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Tumor gangliosides and T cells: A deadly encounter

Dewan Md Sakib Hossain[1], Suchismita Mohanty[1], Pallab Ray, Tanya Das[1], Gaurisankar Sa[1]

[1]Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M, Kolkata 700 054, India

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1. ABSTRACT

Despite major advances in understanding the mechanisms of tumor immunity, its successful translation into effective tumor immunotherapy is hindered by the ability of tumors to foster a tolerant microenvironment and to activate a plethora of immunosuppressive mechanisms. Among different strategies employed by tumors to thwart immune responses, shedding of immunosuppressive molecules, such as sialic acid-containing glycosphingolipids, gangliosides, by the tumor is one important strategy. Aberrant and elevated expression of gangliosides has been demonstrated on the surface of cancer cells. Here we discuss about the molecular mechanisms underneath the contribution of tumor gangliosides in targeting multiple steps of T cell response. We shall also underscore the contribution of T-regulatory, NK and dendritic cells in this immunosuppressive network. Inhibitory effects of gangliosides ultimately converge to T cell apoptosis in receptor-dependent and -independent manners *via* IL-2 deprivation, ROS production, cytochrome c release, NFkappaB inhibition and caspase activation. Current wealth of information promises a future scenario in which synchronized blockade of immunosuppressive mechanisms and removal of inhibitory signals might be effective in overcoming immunological tolerance and promoting tumor regression.

2. INTRODUCTION

T cells are often held in a tug-of-war between opposing signals from tumors, where tumor cells employ an array of mechanisms to paralyze the immune system. T cells - our "defenders" - methodically surround enemy positions and "patrol" until they encounter a tumor cell, which they have previously learned to recognize. They eliminate the tumor cell, and then resume their rounds. Fast movements of the T cells signal either the absence of the adversary, or the defeat of the enemy on the battlefield. Though the immune system is capable of recognizing and eliminating tumors, more often tumors fail to prime, initiate and maintain adequate immune responses, resulting in tumor outgrowth. Moreover, while protecting the host against cancer development, the immune system at times also favors the emergence of tumors with reduced immunogenicity, thereby potentiating immune evasion. Tumor evasion reflects the myriad of influences on immune competence, and several defects have been implicated. They include aberrant antigen processing by tumor cells, anergy/deletion of tumor-specific cytotoxic T cells, recruitment of inhibitory cell types, and recruitment of regulatory T cells (Figure 1) (1-9). Identifying of the opposing team and how they are organized will be important in advancing our understanding of *in vivo* anti-tumor immune function. Burgeoning literature ascribing

