** gMDSC **(E),** and mMDSC **(F)** in primary tumors using flow cytometry. All groups were compared to Lm-Mb+Curc. Flow cytometry profiles of MDSC of each group (saline, Listeria^{at}, Listeria^{at}-Mage-b, Listeria^{at}-Mage-b and curcumin, curcumin) are presented in **the Supplementary Information Fig S3**. This experiment was performed three times with 5 mice per group. Average of 3 experiments. Mann-Whitney $p<0.05$ is significant. *$p<0.05$, **<0.01, ***<0.001, ****<0.0001. ns=not significant.

Figure 4: Effects of Listeria^{at}-Mage-b and curcumin on IL-6 in 4T1 tumor-bearing mice. Curcumin treatment before tumor development followed by immunizations with Listeria^{at}-Mage-b after tumor development (Immunization protocol B), significantly reduced IL-6 levels in primary tumors as shown here by ELISA **(A),** and the intracellular production of IL-6 by gMDSC and mMDSC in primary tumors **(BC)** and by mMDSC in blood **(DE)** as shown here by flow cytometry. In A, the curcumin-containing groups were compare to the saline group, while in BC and ED, the curcumin-containing groups were compared to Lm-Mb. These experiments

were repeated three times with 5 mice per group, and the results were averaged. Mann-Withney $p<0.05$ is significant. Mann-Whitney $p<0.05$ is significant *$p<0.05$, **<0.01, ***<0.001, ****<0.0001. ns=not significant.

Figure 5: The combination of Listeria^{at}-Mage-b and curcumin increased IL-12 production by MDSC and improved T cell responses in 4T1 tumor-bearing mice. Curcumin treatment before tumor development followed by immunizations with Listeria^{at}-Mage-b after tumor development (Immunization protocol B) significantly increased the percentage of gMDSC **(A)** and mMDSC **(B)** producing intracellular IL-12 in blood of 4T1 tumor-bearing mice. This correlated with a significant increase in the percentage of CD4 **(C)** and CD8 T **(D)** cells producing intracellular IFNγ (activation marker for T cells) in blood of 4T1-tumor-bearing mice as shown here by flow cytometry. CD8 T cell responses (extracellular production of IFNγ) were also analyzed in the spleen in vitro upon re-stimulation with Mage-b by ELISPOT, and a significant higher number of CD8 T cells was found in the spleen that received Listeria^{at}-Mage-b and curcumin compared to all other groups **(E).** These experiments were repeated three times with 5 mice per group, and the results were averaged. Mann-Whitney $p<0.05$ is significant. * $p<0.05$, **<0.01, ***<0.001, ****<0.0001.

Figure 6: Curcumin inhibited proliferation and killed 4T1 tumor cells through apoptosis. 4T1 tumor cells were cultured with different doses of curcumin for 72 h, and cell viability was analyzed by MTT **(A).** We also analyzed the Mitotic Index in tumors of mice that received curcumin or saline **(B).** 4T1 tumor cells were cultured with 100 μM of curcumin in vitro for 24h, and subsequently incubated with anti-Annexin-V antibodies for the detection of early apoptosis **(C).** Primary tumors of mice that received curcumin or saline (according Immunization protocol B) were analyzed for the detection of late apoptosis in vivo by the TUNEL assay **(D).** Apoptotic cells in the primary tumor by the TUNEL assay and light

microscopy are shown in **(E).** Representative of two experiments in A, C, D. Average of two experiments in B and D. n= 5 mice per group. Unpaired t test $p<0.05$ is significant. Magnification light microscopy in C and E is 400x. In A, curcumin was dissolved in DMSO and then diluted to the final concentrations of 1-50 uM. The 0 μM represents DMSO without curcumin.

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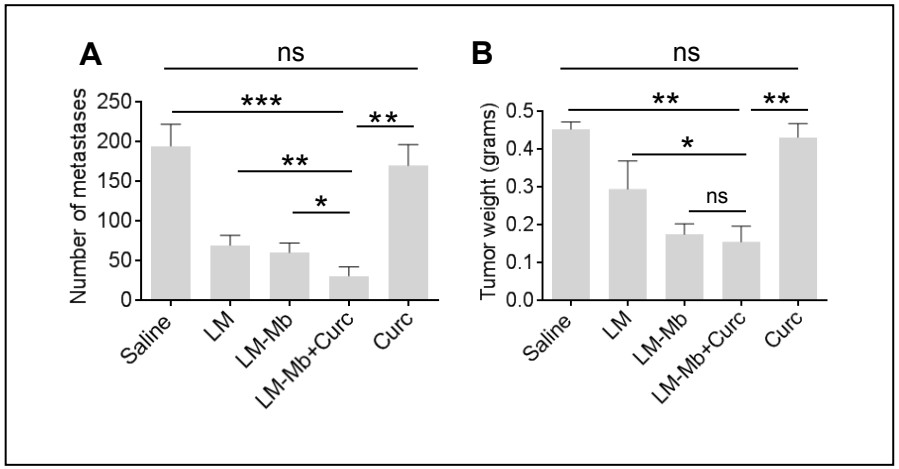
Figure 1AB


A
ns
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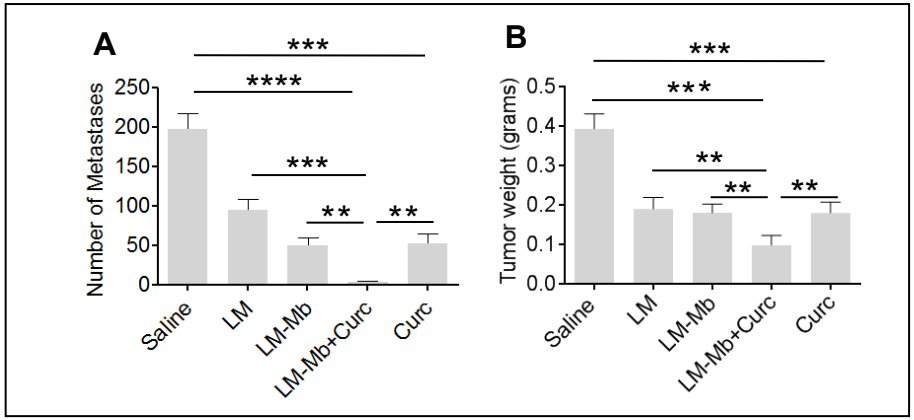
**
**
*
Number of metastases
250
200
150
100
50
0
Saline
LM
LM-Mb
LM-Mb+Curc
Curc
B
ns
**
**
*
ns
Tumor weight (grams)
0.5
0.4
0.3
0.2
0.1
0.0
Saline
LM
LM-Mb
LM-Mb+Curc
Curc

Figure 2AB


A
***
****
***
**
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Number of Metastases
250
200
150
100
50
0
Saline
LM
LM-Mb
LM-Mb+Curc
Curc
B
***
***
**
**
**
Tumor weight (grams)
0.5
0.4
0.3
0.2
0.1
0.0
Saline
LM
LM-Mb
LM-Mb+Curc
Curc

Figure 3ABCDEF

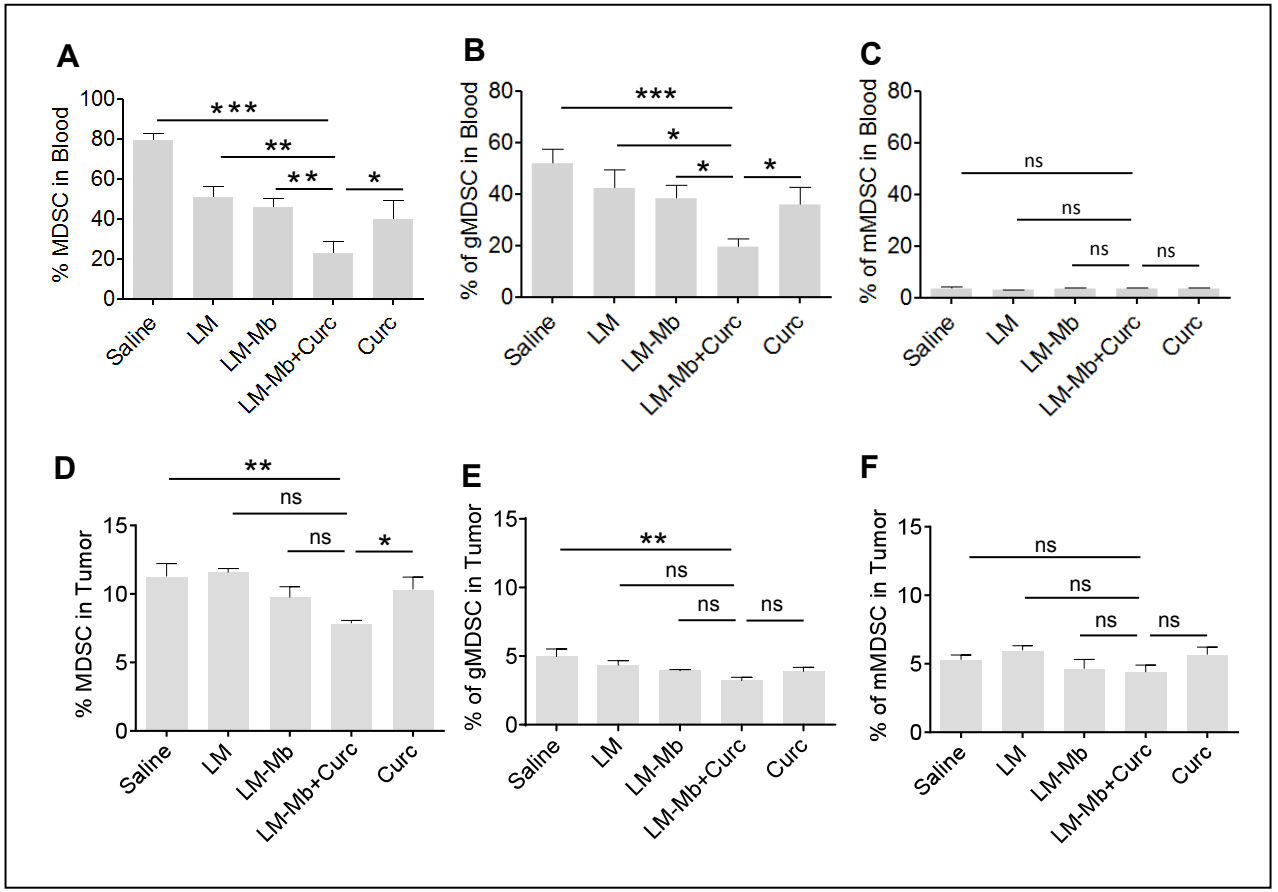
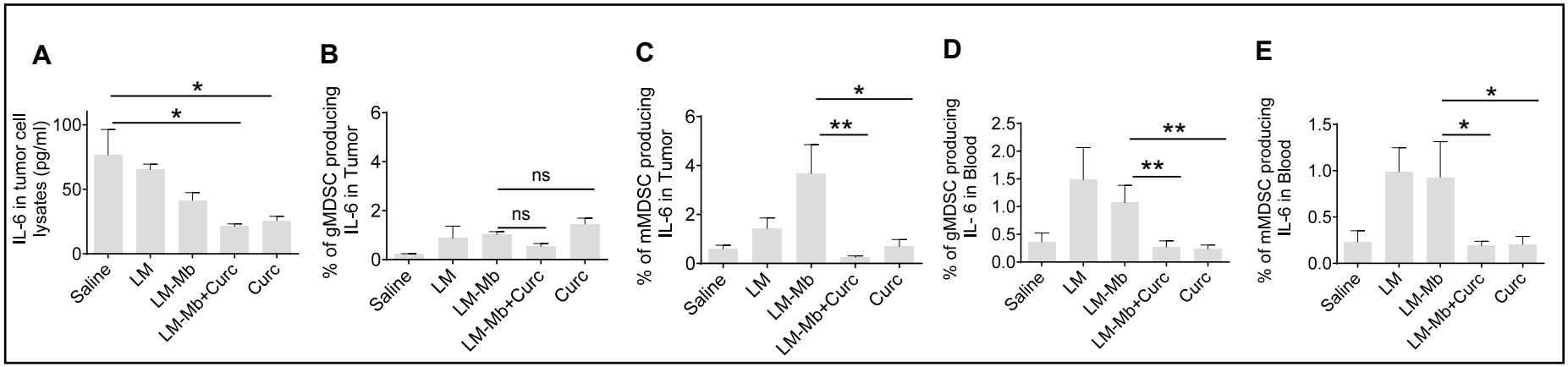


Figure 4ABCDE



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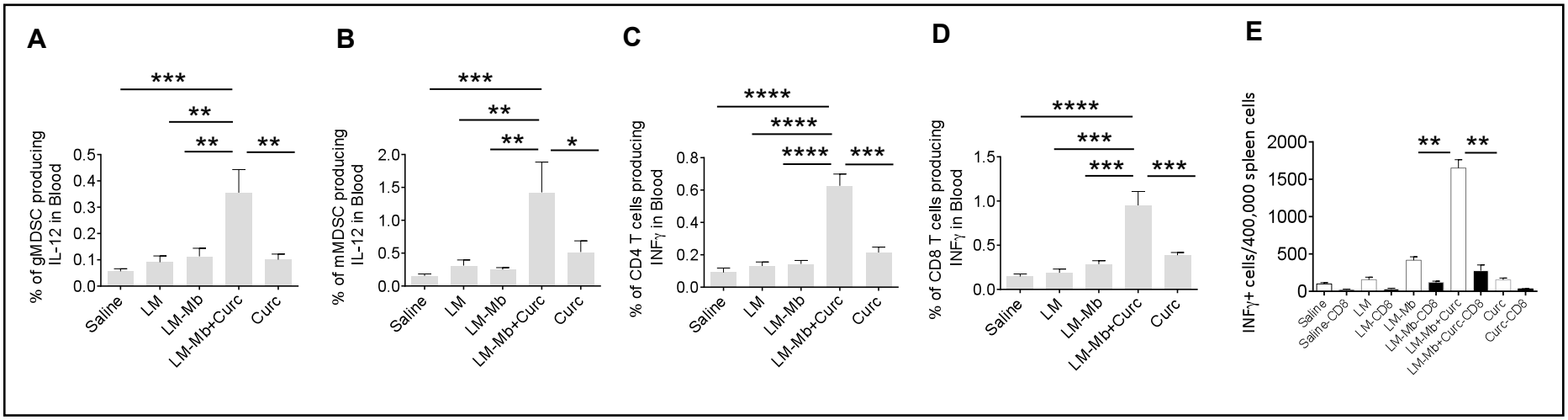

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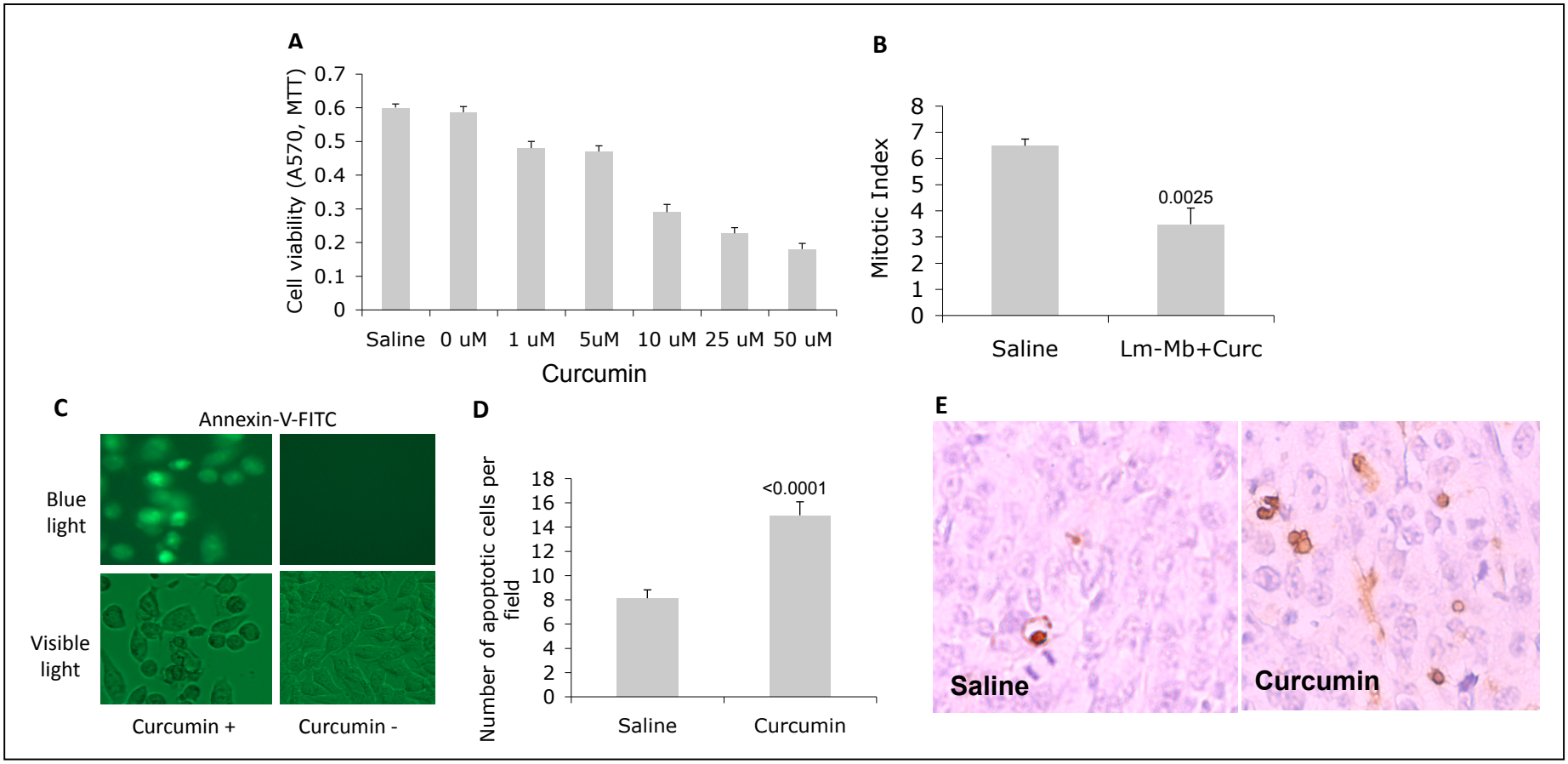

Supplementary information

Title: *Curcumin Improves the Therapeutic Efficacy of Listeria^{at}-Mage-b Vaccine in Correlation with Improved T Cell Responses in Blood of a Triple Negative Breast Cancer model 4T1*

Running Title: Curcumin improves therapeutic efficacy of Listeria^{at}-Mage-b

Authors: Manisha Singh[1], Ilyssa Ramos[1], Denise Asafu-Adjei[1], Wilber Quispe-Tintaya[1], Dinesh Chandra[1], Arthee Jahangir[1], Xingxing Zang[1], Bharat B. Aggarwal[2], and Claudia Gravekamp[1].

