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Tumor and its Environment: Effort by Neem Leaf Glycoprotein to keep it Green

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ABSTRACT

Tumor is not only the accumulation of tumor cells; it is a heterogeneous mass consisting of tumor cells, along with numerous other cells. This includes fibroblasts, inflammatory immune cells (such as, macrophages, smooth muscle cells), blood vessel constituting cells (endothelial cells and pericytes), embedded in an extracellular matrix. In fact, just like seeds, tumor cells are impregnated in soil, i.e., its environment. Environment as well as its secretary factors (VEGF, TGFβ, IL-10 etc.) provide all necessary extrinsic signals to proceed for further malignant growth and metastasis. Tumor microenvironment (TME) takes the critical decision in determining tumor progression versus dormancy or destruction. Accordingly, TME demands research attention to find out way to inhibit the tumor growth. Unfortunately, up to mid 1990s, cancer researchers paid little attention to TME, because of little understanding on the alliance between tumor and its environment causes tumor proliferation, angiogenesis, drug resistance, metastasis, immune paralysis and escape. Available therapeutic measures inhibit tumor growth at the cost of severe pollution in TME in terms of induction of hypoxia, T cell anergy/exhaustion/apoptosis, protumor bias of cytokine/chemokine network and several other tumor promoting activities. Thus, in true sense, targeting of tumor only give transient response and requires normalization of TME to obtain durable tumor restriction. As complicated network of signaling is operational within TME between the tumor and several other tumor associated

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cellular components, normalization of TME requires synchronization of multiple events. In order to do this important job, the first task is to find out the candidate drug(s) for this particular purpose. Obtained experimental and clinical data confirmed that chemotherapeutic drugs and radiotherapeutic measures inhibit tumor proliferation in one hand, but creates protumor microenvironment in other hand. Different medicinal plants have proved worthy for cancer treatment from very early times, and, evaluated in clinical studies or currently being investigated to understand their tumoricidal actions against various cancers. Although, extensive research is waiting, it is expected that plant derived anti-tumor drugs cause limited pollution to TME.

Neem leaf Glycoprotein (NLGP), a glycoprotein from aqueous preparation of neem (Azadirachta indica) leaf is efficient to restrict murine tumor growth. NLGP has no direct tumoristatic or tumorilytic activities, but it offers tumor growth restriction chiefly by immunomodulation as evident from several studies from our group. Such success may not be possible only by activating immunocompetent cells, like, T cells or NK cells, but also requires normalization of TME. TME obtained from NLGP treated sarcoma and melanoma bearing mice (NLGP-TME), is completely different (normalized) from untreated tumor bearing mice (PBS-TME), immunologically as well as biochemically. Proportion of $CD8^+$ T cells increased within NLGP-TME with downregulation of $CD4^+CD25^+Foxp3^+$ Tregs and $CD11b^+Gr1^+$ MDSCs. Analysis of cytokine milieu revealed IL-10, TGF-β, IL-6 rich TME with type-2 characters that switched to create type-1 microenvironment with dominance of IFNγ secretion from cellular components of NLGP-TME. NLGP defends T cells from TME-induced anergy, as indicated by higher level of pNFAT and related downstream signaling. Moreover, low expression of $FasR^+$ cells within $CD8^+$ T cell population denotes prevention of activation induced cell death. Using CFSE as a probe, better migration of T cells was noted within TME from NLGP treated mice than PBS cohort. $CD8^+$ T cells isolated from NLGP-TME exhibited greater cytotoxicity to sarcoma/melanoma cells in vitro and these cells show higher expression of cytotoxicity related molecules like perforin and granzymeB. Adoptive transfer of NLGP-TME exposed T cells inhibited the growth of different tumors. Accumulated evidences strongly suggest that NLGP is able to maintain green of cellular environment by reducing toxic nature of TME that allows T cells to perform optimally to kill the tumor cells. The proposed review would discuss the efforts of NLGP to maintain the normal functions of several components of TME, thereby, to keep the TME green.

Keywords: Neem leaf glycoprotein, Tumor microenvironment, Cytotoxic T cells, Tumor associated macrophages, Dendritic cells, Regulatory cells.

Introduction

Genomic instability and high proliferation rates impose a unique mutational signature to each cancer cell that gives rise to genetic heterogeneity of a tumor (Pedraza-Farina *et al.*, 2006). For the majority of tumors, genetic heterogeneity helps cancer cells to grow and to acquire drug resistance in cancer therapy (Turner *et al.*, 2012). In addition, cellular heterogeneity provides a growth and survival advantage to the cancer cells within a growing tumor. As the cancer cells grow, they are stressed by environmental factors, like, lack of oxygen, nutrients and pressure of the nearby

tissues (Höckel *et al.,* 2001). Without a steady supply of nutrition and oxygen, microscopic colonies of cancer cells ("carcinoma in situ", CIS) cannot grow and transform into invasive carcinoma, thus, tumors require their environmental support for progression. Tumor cells send out chemical signals to modulate the different populations of the host's healthy cells (Whiteside *et al.,* 2006) and to recruit blood-derived immune cells simultaneously (Penn *et al.,* 1973). As tumor develops, these surrounding environment and recruited normal cells co-evolve into an alternatively-activated state through a continuous paracrine as well as autocrine communication, thereby creating a microenvironment that promotes cancer growth and ultimately leads to metastatic disease (Wels *et al.,* 2008). Accordingly, malignant cells create a tolerant neighborhood in which they can function with limited interference. Collectively this neighborhood consisting of connective tissue, extracellular matrix, and the resident non-cancerous cells surrounding the cancer cells are known as tumor stroma. Many researchers prefer the broader term "tumor microenvironment (TME)," instead of "stroma," as it includes infiltrating cells of the immune system (innate and adaptive), and cell-free molecules (proteases and growth factors) in addition to the residential stromal components (Pietras *et al.,* 2010) Although the same basic building blocks constitute the stroma of all tumors, the actual composition of the TME is quite variable, with differences seen between individuals, between different tumor types, and in different areas of the same tumor. Furthermore, the composition of the TME is also altered as the disease progresses (Diaz-Cano., 2012).

Tumor and its Environment

From the beginning of the discovery of cancer to late 1990s, scientists have focused on the cancer cell itself; more recently they begun to think; how the rest of our body or surrounding tissues influence the cancer cells. The microenvironment: the normal cells and molecules that surround a tumor cells- are becoming extremely important in the process of tumorigenesis. Tumor is an assorted mass of different cell types, which includes cancer cells and several other cells, including fibroblasts, inflammatory immune cells (*e.g.,* macrophages), smooth muscle and endothelial cells, all embedded in an extracellular matrix. It also contains special kinds of growth factors, chemical messenger molecules, called chemokines and cytokines, and also chemicals, like oxygen. The communication between the tumor cells and the surrounding cells of the microenvironment helps to drive the process of tumor progression. So transformation from normal to benign, benign to malignant, malignant to metastatic is driven not just by what's happening inside the tumor cell itself but also depends on what's happening around it. Recently, it is becoming more and more clear that TME plays a very important role in assisting tumor cells for metastasis (Chantrain *et al.,* 2008). Interestingly, the association between TME and metastasis is named as "Seed and soil" hypothesis, first time suggested by Stephen Paget over a century ago in 1889 to explain organ preferred metastasis (Langley and Fidler, 2011). This hypothesis suggested that tumor cells (seed) can colonize only to those distant organs (soil), where favorable environment for tumor growth exists. Stephen Paget concluded that metastasis results only when the seed and soil were compatible (Paget 1989). However, only recently Paget's idea received a lot of attention, and TME in

cancer progression, metastasis and drug-response became a very actively investigated topic.

The tumor cells are highly heterogeneous with diverse differentiation grades. The success of tumor cells to grow seems to be dependent on their ability to control and shape the surrounding microenvironment to favor their own survival (de Visser *et al.,* 2006). Eventually the fact is established that tumor progression or metastasis is governed on a systemic level involving various non-malignant (stroma) cells, soluble factors and ECM components (Deryugina and Quigley, 2006). *This research field is still at the very early phase, and the direct contribution of each element of TME in tumor progression or metastasis remains to be elucidated in detail. It has been proposed that targeting tumor cells together with the targeting of implicated TME factors can improve the efficacy of anticancer therapy.*

Tumor Microenvironment (TME) and Associated Factors

A hallmark of chronic inflammatory tissues and adenocarcinomas is the presence of an abundant fibrotic component, *i.e.* desmoplasia (Korc., 2007). The interlinked relationship between tumor cells and its stroma promotes tumor growth and metastasis by supporting activation of fibroblasts (Liao and Luo, 2009). Stromal components are important for creation and homeostasis of the inflammatory TME, consists of infiltrating immune cells, including, neutrophils, dendritic cells (DCs), macrophages, eosinophils, mast cells, and T cells. These cells will also recruit additional immune cells to the tumor milieu by secretion of chemotactic factors. Infiltrating immune cells support tumor progression by the release of growth and survival factors, matrix remodeling factors, and reactive oxygen species etc. (Erez *et al.,* 2010). The composition and characteristics of the TME vary widely and are important in determining the anti-tumor immune responses. For example, certain cells of the immune system, including NK cells, DCs and effector T cells, are capable of driving potent anti-tumor responses. However, several subtypes of immune cells (such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs)) that commonly infiltrate solid tumor harbor immunosuppressive activity and foster tumor development by expression of potent pro-tumor mediators. These cells undoubtedly restrict the effectiveness of anticancer strategies. Formation of new blood vessels, *i.e.,* angiogenesis is essential for the growth of solid tumors beyond 1-2 mm^3 (Bhowmick *et al.,* 2004). The cancer cells could promote angiogenesis directly by secreting VEGF, bFGF, CXCL8, and placenta growth factor (PIGF), and also indirectly by taking over the role of the thrombocytes by attracting immune cells and by the transformation of stellate cells to highly proliferating CAFs.