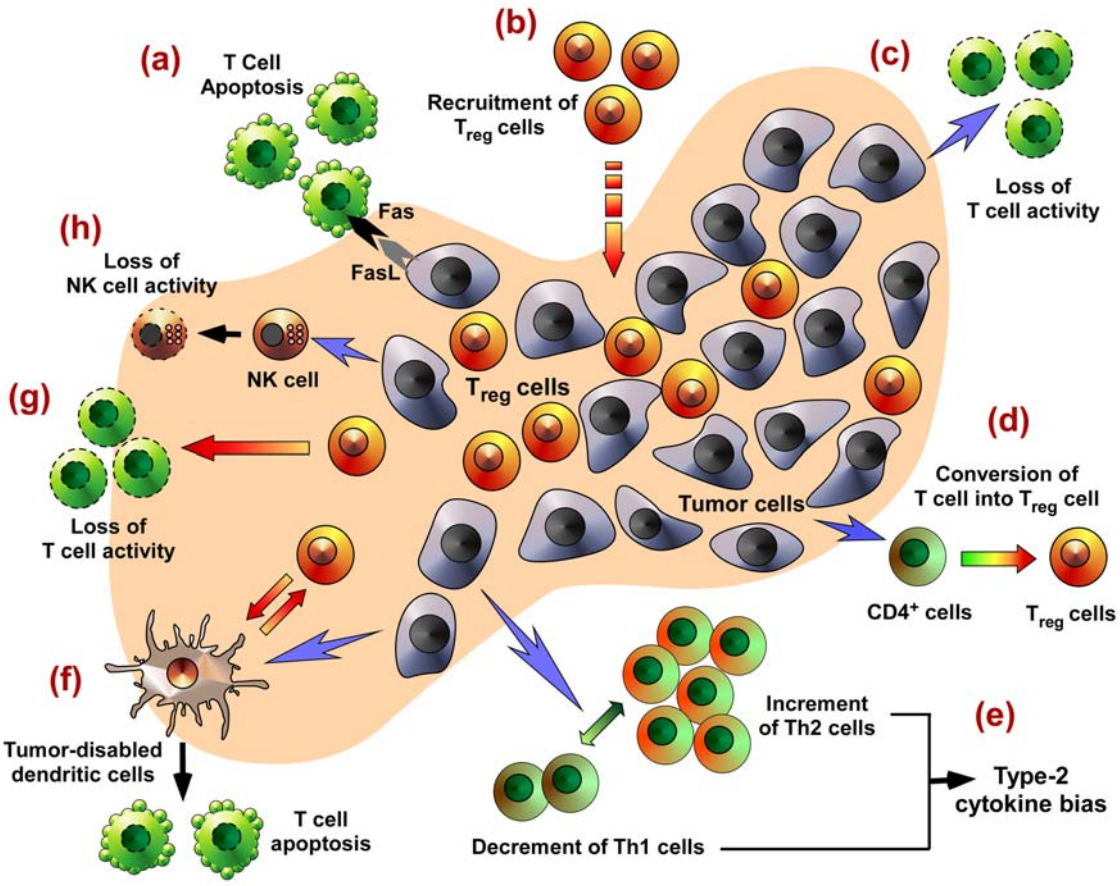

Figure 1. Tumor immune evasion strategies. Tumors have evolved complex mechanisms to subvert cell-mediated immune responses. These include (a) induction of Fas/FasL-mediated T cell apoptosis, (b) recruitment of T_{reg} cells, (c) loss of T cell proliferation and effector functions, (d) differentiation of $CD4^+$ T cells into T_{reg} cells, (e) imbalance between Th1 and Th2 cells resulting in type-2 cytokine bias, (f) impairment of dendritic cell function that leads to DC-mediated T cell anergy and apoptosis, (g) tumor-recruited T_{reg} cells-induced loss of T cell activity and (h) inhibition of natural killer cell activity.

roles for tumor microenvironment in T cell dysfunctions imply that any loss of T cell functions could be highly inductive for tumor cell growth (10). Therefore for tumor flora and tumor-shed immunosuppressive molecules to nullify immune responses, it is essential for them to be capable of overcoming T cell-mediated immunesurveillance mechanisms. Among them tumor gangliosides are best known to dynamically rearrange the battlefield in favor of tumor to resurrect the process of tumorigenesis (11). This review will focus on recent studies providing insight into the mechanisms by which tumor gangliosides jeopardize and down-regulate T cell functions including T cell differentiation, apoptosis, responsiveness and proliferation. Elucidation of these mechanisms will suggest strategies for targeting tumor gangliosides to normalize T cell functions for more pronounced and sustained efficacy of cancer-specific immunotherapy.

3. TUMOR AND T CELLS: ENGAGED IN A DUEL

The encounter between tumor and T cells is decisive to either rejection or progression of a particular tumor. It is during this *tug-of-war* that tumor-specific T cells primarily try to wipe out tumors and their remnants for complete protection of the host. However tumors on the other hand decide whether or not to be victimized by the immune cells. Given below is a brief description of how T cells flawlessly try to execute their functions which regrettably at many times is molded by the tumors for their own gains.

3.1. T cell and immunesurveillance: Wiping out the enemies

Establishing a balance between the rapid generation of effective immunity and the production of overly exuberant or excessively prolonged responses is critical to the maintenance of the equilibrium between health and disease. Evolution has thus provided an immunological system capable of clearance of pathogens and foreign cells but which generally avoids the severe collateral damage that is associated with failure to control immunity (12). Central to this is the contribution of T lymphocytes where different populations of T lymphocytes are meant to work in concert during immune homeostasis.

Table 1. Tumor derived soluble factors in immunosuppression

Factors	Effect on immune system
Gangliosides	↓Antigen presenting and processing ↓T cell proliferation →Th2 cytokine bias →T cell apoptosis ↑T_{reg} cell
TNFα	→ NK cell and T cell apoptosis
TGFβ	↓ MHC II ┤ T cell, NK cell ↑ T_{reg} cell and MDSC
PGE_2	┤ DC Maturation ↑ MDSC; Arginase in MDSC
Arginase	→ Arginine depletion → T cell dysfunction
VEGF, GM-CSF	┤ T cell and DC function ┤ DC maturation ↑ MDSC
IL-10	→ Th2 cytokine bias ↑ T_{reg} cell and MDSC ↓ MHC I and MHC II
IL-6	┤ NK cell and DC maturation/function ↑Th2 cytokine bias

DC: Dendritic cell; MDSC: Myeloid derived suppressor cell; NK cell: Natural killer cell; T_{reg}: T regulatory cell; Th: T helper cell; PGE2: Prostaglandin E2; TGFβ: Transforming growth factor beta; VEGF: Vascular endothelial growth factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; IL-10: Interleukin-10; IL-6: Interleukin-6, TNFα: Tumor necrosis factor-alpha; ↑ : Increase; ↓ : Decrease; ┤ : Inhibition; → : Induction

As in the case of foreign pathogens, accumulating evidence indicates that the innate and adaptive immune systems also participate in the recognition and destruction of tumor cells by a process known as cancer immunosurveillance (13).

Among the T cells, näive $CD4^+$ T helper cells have the potential to differentiate into distinct lineages of effector cells that are primarily defined by the cytokines they produce. These effector T cell lineages orchestrate the adaptive immune response, playing key roles in directing the elimination of tumor cells. For example, effector T helper 1 (Th1) cells producing high levels of interferon-gamma (IFN-gamma) and very low levels of interleukin-4 (IL-4) are highly anti-tumorigenic whereas effector T helper 2 (Th2) cells producing high levels of IL-4 and very low levels of IFN-gamma favor tumor progression (14). This apart Th1 cells prime näive $CD8^+$ T cells to become tumor antigen-specific cytotoxic T-lymphocytes (CTL) that function as the major effectors in the immune response against tumor cells (Figure 2) (15). Generation of CTL response against tumor antigens has been thought to be directed primarily by $CD4^+$ T cell interactions with antigen presenting cells for e.g., dendritic cells (DCs) that convert 'unlicenced' DCs into DCs capable of implementing a full-blown immune response ('licenced' DCs) by inducing CTLs specific for foreign intruders (16). In a further extension of the importance of $CD4^+$ T cells, a paper recently published in *Nature* by Nakanishi *et al.* (17) now paints an even broader picture of the pivotal role of $CD4^+$ T cells in laying the foundation of the immune response. In an unexpected twist Nakanishi *et al.* found that it was $CD4^+$ T cell signals that provide the roadmap to guide effector $CD8^+$ T cells to inflammated tissues where they can destroy foreign cells (17).