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Legends

Figure S1: Significant reduction in the tumor size by preventive administration of curcumin followed by therapeutic immunization with Listeria^{at}-Mage-b in 4T1 tumor-bearing mice. BALB/c mice were treated with curcumin before tumor development and immunized with Listeria^{at}-Mage-b after tumor development (Immunization protocol B), and analyzed for tumor weight during the treatments. This experiment was performed two times with 5 mice per group, and the results were averaged.

Figure S2: The combination therapy of Listeria^{at}-Mage-b and Curcumin is non-pathogenic and non-toxic. BALB/c mice were treated with Curcumin before tumor development and immunized with Listeria^{at}-Mage-b after tumor development (Immunization protocol B). Two days after the last immunization, mice were euthanized and liver sections were stained by H&E, followed by pathological examination. Extramedullary hematopoiesis (black arrow) was mild and multifocal in the liver (possibly tumor related) of both groups, i.e. Saline (negative control) and Listeria^{at}-Mage-b and Curcumin. There were also scattered infiltrates of immune cells (incidental background finding) found in the liver of both groups of mice**.** The boxed areas in the top (magnification 200x) are shown in a larger magnification at the bottom (Light microscopy Magnification: 600x). Representative of two experiments.

Figure S3: The effect of Listeria^{at}-Mage-b and Curcumin on MDSC in 4T1 tumor-bearing mice (Flow cytometry profile). BALB/c mice were treated with Curcumin before tumor development and immunized with Listeria^{at}-Mage-b after tumor development (Immunization protocol B), and analyzed for MDSC ($CD11b^+Gr1^+$)**(A),**

gMDSC ($CD11b^{+}Gr1^{high}$)**(B),** and mMDSC ($CD11b^{+}Gr1^{low}$) **(C)** in blood and for MDSC **(D),** gMDSC **(E),** and mMDSC **(F)** in primary tumors using flow cytometry.

Figure S1

Mann-Whitney

Sal vs LM p=0.2222
Sal vs LM-Mb p=0.0317
Sal vs LM-Mb+Curc p=0.0079
Sal vs Curc p=0.0496

LM-Mb+Curc vs LM-Mb p=0.0162
LM-Mb+Curc vs Curc p=0.0272 p<0.05 is significant

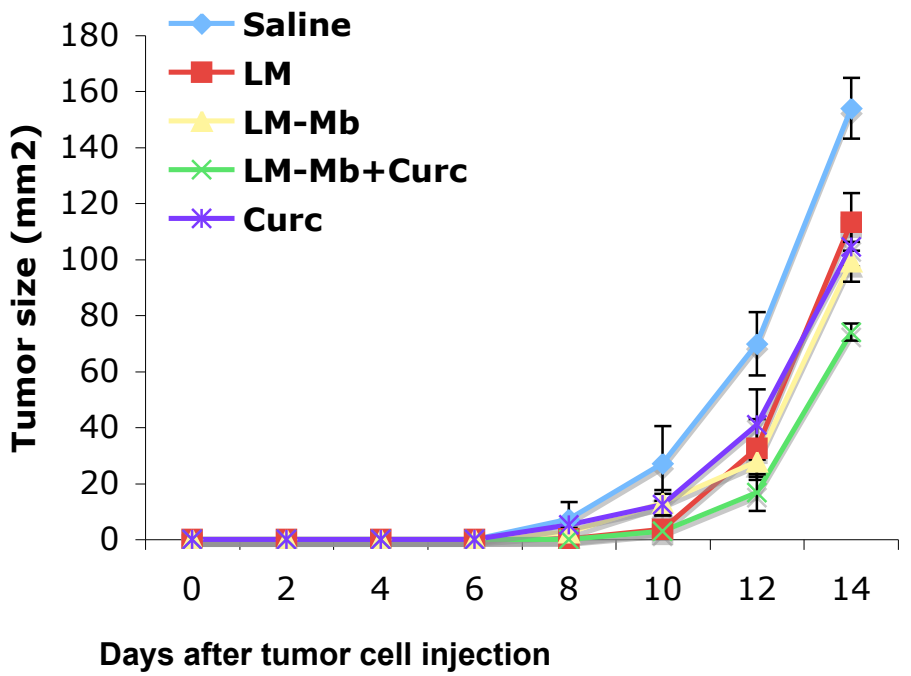
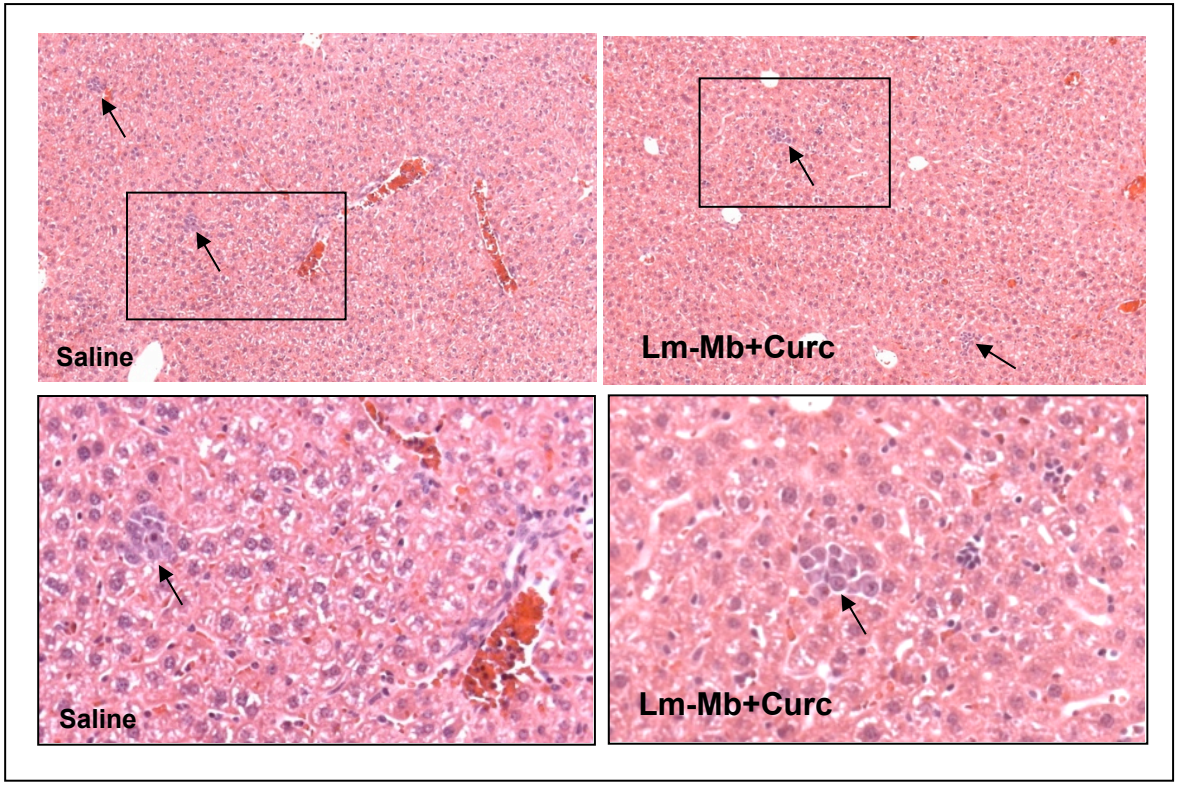


Figure S2



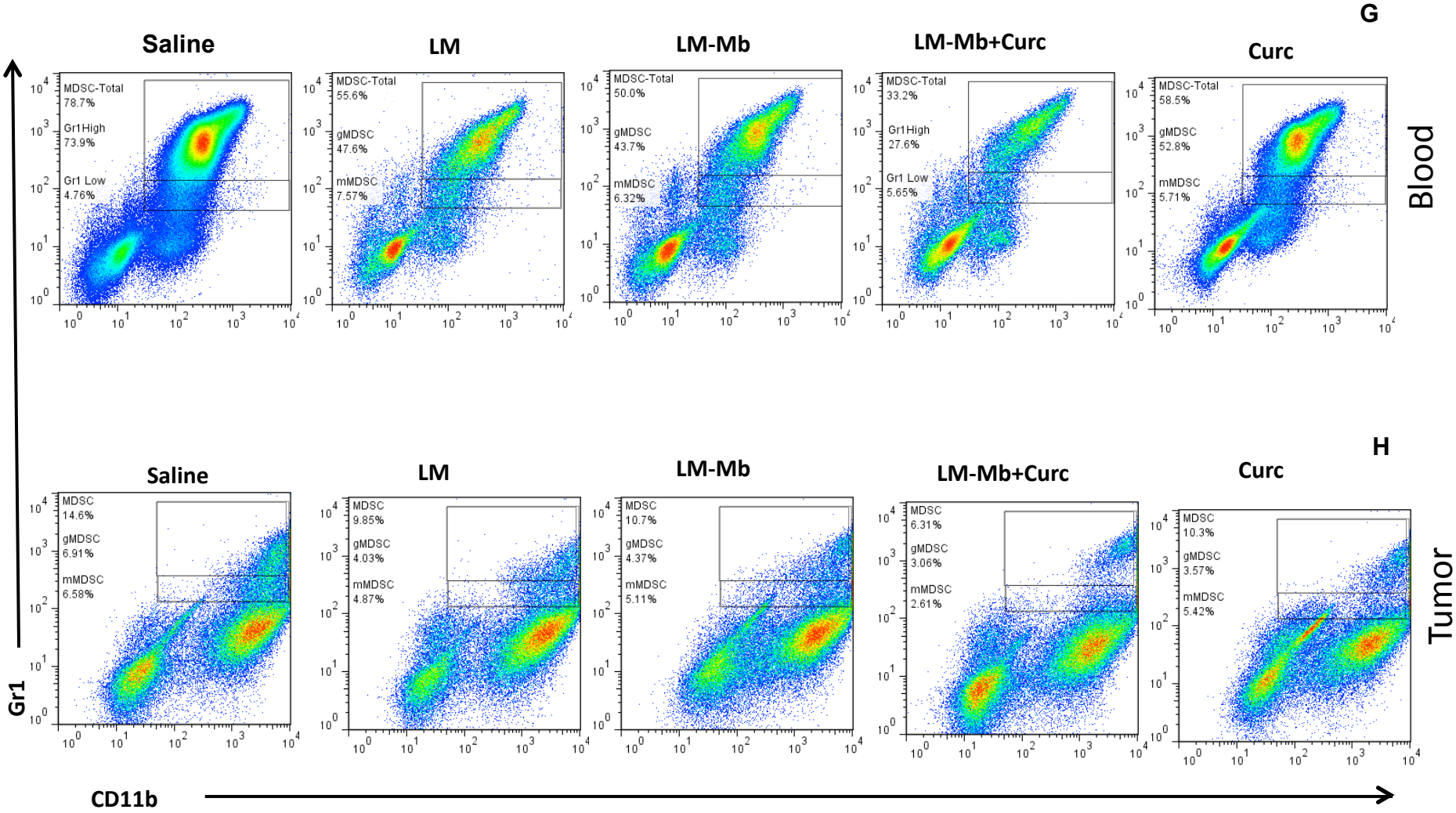

G
Saline
LM
LM-Mb
LM-Mb+Curc
Curc
Blood
MDSC-Total 78.7%
Gr1High 73.9%
Gr1 Low 4.76%
MDSC-Total 55.6%
gMDSC 47.6%
mMDSC 7.57%
MDSC-Total 50.0%
gMDSC 43.7%
mMDSC 6.32%
MDSC-Total 33.2%
Gr1High 27.6%
Gr1 Low 5.65%
MDSC-Total 58.5%
gMDSC 52.8%
mMDSC 5.71%
H
Saline
LM
LM-Mb
LM-Mb+Curc
Curc
Tumor
MDSC 14.6%
gMDSC 6.91%
mMDSC 6.58%
MDSC 9.85%
gMDSC 4.03%
mMDSC 4.87%
MDSC 10.7%
gMDSC 4.37%
mMDSC 5.11%
MDSC 6.31%
gMDSC 3.06%
mMDSC 2.61%
MDSC 10.3%
gMDSC 3.57%
mMDSC 5.42%
Gr1
CD11b

Table S1: Histological examination of tissues after therapeutic treatment with Listeria-Mage-b and curcumin

	Mouse number					Mouse number				
Organ	**S1**	**S2**	**S3**	**S4**	**S5**	**MC1**	**MC2**	**MC3**	**MC4**	**MC5**
Kidney	nsf	nsf	nsf	nsf	nsf	nsf	nsf	nsf	nsf	nsf
Heart	nsf	nsf	nsf	nsf	nsf	nsf	nsf	nsf	nsf	nsf
Lung	icn3	icn1	icn2	icn3	icn1	icn3	icn3	icn1	icn3	icn3
Liver	cn0	cn0	cn0	cn0	cn0	cn1	cn0	cn0	cn0	cn0
Liver	emh3	emh0	emh2	emh3	emh1	emh4	emh4	emh1	emh2	emh3
Liver	ipp1	ipp0	ipp1	ipp1	ipp0	ipp2	ipp2	ipp0	ipp1	ipp2
Liver	gmi1	gmi0	gmi1	gmi1	gmi1	gmi2	gmi3	gmi0	gmi1	gmi0
Spleen	imh2	imh1	imh2	imh3	imh1	imh3-4	imh3-4	imh0	imh4	imh3-4

Nsf=no-significant finding, **icn**=increased circulating neutrophils, **cn**=coagulation necrosis, **emh**=extramedullary hematopoiesis, **ipp**=infiltrate, portal, mixed polymorphonuclear cells, **gmi**=granulomas, mixed inflammation, **imh**=increased myeloid extramedullary hematopoiesis
The numbers after the abbreviations represents the grade. 0=no finding, 1=minimal finding, 2= mild finding, 3=moderate finding, 4=marked finding, 5=severe finding. S=saline, MC=Listeria^{at}-Mage-b and curcumin.

CHAPTER 3

Manuscript prepared for submission to Breast Cancer Research

Title: Direct incorporation of the NKT cell activator α-galactosylceramide improves efficacy and safety of a recombinant *Listeria monocytogenes* breast cancer vaccine

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Abstract

Introduction: Immune suppression in the tumor microenvironment (TME) remains a major limitation to successful immunotherapy of cancer, and a major focus of cancer research is the discovery of new adjuvants and vaccine regimens that overcome this suppression. In the current study, we developed a combination immunotherapy using an attenuated *Listeria monocytogenes*-based vaccine expressing the tumor-associated antigen (TAA) melanoma-associated antigen (Mage)-b (LM-Mb) and the natural killer T (NKT) cell-activating glycolipid α-galactosylceramide (αGC). This glycolipid has been previously identified as a potent immunological adjuvant because its ability to stimulate CD1d-restricted NKT cells, which drives a cascade of immune reactions that include potent stimulation of natural killer (NK) cell effector functions and cross-priming of antigen specific $CD8^+$ T cells.

Methods: Mice with metastatic breast cancer (4T1 model) were therapeutically treated with LM-Mb and αGC (3 immunizations) as separate agents or as a complex of αGC stably incorporated into Lm-Mb (5 immunizations). Two days after the last treatment, the mice were analyzed for the number of metastases, tumor weight, toxicity, and immune responses.

Results: We found that sequential treatments of mice with established 4T1 breast carcinomas using LM-Mb followed by αGC was highly effective at reducing metastases, but was accompanied by severe liver toxicity. In contrast, the simultaneous administration of these two agents, using a method to stably incorporate αGC into the live LM-Mb organisms, resulted in a therapeutic vaccine that reduced the metastases by more than 99% without evidence of toxicity. The anti-metastatic response in mice immunized with LM-Mb incorporated with αGC was associated with a significant increase in IL-12 production, increased NK cell activity and an increase in T cell

responses to Mage-b. This combination therapy did not result in depletion or anergy of NKT cells, which has been repeatedly identified as a problem resulting from treatment of mice with αGC alone.

Conclusions: These results define a highly promising new approach for combining two immunotherapeutic agents in one complex of Lm-Mb and αGC to create an efficacious and non-toxic vaccine regimen for prevention of metastatic breast cancer.

Introduction

Breast cancer is the most common cancer among women world-wide [1], and 30% of women diagnosed with breast cancer will progress to metastatic disease which is difficult or impossible to treat effectively [2]. Current treatment options for metastatic cancer include surgery followed by chemotherapy or radiation, or other adjuvant therapy [3]. Despite aggressive treatment, for most patients the elimination of metastases or residual tumor cells after initial treatment is incomplete, and removal of residual disease by chemotherapy is prevented by chemoresistance [4]. Thus, metastases and not the primary tumor is the most important contributor to breast cancer morbidity and mortality.

It has been shown in mice and humans that vaccines can have a favorable effect on metastases [5],[6],[7],[8],[9],[10],[11], but that vaccine efficacy is strongly reduced by immune suppression in the tumor microenvironment (TME) [12]. In a previous study, we developed a vaccine based on a non-pathogenic strain of *Listeria monocytogenes* (LM) for the delivery of the tumor-associated antigen (TAA) Mage-b (LM-Mb) *in vivo*, (11). LM is an intracellular bacterium which has the capacity to deliver antigens through infection into antigen-presenting cells (APC) such as dendritic cells (DC), monocytes and macrophages with high efficiency [13]. We have also demonstrated that LM infects tumor cells, which can lead to cytolytic effects through a mechanism involving induction

of high levels of reactive oxygen species (ROS), and sensitizes the infected tumor cells for recognition by LM-specific $CD8^+$ T cells [14]. When administered prior to tumor establishment in an aggressive mouse model of metastatic breast cancer (4T1), LM-Mb treatment resulted in strong $CD8^+$ T cell responses to both Mage-b and LM and an almost complete elimination of metastatic disease [11]. However, when administered in a therapeutic immunization regimen (i.e., after establishment of primary 4T1 tumors), LM-Mb treatment was only moderately effective against metastatic breast cancer, and induced relatively weak $CD8^+$ T cell responses to Mage-b [14]. This failure to stimulate adequate $CD8^+$ T cell responses in tumor-bearing hosts is indicative of the chronic immunosuppression associated with the TME, and represents a major problem in cancer vaccination.

To overcome the immune suppression that is characteristic of tumor-bearing hosts, there is an urgent need for development of immunologic adjuvants that can promote robust immune responses in this setting and augment the effects of therapeutic vaccines. Glycolipids of the α-galactosylceramide family (αGC) represent one potentially useful class of adjuvants that have shown promise in preclinical studies for immunotherapy of cancers [15]. These glycolipids mediate their effects on the immune system by binding to an MHC class I-like molecule called CD1d, creating a complex that is recognized by a population of conserved effector lymphocytes known as natural killer T cells (NKT cells) [16],[17]. Several subsets of NKT cells have been defined, with the most abundant being the so-called type 1 or invariant NKT cell (iNKT) subset which is highly responsive to αGC and highly conserved between primates and mice [18],[19]. It has been shown that formation of intracellular complexes of CD1d with αGC in antigen presenting cells initiates rapid NKT cell activation [20], resulting in the production of Th1-associated cytokines such as IFNγ and IL-12p70, maturation of the $CD8\alpha^+$ DCs in the

lymph nodes and subsequent activation of NK and conventional T cells [17]. This cascade of immune reactions that is initiated by NKT cells in response to αGC has been shown in mouse models to generate innate and adaptive immunity against a wide range of cancers and infections [21],[22],[23],[20],[24]. Based on these observations we hypothesized that addition of αGC to the LM-Mb vaccine could improve the vaccine efficacy, in part through enhancement of specific T cell responses to Mage-b.