Inflammation and Cancer

Inflammation is an essential event in the development and progression of tumors and is suggested to be the seventh hallmark of cancer (Colotta *et al.,* 2009). The inflammatory environment is created by the release of proinflammatory cytokines, defined as "alarm cytokines" present early in the carcinogenesis, such as TNFα, IL-1α, and IL-1β. These cytokines initiate inflammatory responses and are secreted by infiltrating leukocytes and malignant cells. Besides being inflammatory initiators,

these cytokines also induce expression of other proinflammatory genes, such as COX-2, inducible nitric oxide synthase (iNOS), chemokines, cytokines, and matrix metalloproteinases (MMPs) (Apte and Voronov, 2008). MMPs are zinc-dependent endopeptidases that function to degrade all kinds of extracellular matrix proteins. The MMPs have been shown to play important roles in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis.

Immune Components of TME

The composition and characteristics of the TME vary widely and are important in determining the anti-tumour immune response. Certain cells of the immune system, including macrophages, NK cells, DCs and effector T cells are capable of driving potent anti-tumour responses. However, tumor cells often induce an immunosuppressive microenvironment, which favors the development of immunosuppressive populations of immune cells, such as MDSCs and Treg cells. Understanding the complexity of immunomodulation by tumors is important for the development of immunotherapy.

Effector Cells

The immune system contains several types of cytotoxic cells, which are able to lyse host and foreign cells. Cytotoxic T-lymphocytes (CTL) appear to play the most important role among the killer cells but other lymphatic cells, such as NK cells and lymphokine-activated killer (LAK) cells as well as macrophages are also highly effective in the lyses of appropriate targets. The various cytotoxic effector cells differ distinctly concerning origin, phenotype, morphology and target cell specificity, but they bear the common feature that they destroy the target cells in a contact-dependent process. CTL recognize specific antigens which are presented in context with molecules of class I major histocompatibility complex (MHC). NK cells, on the other hand, kill the appropriate targets without prior immunization and without requiring recognition of MHC molecules at the target cells. They also bear a typical pattern of surface markers which differ in several aspects from that of CTL. CTL, NK and LAK cells appear to possess similar mechanisms for cytolysis including secretion of pore-forming proteins, serine proteases and other proteins. Macrophages differ substantially from other cytotoxic effector cells concerning morphology, phenotype, kinetics of activation and target cell spectrum. They perform a variety of functions whereby contact-dependent target cell lysis represents only one of their properties. After binding with target cell they release over 20 different molecules such as IL-1 and TNFα as mediators for cytolysis. Within TME, effector cells become paralyzed due to interactions between tumor cell and altered stromal cells, along with suppressive activity of regulatory cells. The tumor cells cannot be targeted by CTL as an efûcient APC within TME is unable to deliver a co-stimulatory signal required for productive interactions with T lymphocytes. These CTLs become anergized and undergo activation induced cell death.

Regulatory Cells

Regulatory cells (Tregs, MDSCs) of immune system down regulate the activity of other immune cells, thus preventing the development of autoimmune diseases or

allergies. Treg cells may secrete suppressor cytokines that can directly inhibit the function of responder T cells and myeloid cells. Treg cells express high CD25, the IL-2 receptor alpha chain, and have the capacity to compete with effector T cells for IL-2 resulting in cytokine-mediated deprivation of the effector cells and Bim-mediated apoptosis. Treg cells also express CTLA4 that mimics CD28 on T cells and promotes crosstalk with APCs by means of B7 molecules and inhibits antigen presentation (André *et al.,* 2009). Activated Foxp3$^+$ Treg cells may function as cytotoxic cells and directly kill effector cells in a manner similar to CD8$^+$ cytotoxic cells. Thus, Tregs within TME play a role in promoting tumor growth and metastasis by inhibiting the immune response against cancer by means of suppression of activation of antigen specific effector immune cells (both CD4$^+$ and CD8$^+$ T cells).

Immunosuppressive functions of other suppressor cells, MDSCs, require direct cell-cell contact (Wang *et al.,* 2012), which suggests that they act either through cell-surface receptors and/or through the release of short-lived soluble mediators like arginase, iNOS, ROS and peroxynitrite (Gabrilovich and Nagaraj, 2009). It also induces development of Treg cells. Some studies also observed that MDSCs abrogated NK cell cytotoxicity against tumor cells and inhibited IFNγ production by NK cells (Jewett and Tseng, 2011).

Non-Immune Component of TME

Besides immune components, TME contains resident non-cancerous cells (fibroblasts, endothelial cells), connective tissue, and extracellular matrix (ECM; components of tissues that provide structural support, such as proteins like collagen). These components are equally important both in tumor initiation and progression. Collectively, these components are known as stroma, required to maintain epithelial tissues. Changes in epithelium cause inevitable alteration in stroma. *In cancer, changes in the stroma drive invasion and metastasis, hallmarks of malignancy.*

Vascular Endothelial Cells

Endothelial cells in direct contact with blood are called vascular endothelial cells (VEC) whereas those in direct contact with lymph are known as lymphatic endothelial cells (Alitalo and Detmar, 2011). VECs use cell adhesion molecules, such as integrins and cadherins to attach themselves to each other and to the vascular extracellular matrix. Tumor cells secrete angiogenic factors (like, VEGF) to stimulate VEC proliferation and induce angiogenesis. These tumor-associated growth factors stimulate the direct interaction between tumor cells and VEC via MAPK and Notch signaling pathways, thereby promoting tumor angiogenesis and tumor growth (Veikkola *et al.,* 2000).

Pericytes

Blood vessels are composed of two interacting cell types. VECs from the inner lining of the vessel wall, and perivascular cells-referred to as pericytes, vascular smooth muscle cells or mural cells-envelop the surface of the vascular tube (Bergers and Song, 2005). VECs in blood vessels under formation are stabilized by the recruitment of pericytes, both in normal tissues and during angiogenesis in

pathological situations, including neoplasia (Franco *et al.*, 2011). In the tumor vasculature, besides supporting the functionality of blood flow, pericytes protect VECs from anti-angiogenic therapies, and have thus been implicated in clinical resistance to vascular targeting drugs (Franco *et al.*, 2011). Recently, another function of pericytes within TME is documented by us, *i.e.*, tumor associated pericytes induce tolerance to $CD4^+$ and $CD8^+$ T cells, as tumors and tumor derived factors alter immunophenotypes and functionality of tumor resident pericytes *(unpublished observation)*.

Fibroblasts

A fibroblast is a type of cell that synthesizes the extracellular matrix and collagen, the structural framework (stroma) for animal tissues, and plays a critical role in wound healing. Fibroblasts are the most common cells of connective tissue in animals. The main function of fibroblasts is to maintain the structural integrity of connective tissues by continuously secreting precursors of the extracellular matrix (Ge *et al.*, 2004). Fibroblasts are the most prominent cell type within the tumor stroma of many cancers (Cirri and Chiarugi, 2011); these populations share some properties collectively leading to their "activation state". The main activation markers, fibroblast specific protein (FSP), PDGFRα and fibroblast activation protein (FAP) have been found over expressed in stromal fibroblasts of solid tumors (Pietras and Ostman, 2010).

Extracellular Matrix (ECM)

ECM constitutes a major component of microenvironment of a cancer cell, plays important roles in cancer development. It is a complex network of macromolecules with distinctive physical and biochemical properties (Lu *et al.*, 2012). ECM has many important roles, collectively measured by its production, degradation, and remodeling. Abnormal ECM affects cancer progression directly by promoting cellular transformation and metastasis. Notably, this abnormal ECM affects character of stromal cells, assist tumor-associated angiogenesis and inflammation, and lead to creation of a tumorigenic microenvironment (Hanahan and Weinberg, 2011).

Cytokines

During carcinoma formation, cancer cells release various cytokines and growth factors into their surroundings and recruit and reprogram many other types of cells in order to establish a TME (Sheu *et al.*, 2008). Consequently, the tumor tissues almost always contain a large number of VECs, fibroblasts, and infiltrating inflammatory cells that in turn fabricate a variety of cytokines, like, IL-10, IL-6, TGFβ (Ma *et al.*, 2012). The cytokines produced by these cells have been positioned as key factors in modulating immune response either against or in favor of tumorigenesis in the microenvironment. The interactions that take place between immune and cancer cells are complex, involving multiple cascades of cytokines, chemokines, and/or growth factors (Coussens and Werb, 2002).

Chemokines

In the immune system, chemokines exert vital homeostatic functions by regulating the organization of lymphoid organs, lymphocyte differentiation, and lymphocyte

homing, thus inuencing inammation and adaptive immune responses (Stein and Nombela-Arrieta, 2005). It has also been realized that tumor cell migration and growth is dependent on direct chemokine signals to tumor cells (Kulbe *et al.,* 2044). During tumor progression chemokines play a paramount role. Both tumor cells and stromal cells elaborate chemokines and cytokines. These act either by autocrine or paracrine mechanisms to sustain tumor cell growth, induce angiogenesis and facilitate evasion of immune surveillance through immunoediting (Gouwy *et al.,* 2005).