Though Mosmann and Coffman (18) proposed 20 years ago that T helper cells could be divided into two distinct subsets, Th1 and Th2, characterized by distinct cytokine profiles and effector functions, the Th1/Th2 paradigm has recently been reevaluated to include a third population of T helper cells, producing IL-17 and designated Th17. Th17 effector functions are distinct from Th1 and Th2-mediated immunity. In addition to Th1 and Th2 responses, Th17 cells appear to be critical to enhance host protection against tumor antigens (19).

Apart from Th1, Th2, Th17 and CTLs, regulatory T cells (T_{reg}) are pivotal for maintenance of immune self-tolerance, and evidence also suggests that they are important for promoting immune homeostasis following response to exogenous antigens, including allergens (20). Being immunosuppressive in nature, T_{reg} cells have a tendency to negate anti-tumor immune responses (21). Most research to date on regulatory T (T_{reg}) cells has focused on the $CD4^+FoxP3^+$ T_{reg} cell population (22). Recently a subset of $CD8^+$ T cells has been shown to suppress $CD4^+$ T cells, however little is known about the exact function of $CD8^+$ T_{reg} cells (23). It was recently shown that infiltration of $CD8^+$ T cells into the immunosuppressive microenvironment of prostate tumors can convert tumor-specific $CD8^+$ effector T cells into regulatory cells (23). In another recent study, $CD8^+CD25^+FOXP3^+$ regulatory T cells were isolated from colorectal cancer tissue and shown to have suppressive activity *ex vivo* (24).

From the above cited literature, we can therefore infer that a proper balance between the several components of T cell population is critical for both clearance of tumor antigens and masking of self-directed responses.

3.2. Tumor immune evasion: The guerrilla attack

Given the existence of cancer immunosurveillance, why then do cancers occur in immunocompetent individuals? In fact, when a growing tumor develops into a full-blown tumor, it is often imprinted by the immunologic environment in which it forms and it may be during this imprinting process it acquires traits to either manipulate the immune system or manipulate itself to ultimately escape the consequences of immunesurveillance machinery. Recently, it has become clear that the immune system not only protects host against tumor development but also sculpts the immunogenic phenotype of a developing tumor and can favor the emergence of resistant tumor cell variants (25). Important in this regard is the ability of both tumor and tumor microenvironment to maneuver the restraints imposed by adaptive immunity. Though a number of reviews have discussed direct mechanisms of tumor-mediated immunosuppression, the contribution of tumor flora and tumor-shed molecules in immune dysfunctional has often gone unnoticed (26-28). Tumor microenvironment comprises of an array of suppressive molecules ranging from cytokines, peptides, growth factors, inhibitory ligands prostaglandins, gangliosides, etc. (Table 1) (29-31). Among them gangliosides are important molecules that can be considered as tumor weapons directed to attack and destroy

immunosurveillance mechanisms devoted to control cancer progression (32-33). Encompassing several studies that have earlier delineated the mechanisms of ganglioside-induced immunesuppression, this review provides a global picture of how gangliosides negatively affect immune system with special emphasis on T cell dysfuction.

4. TUMOR GANGLIOSIDES: THE ARMAMENTS

Tumor gangliosides are a class of biologically active cell surface molecules, synthesized and expressed in high amounts on the plasma membrane of tumor cells and meant for their systemic growth and protection from the host immune system (34).

4.1. Structure

Gangliosides, characteristic complex lipids present in the external layer of plasma membranes, deeply influence the organization of the membrane as a whole and the function of specific membrane associated proteins due to lipid-lipid and lipid-protein lateral interaction (35). Since their discovery in the late 1800s, sphingolipids remain enigmatic, mostly because of their complexity, structural diversity, and cell specificity. However, increasing attention has been given to sphingolipids in recent years after the discovery of their pivotal role in signal transduction. These molecules are distinguished by the presence of a sphingoid long-chain base (usually sphingosine in mammalian cells) that can be acylated at the 2-amino position to form ceramide (36). This simple sphingolipid, a potent signaling molecule by itself, serves as the precursor of more complex sphingolipids such as glycosphingolipids (GSLs) and gangliosides, a subclass of acidic GSLs (36).

Gangliosides are composed of a common hydrophobic ceramide moiety, which acts as a membrane anchor, and a hydrophilic oligosaccharide chain, which varies in length and composition and contains one or more sialic acid residues (Figure 2) (37,38). The presence of sialic acid distinguishes the gangliosides from other glycosphingolipids. Most gangliosides are amphipathic constituents of the outer leaflet of cell membranes and are particularly abundant in the central nervous system, where they are vital for the maintenance of membrane structure and organization (36). A small proportion (10%) is localized in mitochondria and endoplasmic reticulum (36). The hydrophilic carbohydrate residue of gangliosides is projected into the extracellular environment which allows the head group to modulate numerous cell surface events pivotal for biological processes, including cellular recognition and adhesion, receptor signal transduction, growth regulation, and differentiation (39).