Here, we developed a therapeutic immunization protocol for the combination of LM-Mb and αGC, and developed a safe and effective method for delivering this immunotherapy to tumor bearing mice. When these two agents were used as combination therapy in the 4T1 model, αGC significantly improved the therapeutic vaccine efficacy of LM-Mb as demonstrated by the almost complete elimination of the metastases. However, the administration of these two agents sequentially, using a series of LM-Mb injections followed by a series of systemic αGC injections, caused severe and in some cases fatal toxicity to the liver. Therefore, we explored other novel strategies to improve the vaccine efficacy of Lm-Mb but at lower dose of αGC. Drawing on previous experience using direct incorporation of relatively low doses of αGC into live *Mycobacterium bovis* BCG to improve vaccine efficacy [25], we developed a similar approach for direct incorporation of the glycolipid into live LM organisms. Therapeutic immunizations with αGC directly incorporated into live LM-Mb was equally effective against the metastatic breast cancer compared to sequential administration of LM-Mb and αGC as separate agents, but without any apparent toxicity. The powerful anti-metastatic effect of vaccination with LM-Mb modified by direct incorporation of αGC correlated with increased IL-12 production and improved Mage-b-specific $CD8^+$ T cell and NK responses. This novel approach using incorporation of αGC into live LM may

provide a basis for new strategies to improve vaccine efficacy against metastatic cancer through augmentation of multiple innate and adaptive immune mechanisms.

Materials and Methods

Mice

Normal 3 month old female BALB/c mice were obtained from Charles River Laboratories and maintained in the animal husbandry facility at Albert Einstein College of Medicine according to the Association and Accreditation of Laboratory Animal Care (AALAC) guidelines. All mice were kept under biosafety level 2 conditions as required for LM vaccinations.

Cells and cell culture

The TNBC 4T1 cell line, derived from a spontaneous mammary carcinoma in a BALB/c mouse [26], was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM mixed nonessential amino acids, 2 mM L-glutamine, insulin (0.5 USP units/ml), penicillin (100 units/ml) and streptomycin (100 µg/ml).

LM based vaccine

The Lm-Mb was developed in an earlier study [11]. This was constructed in the *prfA* negative XFL-7 strain, which lacks the positive regulatory factor A that is a central mediator of virulence [27]. The vaccine strain was transformed with LM plasmid pGG-34, which encodes prfA and amino acids 311-660 of murine Mage-b fused to a non-cytolytic form of Listeriolysin O (LLO) [28]. Complementation of prfA expression by the plasmid does not fully restore virulence, but enforces retention of the plasmid during infection [27] [28].

Incorporation of α-galactosylceramide into live LM-Mb

The αGC used in this study was [(2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol], also known in previous studies as KRN7000 or αGalCer-C26:0. This was synthesized as previously described [29], and was stored as solvent-free aliquots in glass vials at -20°C. The glycolipid was reconstituted either in 100% DMSO at 100μM for *in vitro* studies, or in aqueous vehicle consisting of PBS with 0.05% Tween 20 and 0.1% DMSO at 500 μM for *in vivo* studies. The incorporation of αGC into live LM was done using a method similar to that described previously for *Mycobacterium bovis* BCG [25]. Briefly, αGC was solubilized at a concentration of 2.3 μM in glass vials by addition of warm (37°C) BHI medium containing 5% tyloxapol, followed by sonication for 5 min, heating at 80°C for 2 min, and vortexing for 1 min. 450 μl of the 2.3 μM solubilized glycolipid was immediately diluted into 50 ml of warm BHI to give the required final concentrations of 20 nM glycolipid and 0.05% tyloxapol. 500 μl of a LM-Mb mid-log phase culture (OD_{600} 0.5– 0.8) was inoculated into this, and grown to mid-log phase (OD_{600} 0.5–1.0), which generally required 4–6 hours. The bacterial culture was aliquoted in 1 ml vials and frozen at -80°C. For subsequent use, the bacteria were thawed, harvested by centrifugation, washed three times with saline and resuspended in saline for injection. In order to determine the incorporation of αGC into LM, we determined the activation of iNKT cell hybridomas by LM or αGC-incorporated LM infected BMDCs *in vitro* as described below. As shown in **Figure S1,** BMDCs infected with αGC-incorporated LM elicit a strong iNKT cell response, confirming the efficiency of the αGC incorporation.

Immunization and tumor challenge

Mice were challenged with 4T1 tumor cells as described previously [11], and then treated using therapeutic vaccination regimens to compare the effects of combined LM-Mb and αGC treatment either as separate agents or as glycolipid-modified bacteria. Briefly, two different immunization protocols were tested. The first immunization protocol consisted of three therapeutic immunizations with LM-Mb and αGC. Mice received 1 x 10^4 4T1 tumor cells in the mammary fat pad on day 0, 1 x 10^4 CFU of LM-Mb or LM or saline i.p. on days 3, 6, and 9, and αGC i.p. at the indicated dosage on days 14,15 and 16. The second immunization protocol consisted of five therapeutic immunizations with LM-Mb modified by direct incorporation of αGC (I-αGC-LM-Mb). Mice received 1 x 10^4 4T1 tumor cells in the mammary fat pad on day 0, and, five therapeutic immunizations with 1 x 10^4 CFU I-αGC-LM-Mb injected i.p. on days 3, 6, 9, 12 and 15. In studies carried out for analysis of the extent of metastatic disease, all mice were euthanized on day 18, which was prior to death in saline treated animals, and analyzed for metastases and tumor growth. Primary tumors extended to the chest cavity lining, and predominantly metastasized to the mesenteric lymph nodes (MLN) (81%), and less frequently to the diaphragm (7%) and portal liver (4%), as well as to the surface of spleen (4%) and kidneys (4%). Metastases were visible to the naked eye as nodules. The total number of metastases per mouse (MLN, diaphragm, liver, kidney and spleen) was determined as previously described [11]. For studies carried out to determine effects on survival, mice were maintained until they succumbed spontaneously, or were terminated upon appearance of severe pre-morbid symptoms requiring euthanasia as specified by our approved animal use protocol.

Activation of iNKT cells *in vivo*

BALB/c mice were immunized i.p with the inert vehicle (PBS plus 0.05% tyloxapol), 1 x 10^4 CFU LM, 1 x 10^4 CFU I-αGC-LM, or 4 nmoles of free αGC (dissolved in PBS plus 0.01% Tween 20 plus 0.1% DMSO). Sera were assayed at the indicated times for IL-4, IL-12p70, and IFN-γ by capture ELISA as previously described [30]. Alternatively, BALB/c mice receiving 4T1 tumor cells and therapeutic immunizations with LM-Mb or I-αGC-LM-Mb (see above), were immunized i.p with 4 nmoles of free αGC 15 days after the injection of tumor cells. After the indicated times, splenocyte single cell suspensions were obtained and stained with anti mouse TCR-FITC (clone H57-597, BD Biosciences) and αGC-loaded mouse CD1d tetramers-APC, prepared as previously described[30] Samples were acquired using a LSR II Flow Cytometer (BD Biosciences) and analyzed using FlowJo software.

Flow cytometry analyses

Cells were isolated from spleen and blood as described previously[31] [32]. Briefly, red blood cells were lysed according standard protocols, and the remaining leukocyte population was used for analysis. Cells were first incubated with an Fc blocker (anti-CD16), and subsequently with specific fluorochrome-conjugated antibodies for the identification of different cell types. Anti-CD49b-PerCP5.5 and anti-CD8-PE antibodies were used to identify NK cells and CD8 T cells, respectively, and anti-Gr1-PerCP5.5, CD11b-Alexa 488 antibodies were used to identify MDSC. Anti-CD45-APC antibody was used to identify the leucocyte population in tumor cell suspensions. To detect the production of intracellular cytokines, the cytofix/cytoperm kit from Pharmingen was used according to the manufacturer's instructions, and antibodies to IL-12p70 and IFNγ, were used. Appropriate isotype controls were used for each sample. Depending on the sample size, data from between 1 x 10^4 and 2 x 10^5 cells were acquired using a FACS

Calibur flow cytometer (BD Biosciences), and analyzed using Flowjo software as described previously [32]. Cell debris and dead cells were excluded from the analyses based on forward and side scatter signals and use of Fixable Blue or Green Live/Dead Cell Stain Kit (Invitrogen). All antibodies were purchase from BD Biosciences.

ELISPOT

Spleen cells were isolated from vaccinated and control mice with 4T1 tumors and analyzed for T cell responses by ELISPOT as described previously [11]. To detect LM-induced immune responses, 2 x 10^5 spleen cells were infected with 2 x 10^5 CFU of LM for 1 hour, and subsequently treated with gentamicin (50 μg/ml) until the end of re-stimulation (72 hrs). To detect TAA-specific immune responses, 4 x 10^5 spleen cells of vaccinated or control mice were transfected with pcDNA3.1-Mage-b and pCMV-GM-CSF using Lipofectamine 2000, as described previously [11]. 72 hrs later, the frequency of IFNγ-producing cells was determined by ELISPOT according to standard protocols (BD Biosciences, San Diego, CA), using an ELISPOT reader (CTL Immunospot S4 analyzer, Cellular Technology Ltd, Cleveland, OH). To determine the $CD8^+$ T cell component of the responses, spleen cells were depleted of $CD8^+$ T cells using magnetic bead depletion techniques according to the manufacturer's instructions (Miltenyi).

Assessment of toxicity

Several parameters were used to analyze the toxicity of therapy. Survival was followed for up to 18 days, and survival curves plotted for the various treatment groups. Liver toxicity was assessed by visual inspection following sacrifice, and a numerical grade was assigned corresponding to the size and number of visible necrotic plaques. The toxicity was graded as follows: 0 = no lesions (normal appearance), 1 = uniform light discoloration and firmness, 2 = white plaques visible covering ~5% of the liver surface, 3

= white plaques covering ~10% of liver surface, 4 = white plaques covering ~20% of liver surface, 5 = white plaques covering ~30% of liver surface. Hematoxylin and Eosin (H&E) staining of thin sections of livers was also done to confirm the presence and extent of hepatic inflammation and necrosis. Briefly, liver tissues were fixed in 10% formaldehyde for 48 hrs, and then kept in 70% ethanol until use. Sections of 1 mm thick were stained with H&E, and analyzed for pathological damage by light microscopy. All pathological analyses were performed by a trained veterinary pathologist in the Histology and Comparative Pathology Core Facility, Albert Einstein College of Medicine.

Statistical analysis

To statistically analyze the effects of Listeria^{at} and αGC on the growth of metastases and tumors and immune responses in the 4T1 model, the Mann-Whitney test and for the survival studies the Mantel-Cox test were used. Values $p<0.05$ were considered statistically significant. *p<0.05, **<0.01, ***<0.001, ****<0.0001 is significant.

Results

Efficacy of combination therapy with LM-Mb and αGC in metastatic breast cancer

The therapeutic effect of combining the Lm-Mb vaccine with αGC treatment on metastatic breast cancer was assessed in the 4T1 transplantable mouse tumor model. This combination was tested with αGC administered as a free glycolipid, and also using a protocol to physically incorporate the glycolipid into the live bacterial vaccine. This latter approach has been shown by us in previous work on the mycobacterial vaccine strain BCG, in which αGC incorporation was successfully achieved, to elicit more robust CD8+ T cell responses [25]. Mice treated with unmodified LM-Mb showed marked

reduction in metastases when sacrificed at day 18 after initial tumor implantation **(Figure 1A)**, consistent with previous studies showing that this treatment induces a variety of tumoricidal mechanisms**.** An improved anti-metastatic effect was observed with LM-Mb compared to LM, suggesting an effect of Mage-b specific immunity, and also with αGC administered as a single agent. Strikingly, the combination of αGC with LM-Mb, either as a separately administered agent (LM-Mb + αGC) or by direct incorporation of the glycolipid into the live bacteria (I-αGC-LM-Mb), gave a significantly better anti-metastatic effect compared to LM-Mb alone or αGC alone. Both of the regimens using combined LM-Mb and αGC treatment reduced the number of macroscopically visible metastases nearly to zero, with only a rare nodule being detected in these mice. Significant reductions in the weights of primary tumors were also observed in all treatment groups, although combination treatment did not show significant improvement over single agent treatment in this parameter **(Figure 1B).**

Direct incorporation of αGC into LM-Mb avoids severe toxicity

Although αGC appeared to be equally efficacious when administered as a free glycolipid following LM-Mb treatment or simultaneously using the direct incorporation approach, analysis of survival of treated mice revealed a striking difference between these two approaches. Whereas all untreated 4T1 bearing mice (i.e., saline injections only) survived at least 18 days from the time of tumor initiation, we observed a significant fatality rate starting around day 11 in animals treated with the sequential administration of LM-Mb and αGC as separate agents. In contrast, no deaths were observed over this time period in any of the other treatment groups, including those which received LM-Mb directly incorporated with αGC (I-αGC-LM-Mb) **(Figure 2A).** Visual inspection revealed obvious white plaques on the surface of the livers only in animals receiving the

separately injected LM-Mb and αGC, suggesting focal areas of hepatic necrosis. This was verified by histologic studies of thin sections of the liver, which revealed foci of necrosis in mice that received the two agents as separate series of injections **(Figure 2B**). Such foci were also observed with lower frequency and smaller size in mice that received αGC alone, but not at all in animals that were treated with I-αGC-LM-Mb or LM-Mb alone **(Figure 2C).** We then carried out a more extended survival study to compare time to death in 4T1 tumor bearing mice receiving therapeutic vaccination with I-αGC-LM-Mb versus mice receiving only saline injections. As shown in **Figure 2D,** while mice that received only sham immunizations with saline all succumbed by day 20, mice that received I-αGC-LM-Mb all survived past day 20 and showed a significant extension (30%) of overall survival. This extension of survival was consistent with the marked anti-metastatic effect and low toxicity of the I-αGC-LM-Mb treatment.

Activation of NKT cells in spleens of 4T1 tumor-bearing mice that received I-αGC-LM-Mb

To determine whether Listeria^{at} bacteria modified by direct incorporation of αGC could activate NKT cells *in vivo*, we assessed the rapid production of IFNγ and IL-4, two cytokines that are charactistically produced by NKT cells. For this purpose we injected naïve mice once with LM, αGC or I-αGC-LM and obtained serum samples at various time points after injection to determine cytokine levels. Previous studies have shown that αGC administered as a free glycolipid induces the production of IFNγ and IL-4 which peak in the serum at approximately 12 hours and 2 hours, respectively [25]. Infection with LM also induces the production of IFNγ by NKT cells and macrophages [33]. We found that I-αGC-LM stimulated a serum IFNγ response that was apparent at 12 hours, and peaked at 24 hours. In contrast, LM infection generated a large transient serum

IFNγ response which was first detected at 24 hours **(Figure 3A).** The accelerated IFNγ production seen with I-αGC-LM was consistent with direct NKT cell activation by the αGC incorporated into the bacteria. Also supporting the conclusion that NKT cells were directly activated, we observed a significant IL-4 response at 2 hours after injection of I-αGC-LM, whereas LM alone did not stimulate detectable IL-4 **(Figure 3B).** To confirm that NKT cells were rapidly activated by treatment with I-αGC-LM-Mb in tumor-bearing mice *in vivo*, we analyzed the percentages of NKT cells in the spleens of 4T1 tumor-bearing mice that were therapeutically immunized with I-αGC-LM-Mb using CD1d tetramers loaded with αGC. The percentage of NKT cells stained with the tetramer reagent detected in the spleens of tumor-bearing mice that had received 5 therapeutic treatments with I-αGC-LM-Mb was either unchanged or slightly increased compared to saline or LM treated controls (**Figure 4AB).** This indicated that administering αGC in this form did not cause systemic depletion of NKT cells, which is a potential problem that has been associated with treatments using systemic repeated administrations of αGC as a free glycolipid. Furthermore, when similarly treated mice were injected with free αGC, their tetramer binding NKT cells showed a transient decrease in the spleen at 12 hours, followed by a rebound to greater than baseline levels at 72 hours **(Figure 4AB).** This pattern was consistent with the normal activation pattern observed for intact NKT cell populations in healthy naïve mice, which is characterized by TCR down modulation leading to loss of tetramer staining at earlier time points followed by TCR re-expression and expansion of the tetramer staining population due to proliferation by 72 hours. In fact, both LM-Mb and I-αGC-LM-Mb showed significant increases in the expansion of NKT cells at 72 hours, with I-αGC-LM-Mb showing the greater effect **(Figure 4AB).**

In summary, these results indicated that NKT cell activation by repeated I-αGC-LM-Mb treatments did not lead to either depletion or anergy of NKT cells *in vivo*,

and may actually have primed NKT cells to respond more vigorously to subsequent stimulation.

The effect of I-αGC-LM-Mb treatment on circulating MDSC

In mice, MDSC express both the myeloid lineage differentiation antigen Gr1 (Ly6C and Ly6G) and the α_M integrin CD11b. Two major groups of MDSC have been described: $CD11b^+Gr1^{high}$ ($CD11b^+Ly6G^+Ly6C^{low}$) with a granulocytic phenotype (gMDSC), and $CD11b^+Gr1^{low}$ ($CD11b^+Ly6G^-Ly6C^{high}$) with a monocytic phenotype (mMDSC) [34, 35]. MDSCs are present in large numbers in blood of mice and humans with cancer [36, 37]. We found that the percentage of MDSC was extremely high in blood of the 4T1 model (~80%), while in non-tumor-bearing mice MDSC hardly detectable (~3%)**(Figure S2AB).**

Since LM has been shown to infect MDSC[38], we analyzed the effect of I-αGC-LM-Mb on the levels of these circulating MDSC. Although our regimen of five therapeutic injections with αGC alone reduced the percentage of total and gMDSC in the blood of tumor-bearing mice by 39% compared to the saline group, all other treatment groups including I-αGC-LM-Mb had little effect on the levels of these cells **(Figure 5A).**

MDSC strongly suppress T cell and NK cell responses in the TME [39],[40]. Moreover, they are associated with the production of immunosuppressive cytokines such as IL-10 [41] and suppression of pro-inflammatory IL-12p70, [17],[42]. Therefore, we analyzed the production of IL-12p70 by gMSDC and mMDSC populations in the blood of 4T1 tumor bearing mice treated with our various regimens. All treatments showed a trend toward increased IL-12p70 production by both circulating myeloid cell subsets, but this rose to a statistically significant level only in mice treated with I-αGC-LM-Mb, and only in mMDSC **(Figure 5B).** We also assessed IL-12p70 production by MDSC isolated from primary tumors of these mice, but significant levels of IL-12 were not observed (data

not shown). Overall, these results indicated a significant effect of I-αGC-LM-Mb treatment on mMDSC, and a shift to more pro-inflammatory and less suppressive function was observed in MDSC outside of the TME.