Importance of Normalization of TME for Anti-Tumor Immunity

It has been widely recognized, though recently, that a tumor grows towards a malignant phenotype by altering its microenvironment in a way that accelerates its malevolent potential (Lorusso and Rüegg, 2008). Consequently, current anti cancer therapeutic strategies target not only the intracellular distortions of a malignant cell but also cause deformation in its extracellular microenvironment (Prager *et al.,* 2012). The remedial strategies are designed with a major aim to optimize the antitumor immunity in the tumor vicinity, as the alterations in the TME are dominantly manifested as an impaired immune response. As tumor is seeded in the soil, *i.e.,* TME, its progression, and, in some cases regression solely depends on the constituents of TME (Witz, 2009). Components of TME interact with each other and contribute significantly in tumor progression. Cells (TAM, MDSCs, tumor associated DCs and tumor cell itself) from TME produce angiogenic factors, like, VEGF and PDGF (Rehman *et al.,* 2003). Moreover, TGFβ, HGF secreted from fibroblasts stimulate tumor cell proliferation (Bhowmick *et al.,* 2004). Tumor conditioned immune cells with type 2 characters, like, Tregs, MDSCs and TAMs synthesize and secrete VEGF, PDGF, HGF, TGFβ etc, to promote tumor progression. As tissue becomes cancerous, pathological interactions between cancer cells and host immune cells in the TME create an immunosuppressive network that protects the tumor from immune attack (Whiteside, 2006) leading to tumor growth, progression, invasion and metastasis (Mbeunkui and Johann, 2009). Thus, normalization of TME is the principle task of cancer immunotherapy.

Plant Products in Normalization of Immune System and Cancer Therapy

Medicinal plants impart significant roles in preventing human being from various pathogenic microorganisms and the diseases like cancer (Singh *et al.,* 2011). In nature there are various medicinal plants, used as immunomodulatory agents (Kannan and Singh, 2010). In Indian medicinal literature a large number of plants are included, that promote defense mechanisms of the body. In other hand a large number of medicinal plants included in Rasayanas have been claimed to possess immunomodulatory activities (Kumar *et al.,* 2011). Medicinal plants are used as an immunomodulator to provide alternative potential to conventional chemotherapy for cancerous diseases, especially in relation to host defense mechanism. The use of plant products like polysaccharides, lectins, peptides, flavonoids and tannins has been established in the modification of immune response in various *in-vitro* models (Mazid *et al.,* 2011).

Natural Products Modulates Tumor Microenvironment

Several plant products, like, Combretastatin A4, Cinnamon extract, Curcumin have been identified for their anti cancer activity and they have utilized a variety of mechanisms. However, few of these compounds were tested in context of modulation or normalization of components of TME.

Combretastatin A4

Isolated from the stem wood of the South African tree *Combretum caffreum*, is a vascular disruptive agent, thus, playing an important role in normalization of angiogenesis in cancer. It inhibits tumor blood vessel growth, causing tumor cell death and necrosis. Phase I trials have shown some clinical activity of combretastatin A4 and a favourable toxicological profile (Rustin *et al.*, 2003).

Cinnamon Extract

Cinnamon is one of the most widely used herbal medicines with diverse bioactive effects (Yu *et al.*, 2010). However, little evidence has been reported about the potential anti-tumor effects of cinnamon. Cinnamon may act by normalization of TME by means of reduction of excessive blood flow within tumors. In vitro (in melanoma cells) and in vivo (in experimental melanoma model) studies showed that cinnamon treatment strongly inhibited the expression of pro-angiogenic factors and master regulators of tumor progression (Kwon *et al.*, 2009). In addition, cinnamon treatment increased the anti-tumor activities of $CD8^+$ T cells by increasing the levels of cytolytic molecules (Kwon *et al.*, 2009). Cinnamon extract has the potential to be an alternative medicine for tumor treatment. Anti-tumor effect of cinnamon extracts is directly linked with enhanced pro-apoptotic activity and inhibition of NFkB and AP1 activities and their target genes (Kwon *et al.*, 2010). However, no report is available regarding their effects on other components of TME.

Curcumin

Curcumin is one of the most studied chemopreventive agents (Duvoix *et al.*, 2005). It is a natural compound extracted from the rhizome of *Curcuma longa* L. that allows suppression, retardation or inversion of carcinogenesis (Singh and Singh, 2011). Recent evidences suggest that curcumin exhibits strong anti-inflammatory and antioxidant activities and modulates the expression of transcription factors, cell cycle proteins, and signal transducing kinases etc (Surh *et al.*, 2001). All of these functions are major challenges within TME, thus, curcumin may work in the direction of TME normalization. Curcumin has different immunomodulatory effects on the development of immune responses, which include its effect on lymphoid cell populations, antigen presentation, humoral and cell-mediated immunity, and cytokine production (Bill *et al.*, 2009). Although, studies are coming up to measure the modulation of intratumoral immunity by curcumin, it may be hypothesized that anti tumor activity of curcumin may be mediated by TME normalization.

Resveratrol

Resveratrol, a natural compound found in red wine, some fruits and nuts, has shown anti-tumor activity in concentrations and regimens that appear to show

minimal toxicity to normal cells (Van Ginkel *et al.*, 2007). It slows tumor growth and, depending on the dose and route of administration, can also lead to the regression of local tumors (Van Ginkel *et al.*, 2007, Chen *et al.*, 2004). The anti-tumor action of Resveratrol has been tested in mice bearing different types of malignant tumors such as breast, lung, liver, melanoma and neuroblastoma (Busquets *et al.*, 2007). Indirect evidences suggest that Resveratrol has some effect on TME or TME components. Positive effects of Resveratrol in ameliorating inflammatory conditions such as colonic inflammation, (Martín *et al.*, 2004) lipopolysaccharide-induced airway inflammation and osteoarthritis, and even in preventing allograft rejection (Wu *et al.*, 2005) have been reported. Resveratrol has been shown to inhibit activation of the transcription factor NF-êB induced by IL-1β (Estrov *et al.*, 2003) and by TNFα, and also to inhibit activation of AP-1, MAPK and c-Jun N-terminal kinase (Manna *et al.*, 2000). PBMC exposure to Resveratrol produced a biphasic effect on the anti-CD3/anti-CD28-induced development of both IFNγ/IL-2 and IL-4 producing $CD8^+$ and $CD4^+$ T cells, with stimulation at low Resveratrol concentrations and suppression at high concentrations. Similarly, the compound was found to induce a significant enhancement at low concentrations and suppression at high concentrations of both CTL and NK cell cytotoxic activity.

We have attempted to present an overview on TME in the earlier discussion and it is understandable that TME has great influence on cancer progression and metastasis. Thus, targeting of only tumor cells may not offer maximum clinical benefit. Effort to normalization of TME would be crucial to design future medicine. So far, very little attempt has been done in this direction of research and we have found few reports using natural products to normalize TME or its components. We are extensively exploring the role of neem leaf glycoprotein (NLGP) in cancer immunotherapy. Now, we are describing the effect of NLGP on various tumor components to predict the possible ways to restrict the tumor growth by restoring the environment of tumor towards normal as much as possible.

Neem Traditional View

The neem tree has been found as a traditional medicinal plant from ancient Harappa and Mohenjo-Daro civilizations in Indian subcontinent. The medical practitioners of that age studied a variety of plants and trees having therapeutic values and neem tree was one of them. The earliest indication of neem tree being used for its medicinal properties in households began nearly 5000 years ago. Neem is said to have been widely used in traditional systems of medicine like Ayurveda and this is also mentioned in earliest Indian scriptures of medicine- the Charak Samhita and Sushruta Samhita. Hence neem received the key interest in traditional medicine as SARBOROGANIBARANI (can cure all forms of diseases) and eight parts of this plant have wonderful biological actions (Biswas *et al.*, 2002). In 1992, US National Academy of Science designated this tree, as 'A tree sloving global problem' (National Research Council, 1992). Our group identified the tumor restricting property of an aqueous preparation from neem leaf, termed Neem Leaf Preparation (NLP) (Baral and Chattopadhyay, 2004) and a glycoprotein, named Neem Leaf Glycoprotein (NLGP), which appeared as an active principal for tumor growth restriction and

associated immunomodulation (Chakraborty K *et al.*, 2008). NLGP is totally nontoxic in nature and have potential efficacy to regress tumor in different cancer models (Haque *et al.*, 2006; Mallick *et al.*, 2012).

NLGP from Chemical Viewpoint

NLGP is a purified glycoprotein isolated from aqueous preparation of neem leaf. Purified NLGP appeared in nondenatured PAGE as a single band and a single peak in HPLC, whereas, three bands having molecular weight of 15 kd, 23 kd and 47 kd in SDS-PAGE. This data indicates its hetero-trimeric nature. This glycoprotein constitutes of the carbohydrate moiety of about 33 per cent sugar, consisting of arabinose, galactose and glucose. Protein moiety of glycoprotein consists of sixteen amino acids, except arginine. Tumor restricting activity of NLGP is dependent on both glycol- part as well as protein nature of the NLGP. This hypothesis was proved by exposing NLGP in an array of temperature (100ºC), pH and enzymes. Exposure to the adverse temperature (100ºC), pH (5.7) and enzymes (papain, neuraminidase), resulted complete disappearance of the tumor growth restricting activity of NLGP (Baral *et al.*, 2010). Further works to characterize the protein are in progress.