4.2. Biosynthesis

Ganglioside biosynthesis takes place in the golgi apparatus and endoplasmic reticulum, where glucosylceramide is glycosylated by sequential addition of galactose, sialic acid, and *N*-acetylgalactosamine (40,41). Among the gangliosides, the ganglioside GM3 (Neu5Acalpha2,3 Galbeta1,4Glcbeta1,1Cer), one of the main components of the total gangliosides in many cell types, is synthesized in the first step of ganglioside biosynthesis, and all other ganglio-series gangliosides are synthesized from GM3 by linkage-specific glycosyltransferase (Figure 3) (42). The expression mechanism of these gangliosides in terms of the status of their respective glycosyltransferase genes has been extensively studied. They are biosynthesized by the sequential action of a series of specific glycosyltransferases and sialyltransferases (39). To date, a large number of glycosyl-transferase and sialyltransferase genes have been cloned and characterized, and studies of these genes have shown that various expression patterns of gangliosides are determined basically by a combination of activated glycosyltransferase genes (39). The amount and composition of gangliosides in a cell is species- and cell type-specific and may vary dramatically during development and changes in the metabolic state of the cell (36). Owing to their amphiphilic nature and the unique composition of their hydrophobic portion, gangliosides can either distribute asymmetrically or segregate with cholesterol and other membrane proteins in specialized microdomains or clusters (e.g., lipid rafts and caveolae) whose biological activities are greatly influenced by their lipid content (43).

4.3. Function

Accumulating evidence indicates that cellular function and phenotype are highly influenced by gangliosides. Gangliosides have been shown to have crucial regulatory roles in the normal physiological process, such as cellular recognition and adhesion, receptor signal transduction, growth regulation, differentiation and embryogenesis, as well as in pathological conditions, including tumor onset and progression (44-46). Many recent studies indicate that tumor-associated gangliosides are a result of initial oncogenic transformation and these molecules play a key role in the induction of invasion and metastasis (46). The concept of ganglioside-dependent promotion of tumor progression has been developed in conjunction with clinic-pathological studies that have shown that there are some ganglioside species with relatively simple structures that show very restricted expression in normal tissues and markedly enhanced expression in a particular malignant tumor (46). GD3, which has been identified as a melanoma-specific antigen, is an example of such a ganglioside (47). All primary melanoma tissues as well as established melanoma cell lines contain high amounts of GD3 as a major ganglioside component (48). In contrast, human melanocytes, the normal counterpart of melanoma cells, expressed no or minimal levels of GD3 (49). Furthermore, highly metastatic cells show an increase in ganglioside content and express more GD3 or complex gangliosides than poorly metastatic cells (50-51). These finding suggest that GD3 might play an important role in the transformation of melanocytes into melanomas and also in the maintenance of malignant characteristics in melanoma cells. Glycosphingolipids, including gangliosides, have been shown to be major components in some types of micro-domains associated with various functional membrane proteins involved in cell adhesion and cell signaling at the cell extracellular matrix interface (52). Studies have shown that microdomains have