Improved T cell and NK cell responses in mice vaccinated with I-αGC-LM-Mb

The activation of NKT cells has been frequently shown to lead secondarily to increased $CD8^+$ T cell cross-priming and to NK cell activation, and both of these processes are assisted by IL-12p70 [17]. Given that NKT cell activation and IL-12p70 production by mMDSC were both augmented by treatment of tumor bearing mice with I-αGC-LM-Mb, we analyzed whether this treatment could also enhance $CD8^+$ T cell and NK cell responses using the production of IFNγ as an activation marker. First, we analyzed $CD8^+$ T cell responses to Mage-b in the spleens of I-αGC-LM-Mb treated and control mice upon re-stimulation with Mage-b *in vitro* by measuring extracellular IFNγ using ELISPOT. This showed a significantly higher number of Mage-b-specific $CD8^+$ T cells producing IFNγ in the spleens of mice treated with I-αGC-LM-Mb than in mice treated with either LM-Mb or αGC alone **(Figure 6A).** Similarly, by enumerating CD8 T cells producing intracellular IFNγ (as a results of the *in vivo* treatments) in peripheral blood samples by flow cytometry (without *in vitro* re-stimulation), a higher response was evident in mice treated with I-αGC-LM-Mb compared to mice treated with Lm-Mb or αGC alone **(Figure 6B).** These results indicated a superior effect of I-αGC-LM-Mb on stimulating the cross-priming of CD8+ T cells specific for tumor associated antigens.

We also analyzed NK cell responses by measuring extracellular IFNγ using ELISPOT in I-αGC-LM-Mb treated and control mice following *in vitro* infection of splenocytes with LM. This showed significant increase in the number of IFNγ-producing NK cells in I-αGC-LM-Mb treated mice compared to mice treated with saline, or with LM-

Mb or αGC alone **(Figure 6C).** In addition, analysis of the intracellular IFNγ production by NK cells in blood (as a result of *in vivo* treatments) by flow cytometry (without any re-stimulation *in vitro*), showed that the percentage of activated circulating NK cells was significantly higher in mice that received I-αGC-LM-Mb than LM-Mb or αGC alone, or saline **(Figure 6D).**

Discussion

While treatment with αGC as a free glycolipid injection has shown remarkable anti-tumor activity in a variety of mouse models of cancer, phase I clinical trials of this approach in human cancer patients have not shown clear evidence of therapeutic benefit[43] Improvements in this approach by using immunizations with αGC pulsed autologous DCs, or by infusing *ex vivo* expanded NKT cells, are currently being studied. While these approaches may increase the ability to harness anti-tumor properties of NKT cells for cancer immunotherapy, they are complicated and difficult to administer. In the study presented here, we have developed a simple approach for combining αGC with a *Listeria monocytogenes*-based tumor vaccine to achieve strong synergistic effects, particularly in the suppression of metastatic disease. We showed that αGC significantly improved therapeutic efficacy of a LM-based vaccine expressing the TAA Mage-b in a highly metastatic mouse breast tumor model using the transplantable 4T1 cell line. The combination of LM-Mb with αGC, with the glycolipid administered either as a separate series of injections or directly incorporated into the LM-Mb bacteria, was highly effective at reducing the number of metastases, and almost completely eliminated grossly visible metastatic nodules. Most significantly, while the administration of LM-Mb and αGC as separate injections in sequential fashion was associated with marked toxicity due to

hepatic necrosis, we found that the direct incorporation of the glycolipid into LM-Mb completely eliminated the toxicity while still preserving the marked clinical benefit.

The potential for αGC to induce foci of necrosis in the liver of mice is well documented, and is believed to be related to the very high frequency of NKT cells that are resident in the liver in this species [44]. Humans have a much lower frequency of NKT cells in liver tissue [45, 46], and hepatic toxicity following administration for αGC to humans has not been observed [47, 48]. Nevertheless, the combination of LM-Mb and αGC has never been tested in humans, and the possibility that infection with LM could prime the liver to become sensitive to toxic effects of αGC is an important consideration. Therefore, our alternative method using live LM-Mb with αGC directly incorporated into the bacteria (I-αGC-LM-Mb) represents an extremely practical and potentially safer approach to administering this combination immunotherapy. To produce I-αGC-LM-Mb, we used an approach that was similar to that used previously to successfully incorporate αGC into live *M. bovis* BCG organisms [25]. This method involves simply growing the bacteria in suitable protein-free medium in the presence of low concentrations of a detergent (tyloxapol) and the synthetic glycolipid. In studies with BCG, we found that approximately 25-35% of the glycolipid became stably associated with the bacteria, most likely through direct intercalation into the bacterial cell wall and membrane. Although the extent and mechanism of association of αGC with LM bacteria modified in this way have not been studied, the glycolipid incorporation resisted extensive washing of the modified bacteria suggesting possible intercalation into the bacterial membrane. The LM organisms modified in this way maintained full viability, and acquired the ability to rapidly activate NKT cells in culture (data not shown) and in vivo (Figure 3).

Although the incorporation of αGC into LM-Mb was similar to the *M. bovis* BCG, their mechanisms in activating T cells may be different. Since LM infects CD8α+DC [49],

we expect that I-αGC-LM-Mb also infects and matures these DC. Moreover, it is likely that the high efficiency of infection of CD8α+DC with LM, resulted in a much higher intracellular concentration of αGC compared to the uptake of free αGC. In addition to monocytes, macrophages and CD8α$^+$ DC, LM also infects MDSC [38], which is particularly important in cancer because they are present in large numbers in blood of tumor bearing humans and mice [36], including the 4T1 model as we have shown in this study. These cells are known for their strong suppression of T cells and NK cells in the TME through the production of cytokines such IL-6, IL-10, and TGFβ, or factors such as arginase and iNOS [39], [12]. Our analyses of circulating MDSC populations in mice immunized with I-αGC-LM-Mb suggested a conversion of mMDSC, and less pronounced of gMDSC, to an immune-stimulating phenotype producing IL-12p70. This correlated with improved CD8$^+$ T cell responses to Mage-b and increased NK cell activation *in vitro* and *in vivo*. Others have shown that αGC or LM increases the production of IL-12p70 in naïve mice, resulting in improved T cell responses [42]. Our results suggest that the IL-12p70 induced by I-αGC-LM-Mb in the 4T1 model may have improved the T cell responses to Mage-b and NK cell responses to LM. The metastases and primary tumor highly express Mage-b and are therefore a target for Mage-b-specific CD8 T cells. In a previous study we have shown that LM also infects tumor cells and activates CD8 T cells and NK cells[14]. Therefore, LM-infected tumor cells are a sensitive target for LM-activated T cells [14], which may also have contributed to tumor cell destruction *in vivo*.

While I-αGC-LM-Mb was extremely effective against the metastases, it was less effective against the primary tumors. In humans and mice the number of MDSC in the blood is much higher than in the primary tumor [36]. We observed increased levels of IL-12p70 in mMDSC by I-αGC-LM-Mb in the blood, but not in the primary tumors. Similarly, improved T cell activation by I-αGC-LM-Mb was detected in the blood but not in the

primary tumors. These may be the reasons why our I-αGC-LM-Mb treatment were more effective against the metastases, that usually spreads via the blood stream, than against the primary tumors.

Conclusions

In summary, we demonstrated that a novel combination of a recombinant LM expressing Mage-b and directly incorporated with αGC almost completely eliminated metastases in the 4T1 model without toxicity. Our results suggest that activation of NKT cells, NK cells and CD8 T cells, as well as the interaction between I-αGC-LM-Mb and mMDSC have contributed to this success. The almost complete elimination of the metastases is of crucial importance because patients usually die of metastases and not of their primary tumor. Moreover, standard therapies such as surgery, chemotherapy and radiotherapy are quite successful against primary tumors, but not against metastases. Therefore, standard therapy to eliminate the primary tumor combined with a treatment of I-αGC-LM expressing and appropriate TAA to eliminate metastases could be a promising new approach to treat metastatic breast and perhaps other metastatic cancers.

Abbreviations

APC: antigen-presenting cell; αGC: alphagalactosylceramide; CTL: cytotoxic T lymphocytes; DC: dendritic cell; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; gMDSC: granulocytic myeloid-derived suppressor cells; iNOS: inducible nitric oxide synthetase; IL: interleukin; I-αGC-LM-Mb: LM-Mb incorporated with αGC; IFN: interferon;LLO: listeriolysin O; LM: listeria monocytogenes; LM-Mb: Listeria-Mage-b;

MAGE: melanoma-associated antigen; MDSC: myeloid-derived suppressor cells; mMDSC: monocytic myeloid-derived suppressor cells; MHC: major histocompatibility complex; NK: natural killer; NKT: natural killer T; prfA: positive regulatory factor; ROS: reactive oxygen species; TME: tumor microenvironment; TAA: tumor-associated antigen; TAM: Tumor-associated macrophages; TN: triple-negative; TNBC: triple negative breast cancer

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Figure Legends

Fig 1: Therapeutic immunizations with Lm-Mb+αGC or I-αGC-LM-Mb are equally effective against metastatic breast cancer (4T1 model). BALB/c mice were challenged with 4T1 tumor cells in the mammary fat pad and immunized therapeutically (ip) three times with Listeria^{at}-Mage-b (LM-Mb) and α-galactosylceramide (αGC) as combination (LM-Mb+αGC) or five times with LM-Mb incorporated with αGC (I-αGC-LM-Mb). Mice were euthanized one day after the last immunization and analyzed for the frequency of metastases **(A)** and tumor weight **(B).** This experiment was performed three times with 5 mice per group, and the results were averaged. Mann-Whitney $p<0.05$ is significant. *<0.05, **<0.01, ***<0.001, ****<0.0001. The combination of LM-Mb+αGC or I-αGC-LM-Mb was compared to the LM-Mb or αGC alone, and I-αGC-LM-Mb was compared to LM-Mb+αGC. All groups were significantly different from the saline group. The error bars represent the standard error of the mean (SEM).

Fig 2: αGC is toxic in combination with LM-Mb but not when incorporated into LM-Mb in 4T1 tumor bearing mice.

BALB/c mice were challenged with 4T1 tumor cells and immunized with LM-Mb+αGC or I-αGC-LM-Mb and controls as described in Fig. 1. The percentage of live animals was determined in all groups 18 days after tumor cell injection. Mice treated with LM-

Mb+αGC only died between 12 and 18 days **(A).** This experiment was repeated three times with 5 mice per group and the results were averaged. Mantel-Cox $p<0.05$ is significant. Sections of the liver of mice that received LM-Mb+αGC or I-αGC-LM-Mb were stained with H&E and analyzed by light microscopy. Foci of necrosis were found in mice immunized with LM-Mb+αGC (see black arrow) but not in mice that received with I-αGC-LM-Mb (**B).** The boxed areas on the left side of the figure are shown in larger magnification on the right side. Graph is a representative of 2 experiments. The toxicity grade in the liver was determined by the naked eye **(C).** The toxicity graded was quantified as follow: 0=no toxicity, 1=uniformly light tanned and firm, 2=5% of the liver is covered by white plaques, 3=10% of the liver is covered by white plaques, 4=20% of the liver is covered by white plaques, 5=30% of the liver is covered by white plaques. This experiment was repeated three times with 5 mice per group and the results were averaged. In addition, the survival time of mice that received I-αGC-LM-Mb or saline were compared. For this purpose, BALB/c mice were challenged with 4T1 tumor cells and immunized with I-αGC-LM-Mb and saline as described in Fig. 1, but in this experiment mice received 7 instead of 5 immunizations. I-αGC-LM-Mb-treated 4T1 tumor-bearing mice lived significantly longer than the saline control group **(D).** This experiment was performed once with 8 mice per group. Mantel-Cox $p<0.05$ is significant.

Fig. 3: Immunizations with I-αGC-LM in naïve mice induce a rapid production of IFNγ and IL-4. Naïve BALB/c mice were injected once with LM, αGC or I-αGC-LM and serum samples were obtained at various time points after injection to determine the IFNγ levels **(A)** or at 2 hours after injection to determine the IL-4 levels **(B)** by ELISA. Graphs is representative of three experiments.

Fig. 4: Repeated therapeutic immunizations with l-αGC-LM do not anergize NKT responses in the spleen of 4T1-tumor-bearing mice. BALB/c mice were challenged with 4T1 tumor cells and immunized with l-αGC-LM-Mb as described for Fig. 1. After 15 days of the first immunization, splenocytes were obtained and stained for iNKT cells using αGC-loaded CD1d tetramers (0 hrs time point). In parallel, a group of mice receiving the same treatment was injected i.p. with free αGC and splenocytes were stained for iNKT cells after 12 and 72 hours. Representative dot plots showing TCR and αGC-loaded CD1d tetramers staining are shown in **(A),** and the summarized data with three mice per group is shown in **(B).** Mann-Whitney * and ** represent p<0.05 and p 0.01, respectively.

Fig 5: Therapeutic immunizations with l-αGC-LM-Mb reduce the percentage of MDSC and improve IL-12 production by MDSC in blood of 4T1 tumor-bearing mice. BALB/c mice were challenged with 4T1 tumor cells and immunized with l-αGC-LM-Mb as described in Fig. 1. The total MDSC population ($CD11b^{+}Gr1^{+}$), the gMDSC population ($CD11b^{+}Gr1^{high}$) and mMDSC population ($CD11b^{+}Gr1^{low}$) were gated within total live leukocyte population in blood and analyzed by flow cytometry. This experiment was repeated 3 times with n=3-5 mice per group and the results were averaged **(A).** Mann-Whitney p<0.05 is significant. *<0.05, **<0.01, ***<0.001, ****<0.0001. The production of IL-12 by mMDSC and gMDSC was analyzed in blood by flow cytometry**.** *A representative example of gating MDSC populations by flow cytometry is provided in the Supplementary Information (**Figure S3A).*** The mMDSC and gMDSC producing IL-12 were gated in the live MDSC population. This experiment was repeated 3 times with n=3-5 mice per group and the results were averaged **(B).** Mann-Whitney p<0.05 is significant. *<0.05, **<0.01, ***<0.001, ****<0.0001. In both figures l-αGC-LM-Mb was compared to LM-Mb or αGC alone. The error bars represent the SEM. *A representative*

flow cytometry profile of MDSC producing IL-12 is provided in the Supplementary Information ***(Figure S3B).***

Fig 6: Therapeutic immunizations with I-αGC-LM-Mb improve CD8 T cells and NK cells responses in blood and spleen of 4T1 tumor-bearing mice. BALB/c mice were challenged with 4T1 tumor cells and immunized with I-αGC-LM-Mb as described in Fig. 1, and analyzed for Mage-b-specific T cell responses and NK cell responses to LM *in vitro* and *in vivo*. For Mage-b-specific CD8 T cell responses *in vitro*, the number of CD8 T cells producing extracellular IFNγ per 400,000 spleen cells of I-αGC-LM-Mb and control mice was determined by ELISPOT after re-stimulation with Mage-b **(A).** CD8 T cells were depleted by magnetic beads technique. Spleens of 5 mice per group were pooled. This experiment was repeated 2-3 times and the results were averaged. I-αGC-LM-Mb was compared to LM-Mb or αGC alone. Mann-Whitney $p<0.05$ is significant. *<0.05, **<0.01, ***<0.001, ****<0.0001. The error bars represent the SEM. For in CD8 T cell responses *in vivo*, the percentage of CD8 T cells producing intracellular IFNγ was determined by flow cytometry in blood of I-αGC-LM-Mb and control mice without any re-stimulation **(B).** The CD8 T cells were gated within total live lymphocyte population in blood. Mice were analyzed individually. n=5 mice per group. This experiment was repeated 3 times and the results were averaged. I-αGC-LM-Mb was compared to LM-Mb or αGC alone. Mann-Whitney $p<0.05$ is significant. *<0.05, **<0.01, ***<0.001, ****<0.0001. The error bars represent the SEM. For NK cell responses to LM *in vitro*, the number of NK cells producing extracellular IFNγ per 200,000 spleen cells of I-αGC-LM-Mb and control mice was determined by ELISPOT after infection with LM **(C).** NK cells were depleted by magnetic beads technique. Spleens of 5 mice per group were pooled. This experiment was repeated 2-3 times and the results were averaged. I-αGC-LM-Mb was compared to LM-Mb or αGC alone. Mann-Whitney $p<0.05$ is significant. *<0.05,

<0.01, *<0.001, ****<0.0001. The error bars represent the SEM. For NK cell responses *in vivo*, the percentage of NK cells producing intracellular IFNγ was determined by flow cytometry in blood of I-αGC-LM-Mb and control mice without any re-stimulation **(D).** The NK cells were gated in the total live lymphocyte population. I-αGC-LM-Mb was compared to LM-Mb or αGC alone. This experiment was performed 3 times n=3-5 mice per group and the results were averaged. Mann-Whitney $p<0.05$ is significant. *<0.05, **<0.01, ***<0.001, ****<0.0001. The error bars represent the SEM.