NLP, NLGP and Restriction of Murine Tumors

We have started our work with NLP to see its effect on tumor growth restriction. In that effort, mice were grouped into two. Mice (Swiss and C57BL/6) of first group were subcutaneously injected with NLP weekly for four weeks in total and other group received PBS or extract obtained from leaf of the tree belongs to same Melliesse family (Mehagani). Following immunization carcinoma and melanoma cells were inoculated in Swiss and C57BL/6 mice respectively of both groups. In NLP immunized group, tumor growth was restricted significantly (Haque and Baral, 2006). Repetition of such experiments several times yielded identical results. Our in vitro studies with several murine and human cancer cells, along with normal cells, revealed that NLP has no cytotoxic effect on neither cancer cells nor normal cells. However, inoculation of spleen cells of NLP treated mice mixed with B16Mel cells in a group of mice, revealed significant reduction of tumor growth and increased survivability of the tumor hosts in comparison to mice inoculated with tumor along with normal spleen cells. This observation compelled us to think about a strong immune-boosting ability of NLP. Similar studies were conducted with NLGP and identical results were obtained. Most recently, we have observed that use of NLGP in therapeutic settings effectively restricts the growth of murine sarcoma (Mallick *et al.*, 2012), carcinoma and melanoma (*unpublished observation*) *in vivo*. Pattern of immunomodulation by NLGP on different immune cells, mostly in periphery, was elucidated in a series of investigations. These observations help to investigate their behaviour within TME in close vicinity of other TME components, like, vascular endothelial cells, pericytes, fibroblast etc.

Immunomodulation by NLP/NLGP

Immunomodulatory effect of NLP and NLGP on macrophages, DCs, NK cells, NK-T cells, T cells, regulatory T cells, B cells, etc. and secretion of various cytokines

and chemokines was discussed in a series of studies. Principal observations are discussed below.

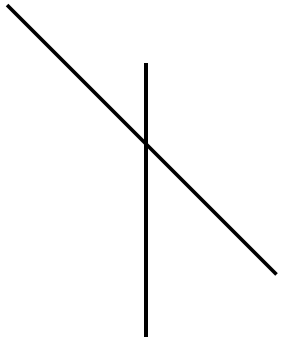
Regulation of Macrophages, NK cells, NK-T cells by NLP/NLGP in Periphery

In the initial phase of our research on NLP, we observed that NLP was found to activate PBMC from either healthy subjects or HNSCC patients, demonstrating significant enhancement in the cytotoxicity of tumor cells. We found $CD56^+$ and $CD16^+$ population increases within the PBMC fraction obtained from healthy and HNSCC patients upon NLP stimulation suggesting the participation of NK cells in cytotoxic function. NLP efficiently upregulated cytotoxic efficacy of NK cells ($CD16^+$ and $CD56^{dim}$) by means of enhanced killing of NK-sensitive K562 cells. Such killing is dependent on IL-12. Moreover, NLP upregulated the expression of cytotoxic molecules, perforin-granzyme B and FasL. Studies using specific inhibitors suggested that NLP-mediated induction of cytotoxicity is chiefly dependent on perforin-granzyme B system (Bose and Baral, 2007).

In addition, NLP unregulated the expression of CD40 on $CD14^+$ monocytes and CD40L in $CD56^+$ lymphocytes. Neutralization of CD40 and CD40L in NLP-stimulated PBMC culture resulted in significant downregulation of IL-12 release and cytotoxicity of NK cells; this clearly suggested that IL-12 is the key regulator of the NLP-mediated NK cell cytotoxicity, demonstrating the role of CD40-CD40L interaction in the observed functions (Bose and Baral, 2007). After identification of NLGP as an active component, similar experiments with NK cell functions were performed and identical results were obtained. Within TME NLGP may activate suppressed NK cell functions, but experiments in context of TME are yet to be done. After identification and purification of active principle of NLP *i.e.*, NLGP, we have demonstrated augmentation of the NK ($CD3^-CD56^+$) and NK-T ($CD3^+CD56^+$) cells mediated tumor cell cytotoxicity. These NK cells were isolated from the peripheral blood of HNSCC patients with a state of immunosuppression (Bose *et al.*, 2009). In addition to human system, we have also observed that mononuclear cells from blood and spleen of NLP-activated Swiss and C57BL/6 mice cause enhanced cytotoxicity to murine tumor cells in vitro. Flow cytometric analysis revealed in both blood and spleen, NK cells ($DX5^+$ or $NK1.1^+$) and NK-T cells ($CD3^+/DX5^+$ or $CD3^+/NK1.1^+$) were increased in number in Swiss, C57BL/6 and athymic nude mice those were NLP pretreated and tumor bearing. NLP-stimulated spleen cells showed greater secretion of TNFα and IFNγ. Thus, NLP-activated NK and NK-T cells in mice may regulate tumor cell cytotoxicity by enhancing the secretion of different cytotoxic cytokines (Haque and Baral, 2006).

Regulation of macrophages, NK cells, NK-T cells by NLP/NLGP in TME

Within solid tumors marked myeloid cell infiltrates are seen and activation of these, chiefly, tumor associated macrophages (TAMs), is now known to play a key role in tumor progression. Monocytes enter tumors through blood vessels during the life span of tumors, from early-stage tumor nodules that are beginning to vascularize to late-stage tumors that are invasive and metastatic. A number of tumor-derived



chemoattractants are thought to ensure this ongoing recruitment, including colony-stimulating factor-1 (CSF-1 also known as M-CSF), the CC chemokines, CCL2, CCL3, CCL4, CCL5, CCL8 and VEGF. After recruitment according to the local tissue microenvironment these monocytes are either polarized into proinflammatory anti-tumorous M1 type or anti-inflammatory protumorous M2 form. We studied the effect of TME on the generation of alternate M2 type macrophages in B16 melanoma system, in association with the immunoregulatory function of NLGP.

Our *in vitro* and *in vivo* studies confirmed that B16 melanoma TME polarizes macrophages in alternative M2 form where they show IL-12^{low}, IL-10^{high}, Nitric Oxide^{low}, Arginase^{high}, F4/80^{high}, ManR^{high}, Fizz1^{high}, YM1^{high}, CCR5^{low}, CCR7^{low} phenotypic profile and poor antigen presenting ability that are in contrary to that of the classical phenotype of normal peritoneal macrophages. Results further confirm that macrophage alteration does not only stay restricted to genotypic and phenotypic level as these alternatively activated macrophages show altered functional property and subvert normal immunological functioning in a way by paralyzing T effector cell functions, secrete immunosuppressive cytokines, augment Treg population, slows and/or stops DC maturation, negatively affect activated NK cell status. NLGP was found to partially modulate these M2 characteristics of TAMs and polarize them towards M1 version specifically by downregulating the upregulated status of STAT3 in M2 macrophages. In particular, to assess the immunomodulatory role of NLGP, we isolated TAMs from both tumor core and peripheral regions and have found that NLGP has prominent effect in downregulating the M2 status in tumor core by means of impeding the IL-10 signaling pathway that upon activation causes upregulation of STAT3 responsible for giving type 2 genotypic expression. Therefore, NLGP can be used as a natural contrivance to reduce M2 load from tumor that in majority of the cases **is** responsible for poor prognosis.

Studies on NLGP regulation of NK and NK-T cell functions within TME is awaiting.

Regulation of T Cells by NLP/NLGP in Periphery

NLP helps to increase CD4$^+$ and CD8$^+$ T cell population upon its treatment in both tumor bearing and normal mice. Significant increase in lymphocyte count both in blood as well as in spleen of NLP treated mice suggested that NLP is effective in inducing lymphocytosis. Increase in the number of CD4$^+$ and CD8$^+$ T lymphocyte subpopulation in spleen and blood of the NLP treated mice also suggested NLP induced proliferation of T cell subpopulation, which may play a major role in restricting tumor growth in the NLP treated mice (Baral and Chattopadhyay, 2004).

After identification and purification of active principle of NLP *i.e.,* NLGP, we have demonstrated increase in the number of T cells (CD3$^+$CD8$^+$CD56$^-$) mediated tumor (KB) cell cytotoxicity. These T cells were isolated from the peripheral blood of HNSCC patients with a state of immunosuppression. NLGP induces TCRαβ-associated CTL reaction to kill KB cells. This CTL reaction is assisted by NLGP-mediated up-regulation of CD28 on T cells and HLA-ABC, CD80/86 on monocytes. CTL-mediated killing of KB cells is associated with activation of these cells by NLGP. This activation is evidenced by increased expression of early activation marker CD69

with altered expression of CD45RO/CD45RA. NLGP is a strong inducer of IFNγ from T cells. This NLGP-induced cytotoxicity is dependent on up-regulated perforin/granzyme B expression in killer cells, which is again IFNγ dependent in T cells. Although, FasL expression is increased by NLGP, it may not be truly linked with the cytotoxic functions, as brefeldin A could not block such NLGP-mediated cytotoxicity, like, concanamycin A, a perforin inhibitor. On the basis of these results, we conclude that NLGP might be effective to recover the suppressed cytotoxic functions of T cells from HNSCC patients.

Regulation of T Cells by NLP/NLGP in TME

In order to evaluate the mechanism of tumor growth restriction in therapeutic settings, we have analyzed TME from sarcoma and melanoma bearing mice with NLGP therapy. A significant increase of $CD8^+$ T cells was observed within TME from NLGP treated mice (NLGP-TME), in comparison with control tumor. $CD8^+$ T cells isolated from NLGP-TME exhibited greater cytotoxicity to sarcoma/melanoma cells in vitro and these cells show higher expression of cytotoxicity related molecules, perforin and granzyme B. Anergy and AICD are the terminal fates of impaired T cells; thus, we studied these phenomena within NLGP-TME and PBS-TME. Anergy is a hyporesponsive state of T cells commonly encountered at tumor vicinity that is mediated by poor co-stimulation in absence of IL-2, a condition that upregulates various anergy related genes cbl-b, egr2, egr3, itch, GRAIL and DGKα in T cells. Negligible expression of these anergy related genes along with higher level of phosphorylated NFAT in T cells incubated within NLGP-TME as well as those recovered from in vivo NLGP treated tumors in comparison to PBS-TME and ionomycin treated controls is a noteworthy observation. AICD causes premature turnover of T cells at tumor site and is marked by enhanced expression of FasR, caspase 3 and 8. T cells incubated with NLGP-TME showed a diminished expression of these markers and decreased expression of FasR on $CD8^+$ T cells isolated from NLGP treated tumors compared to control. Accumulated evidences strongly suggest that NLGP mediated normalization of TME allows T cells to perform optimally to inhibit the tumor growth.