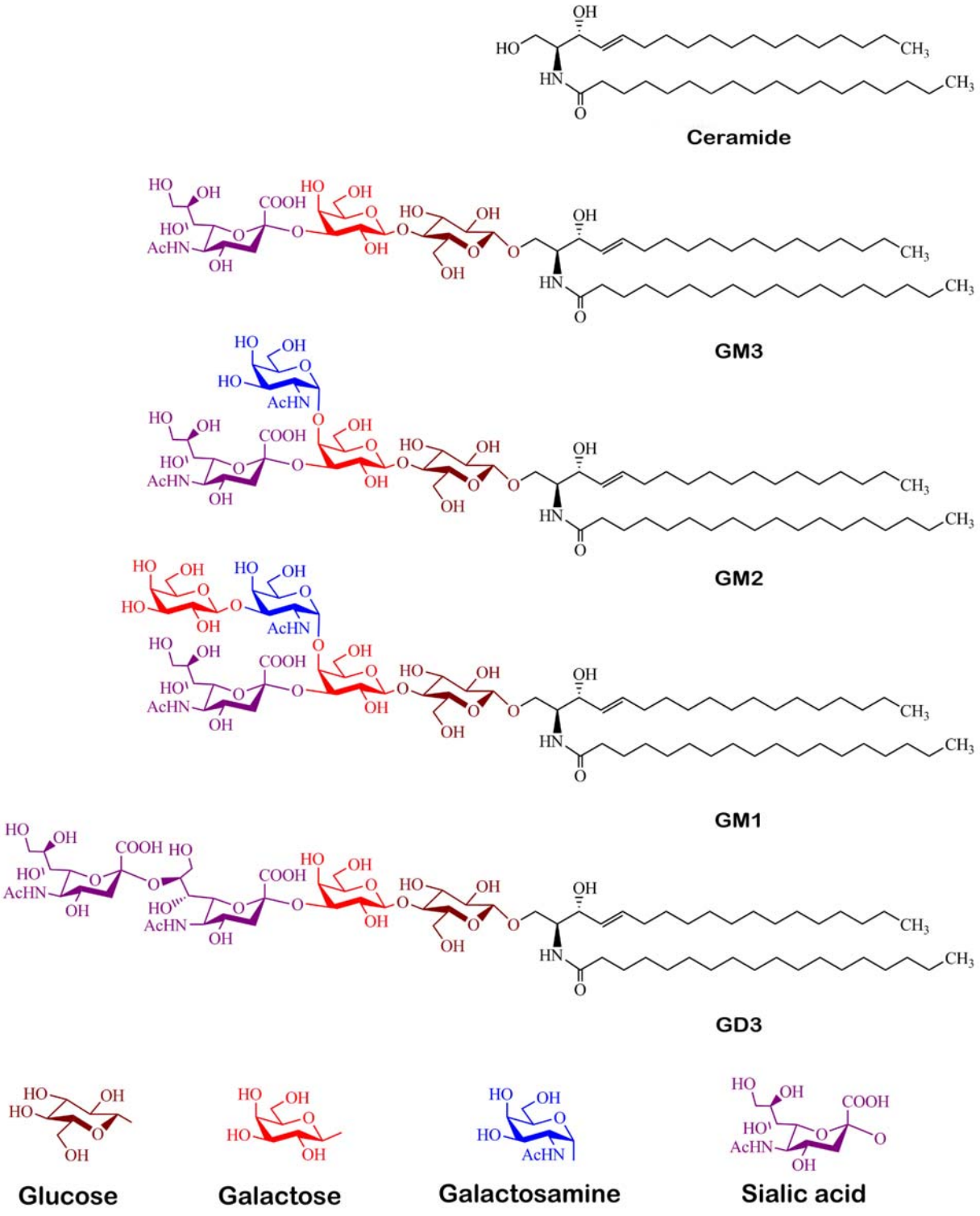

Figure 2. Chemical structure of some immunosuppressive gangliosides. GM3, GM2, GM1 and GD3 are most commonly involved in tumor-mediated immune dysfunctions. All gangliosides are composed of a common hydrophobic ceramide moiety along with a hydrophilic oligosaccharide chain, which varies in length and composition and contains one or more sialic acid residues.