Figure 1AB

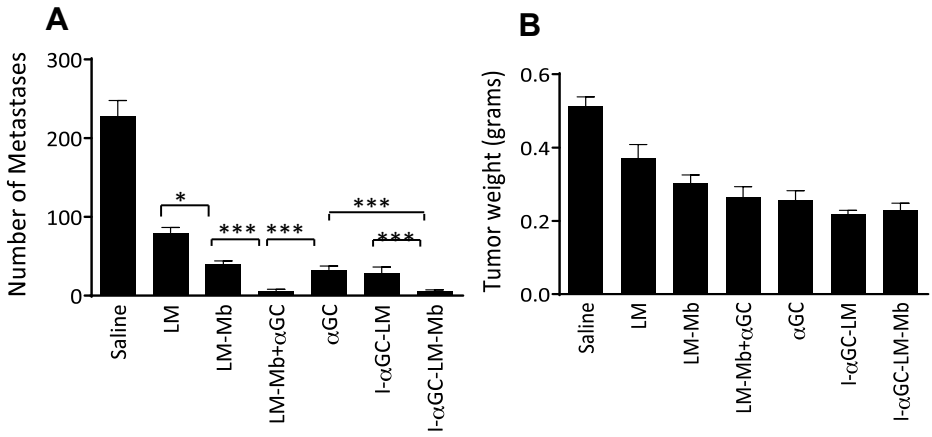

A
Number of Metastases
300
200
100
0
*
***
***
***
***
Saline
LM
LM-Mb
LM-Mb+αGC
αGC
I-αGC-LM
I-αGC-LM-Mb
B
Tumor weight (grams)
0.6
0.4
0.2
0.0
Saline
LM
LM-Mb
LM-Mb+αGC
αGC
I-αGC-LM
I-αGC-LM-Mb

Figure 2ABCD

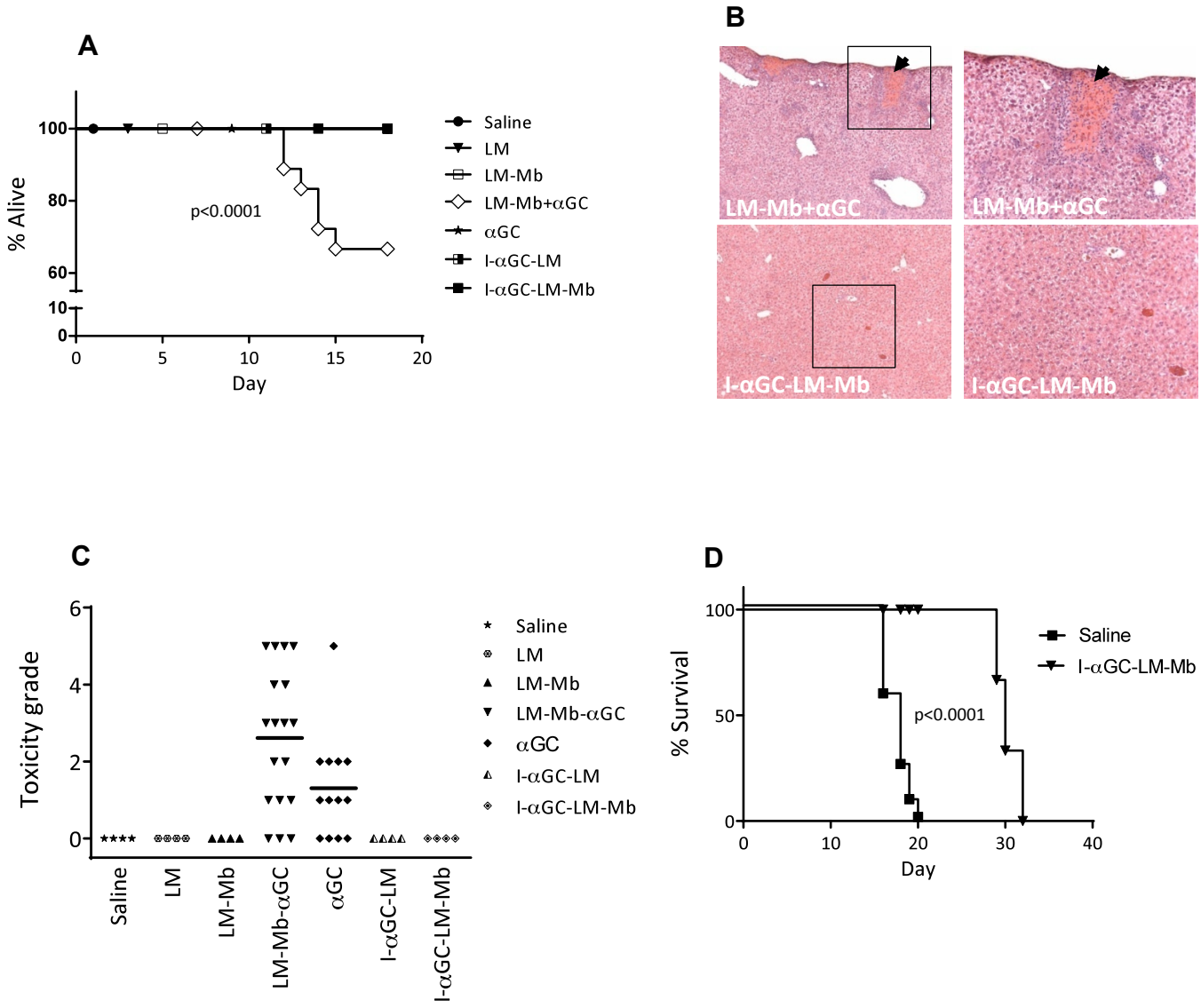


Figure 3

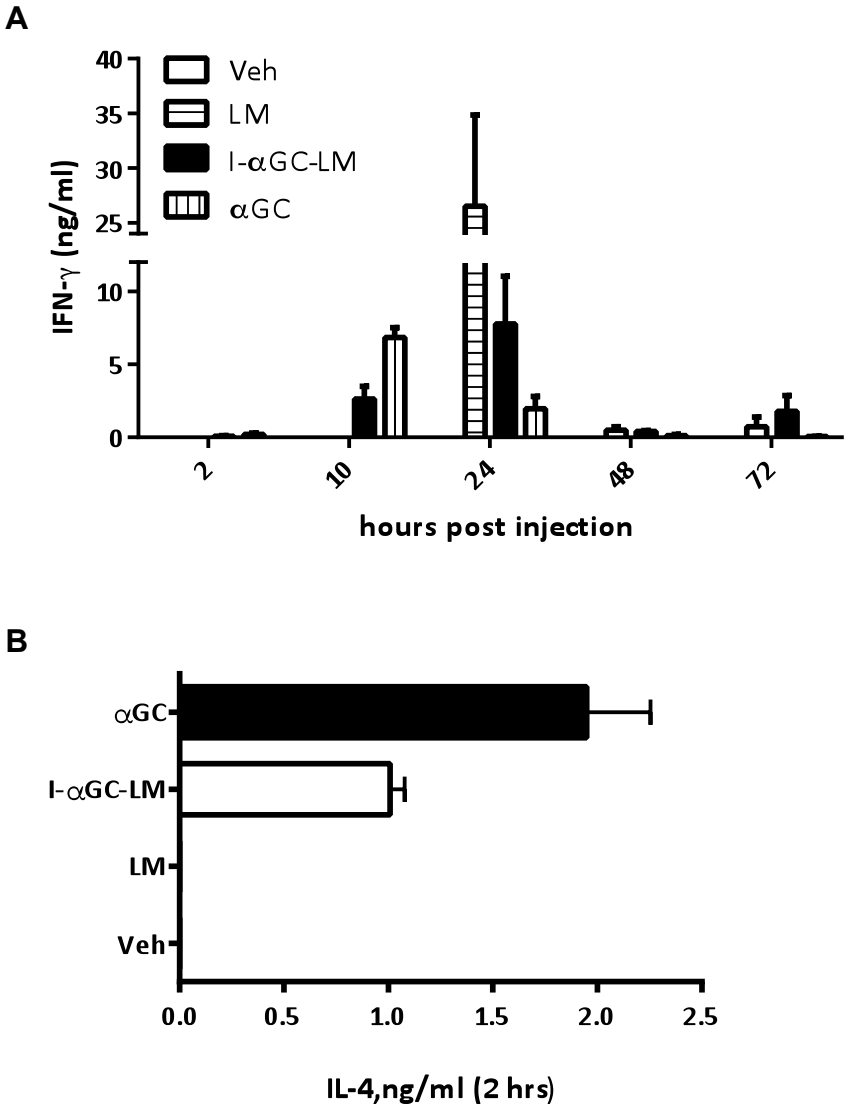
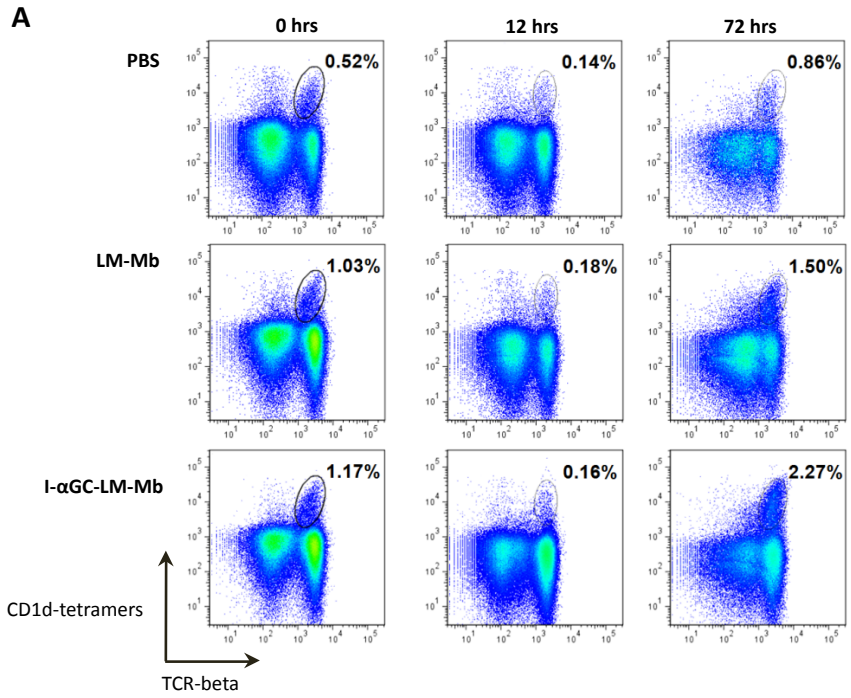

A
Veh
LM
I-αGC-LM
αGC
IFN-γ (ng/ml)
40
35
30
25
10
5
0
2
10
24
48
72
hours post injection
B
αGC
I-αGC-LM
LM
Veh
0.0
0.5
1.0
1.5
2.0
2.5
IL-4,ng/ml (2 hrs)

Figure 4


A
0 hrs
12 hrs
72 hrs
PBS
LM-Mb
I-αGC-LM-Mb
0.52%
0.14%
0.86%
1.03%
0.18%
1.50%
1.17%
0.16%
2.27%
CD1d-tetramers
TCR-beta

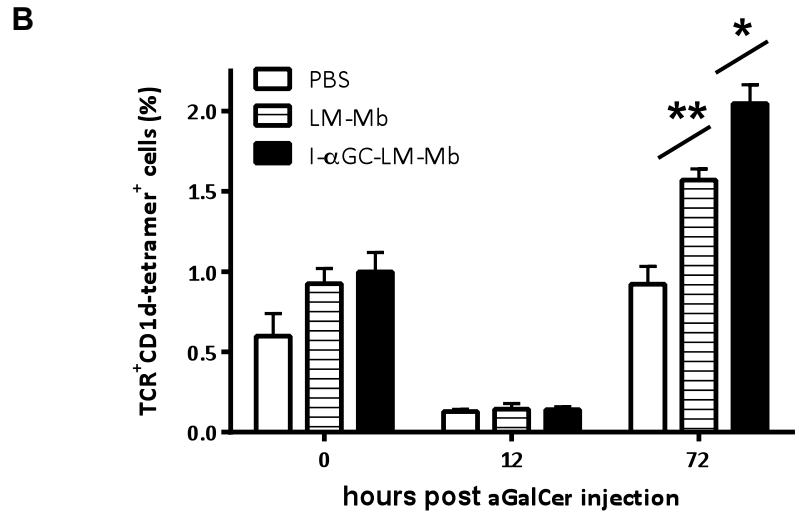

B
PBS
LM-Mb
I-αGC-LM-Mb
TCR+CD1d-tetramer+ cells (%)
2.0
1.5
1.0
0.5
0.0
0
12
72
hours post aGalCer injection
**
*

Figure 5

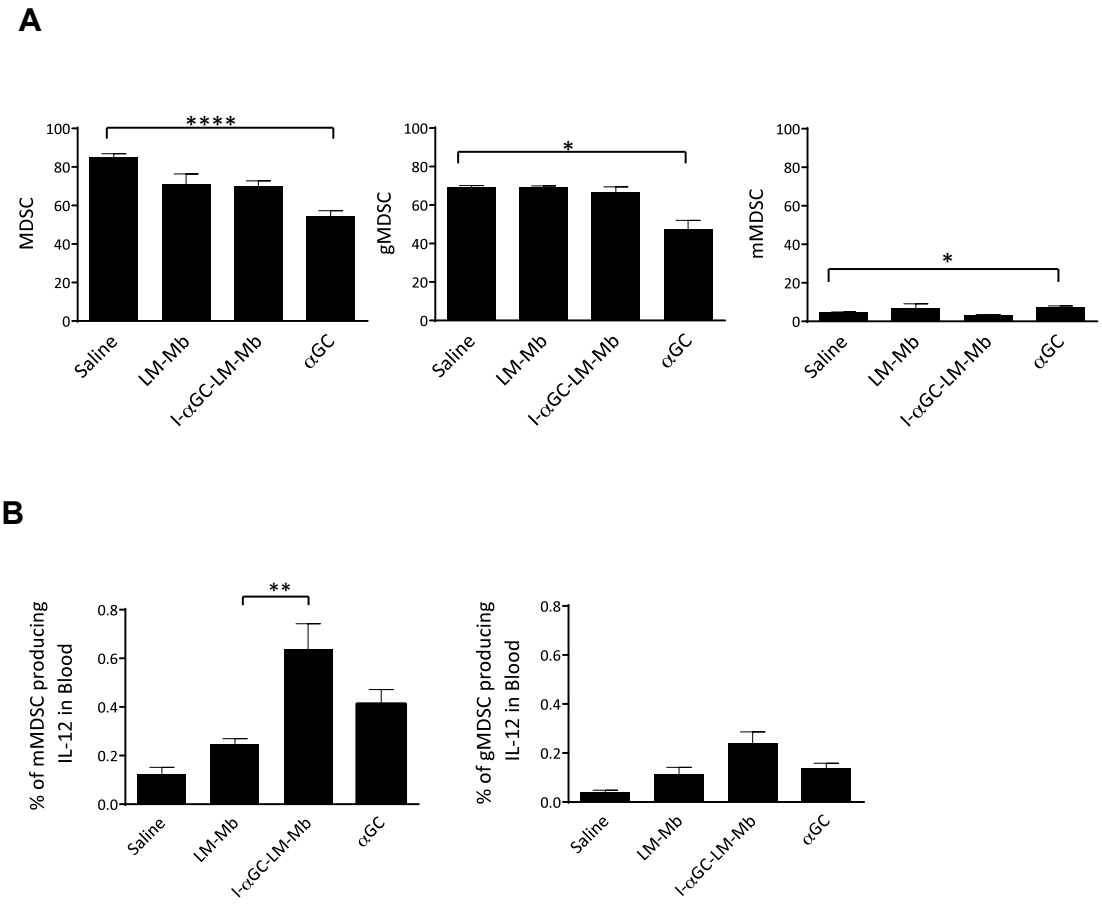
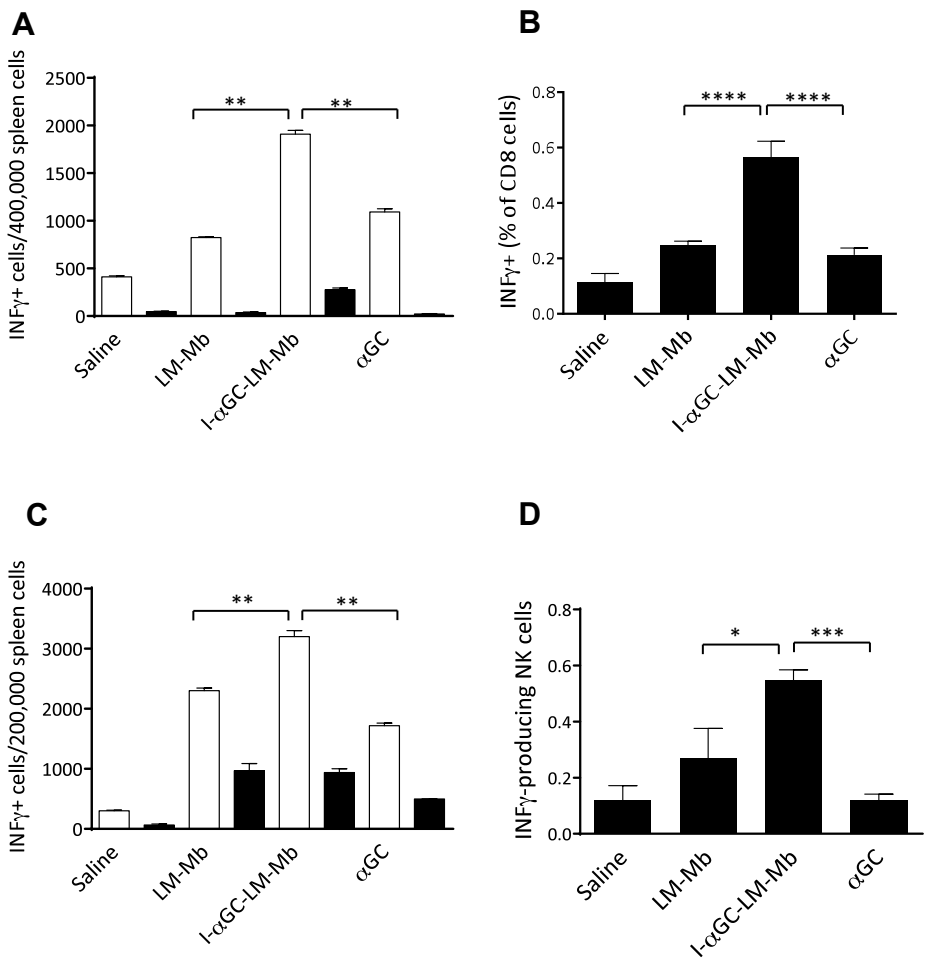

A
MDSC
gMDSC
mMDSC
****
*
*
100
80
60
40
20
0
Saline
LM-Mb
I-αGC-LM-Mb
αGC
B
% of mMDSC producing IL-12 in Blood
% of gMDSC producing IL-12 in Blood
**
0.8
0.6
0.4
0.2
0.0

Figure 6


A
INFγ+ cells/400,000 spleen cells
2500
2000
1500
1000
500
0
**
**
Saline
LM-Mb
I-αGC-LM-Mb
αGC
B
INFγ+ (% of CD8 cells)
0.8
0.6
0.4
0.2
0.0
****
****
Saline
LM-Mb
I-αGC-LM-Mb
αGC
C
INFγ+ cells/200,000 spleen cells
4000
3000
2000
1000
0
**
**
Saline
LM-Mb
I-αGC-LM-Mb
αGC
D
INFγ-producing NK cells
0.8
0.6
0.4
0.2
0.0
*
***
Saline
LM-Mb
I-αGC-LM-Mb
αGC

SUPPLEMENTARY INFORMATION

Title: Direct incorporation of the NKT cell activator α-galactosylceramide improves efficacy and safety of a recombinant *Listeria monocytogenes* breast cancer vaccine

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Figure S1: Bone marrow dendritic cells infected with l-αGC-LM induced efficient iNKT cell activation in vitro. BALB/c derived BMDCs were infected with LM or l-αGC-LM at different multiplicity of infection, for 3 hours and then co-cultured with an iNKT cell hybridoma for 24 hours. As a control, BMDCs were pulsed with 100 nM of free αGC and then co-cultured with the iNKT cell hybridoma for 24 hours. After the co-culture, IL-2 levels were determined in the supernatants by ELISA. For more detailed information see M and M below.

Figure S2: MDSC were present in large numbers in tumor-bearing mice but not in naïve mice. MDSC populations were analyzed in blood of tumor-bearing and naive BALB/c mice. MDSC ($CD11b^{+}Gr1^{+}$)(top and bottom box), gMDSC ($CD11b^{+}Gr1^{high}$)(top box) and mMDSC ($CD11b^{+}Gr1^{low}$)(bottom box) were gated within total live leukocyte population in blood and analyzed by flow cytometry **(A).** n=3 mice per group. This experiment was performed 2-3 times, and the results were averaged and summarized **(B).**

Figure S3: A representative flow cytometry profile of MDSC populations and MDSC producing IL-12. BALB/c mice were challenged with 4T1 tumor cells and immunized with l-αGC-LM-Mb as described in Fig. 1 of the manuscript. MDSC ($CD11b^{+}Gr1^{+}$)(top and bottom box), gMDSC ($CD11b^{+}Gr1^{high}$)(top box) and mMDSC ($CD11b^{+}Gr1^{low}$)(bottom box) were gated within total live leukocyte population in blood and analyzed by flow cytometry **(A).** The mMDSC and gMDSC producing IL-12 were gated in the live MDSC population **(B).** This experiment was repeated 3 times with n=3-5 mice per group.