Regulation of Treg Cells by NLP/NLGP in Periphery

Downregulation of Tregs is considered as a promising cancer immunotherapeutic approach due to its immense immune-suppressive nature. As NLGP restricts tumor growth in mice by immune activation, we were interested to see whether NLGP can modulate Tregs in association with tumor growth restriction. We have observed that NLGP down regulates $CD4^+CD25^+Foxp3^+$ Tregs within tumors. NLGP-mediated down regulation of CCR4 along with its ligand CCL22 restricts Treg migration at the tumor site. NLGP is not apoptotic to Tregs but significantly downregulates the expression of Foxp3, CTLA4 and GITR in vitro. It also reverses the functional impairment of T effector cells by Tregs, in terms of IFNγ secretion, cellular proliferation and tumor cell cytotoxicity. NLGP also facilitates reconditioning of TME by increasing IFNγ and IL-12 but decreasing IL-10, TGFβ, VEGF and IDO, creating an antitumor niche. Interaction between Foxp3, *p*NFATc3 and *p*Smad2/3, needed for successful Treg function, is also inhibited by NLGP (Chakraborty *et al.*, 2011).

In separate observation we have shown that NLGP inhibits the Treg induced suppression of tumoricidal functions of $CD14^+CD68^+$ monocyte/macrophages (MO/Mö) from human peripheral blood. Cytotoxic efficacy of MO/Mö toward macrophage sensitive cells, U937, is decreased in presence of Tregs (induced), however, it was increased further by treatment of NLGP in vitro. Associated Treg mediated inhibition of perforin/granzyme B expression and nitric oxide release from MO/Mö was normalized by NLGP. Altered status of signature cytokines, like, IL-12, IL-10, IL-6, TNFα from MO/Mö under influence of Tregs is also rectified by NLGP. Tregs significantly enhanced the expression of altered marker, mannose receptor (CD206) on $CD68^+$ cells that was downregulated upon NLGP exposure. Treg mediated inhibition of MO/Mö chemotaxis in contact dependent manner was also normalized partially by NLGP, where participation of CCR5 was documented. Overall results suggest that Treg influenced pro-tumor MO/Mö functions are rectified in a significant extent by NLGP to create an anti-tumor immune environment. All of these coordinated events might result in inhibition of Treg associated-tumor growth and therefore increased survivability of mice having NLGP treatment before or/and after tumor inoculation.

Regulation of Treg Cells by NLP/NLGP in TME

In cancer, enhanced frequency of suppressor cells, like, Tregs make the situation more favorable for tumorigenesis. These cells negatively interfere in optimum anti-tumor T cell functions. Previously we have observed that NLGP treatment efficiently down regulates Treg generation, but in vivo data suggest that NLGP does not reduce $CD4^+CD25^+$ T cells within TME of NLGP treated mice. NLGP may reduce the Treg functionality within TME. Investigation in this direction is under progress. Western blot analysis showed the down regulation of Foxp3 within NLGP-TME. In addition, it was observed that TME from NLGP treated mice showed lower mRNA expression of IDO1 and CTLA-4 in comparison to PBS-TME. Minimization of the suppressor functions within TME by NLGP would be translated into inhibition of tumors in therapeutic settings. As optimum therapeutic outcome may depend on the balance between effector to regulatory T cell ratios, we determined the ratio between $CD8^+$ effector T cells and $CD4^+CD25^+$ regulatory T cells within TME, the obtained data revealed increment in such ratio after NLGP treatment in tumor bearing mice, favoring the T cell mediated anti-tumor immunity.

Regulation of B Cells by NLP/NLGP in Periphery

The conditional tumor growth retardation, observed in mice treated with NLP before tumor inoculation, may be regulated by NLP mediated immune activation, having prominent role in the humoral and cellular immune functions of the tumor host. The first evidence to regulate B- cells by NLP was found when we observed mice (Swiss and Balb/c) and rats (Sprague Dawley) immunized with BTAA (identified and partially characterized from human breast tumors) along with NLP have a higher IgG antibody response and a lower IgM response than mice immunized with poorly immunogenic Breast Tumor Associated Antigen (BTAA) alone. Antibody generated by immunization with BTAA and NLP can induce antibody-dependent cellular cytotoxicity (ADCC) and CTL response towards BTAA-expressing MCF-7 cells

whereas a poor cytotoxic response was found, when serum from BTAA control mice was used. The occurrence of ADCC and CTL response induced by BTAA plus NLP vaccination was possibly assisted by the induction of a Th1 response, as evidenced by the enhanced secretion of IFNγ and decreased release of IL-10 from spleen cells and the greater production of IgG2a antibody in immunized mice (Mandal *et al.*, 2007). Similar observation was found when a significant higher antibody (IgG2a) titre was found in mice immunized with B16MelSAg + NLP, in comparison to B16MelSAg alone (Baral *et al.*, 2005). After identification of NLGP from NLP, adjuvant function of it was studied in context to carcinoembryonic antigen (CEA) expressing tumors. CEA was presented using macrophages, in combination with NLGP and such vaccination generates significantly higher antibody (IgG2a) and T cell response than the immunization protocol without NLGP. NLGP controls the function of both B cells and macrophages by altering the expressions of various regulatory molecules, like, CD19, CD11b, etc. NLGP also directs CEA vaccination towards Th1 bias, by modulating cytokine secretion. This NLGP-generated anti-CEA immune response would be effective as a vaccine to lyse CEA^+ tumors in vitro and in vivo (Sarkar *et al.*, 2008).

Regulation of B Cells by NLP/NLGP in TME

B cell components within TME might be in some dysregulated state. Role of NLGP in rectification of B cell functions within TME is not studied. However, enormous scope is lying in the research in this direction.

Regulation of Dendritic Cells by NLP/NLGP in Periphery

Previously we observed NLGP regulates the function of both B cells and macrophages by altering the expression of various regulatory molecules, like, CD19, CD11b, etc. as well as directs type 1 skewness, by modulating cytokine secretion. These results provided an indication that it might have a role on generation and functioning of DCs, a major antigen presenting cell population. Interestingly, we observed that GMCSF/IL-4 differentiated $CD14^+$ monocytes were matured with NLGP. NLGP matured DCs (NLGP-DCs) show upregulated expression of CD83, CD80, CD86, CD40 and MHCs, in a comparable extent of control, LPS. NLGP-DCs secrete high amount of IL-12p70 with low IL-10. NLGP upregulates the expression of crucial transcription factor, ikaros, indicating maturation towards DC1 phenotype. Increased expression of CD28 and CD40L on T cells following co-culture with NLGP-DCs was noticed to promote DC-T cell interactions. As a result, T cells secrete high amount of IFNγ with low IL-4 and generates anti-tumor type 1 immune microenvironment. Such NLGP-DCs present CEA effectively to T cells to increase CTL mediated cytotoxicity of CEA^+ tumor cells in vitro and in vivo (Goswami *et al.*, 2010). In other experimental settings, we have observed that vaccination with NLGP matured CEA^+ pulsed DCs enhances antigen-specific humoral and cellular immunity against CEA and restricts the growth of murine CEA^+ tumors as it helps better CEA uptake, processing and presentation to T/B cells. This vaccination (NLGP matured CEA pulsed DCs (DCNLGPCEA)) elicits mitogen induced and CEA specific T cell proliferation, IFNγ secretion and induces specific cytotoxic reactions to CEA^+ colon tumor cells. In addition to T cell response, DCNLGPCEA vaccine generates anti-CEA

antibody response, which is principally IgG2a in nature. This antibody participates in cytotoxicity of CEA^+ cells in antibody-dependent manner, which helped to protect mice from tumor development and these mice remained tumor free following second tumor inoculation suggesting the generation of memory response (Sarkar *et al.*, 2010).

In cancer, dysfunctional maturation of DCs is a common cause of dysregulated anti tumor T-cell functions. We have observed that in myeloid-derived DCs generated from monocytes obtained from stage IIIB cervical cancer (CaCx IIIB) patients instruct poor T cell functions. Accordingly, we were interested to see whether NLGP could rectify DC maturation at least partially in CaCxIIIB patients. In vitro studies showed that NLGP treatment of immature DCs (iDCs) obtained from CaCx IIIB patients results in upregulated expression of various cell surface markers (CD40, CD83, CD80, CD86, and HLA-ABC), which indicates DC maturation. Consequently, NLGP-matured DCs displayed balanced cytokine secretions, with type 1 bias with remarkable functional properties. These DCs displayed substantial T cell allostimulatory capacity and promoted the generation of CTLs. Although NLGP-matured DCs derived from CaCx monocytes are generally subdued compared to those with a healthy monocyte origin, considerable revival of the suppressed DC-based immune functions is noted in vitro at a fairly advanced stage of CaCx, and thus, further exploration of ex vivo and in vivo DC-based vaccines is proposed. Moreover, the DC maturating efficacy of NLGP might be much more effective in the earlier stages of CaCx, where the extent of immune dysregulation is less and, thus, the scope of further investigation may be explored (Roy *et al.*, 2011).