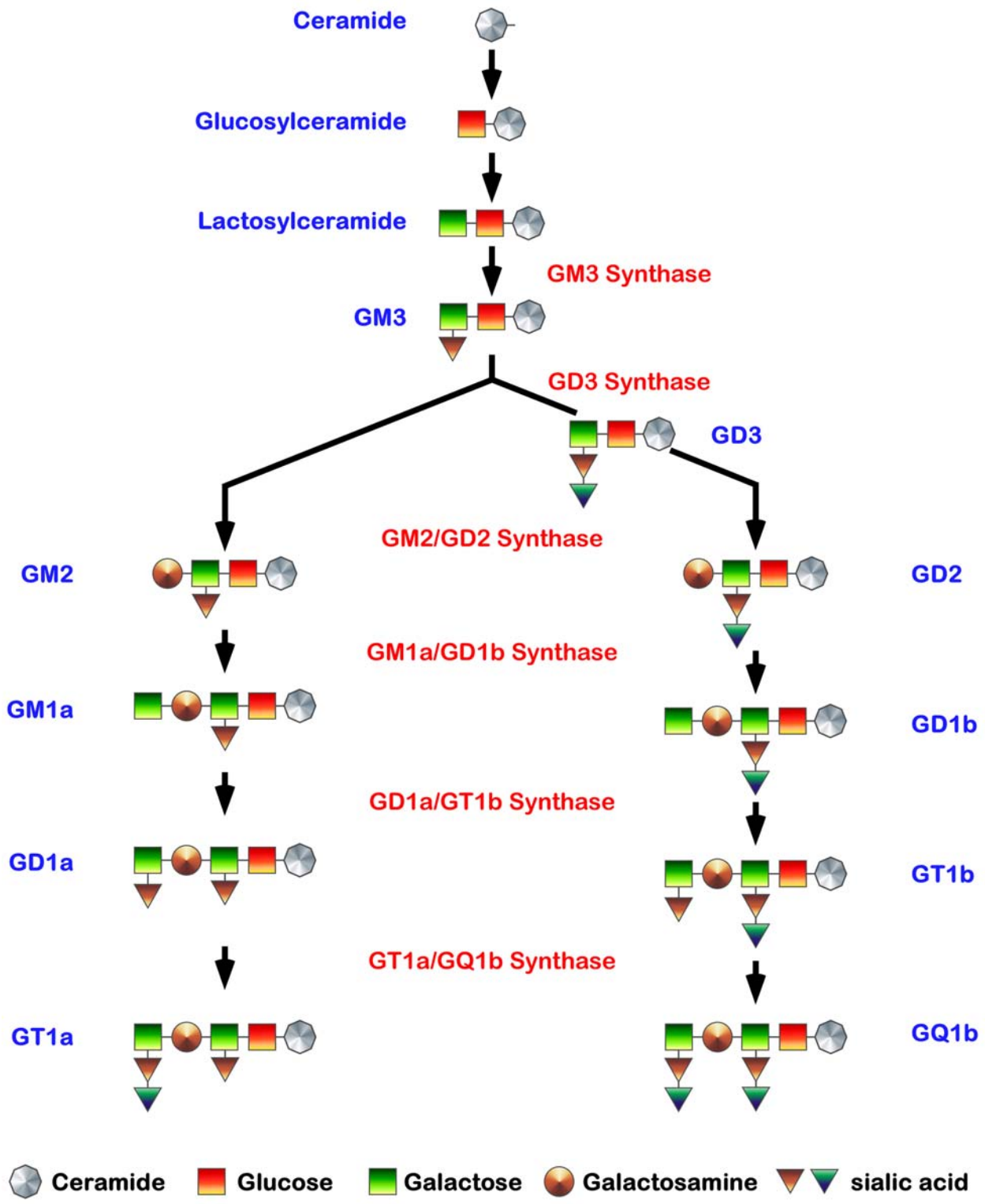

Figure 3. The ganglioside biosynthesis pathway for the ganglio-series of glycosphingolipids. Ceramide, the starting building block of all gangliosides is further processed to lactosylceramide. Lactosylceramide is a substrate for GM3 synthase resulting in production of GM3 which is a precursor for the synthesis other gangliosides. Parallel steps in both the pathways of ganglioside synthesis ("a" series and "b" series) are catalysed by the same glycosyltransferases of the golgi apparatus, i.e., GD3 synthase, GM2/GD2 synthase, GD1b/GM1a synthase, GT1b/GD1a synthase and GQ1b/GT1a synthase.

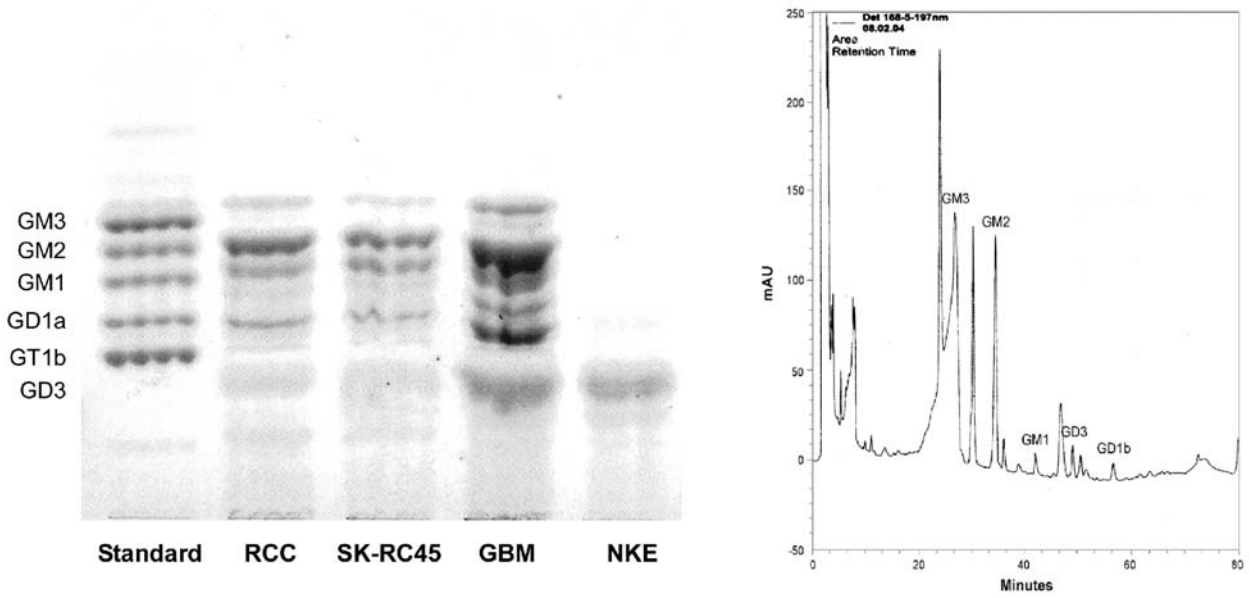

Figure 4. Tumors shed various gangliosides. Tumor-shed various gangliosides were identified by HP-TLC and LC-MS. Standard: standard gangliosides; RCC: primary renal cell carcinoma; SK-RC-45: renal cell carcinoma cell line; GBM: primary glioblastoma; NKE: normal kidney epithelial cell.

different physical properties and specialized functions. The potential roles of gangliosides and the structural variety of glycoclusters in microdomains have been recently reviewed (52). Gangliosides are also actively shed from the tumor (Figure 4) to their microenvironment in the form of micelles, monomers, and membrane vesicles (53,54). Shed gangliosides are able to bind and interact with a wide variety of proteins, including signaling molecules present in the tumor microenvironment (55). Furthermore, because shed gangliosides can be incorporated into the membrane of neighboring host cells, it is possible that the shed gangliosides modulate tumor-host cell interactions (56-57). The recent development in many laboratories of specific monoclonal antibodies directed against the carbohydrate moieties of gangliosides has provided new insights into the biological functions of the molecules exposed on the cell surface. For instance, monoclonal antibodies reacting with ganglioside GM2 were shown to induce necrosis *in vitro* in spheroid cultures of a GM2-rich human glioma cell line, anti-GD3 monoclonal antibodies were demonstrated to inhibit the growth of human melanoma cells both *in vitro* and *in vivo* and monoclonal antibodies against ganglioside GD2 were shown to induce apoptosis in small cell lung cancer cells (58-61). The use of monoclonal antibodies against tumor-associated gangliosides has provided indirect evidence that tumor-associated gangliosides should have an important function in tumorigenesis.