Materials and Methods

Activation of iNKT cell hybridoma *in vitro*

Day 9 BMDCs were infected with different MOIs (0.1, 0.5 and 1) of LM or I-αGC-LM during 3 hours at 37°C, treated with gentamicin (100 ug/ml) for 1 hour to remove extracellular bacteria and cultured overnight. Then, BMDCs were co-cultured with DN3A4-1.2 mouse iNKT cell hybridomas (5 x 10^4 BMDCs and 5 x 10^4 iNKTs/well in 96 well plates). BMDCs treated with 100 ng/ml of αGC were included as positive control. After 24 hours of co-culture, IL-2 secretion in the supernatants was determined by capture ELISA as described previously[1]

1 Castro, F. *et al.* Vaccination with Mage-b DNA induces CD8 T-cell responses at young but not old age in mice with metastatic breast cancer. *Br J Cancer* **101**, 1329-1337, doi:10.1038/sj.bjc.6605329 (2009).

Figure S1

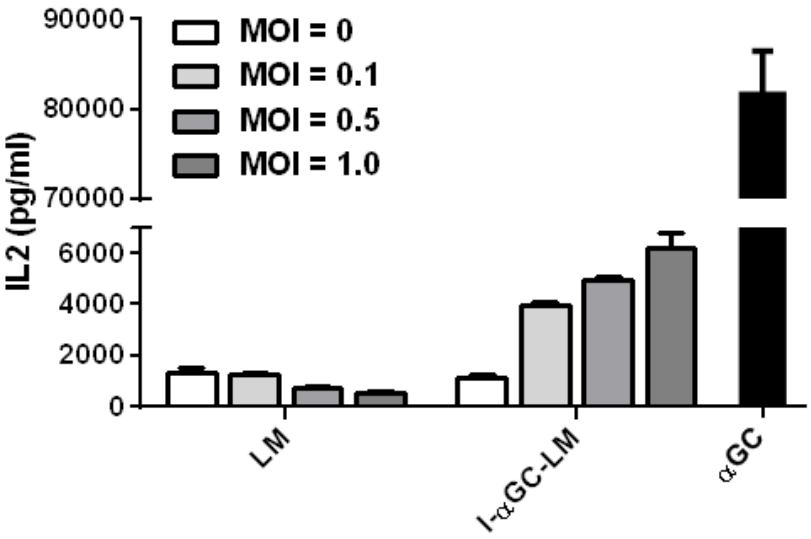

90000
80000
70000
6000
4000
2000
0
IL2 (pg/ml)
MOI = 0
MOI = 0.1
MOI = 0.5
MOI = 1.0
LM
I-αGC-LM
αGC

Figure S2

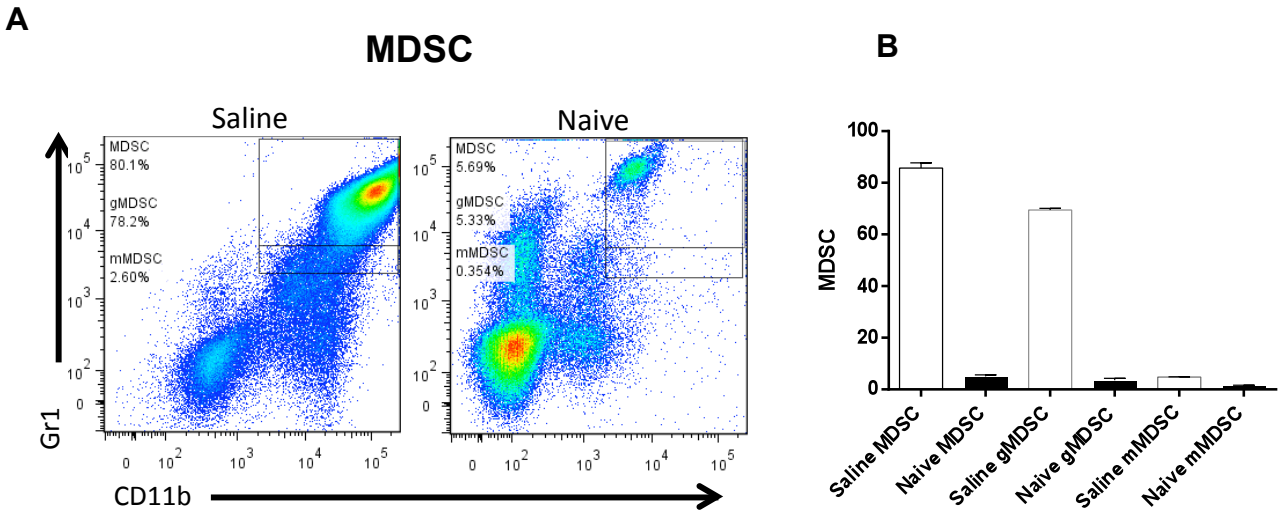
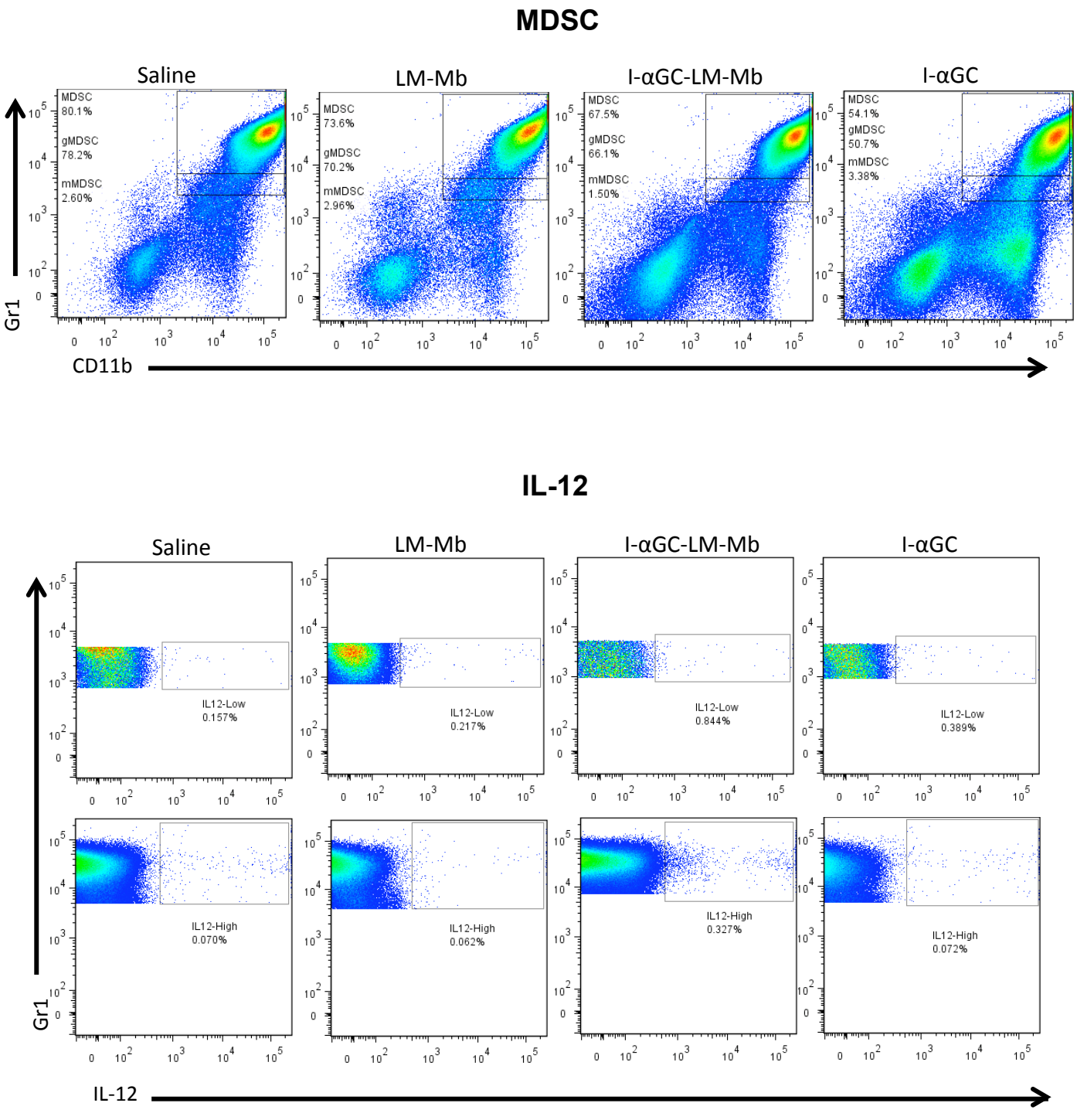

A
MDSC
Saline
Naive
MDSC
80.1%
gMDSC
78.2%
mMDSC
2.60%
MDSC
5.69%
gMDSC
5.33%
mMDSC
0.354%
Gr1
CD11b
B
MDSC
100
80
60
40
20
0
Saline MDSC
Naive MDSC
Saline gMDSC
Naive gMDSC
Saline mMDSC
Naive mMDSC

Figure S3


MDSC
Saline
LM-Mb
I-αGC-LM-Mb
I-αGC
MDSC
80.1%
gMDSC
78.2%
mMDSC
2.60%
MDSC
73.6%
gMDSC
70.2%
mMDSC
2.96%
MDSC
67.5%
gMDSC
66.1%
mMDSC
1.50%
MDSC
54.1%
gMDSC
50.7%
mMDSC
3.38%
Gr1
CD11b
IL-12
Saline
LM-Mb
I-αGC-LM-Mb
I-αGC
IL12-Low
0.157%
IL12-Low
0.217%
IL12-Low
0.844%
IL12-Low
0.389%
IL12-High
0.070%
IL12-High
0.062%
IL12-High
0.327%
IL12-High
0.072%
Gr1
IL-12

CHAPTER 4

GENERAL DISCUSSION

Problems and promises of cancer vaccination

Immune suppression is a major problem in cancer vaccination or cancer immunotherapy. Various approaches have been used in mice and humans to reduce immune suppression in cancer. This includes elimination of immune cells that induce immune suppression such as T_{reg} (using anti-CD25 antibodies) and MDSC (using anti-Gr1 antibodies), or by agents such fish oil and selenium (eliminates T_{regs} and MDSC), or a streptococcal extract OK432 (eliminates T_{regs}), a glycan-binding protein Galectin-1 (eliminates T_{regs}), or antibodies that can block co-inhibitory molecules such anti-CTLA-4 or anti-PD-1[138-143]. Since immune suppression involves a combination of inhibitory cytokines, factors, receptor-ligand interactions, various approaches will be necessary to sufficiently reduce the immune suppression until a level that allows T cells to react to the tumor cells. Also different tumors may induce different types of immune suppression. Therefore, depending on the type of immune suppression a suitable combination therapy could be designed to improve cancer vaccination through reduction of immune suppression.

In the thesis presented here we focused on the immune suppression in metastatic breast cancer. The production of IL-6 is one of the main cytokines that play an important role in immune suppression in breast cancer, and particularly in TNBC[125,144]. TNBC are enriched for stem-like breast cancer cells (CD44+/CD24-/low), which are typically aggressive and highly resistant to current therapies[145-147]. These stem-like breast cancer cells produce high levels of IL-6, and have the capacity to metastasize[137]. Moreover, IL-6 is capable of converting dormant breast cancer cells into an actively growing tumor.

While most breast cancers in an early stage are curable, TNBC is incurable. It lacks the expression of ER, PR, and HER2, which leaves highly toxic therapies as only option, and ultimately does not cure the cancer. Therefore, in this thesis project we selected a metastatic mouse tumor model 4T1 with TNBC, which is highly aggressive and metastatic, and their tumors and metastases produce high levels of IL-6[148].

Increasing the success rate of cancer vaccination or immunotherapy by reducing IL-6

As mentioned above, one of the main problems with treatments against TNBC is the high toxicity. Therefore, we focused in this thesis on non-toxic vaccine therapies against metastatic breast cancer using a TNBC model 4T1. As shown here in this thesis, the combination therapy of Listeria^{at}-Mage-b and curcumin is non-toxic, but induced primarily inflammatory responses in the spleen and liver. Curcumin was selected because it is known for reducing IL-6, and IL-6 is one of the main contributors to immune suppression in the TME, particularly in TBNC. In this thesis (see Chapter 2), we tested whether curcumin could improve the efficacy (effect on metastases and tumor) of Listeria^{at}-Mage-b vaccine, through reduction of IL-6, and improved T cell responses to Mage-b. Various immunization protocols have been tested. In the first immunization protocol with Listeria^{at}-Mage-b and curcumin both therapeutically administered, we found that curcumin significantly improved the efficacy of Listeria^{at}-Mage-b vaccination resulting in a strong reduction in the number of metastases compared to all control groups. Also Listeria^{at}-Mage-b alone reduced the number of metastases and tumor growth significantly but less robust than the combination, while curcumin alone did not have much effect on the metastases or tumors. Others found an anti-tumor effect of curcumin on tumor cells in vitro[117-121]. However, tumor cells may react differently in vitro to curcumin than in vivo because in vitro bioavailability and the immune system does not

play a role and higher concentrations can be obtained in vitro compared to the in vivo situation. Also, the time point of administering curcumin, the concentration of curcumin, and the type of cancer may determine the anti-tumor effect of curcumin. For instance, others reported that the time point of administration of curcumin is important[149]. We found that administering curcumin before tumor development was more effective than after tumor development. Since curcumin has been consumed in food all over the world we tested administration of curcumin before and Listeria^{at}-Mage-b after tumor development. This combination therapy was significantly more effective against the metastases compared to the administration of curcumin and Listeria^{at}-Mage-b after tumor development. Moreover, administration of curcumin before tumor development was more effective against metastases and tumor weight than curcumin administered after tumor development.

The almost complete elimination of the metastases and strong reduction in tumor weight by curcumin before and Listeria^{at}-Mage-b after tumor development correlated with reduction in IL-6 levels in the primary tumors and in MDSC in blood, and significantly improved T cell responses to Mage-b in vivo and in vitro. However, the effect on primary tumor was less compared to metastases, in correlation with lower T cell responses. Not only curcumin reduced the IL-6 levels in the tumor cell lysates, but also Listeria^{at}-Mage-b, and the strongest reduction was observed with the combination of Listeria^{at}-Mage-b and curcumin. In addition, we found that the combination therapy significantly reduced the number of MDSC and converted a remaining sub population of MDSC into an immune stimulating phenotype in blood but not in primary tumors, producing IL-12. IL-12 is known for activating naïve and mature T cells[62,150]. The combination therapy significantly increased CD8 T cell responses to Mage-b in vitro and in vivo. Most interesting was that the combination therapy significantly reduced the

percentage of MDSC in blood but not in primary tumors, and may have contributed to the more significant effect on metastases than on primary tumors.

In summary, the results accumulated in this study, i.e. (1) reduction of IL-6 in the primary tumor and MDSC, (2) increase in IL-12 production by MSDC, (3) reduction in the percentage of MDSC, and (4) improved CD8 T cell responses to Mage-b in vitro and in vivo, strongly suggest that our combination therapy of Listeria^{at}-Mage-b and curcumin improved the therapeutic effect of Listeria^{at}-Mage-b through improvement of T cell responses to Mage-b in a TNBC model 4T1. The potential pathways of Listeria^{at}-Mage-b and curcumin involved in anti-tumor responses analyzed in this study are summarized in **Fig 1.** While curcumin alone may have less therapeutic value, as adjuvant with vaccines it is a powerful tool to reduce immune suppression in the TME, and show great promise for clinical application in other IL-6-producing cancers.

Creating an immune-stimulating environment with αGC to help adaptive and innate immune responses against metastatic breast cancer

One way to improve T cell responses is by reducing immune suppression in the TME. Here we used another approach. αGC is a glyclipid that is a strong activator of NKT cells, resulting in a cascade of Th1 cytokines such as IFNγ and IL-12 among others (IL-4, IL-13), and basically converts an immune suppressing into an immune-stimulating environment. Initially we started testing the combination of free αGC and Listeria^{at}-Mage-b in the TNBC model 4T1. While the effect on metastases was very impressive, i.e. we observed an almost complete elimination of the metastases, it appeared that the combination was highly toxic in the liver. Therefore, we used a novel approach by incorporating the αGC into the cell wall of live Listeria^{at}-Mage-b (I-αGC-LM-Mb). This approach was successfully used earlier for Mycobacteria showing improved CD8 T cell

stimulation[151]. While the administration of LM-Mb and αGC as separate injections in sequential fashion was associated with marked toxicity due to hepatic necrosis, we found that the direct incorporation of the glycolipid into LM-Mb completely eliminated the toxicity while still preserving the marked clinical benefit. Repeated immunizations with low dose of I-αGC-LM-Mb almost completely eliminated all metastases without any liver toxicity. This correlated with the activation of NKT cells, the production of IL-12p70 by MDSC, and the subsequent activation of NK cells and Mage-b-specific CD8 T cells in blood in vivo.

Obvious was that the I-αGC-LM-Mb was less effective on CD8 T cells and NK cells in the primary tumor. In humans and mice the number of MDSC in the blood is much higher than in the primary tumor[152]. We observed the increased levels of IL-12p70 in myeloid cells in the blood, but not in the primary tumors. Similarly, improved T cell and NK cell activation was detected in the blood but not in the primary tumors. These may be the reasons why our I-αGC-LM-Mb treatment were more effective against the metastases, that usually spreads via the blood stream, than against the primary tumors.

In summary, we demonstrated that a novel combination of a recombinant LM expressing Mage-b and directly incorporated αGC almost completely eliminated metastases in the 4T1 model without toxicity. This is of crucial importance because patients usually die of metastases and not of their primary tumor. Moreover, standard therapies such as surgery, chemotherapy and radiotherapy are quite successful against primary tumors, but not metastases. Therefore, standard therapy to eliminate the primary tumor combined with a treatment of I-αGC-LM-Mb expressing and appropriate TAA to eliminate metastases, could be a promising new approach to treat metastatic breast and perhaps other metastatic cancers. The potential pathways of I-αGC-LM-Mb involved in anti-tumor responses, analyzed in this study are summarized in **Fig 2.**

Our previous and current results strongly suggest that not only Mage-b-specific T cells cause tumor cell destruction. We have shown that Listeria^{at} infected tumor cells and killed tumor cells through high levels of ROS, and that infected tumor cells were sensitive targets for LM-activated T cells[114]. The multiple pathways of Listeria^{at}, i.e. delivering TAA and αGC with extreme high efficiency into APC, infecting and converting MDSC when combined with curcumin or incorporated with αGC into an immune-stimulating phenotype producing IL-12, and infecting and killing tumor cells through ROS, with practically no side effects, makes Listeria^{at} highly attractive for cancer immunotherapy.