Regulation of Dendritic Cells by NLP/NLGP in TME

From our observations on biology of DCs in periphery of healthy individuals and cancer patients, it was proved that NLGP optimizes its maturation and establishes DC1 phenotypes. At the same time, we observed that Th1 bias within TME from NLGP treated sarcoma and melanoma bearing mice. Higher level of secretory IL-12 within TME suggests the presence of mature DCs in a greater percentage. Our preliminary immuno-histochemical observation supported the presence of significant $CD11c^+$ cells in TME from NLGP treated melanoma bearing mice *(unpublished observation)*. This observation indicates that NLGP not only rectifies DC's property but also helps in migration of DCs within TME. Further research is required in this direction.

Regulation of Cytokine/Chemokine Signaling by NLGP in Periphery

Obtained supernatant from NLP stimulated human PBMC, inhibits tumor cell proliferation and induces apoptosis. Presence of cytotoxic cytokines, IFNγ and TNFα in such supernatant was detected by ELISA and inhibition of secretion of IFNγ and TNFα caused a significant decrease in tumor cell apoptosis (Bose *et al.*, 2007). In other study, NLP was found to activate NK cells to enhance their tumor cell cytotoxic ability and to stimulate the release of IL-12 from macrophages from healthy individuals and HNSCC patients. In addition, NLP upregulated the expression of CD40 and CD40L on $CD14^+$ monocytes and $CD56^+$ lymphocytes respectively, which is directly related to IL-12 release (Bose and Baral, 2007).

We have analyzed immune activation and associated immune polarization upon NLGP treatment. NLGP-activated T cells secrete greater amount of signature Th1 cytokines IFNγ and a lower amount of the Th2 cytokine, IL-4. Similar type-1 directiveness is also observed in antigen-presenting monocytes and DCs by upregulation of IL-12, TNFα and downregulation of IL-10. This type-1 biasness is also assisted by NLGP-induced downregulation of $Foxp3^+$ Treg cells and upregulation of type 1-specific transcription factor, T-bet. In order to find out the mechanism behind it, we have found increased phosphorylation of STAT1 and STAT4 with decreased phosphorylation of STAT3. Overall, it was observed from our studies on human and mouse systems that NLGP is effective to release type-1 cytokines, like, IFNγ, IL-12, IL-2, TNFα and inhibited secretion of IL-10, IL-6 and TGFβ (Bose *et al.*, 2009).

Effect of NLGP in rectification of the dysregulated IFNγ dependent chemokine and its receptor CXCR3 splice variants was investigated. Upregulated expression of CXCR3B in HNSCC-PBMC was downregulated following in vitro NLGP treatment. Unchanged expression of CXCR3A+B by NLGP with downregulation of the CXCR3B indirectly suggests the upregulation of the CXCR3A, responsible for cellular migration. However, stimulation of healthy-PBMC with NLGP maintains physiological homeostasis of CXCL10 and increases IFNγ secretion. The suppressed chemotaxis of HNSCC-PBMC could be restored either by *in vitro* treatment with NLGP or during use of NLGP stimulated PBMC supernatant as a chemoattractant. Ligands (RANTES, MIP1α and MIP1β) of this chemokine receptor were also secreted in lesser quantity from MO/Mw of HNSCC patients in comparison with healthy individuals (Chakraborty *et al.*, 2008). In a separate experiment, we found that NLGP upregulated CCR5 expression, as evidenced from studies on MO/Mw of peripheral blood from HNSCC patients as well as healthy individuals. Expression of RANTES, MIP1α and MIP1β was also upregulated following NLGP treatment of these cells *in vitro*. Interestingly, NLGP has little effect on the expression of CCR5 and the ligand RANTES in oral cancer cells. This restored CCR5 receptor-ligand signaling seen in MO/Mw was reflected in improved CCR5-dependent, p38MAPK mediated migration of MO/Mw after NLGP treatment to a standard chemoattractant. In addition, NLGP-treated MO/Mϕ-primed T cells can effectively lyse tumor cells in vitro (Chakraborty *et al.*, 2010). These results suggest a new approach in cancer immunotherapy by modulating dysregulated CCR5 signals from MO/Mϕ.

Regulation of Cytokine/Chemokine Signaling by NLGP within TME

As TME is dominated with immunosuppressive cytokines, like, IL-10, TGFβ etc., along with low IFNγ and IL-12, normalization of TME is desired to initiate antitumor immune response, especially T cell response. To investigate the role of NLGP in TME normalization, two groups of mice with established sarcoma (average tumor volume, 100 mm^3) were treated with NLGP and PBS respectively. Tumors were harvested in three different days (n=3, in each time point) following initiation of the NLGP treatment and cytokine secretory status was assessed from cellular contents by ELISA and immunoblotting. Tumors of NLGP treated mice exhibited significant upregulated level of IFNγ, IL-2 and IL-12 in day dependent manner and reduced release of IL-6, IL-10 and TGFβ within TME, in comparison to mice having PBS injection.

Chemokine-mediated T cell migration is essential for optimal anti-tumor immune response (Lu *et al.*, 2012). Similar to the cytokine network, chemokine milieu is also dysregulated within TME, thus, hampering movement of effector $CD8^+$ T cells in tumors. In an objective to know the status of chemokine receptor-ligand profile, we have analyzed different genes related to chemokine ligands (*ccl3*, *ccl4*, *ccl5*, *ccl8*, *cxcl9*, *cxcl10* and *cxcl12*) and chemokine receptors (*ccr5*, *cxcr3* and *cxcr4*) at transcriptional level. We observed chemokine receptors, *cxcr3* and *ccr5*, were upregulated in NLGP treated tumors. At the same time it was found that ligands for *ccr5* like *ccl3*, *ccl4*, *ccl5* and *ccl8* were upregulated within NLGP-TME, although extent of upregulation is varied. Same trend of upregulation was observed in case of *cxcr3* ligands, *cxcl9*, and *cxcl10*. We also observed a marked downregulation of *cxcr4* and *cxcl12* in case of NLGP treated tumor than PBS controls *(unpublished observation)*.

Earlier discussion demonstrated the recruitment of enhanced number of type 1 polarized $CD8^+$ T cells within TME. Such recruitment to tumor sites occur via CXCR3 mediated chemotaxis in response to the CXCL9-11 chemokines, produced within the TME. Accordingly in order to determine whether Tumor draining lymph nodes (TDLN)/Tumor derived T cells in NLGP treated animals are expressing more chemotactic components over PBS mice, we assessed $CD8^+$ T cells for their expression of CXCR3 and corresponding ligands on day 21 sarcoma mice. We found that $CXCR3^+$ subpopulation of $CD8^+$ T cells as well as *cxcr3* transcripts were significantly increased in TDLN of NLGP treated mice. Expression of mRNAs corresponding to *cxcl9* and *cxcl10* also increased within TILs from tumors (Mallick *et al.*, 2012). We also found upregulation of $CCR5^+$ cells and enhanced *ccr5* expression at mRNA levels. Though CCR5 is expressed generally on monocytes, it is also involved in T cell migration (Hanahan *et al.*, 2011). Consistently, we found higher expression of its corresponding ligands, *ccl3*, *ccl4*, *ccl5* and *ccl8* within TME. Similar result was obtained in case of TDLN for *ccl4* and *ccl5*, without noticeable upregulation in *ccl3* and *ccl8*. Interestingly, CXCR3 and CCR5 expression was unchanged on sarcoma cells upon *in vitro* NLGP treatment. These data suggest that NLGP therapy not only stimulates T cell expansion, but also licenses these cells for trafficking to peripheral tissues in which CXCR3/CCR5 ligands are expressed, such as TME.

Regulation of Altered Angiogenesis within TME by NLGP

The growth of primary and metastatic tumors to larger than a 2-3 millimeters requires the recruitment of neighboring blood vessels and vascular endothelial cells to support their metabolic requirements. Tumors utilize a number of mechanisms to promote their vascularization, and in each case they subvert normal angiogenic processes to suit this purpose. Tumor angiogenesis is a complex process involving many different cell types that must proliferate, migrate, invade, and differentiate in response to signals from the TME. VECs sprout from host vessels in response to VEGF, bFGF, Ang2 and other proangiogenic stimuli within TME. Sprouting is stimulated by VEGF/VEGFR2, Ang2/Tie-2, and integrin/extracellular matrix interactions. Tumor cells themselves may directly form parts of vascular channels within TME. The pattern of vessel formation is haphazard: vessels are tortuous, dilated, leaky, and branched in random ways. This leads to uneven blood ow within

the tumor, with areas of acidosis and hypoxia (which stimulate release of angiogenic factors) and high intratumoral pressures that inhibit delivery of therapeutic agents, leading to tumor growth. Accordingly, normalization of angiogenic vasculature as well as tumor microenvironment is the essential focus in current cancer therapeutic research. We have observed more normalized blood vasculature within and surrounding tumors of carcinoma and melanoma bearing mice having prophylactic or therapeutic NLGP treatment. NLGP may act directly on VECs of blood vessels as evidenced by downregulation of CD31, VEGFR2, whereas, expression of VEGFR1 is unaltered. NLGP also stabilized blood vessels by normalizing pericytes, as evidenced by downregulation of NG2. At the same time, NLGP make significant downregulation of VEGF, PDGF, IL-10, TGFβ, IDO as detected by Western blotting, RT-PCR and immunohistochemical analysis. Downregulation of HIF 1α by NLGP also indicates reduction of hypoxia and normalization of the vascular interstitial pressure. All of these synchronized events may promote infiltration of antitumor immune competent cells and therapeutic regimens within tumors that ultimately correlated with tumor growth inhibition.