5. PREPARING THE BATTLEFIELD: TUMOR GANGLIOSIDES IN ACTION

The hypothesis that gangliosides may be active in the suppression of the anti-tumor immune response is supported by studies demonstrating that tumor cells synthesize and shed gangliosides into their microenvironments and that these shed gangliosides directly bind to target cells *in vitro* (62,63). Coupled with these observations, many studies have shown that exogenous or tumor-derived gangliosides inhibit multiple steps in the cellular immune response *in vitro* (Figure 5) (64). Studies by Ladisch *et al.* (65) showed that in neuroblastoma patients, the level of shed gangliosides detectable in serum was directly related to the incidence of tumor recurrence and the rapidity of progression. A later analysis by the same laboratory indicated that when assessed by multiple criteria, gangliosides from FBL-3 erythroleukemia cells inhibited tumor-specific secondary proliferative responses and CTL generation *in vitro* (64). The inhibitory effects of tumor-derived gangliosides have also been well demonstrated *in vivo* as evidenced in a syngeneic animal model (FBL-3 erythroleukemia cells, C57Bl/6 mice, and highly purified FLB-3 cell gangliosides) (65). The immunosuppressive activity of the gangliosides has been demonstrated to be quantitatively affected by their molecular structure. Variations in either the carbohydrate or the ceramide structure can lead to various degrees of activity. For example, gangliosides with a terminal sialic acid linked to a compact neutral oligosaccharide had the greatest immunosuppressive activity (66). Other studies indicated that tumor gangliosides were frequently more immunosuppressive than corresponding normal human brain gangliosides of an identical carbohydrate structure (67). These differences were inversely related to the length of the ceramide fatty acyl chain (67). The mechanisms of immunosuppression caused by tumor shed gangliosides are most likely multiple and remain to be fully elucidated. Dr. Stephen Ladisch has extended Black's work by publishing on the ability of gangliosides to inhibit specific components of the immune response, thereby enhancing tumor formation and progression. For instance, tumor-derived

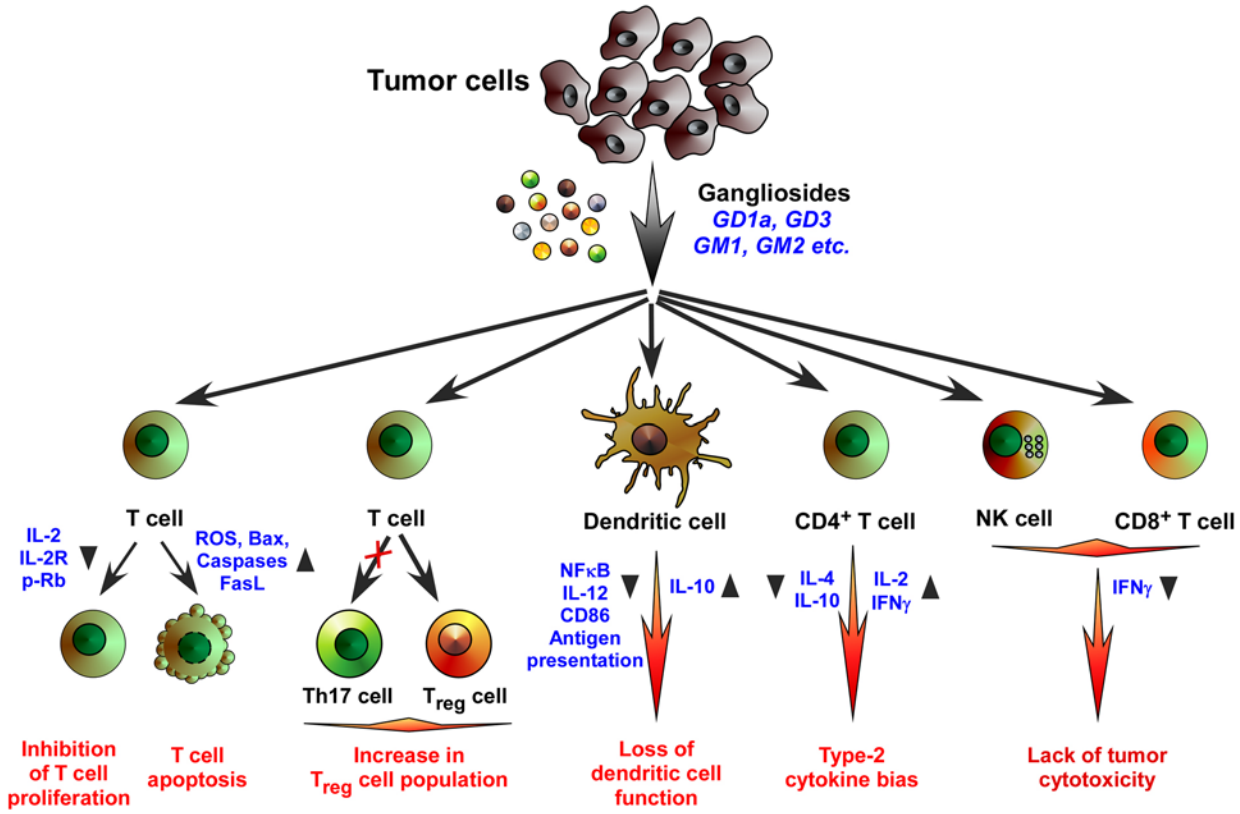

Figure 5. Immunosupressive activities of tumor-derived gangliosides. Tumor-derived gangliosides mediate immune dysfunctions *via* inhibition of T cell proliferation, induction of T cell apoptosis, augmentation of T_{reg} cells, induction of type-2 cytokine biasness and loss of DC, NK cell and cytotoxic T lymphocyte functions.