Future prospects of cancer vaccination

As we have shown here in mice with metastatic breast cancer (4T1 model), vaccines can be effective against metastases, but only if the immune suppression is reduced or converted into immune stimulation. Also important is that both combination therapies are non-pathogenic and non-toxic. This opens up the possibility to use these combination therapies as second line therapy after removal of the primary tumor by surgery, chemotherapy or radiation. Often, the second line therapy is administered after metastases have been detected, because of its high toxicity. Our non-toxic combination therapies could be administered in an early phase after first line therapy (without knowing if metastases will develop), to prevent recurrence or the development of metastases.

One question that arose in both studies, i.e. with Listeria^{at}-Mage-b and curcumin or with l-αGC-LM-Mb, what the value was of the in vitro re-stimulation assays with Mage-b versus the in vivo analysis of CD8 T cells by flow cytometry without any re-stimulation. As described above, it was obvious that in both studies the in vivo analysis by flow cytometry showed CD8 T cell activation in blood but not in the primary tumors,

and that this correlated with an almost complete eradication of the metastases but a moderate to minor effect on the primary tumors. In contrast, the in vitro re-stimulation assay showed strong CD8 T cell responses to Mage-b and did not correlate with the effect of the combination therapy on the primary tumors at all. It is interesting that in many vaccine studies the in vitro re-stimulation assay is often used as measurement for effective vaccines, i.e. that they are able to induce CD8 T cell activation. However, this is only true in a non-tumor environment but as soon as these T cells reach the tumor, their function will be shut down by the immune suppression in the TME. Therefore, it is important to analyze the vaccine-induced immune responses in vivo in mice or humans with tumors.

Another obvious result was that both combination therapies were more effective against the metastases than primary tumors. One of the reasons may be that the blood (~80%) contains many more MDSC than the primary tumors (~10%). However, primary tumors can be removed by surgery for most tumors, or by chemotherapy or radiation. In addition, our laboratory is investigating other non-toxic approaches such as killing primary tumor through cryoablation (freezing and thawing of tumor cells). We were able to completely eradicate primary tumor by this technique, and it appeared to be equally effective in humans (unpublished results).

It is clear that one type of therapy will not be sufficient to eliminate metastatic cancer, and that multiple combination therapies, selected for the various types of cancer, and/or patient will be the future to combat metastatic cancer. The multiple pathways of Listeria^{at}, i.e. delivering TAA and αGC with extreme high efficiency into APC, infecting and converting MDSC when combined with curcumin or incorporated with αGC into an immune-stimulating phenotype producing IL-12, and infecting and killing tumor cells through ROS, with practically no side effects, makes Listeria^{at} highly attractive for cancer immunotherapy. Since Listeria^{at} infects tumor cells, also other approaches are possible

such as delivery of anti-tumor compounds in the tumor cells by Listeria^{at}. Indeed, our laboratory in collaboration with the laboratory of Dr. Dadachova (Microbiology and Immunology, Albert Einstein College of Medicine) has recently developed a radioactive Listeria^{at}, and we showed that Listeria^{at} efficiently delivered the radioactivity to the metastases, resulting in an almost complete elimination of the metastases in pancreatic cancer without noticeable side effects (Quispe-Tintaya et al, A non-toxic radioactive Listeria^{at} is a highly effective therapy against metastatic pancreatic cancer, PNAS, accepted).

Last but not least, the age factor is an important issue because most cancer patients are old and elderly react les efficient to vaccines than young adults, due to T cell unresponsiveness[91]. This is mainly caused by lack of naïve T cells and to an increase in the number of MDSC in the TME at older age[153-159]. Paradoxically, the age factor is totally ignored in clinical trials and may partly be responsible for the absence of a real breakthrough in cancer vaccination. Since both combination therapies tested in this thesis targets MDSC and both are non-toxic, these combination therapies may be especially effective at older age. However, it is also clear that we still have a long way to go for the complete elimination of metastatic cancer at all ages.

Figure 1 Potential pathways of Listeria-Mage-b and curcumin involved in anti-tumor responses in mice with metastatic breast cancer (4T1 model). Mice will be treated with curcumin before and immunized with Listeria-Mage-b after tumor development. Curcumin will kill tumor cells and reduce IL-6 production by tumor cells and MDSC. Reduction in IL-6 production will reduce immune suppression and restore T cell responses. Listeria-Mage-b will infect MDSC, tumor cells and APC (DC, macrophages, monocytes). Mage-b antigen will be delivered by Listeria inside the APC, then secreted at high levels in the cytoplasm and processed for assembling with MHC class I molecules for presentation on the membrane to CD8 T cells. Simultaneously, the combination of Listeria-Mage-b and curcumin will reduce the number of MDSC, and induce IL-12 production in a sub population of MDSC in blood. Reduction in the number of MDSC will result in less immune suppression. IL-12 will induce T cell activation, resulting in the production of IFNγ. Activated T cells will kill the tumor cells. In addition to this cascade of immune reactions the Listeria bacteria will infect and kill tumor cells through high levels of ROS, and infected tumor cells will be killed by Listeria-activated CD8 T cells (not shown).

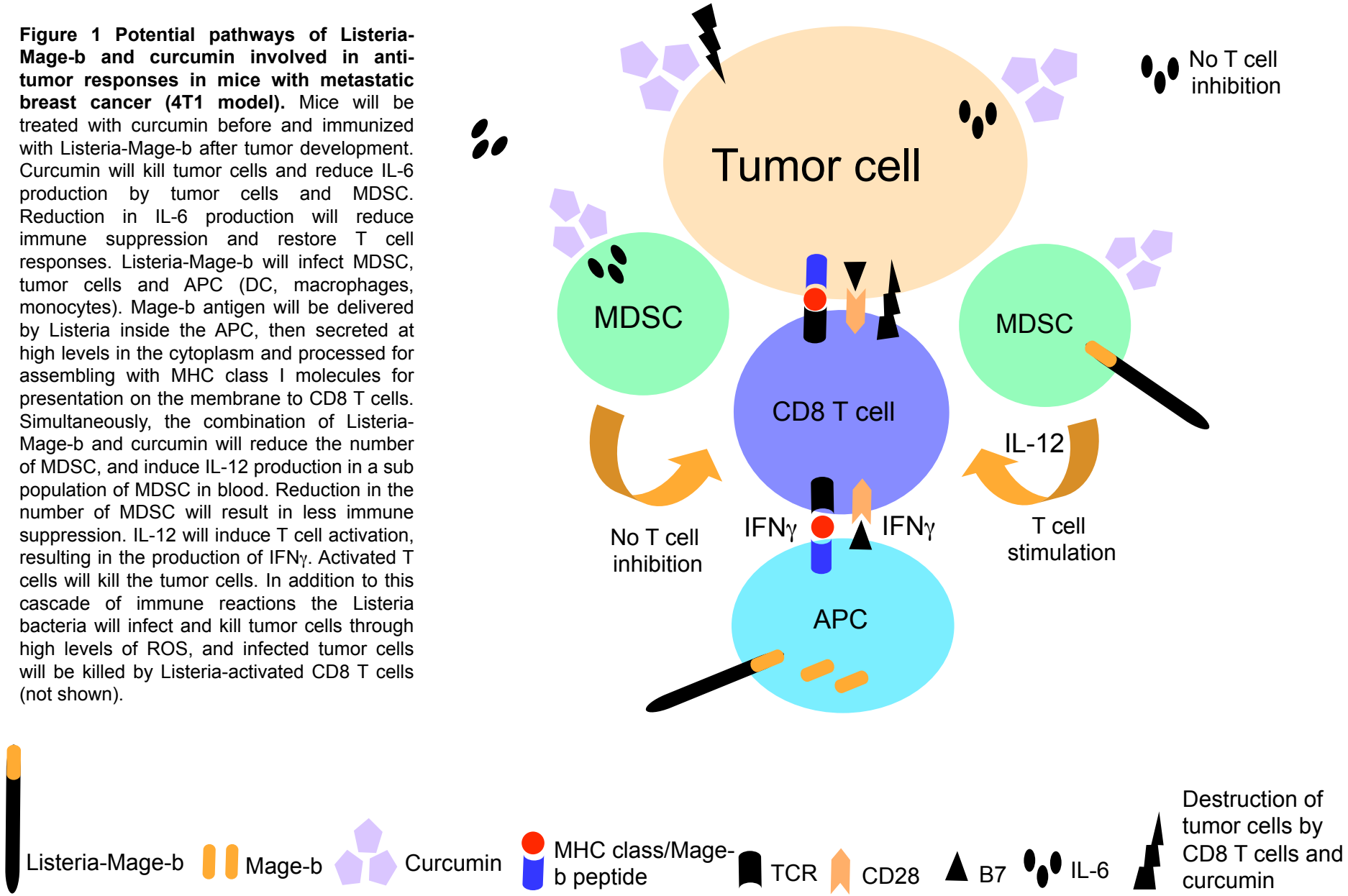
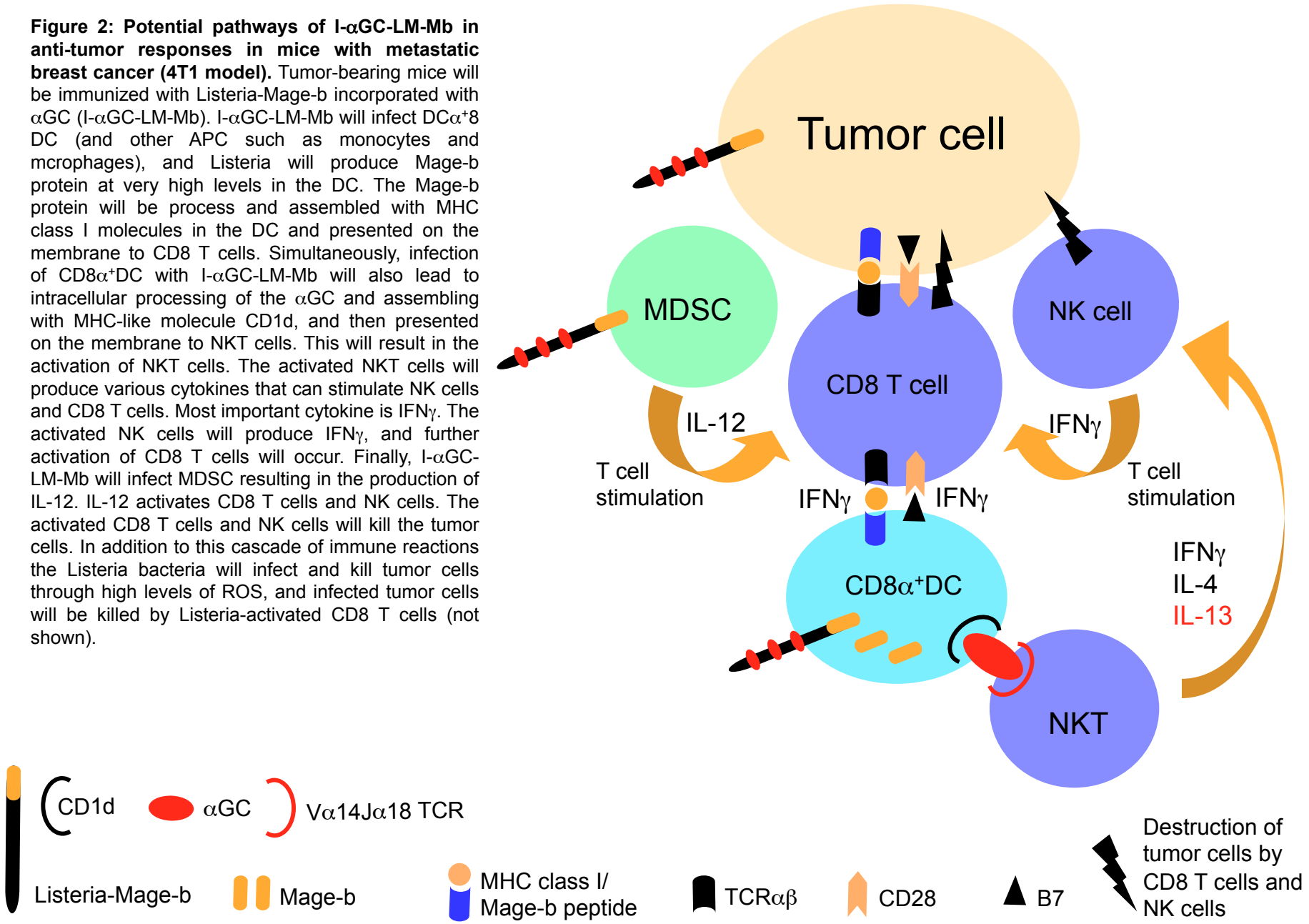


Figure 2: Potential pathways of I-αGC-LM-Mb in anti-tumor responses in mice with metastatic breast cancer (4T1 model). Tumor-bearing mice will be immunized with Listeria-Mage-b incorporated with αGC (I-αGC-LM-Mb). I-αGC-LM-Mb will infect DCα⁺8 DC (and other APC such as monocytes and mcrophages), and Listeria will produce Mage-b protein at very high levels in the DC. The Mage-b protein will be process and assembled with MHC class I molecules in the DC and presented on the membrane to CD8 T cells. Simultaneously, infection of CD8α⁺DC with I-αGC-LM-Mb will also lead to intracellular processing of the αGC and assembling with MHC-like molecule CD1d, and then presented on the membrane to NKT cells. This will result in the activation of NKT cells. The activated NKT cells will produce various cytokines that can stimulate NK cells and CD8 T cells. Most important cytokine is IFNγ. The activated NK cells will produce IFNγ, and further activation of CD8 T cells will occur. Finally, I-αGC-LM-Mb will infect MDSC resulting in the production of IL-12. IL-12 activates CD8 T cells and NK cells. The activated CD8 T cells and NK cells will kill the tumor cells. In addition to this cascade of immune reactions the Listeria bacteria will infect and kill tumor cells through high levels of ROS, and infected tumor cells will be killed by Listeria-activated CD8 T cells (not shown).



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SUMMARY

The diagnosis of breast cancer has a reasonable prognosis, particularly when it is diagnosed in an early phase. Eliminating primary tumors by surgery, chemotherapy, or radiation is quite successful. However, for metastases there is no cure. Thirty percent of the breast cancer cases will progress into metastatic disease, but they cannot be removed by surgery or radiation, and usually become chemoresistant. However, preclinical and clinical studies have shown that cancer vaccination has an effect on metastases but that a real breakthrough is hampered by the strong immune suppression in the tumor microenvironment (TME). Therefore, in this thesis we focused on the treatment of metastatic breast cancer using an attenuated *Listeria monocytogenes* (Listeria^{at})-based vaccine expressing tumor-associated antigen (TAA) Mage-b (developed earlier in our laboratory), combined with adjuvants to reduce immune suppression in the TME in order to improve the vaccine efficacy of Listeria^{at}-Mage-b. Listeria^{at} is a non-pathogenic bacterium, and was selected because of its great capability to deliver TAA into APC in vivo with high efficiency, its minimal side effects, and its ability to activate innate and adaptive immune responses. Listeria^{at} also kills tumor cells directly without harming normal cells. Moreover, the Listeria^{at} is naturally cleared by the immune system in normal tissues within 3-5 days. Mage-b was selected because Mage is homologous with its human homologue MAGE, which is expressed in 90% of all breast cancers. Here we have tested two different combination therapies in mice with metastatic breast cancer (4T1 model), and results demonstrated great promise of our approaches for the battle against metastases.

The first combination therapy consisted of Listeria^{at}-Mage-b and curcumin. Curcumin is an Indian spice and able to reduce the production of interleukin (IL)-6. IL-6 is abundantly

produced by breast tumor cells and immune cells, and particularly in patients with metastatic breast cancer. IL-6 is one of the most important contributors to suppression of T cell responses in the TME. Curcumin also kills tumor cells directly. The second combination therapy consisted of Listeria^{at}-Mage-b and alphagalctosylceramide (αGC). αGC is a glycolipid that stimulates natural killer T (NKT) cells through its interaction with CD1d on CD8α+ dendritic cells (DC), which in turn generates a cascade of cytokine production that activates the innate and adaptive immune system, including the Mage-b-specific T cells.

In Chapter 2, we tested the combination of Listeria^{at}-Mage-b and curcumin therapeutically in mice with metastatic breast cancer, and found a dramatic reduction in the number of metastases by 85%, and reduced tumor growth by 60%, compared to non-treated mice. Since curcumin is consumed through food all over the world, we also tested whether administration of curcumin preventively (before tumor development) could have an advantage over curcumin therapeutically (after tumor development). It appeared that administration of curcumin before tumor development and Listeria^{at}-Mage-b after tumor development resulted not only in a stronger decrease in the number of metastases (99%), but also significantly reduced tumor growth (75%), compared Listeria^{at}-Mage-b and curcumin after tumor development. Also curcumin alone preventively administered significantly reduced the number of metastases and tumor growth, although the combination was more effective. This suggests that consuming curcumin before tumor development may have an advantage over consuming curcumin after tumor development in the battle against metastatic breast cancer. This correlated with a significant reduction in the production of IL-6 by the primary tumors and immune cells in blood. Moreover, a sub population of myeloid-derived suppressor cells (MDSC), one of the most important contributors to immune suppression in the TME, was

converted by the combination therapy into an immune-stimulating phenotype producing IL-12, in correlation with significant improvement in T cell responses to Mage-b in the tumor-bearing mice. This combination therapy was not only highly effective against the metastases but also non-toxic as confirmed by pathological examination of spleen, kidney, liver, heart and lungs.

In Chapter 3, Listeria^{at}-Mage-b and αGC was therapeutically tested in mice with metastatic breast cancer, and we found a dramatic reduction in the number of metastases (99%) compared to non-treated mice. However, this was accompanied with strong toxicity in the liver, which was not observed with Listeria^{at}-Mage-b or αGC alone. To address this problem we incorporated αGC into the cell wall of live Listeria^{at} bacteria expressing Mage-b (I-αGC-LM-Mb). Here, we demonstrate that repeated low dose administrations with I-αGC-LM-Mb in mice with metastatic breast cancer was equally effective against the metastases compared to vaccination with LM-Mb and αGC as a mixture, but without any visible toxicity as confirmed by pathology and survival studies. This was correlated with the activation of NKT cells, the generation of IL-12 production in MDSC, and significantly improved CD8 T cell responses to Mage-b and NK cell responses. Also tumor growth was significantly reduced but the combination was not better than the Listeria^{at}-Mage-b or αGC alone. Here we demonstrate for the first time that therapeutic treatment with live attenuated Listeria^{at} incorporated with αGC is highly effective against breast cancer metastases without toxicity.