Concluding Remarks

In view of the **'Triple E'** (**E**limination, **E**quilibrium and **E**scape) theory of cancer, malignant tumor can be formed when cancer cells escaped from the immune surveillance, otherwise, they can be eliminated by immune system. In the process of escape, TME helps tumor enormously. Again, in the creation of TME tumors play an active role. Immune and non-immune components of TME help tumors to grow in different ways, thus, normalization of TME would be an effective choice for anti-tumor therapy. TME induces type-2 immunity by active contribution of different TME components, like, TAM, that promotes tumor. We have reported earlier that NLGP is able to maintain type-1 immunity in tumor host (Bose *et al.,* 2009) and it also can increase the type 1 cytokines within TME. This is a big step towards keeping the TME green by NLGP. TME derived factors also establish a chemokine gradient that can pool suppressor cells within TME, in spite of effector cells. We have specially accumulated data on the efficacy and migration of $CD8^+$ T cells under the influence of NLGP within TME (Mallick *et al.,* 2012). It is clear that NLGP activated T cells can be more cytotoxic and secretes type 1 cytokines (Bose *et al.,* 2009), even within TME. Abnormal functioning of antigen presenting cells, like, macrophages, DCs, are nicely tackled by NLGP (Sarkar *et al.,* 2008; Goswami *et al.,* 2010; Roy *et al.,* 2011), so that T cells obtain required antigenic activation (Sarkar *et al.,* 2009). Suppressor cells and their secreted cytokines, like, IL-10, TGFβ make immune system dysfunctional and it is clearly demonstrated that NLGP downregulates IL-10, TGFβ, thereby helping other immune cells to become more functional (Chakraborty *et al.,* 2011). Metastasis promoting alteration of TME also corroborated with dysregulated degradation of extracellular matrix by MMPs, thus, expression of those proteins increased significantly. NLGP may inhibit metastasis by downregulating MMPs. Effect of NLGP on extracellular matrix is yet to be elucidated. Hypoxic environment of TME promotes angiogenesis and vasculature becomes chaotic, tortuous, leaky, thus, creating increased interstitial pressure and uneven blood flow. This angiogenic character prevents the normal flow of immune cells within tumor. It also prevents drug delivery

within tumor, if patients are under chemotherapeutic treatment. Evidences are already obtained that NLGP downregulates angiogenesis related molecules, like, VEGF, HIF1α and make the blood flow normalized *(unpublished observation).* We also initiated studies on mesenchymal stem cells that has significant suppressive role on immune functions. NLGP also shows good promise in this regard. In addition to these, several other complicated interactions are always ongoing within TME and role of NLGP on those aspects should be investigated to understand the mechanism by which NLGP attempts to keep the TME green. Maintenance of pollution free green environment is essential as TME not only attenuates the efficacy of endogenous antitumor immune responses, but also represents a barrier to therapeutic modalities, including chemotherapy and immunotherapy.

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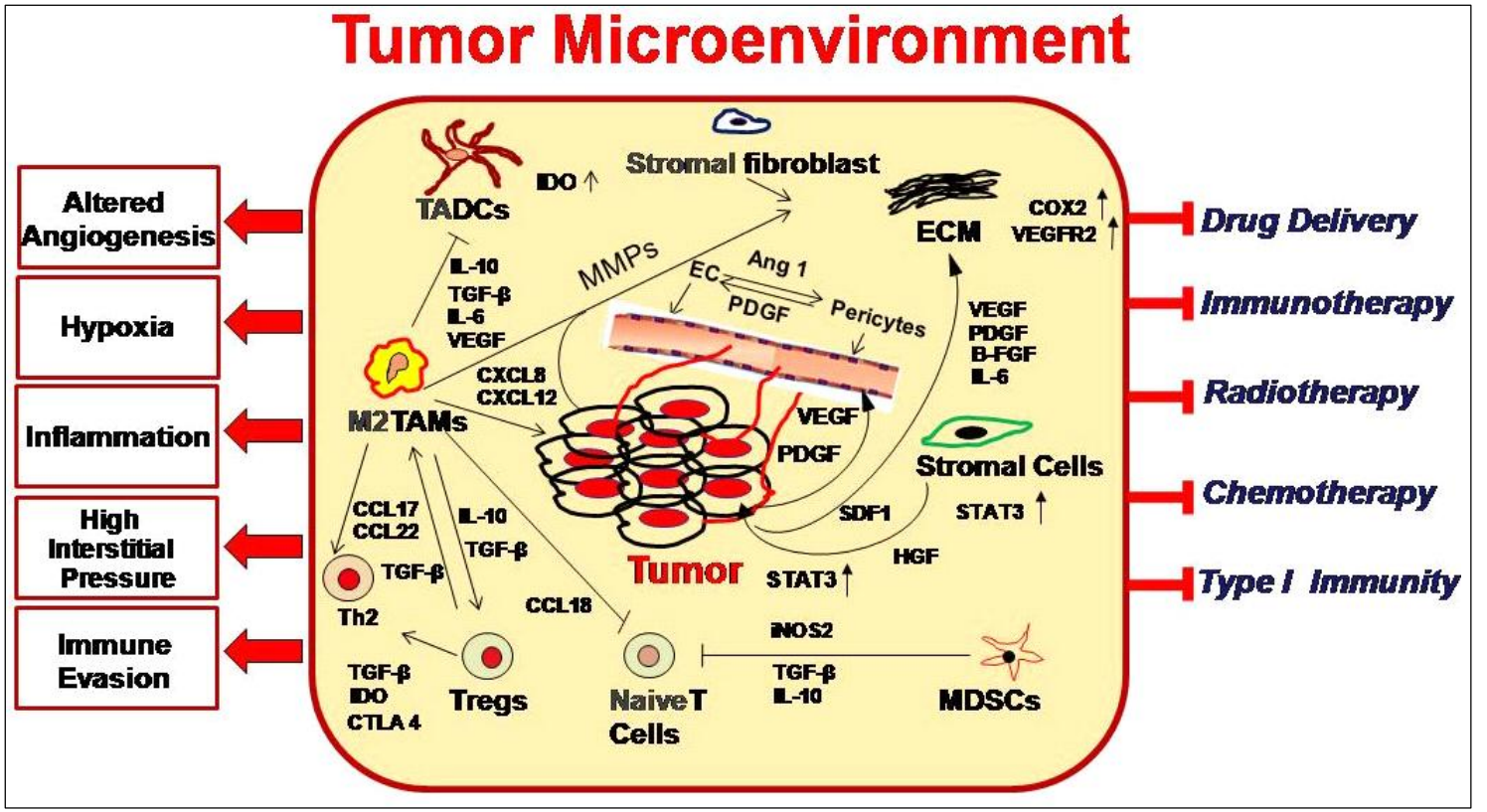

Figure 2.1: Immune and non-immune components of Tumor Microenvironment (TME). *Left panel* describes hallmarks of TME. *Right panel* exhibits obstacles in anti-tumor immunity and therapy due to TME. *Middle panel* represents interactions between tumor cells and other immune and non-immune cells through cytokines, chemokines etc. TADC, Tumor associated dendritic cells; M2TAM, M2 Tumor associated macrophages; ECM, Extracellular matrix; Tregs, regulatory T cells; MDSCs, Myeloid derived suppressor cells (Barik *et al.*).

Figure 2.2: Representative demonstration of modulation of immune components in periphery (I) and TME (II) of tumor host.

Periphery (I). *T cells.* Alterations in blood $CD4^+$ and $CD8^+$ T cells of sarcoma bearing mice having NLGP treatment (A.1). Upregulation of cytotoxic molecules, perforin and granzyme B (A.2), IFNg (A.3) and type 1 specific transcription factor, T-bet (A.4) in $CD8^+$ T cells from HNSCC patients after in vitro NLGP treatment. *Treg cells.* Downregulation of $CD4^+CD25^+Foxp3^+$ Treg cells from HNSCC patients after in vitro NLGP treatment (B.1). Inhibition of tumor supernatant mediated migration of Treg cells by NLGP (B.2). Reduced phosphorylation of STAT3 in tumor conditioned T cells under influence of NLGP (B.3). *Dendritic cells.* Morphology of NLGP matured DCs (C.1). Expression of MHC-I, MHC-II, CD80, CD86 and CD83 on NLGP matured DCs (C.2). IL-12 and IL-10 gene expression in NLGP matured DCs (C.3). *Macrophages.* Increased number of phagocytic cells after NLGP treatment (D.1). Secretion of IL-12 and IL-10 from peritoneal macrophages of colorectal carcinoma bearing mice (D.2). Increased nitric oxide release from peritoneal macrophages of NLGP treated melanoma bearing mice (D.3).