In conclusion, we have demonstrated that therapeutic vaccination with Listeria^{at}-Mage-b, when combined with adjuvants such as curcumin or αGC inhibits or converts immune suppression into immune stimulation, strongly reduced the number of metastases in

mice with metastatic breast cancer, in correlation with improved T cell responses to Mage-b and NK cell responses, without any visible toxicity. While the effect of both combination therapies was less effective against primary tumors, they could be of great values for second line therapy (after surgery, chemotherapy or radiation to remove the primary tumor), to prevent recurrence and the development of metastases. Because of the low toxicity, starting second line treatment in an early phase may further improve the clinical outcome.

SAMENVATTING

De diagnose van borstkanker heeft een redelijke prognose, met name wanneer het is gediagnostiseerd in een vroege fase. Het elimineren van primaire tumoren door chirurgie, chemotherapie of bestraling is redelijk succesvol. Voor metastasen is er echter geen remedie. Dertig procent van de gevallen van borstkanker wordt uiteindelijk metastatisch. Metastasen kunnen niet worden verwijderd door chirurgie of bestraling, en worden meestal chemoresistant. Preklinische en klinische studies hebben echter aangetoond dat kanker vaccinatie een effect heeft op metastasen maar dat een echte doorbraak wordt belemmerd door de sterke immuun suppressie in de directe omgeving van de tumor. Dus, in deze thesis hebben we ons gericht op de behandeling van uitgezaaide borstkanker door gebruik te maken van een verzwakt *Listeria monocytogenes* (Listeria^{at})-gebaseerd vaccin dat codeert voor de tumor-geassocieerde antigeen (TAA) Mage-b (dit vaccin was al eerder gemaakt in ons laboratorium), en we hebben het Listeria^{at}-Mage-b vaccine gecombineerd met specifieke stoffen om de immuun suppressie te kunnen onderdrukken ter verbetering van de werkzaamheid van het vaccin. Listeria^{at} is een verzwakte niet-pathogene bacterie, en wij hebben Listeria^{at} geselecteerd vanwege zijn grote capaciteit om TAA in antigen-presenterende cellen (APC) te leveren met hoge efficiëntie in vivo, zijn minimale bijwerkingen, en het vermogen om adaptieve en innate immuun responsen te activeren. Listeria^{at} doodt ook tumor cellen rechtstreeks zonder nadelige gevolgen voor normale cellen. Bovendien wordt de verzwakte Listeria^{at} geelimineerd door het immuun systeem in normale weefsels binnen 3-5 dagen, maar niet in the tumoren en metastases, omdat daar de Listeria^{at} beschermd worden door de sterke immune suppressie. Mage-b is geselecteerd in ons muizen model met metastatische borst kanker (4T1) omdat muizen Mage homoloog is met de menselijke MAGE, en omdat MAGE in 90% van alle borst kankers

voorkomt. Wij hebben hier twee verschillende combinatie therapieën in muizen met uitgezaaide borstkanker (4T1 model) getest en de resultaten suggeren dat onze aanpak veelbelovend is voor het bestrijden van metastasen. De eerste combinatietherapie bestaat uit Listeria^{at}-Mage-b en curcumin. Curcumin is een Indische specerij en is bekend vanwege het vermogen om de productie van het cytokine interleukine (IL)-6 te verminderen. IL-6 wordt overvloedig geproduceerd door borst tumor cellen en immuun cellen, en met name in patiënten met metastatische borst kanker. IL-6 is een van de belangrijkste cytokines die bijdragen aan de onderdrukking van T cell aktivatie in de directe omgeving van primary tumoren en metastases. Curcumin doodt ook tumor cellen rechtstreeks. De tweede combinatie therapie bestaat uit Listeria^{at}-Mage-b en alphagalctosylceramide (αGC). αGC is een glycolipid die natuurlijke killer T (NKT) cellen stimuleert door de interactie of αGC met CD1d in CD8α^{+} dendritische cellen (DC). Geaktiveerde NKT cellen kunnen een cascade van cytokine productie genereren, die op hun beurt innate and adaptive responsen activeert, inclusief the Mage-b-specific T cellen.

In hoofdstuk 2, hebben we de combinatie van Listeria^{at}-Mage-b en curcumin therapeutisch (na de tumor ontwikkeling) getest in muizen met uitgezaaide borst kanker (4T1 model), en een drastische vermindering van het aantal metastasen (85%) en verminderde tumor groei (60%) gevonden in vergelijking met de onbehandelde muizen. Echter had curcumin zonder Listeria^{at}-Mage-b maar weinig therapeutisch effect. Aangezien curcumin over de hele wereld wordt geconsumeerd via voedsel, hebben wij ook getest of het preventief consumeren van curcumin een voordeel zou kunnen hebben in vergelijking met het therapeutisch consumeren van curcumin. Het bleek dat preventief consumeren van curcumin in combinatie met het toedienen van Listeria^{at}-Mage-b in een therapeutisch setting niet alleen resulteerde in een sterke daling van het aantal

metastasen (99%), maar ook aanzienlijk tumor groei verminderde (75%), in vergelijking met curcumin and Listeria^{at}-Mage-b beiden therapeutisch toegediend. Ook had curcumin zonder Listeria^{at}-Mage-b een significant preventief effect op de metastases and tumoren. Dit suggereert dat het preventief consumeren van curcumine een voordeel zou kunnen hebben ten opzichte van het therapeutisch consumeren van curcumine in de strijd tegen uitgezaaide borst kanker. Dit resultaat correleerde met een aanzienlijke vermindering in de productie van de IL-6 in de primaire tumoren en immune cellen in het bloed. Bovendien werd een sub-populatie van myeloide-afgeleide suppressor cellen (MDSC), een van de belangrijkste immuun cellen die bijdragen aan immuun suppressie in the direct omgeving van de tumor, veranderd door onze combinatie therapie in immuun-stimulerende cellen door het produceren van IL-12 (activeert T cellen), in correlatie met een significante verbetering in T cel responsen tegen het Mage-b antigen in de tumor cellen of primaire tumoren en metastases. Deze combinatie therapie was niet alleen een zeer effectief middel tegen de metastasen maar was ook niet-toxisch, zoals bevestigd door pathologisch onderzoek van de milt, de nieren, lever, hart en longen.

In hoofdstuk 3, hebben we Listeria^{at}-Mage-b en αGC therapeutisch getest in muizen met uitgezaaide borst kanker en ook hier vonden we een drastische vermindering in het aantal metastasen. Dit ging echter gepaard met een sterke toxiciteit in de lever, wat niet werd waargenomen wanneer Listeria^{at}-Mage-b of αGC gescheiden werd toegediend. Om dit probleem aan te pakken hebben we αGC geincorporeerd in de cel wand van de verzwakte Listeria^{at} bacteriën die Mage-b expresseren (I-αGC-LM-Mb). Hier laten we zien dat herhaalde toedieningen van lage dosis I-αGC-LM-Mb in muizen met uitgezaaide borst kanker net zo effectief was tegen de metastasen als het mengsel van LM-Mb en αGC, maar nu zonder enige zichtbare toxiciteit. Dit was gecorreleerd met het aktiveren van de NKT cellen, het genereren van de IL-12 productie in MDSC, en een aanzienlijk

verbetering in de CD8 T cel en NK cel responses in bloed. Ook de tumor groei was aanzienlijk verminderd, maar I-αGC-LM-Mb was niet significant beter dan de Listeria^{at}-Mage-b of αGC alleen. Hier tonen we voor de eerste keer aan dat de therapeutische behandeling met levende maar verzwakte Listeria^{at} geincorporeerd met αGC een zeer effectief middel is tegen de borst kanker metastasen zonder toxiciteit.

Samengevat, wij hebben aangetoond dat therapeutische vaccinatie met Listeria^{at}-Mage-b, wanneer gecombineerd met curcumin of geincorporeerd met αGC, de immuun suppressie kunnen remmen of veranderen in immuun stimulatie, en het aantal metastasen in muizen met uitgezaaide borst kanker sterk vermindert, in correlatie met significant verbeterde T cel responses tegen Mage-b en NK cel responses, zonder enige zichtbare toxiciteit. Terwijl het effect van beide combinatie therapieën minder effectief is tegen primaire tumoren, kunnen ze van grote waarden zijn voor “tweede lijn” therapie (na door chirurgie, chemotherapie of straling verwijderen van de primaire tumor), ter voorkoming van de ontwikkeling van metastasen. Vanwege de lage toxiciteit, kan het starten van de tweede lijn behandeling in een vroege fase beginnen om daardoor de klinische resultaten verder te verbeteren.

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EDUCATION:

2006 **Master of Arts**
Molecular Biology and Microbiology
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1999 **Master of Science**
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PROFESSIONAL EXPERIENCE:

2009-present **Research Trainee (PhD Student)** – Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York.

Mentors: Claudia Gravekamp, PhD and Steve Porcelli, PhD

- Explored therapies against metastatic breast cancer based on the effect of Listeria and curcumin.
- Investigating the therapeutic effect of Listeria and alpha-GalCeramide in a metastatic mouse breast tumor model in relation to innate immune responses at young and old age.

2006-2009 **Research Assistant** - California Pacific Medical Research Institute, San Francisco, CA. Advisor: John L. Muschler, PhD.

- Dystroglycan processing in normal and cancerous cells: structural modeling and mutagenesis.
- Nuclear trafficking of beta-dystroglycan: GFP fusion protein tracking and cell fractionation.
- The role of endocytosis in turnover of basement membrane proteins: flow cytometric analyses of protein trafficking.
- Function of dystroglycan in mammary epithelial cells: histochemical analysis of tissues from dystroglycan knockout mice.

2005-2006 **Research Assistant /Visiting Scholar** - Department of Developmental Biology. Stanford University, Stanford, CA; Project Advisor: Seung Kim, PhD. Research Project: "Molecular, genetics and bioinformatics analyses of insulin orthologs in hydra, Drosophila, and mouse"

2005-2006 **Teaching Assistant** - Department of Biological Sciences, San Jose State University, San Jose, CA; Advisor: David Brook, PhD.
Teaching Human biology and Cell biology lab classes.

2003-2005 **Graduate Assistant** - Department of Biological Sciences, San Jose State University, San Jose, CA; Supervisor: David J Matthes, PhD.
Research Project: "Genetic analysis of Drosophila *sema1b* gene".

2003-2004 **Graduate Student Researchers** - Department of Biological Sciences, San Jose State University, San Jose, CA; Supervisor: John Boothby, Ph.D.
Research Project: "Production of monoclonal antibody against *Listeria monocytogenes*."

PUBLICATIONS:

PEER RIEWED RESEARCH ARTICLES

- Leonoudakis D, **Singh M**, Mohajer P, Fata JE, Campbell KP, Muschler JL. (2010).Dystroglycan controls signaling of multiple harmones through modulation of STAT5 activity. J Cell Sci. 123:3683-3692.
- Oppizzi ML, Akhavan A, **Singh M**, Fata JE, Muschler JL.(2008). Nuclear Translocation of beta-Dystroglycan Reveals a Distinctive Trafficking Pattern of Autoproteolyzed Mucins. Traffic. 9:2063-2072.
- Akhavan A, Crivelli SN, **Singh M**, Lingappa VR, Muschler JL. (2008). SEA domain proteolysis determines the functional composition of dystroglycan. FASEB J. 22:612-621.

MANUSCRIPTS UNDER REVISION OR IN PREPARATION

- **Singh M**, Ramos I, Adjei D, Quispe-Tintaya W, Chandra D, Jahangir A, Zhang X, Aggarwal B, Gravekamp C. Curcumin Improves the Therapeutic Efficacy of Listeria^{at}-Mage-b Vaccine in correlation with improved T cell responses in blood of a triple negative breast cancer model 4T1. (Under revision)
- **Singh M**, Quispe W, Chandra D, Jahangir A, Gravekamp C. Direct incorporation of the NKT cell activator α-galactosylceramide improves efficacy and safety of a recombinant *Listeria monocytogenes* breast cancer vaccine. (Prepared for submission)
- Quispe-Tintaya W, Jahangir A, Chandra D, **Singh M,** Gravekamp C. Novel use of memory T cells to combat metastatic breast cancer. (In preparation)
- Jahangir A, Chandra D, Quispe-Tintaya W, **Singh** M. Gravekamp C. T cell responses induced by Listeria-based immunotherapy in old mice with metastatic breast cancer. (In preparation)

CONFERENCE CONTRIBUTIONS:

- **Singh M**, Ramos I, Adjei D, Quispe W, Chandra D, Jahangir A, Aggarwal B, Gravekamp C. "Improvement of efficacy by Listeria-Mage-b Vaccination through Curcumin." New York Cancer Consortium Developmental Therapeutics Symposium. October 28, 2011. New York, NY.
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- **Singh M**, Quispe W, Chandra D, Jahangir A, Gravekamp C. "Effect of Listeria and curcumin in various metastatic mouse breast tumor models in relation to adaptive immune responses at young and old age." 15th Annual Julius Marmur Symposium. March, 2011. Bronx, NY.
- **Singh M**, Grosveld F. "The role of Filamin A in Congenital Brain Disease". MGC Graduate Student Workshop. June, 2010. Cologne, Germany.
- Fata JE, Mohajer R, **Singh M,** Leonoudakis D , Kevin Campbell, Muschler JL. "A key role for dystroglycan in mammary epithelial morphogenesis and function, established in vivo." The American Society for Cell Biology Meeting. December, 2008. San Francisco, CA.
- Fata JE, Mohajer R, **Singh M** , Leonoudakis D , Kevin Campbell, Muschler JL. "A key role for dystroglycan in mammary epithelial morphogenesis and function, established in vivo." The Basement Membrane Gordon Conference. June, 2008. Biddeford, ME.
- Fata JE, Mohajer R, **Singh M**, Muschler JL."Dissecting key determinants of breast epithelial architecture" The Era of Hope Meeting for the Department of Defense Breast Cancer Research Program. June, 2008. Baltimore, MD.

PROFESSIONAL MEMBERSHIP:

- Member of American Society for Microbiology
- Member of American Society for Cell Biology
- Member of New York Academy of Sciences
- Presentation-SEA domain proteolysis and dystroglycan function, CPMC, April 07
- Volunteered at 16th Annual CSU Biotechnology Symposium (Jan. 2004)
- Purvanchal University Graduate Student Association (PUGSA) Representative (1998)

SKILLS:

TEACHING/MANAGEMENT SKILLS:

- Trained and mentored new lab members in learning molecular techniques at CPMCRI.
- Teaching Assistant for the cell biology, human biology laboratory and general genetics classes at SJSU.

TECHNICAL SKILLS:

- **Molecular Biology:** Recombinant DNA and RNA techniques including purification, PCR, cloning, mutagenesis, cDNA library construction and screening.
- **Cell Biology:** Fluorescence Activated Cell Sorting (FACS).
- **Cell Culture:** Expertise in the growth, maintenance, and transfection of both primary cells (mammary epithelial cells) as well as established cell lines.

- **Biochemistry:** Extensive use of protein purification, affinity chromatography; immunoaffinity chromatography techniques. Protein analysis by SDS-PAGE, western blotting, overlay protein-protein interaction assays.
- **Histology / Immunohistochemistry:** Expertise in the preparation of histological slides from tissue for microscopic examination using a cryostat to cut frozen sections. Performed immunofluorescence, immunohistochemical and histochemical staining of tissue sections.
- **Immunological Techniques:** ELISPOT, FACS, Extracellular and Intracellular staining of cells in blood, spleen, lymph nodes, tumors and metastasis for FACS analysis.
- **Microscopy:** Experience in preparation and imaging of cultured cells and sectioned tissue samples.

  - Confocal Microscopy: Experience with Nikon confocal microscopes and associated software with applications for subcellular protein localization and co-localization using double and triple-labeling techniques.

  - Epifluorescence: Expertise in multichannel indirect immunofluorescence microscopy of cultured cells and application to cell surface protein quantification.
- **Animal handling**: Experience in handling mice, immunizing mice (intraperitoneally and orally), dissecting all organs and preparing single cell suspension of spleen, lymph nodes, tumors, metastasis and blood for various immunological analyses.

COMPUTER SKILLS:

- **Bioinformatics**: Plasmid annotation, primer design, virtual PCR and cloning using Vector NTI software, promoter prediction and analysis, secondary structure prediction and tertiary structure visualization, comparison of orthologus genes using multiple sequence alignment tools like CLUSTALW, sequence assembly & analysis, sequence database searching with BLAST, DNA and protein homology analysis. Experience with Biology Workbench, NCBI, Expasy and other public domain databases and software.
- Experience with Windows & Unix Operating systems; Knowledge of Metamorph, Microsoft Word, Excel, PowerPoint, Perl, C, Adobe Acrobat, Illustrator and Photoshop.

LIST OF PUBLICATIONS

This thesis

Singh M, Ramos I, Adjei D, Quispe-Tintaya W, Chandra D, Jahangir A, Zhang X, Aggarwal B, Gravekamp C. Curcumin Improves the Therapeutic Efficacy of Listeria^{at}-Mage-b Vaccine in correlation with improved T cell responses in blood of a triple negative breast cancer model 4T1. (Under revision Cancer Medicine)

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Quispe-Tintaya W, Jahangir A, Chandra D, **Singh M,** Gravekamp C. Novel use of memory T cells to combat metastatic breast cancer. (In preparation)

Jahangir A, Chandra D, Quispe-Tintaya W, **Singh** M. Gravekamp C. T cell responses induced by Listeria-based immunotherapy in old mice with metastatic breast cancer. (In preparation)

Previous work

Leonoudakis D, **Singh M**, Mohajer P, Fata JE, Campbell KP, Muschler JL. (2010).Dystroglycan controls signaling of multiple harmones through modulation of STAT5 activity. J Cell Sci. 123:3683-3692.

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Akhavan A, Crivelli SN, **Singh M**, Lingappa VR, Muschler JL. (2008). SEA domain proteolysis determines the functional composition of dystroglycan. FASEB J. 22:612-621.

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List of Abbreviations

APC	Antigen-presenting cell
αGC	Alphagalactosylceramide
CCL	Chemokine (C-C) motif ligand
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle's Medium
ER	Estrogen Receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
gMDSC	Granulocytic myeloid-derived suppressor cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
iNOS	Inducible nitric oxide synthetase
IL	Interleukin
I-αGC-LM-Mb	LM-Mb incorporated with αGC
IDC	Invasive ductal carcinomas
ILC	Invasive lobular carcinoma
IBC	Inflammatory breast carcinoma
IFN	Interferon
LLO	Listeriolysin O
LM	Listeria monocytogenes
LM-Mb	Listeria-Mage-b
LCIS	Lobular carcinoma in situ
MAGE	Melanoma-associated antigen
MDSC	Myeloid-derived suppressor cells
mMDSC	Monocytic myeloid-derived suppressor cells
MCA	Methylcholantrene
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MMP	Matrix metalloproteinase
NK	Natural killer
NKT	Natural killer T
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PDGF	Plateled-derived growth factor
PR	Progesterone receptor
PRR	Pattern recognition receptors
prfA	Positive regulatory factor
ROS	Reactive oxygen species
STAT3	Signal transducer and activator of transcription 3
TME	Tumor microenvironment
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophages
TN	Triple-negative
TNBC	Triple negative breast cancer
TGF-β	Transforming growth factor beta
VGEF	Vascular endothelial growth factor