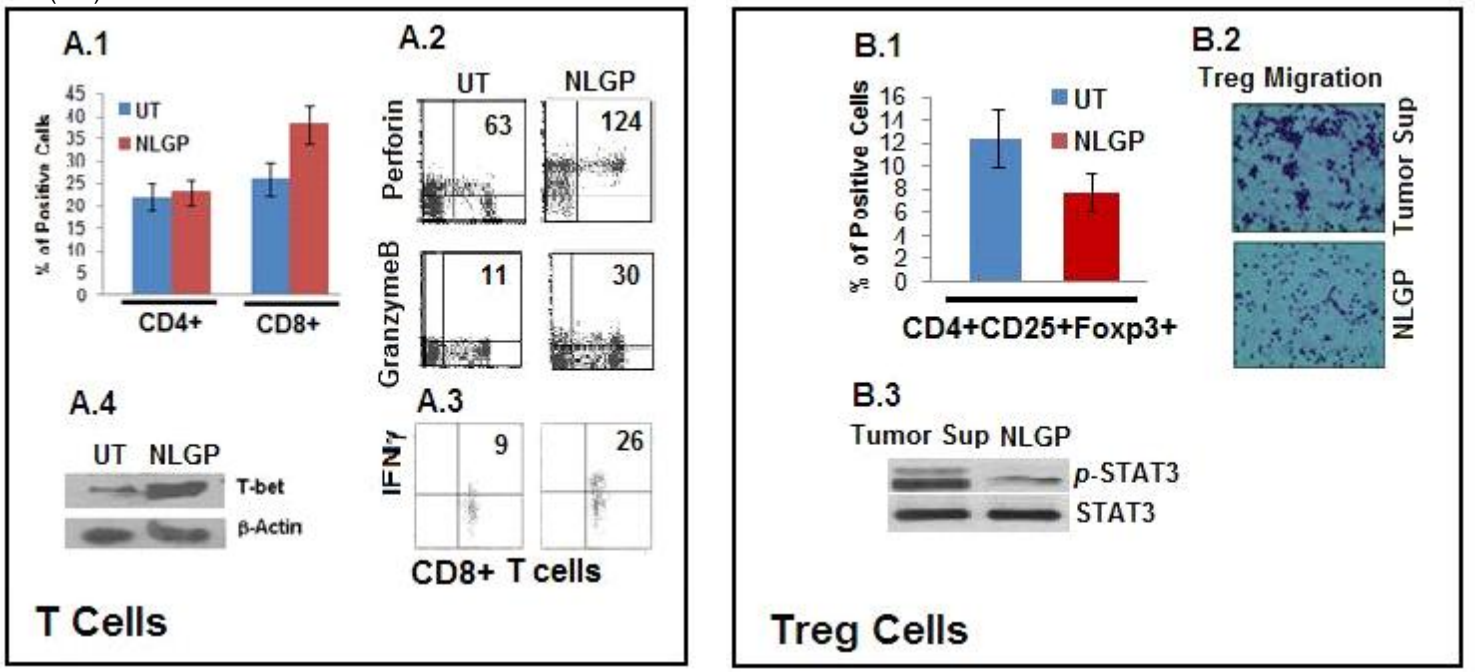

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Figure 2.1 Periphery (I)–*Contd...*

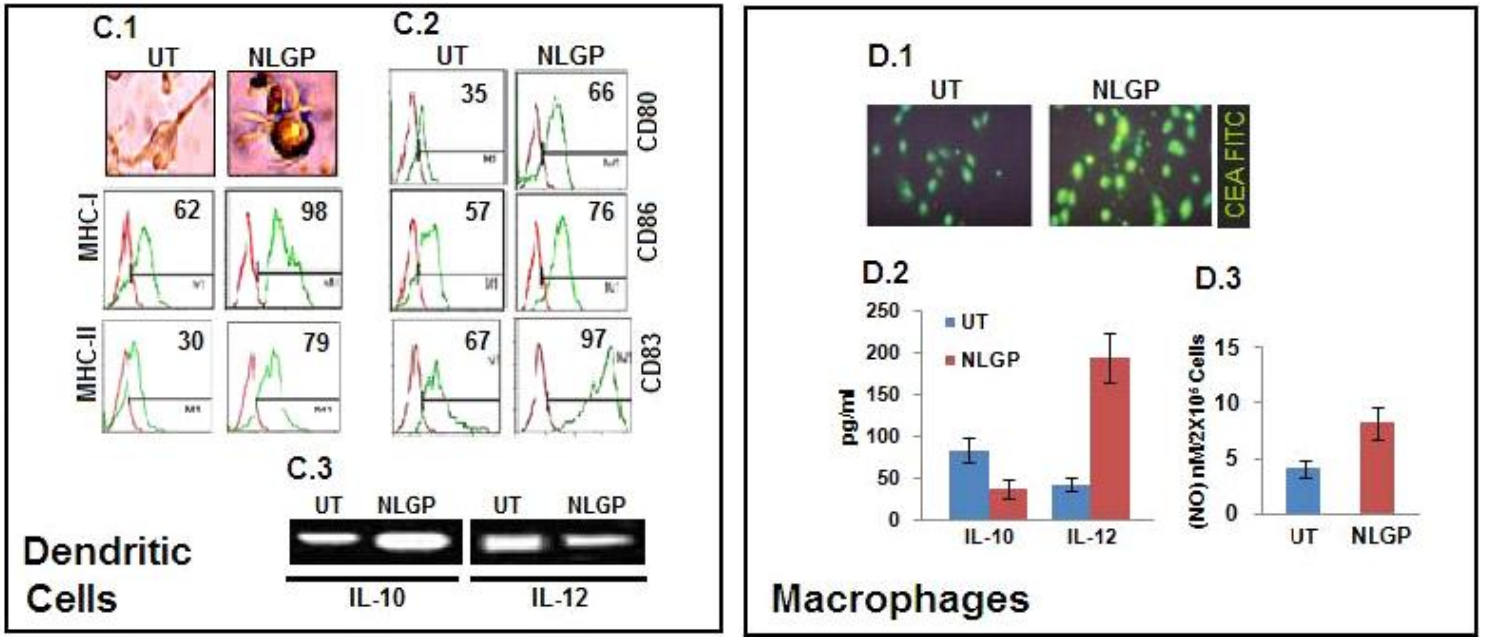

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Figure 2.1–*Contd...*

TME (II). *T cells.* Increased number of $CD8^+$ T cells within sarcoma tumor from NLGP treated mice (E.1). $CD8^+$ T cells expressed greater number of early activation marker CD69 in tumor draining lymph node cells and tumor infiltrating lymphocytes from NLGP treated sarcoma bearing mice (E.2). NLGP mediated therapeutic tumor growth restriction is withdrawn after $CD8^+$ T cell depletion (E.3). Exhaustion of T cells with TME is significantly less in NLGP treated mice, as shown by reduced expression of exhaustion markers, lag3, pd1 and tim3 (E.4). *Treg cells.* Decreased number of $CD4^+Foxp3^+$ Treg cells within tumor from NLGP treated mice (F.1). Treg influenced growth of mouse tumor is inhibited by NLGP treatment (F.2). Reduced expression of transcription factor, Foxp3, and, CCR4 ligand, CCL22 within tumor from NLGP treated mice (F.3). *Dendritic cells.* Increased frequency of $CD11c^+$ DCs within NLGP treated mouse melanoma (G.1). Increased expression of MHC-I, CD80 and CD86 within $CD11c^+$ DCs after NLGP treatment of melanoma bearing mice (G.2). *Macrophages.* Reduced frequency of F4/80 positivity (M2) within $CD11b^+$ cells from NLGP treated murine melanoma (H.1). Increased expression of IL-12 and reduced expression of IL-10 in same tumor from NLGP treated mice (H.2). *Angiogenesis.* Decreased number of $CD31^+$ cells within mouse melanoma after NLGP treatment (I.1). Downregulated VEGF gene (I.2) and protein (I.3) expression in NLGP treated B16 melanoma.

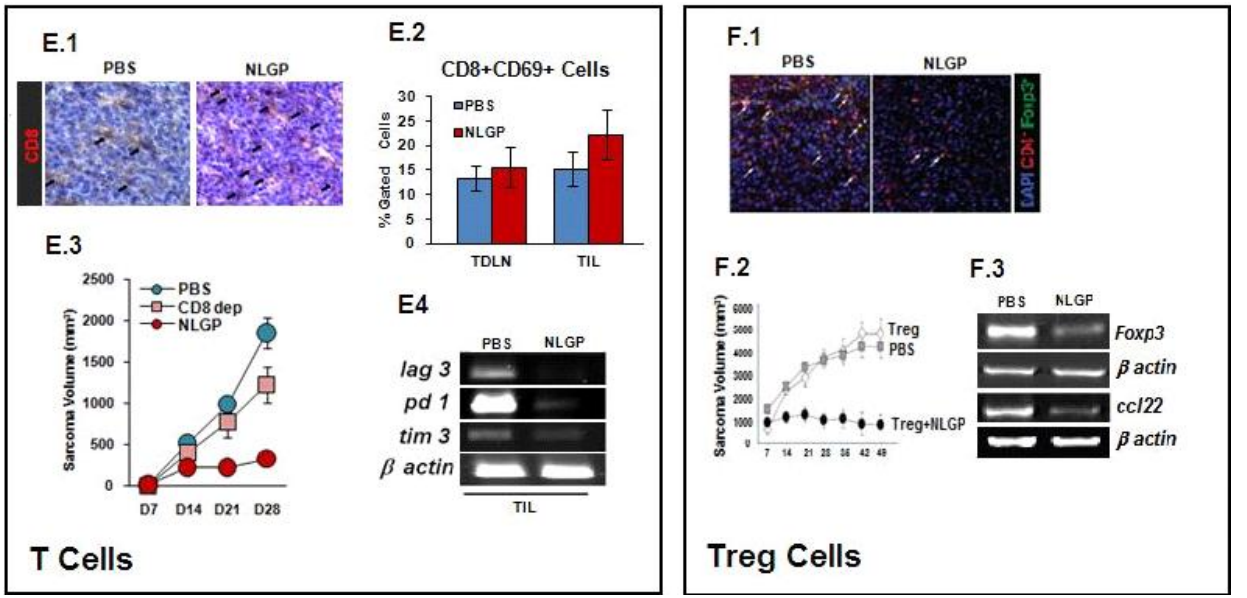

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Figure 2.1 TME (II)–*Contd...*