gangliosides inhibit the activity of several immune cells, including helper T cells, natural killer cell cytotoxicity, and antigen- and mitogen-stimulated T and B cells (46). Gangliosides have also been shown to block the production of TNFalpha as well as antigen presentation by human monocytes (46). In turn they cause down-regulation of constitutive and IL-8 inducible expression of major histocompatibility complex (MHC) class I and II molecules on astrocytes and inhibit the generation of functionally active dendritic cells (DCs) (46).

However the scope of this review is restricted to describe the mechanisms of ganglioside-mediated T cell dysfunctions with particular emphasis on T cell apoptosis, T cell proliferation and Th2-cytokine bias during tumor progression.

5.1. T cell proliferation: Restraining survival

Over the past two decades, there have been numerous reports on the ability of these gangliosides to negatively modulate T cell proliferation, both *in vitro* and *in vivo*. Studies by Bharti *et al* suggest that *in vivo* or *in vitro* treatment of bone marrow cells with gangliosides of Daltons lymphoma results in inhibition of proliferative ability and alteration of colony-forming ability of bone marrow cells (68). Total melanoma gangliosides in micelles inhibited proliferation of peripheral blood mononuclear cells stimulated by various mitogens, modulated lymphocyte surface molecules CD2, CD3, CD4, CD5 and CD8 and inhibited the production of interleukin-1beta (IL-1 β), tumor necrosis factor-alpha (TNF-alpha) and IL-6 by stimulated adherent cells (69). The autocrine production of IL-2, upon TCR activation, is critical to the normal proliferation of activated T cells (70). Bovine brain gangliosides reduced IL-2-dependent T-cell particularly cytotoxic T cell proliferation (71). Among the gangliosides, GM2 was most inhibitory (I_{50} =15μM) (71). GD1a and GD1b were somewhat less potent, whereas GM1 and GM3 were only weakly inhibitory (71). Apart from inhibiting IL-2 production gangliosides appear to act exclusively at the second stage of the process, during which IL-2 binds to high-affinity IL-2R, thus transducing a signal for cellular proliferation (71). The major mechanism by which gangliosides inhibit this stage of the activation process involves competition between gangliosides and IL-2R for binding of available IL-2 (71). The direct interaction of ganglioside micelles with IL-2 reduces the amount of lymphokine available for binding to both the p55 and p75 subunits of the IL-2R (71). This may help explain why gangliosides block the proliferation of IL-2-dependent cell lines that do not themselves make IL-2. A third inhibitory effect of gangliosides is their capacity to block the entry of activated T cells into the cell cycle (72). This correlates with their ability to prevent phosphorylation of Rb

(Retinoblastoma), a protein whose phosphorylation state is an important regulator of the transition from GI- to S-phase (72). This effect on Rb phosphorylation is unlikely to be a downstream response to a more proximal interruption of IL-2R signaling, since gangliosides also block the proliferation of the murine thymoma cell line EL4, which does not require IL-2 for growth (72). Lastly studies by Chu and Sharom indicate that gangliosides act as potent suppressors of IL-4-dependent processes in lymphocytes, and that their mechanism of action involves direct interaction with IL-4, thus preventing IL-4 binding to high-affinity IL-4 receptors (71). Gangliosides were highly effective inhibitors when added to G0-G1-synchronized HT-2 cells during the first 6 hr after IL-4 stimulation, indicating that they act early in the IL-4 signalling pathway (71). Various gangliosides inhibited IL-4-stimulated DNA synthesis in HT-2 with IC_{50} values in the range 26-60 µg/ml (71). However, the proliferation of four lymphokine-independent cell lines was unaffected by 500 µg/ml gangliosides (71).

Apart from directly targeting T cell proliferation, gangliosides are also known to inhibit T cell indirectly by negatively regulating accessory components of immune system involved in T cell priming and proliferation. Because the earliest stage of the cellular immune response known to be affected by exposure to gangliosides is antigen presentation, it is tempting to speculate that the initial step in tumor ganglioside-induced immunosuppression *in vivo* is interference with the functions of antigen presenting cells (APC). Dendritic cells are bone marrow-derived professional APC characterized by their unique capacity to elicit specific immune responses from näive T cells (73). They are found as a trace population in most tissues and fluids, where they exist at different differentiation/maturation stages interconnected by defined pathways of circulation. DCs are capable of recognizing, processing and presenting tumor antigens to T cells, in turn initiating a tumor antigen-specific immune response (73). The transfer of antigens from tumor cells to DC, or cross-priming, is thought to be mandatory for processing of tumor-associated antigens and presentation by MHC class I to T cells (73). Conversely, tumor progression is associated with increased immunosuppression involving impairment of the DC system (73). With respect to tumor gangliosides and DC dysfunction, numerous findings have identified ganglioside interference with DC-mediated T cell priming at different stages including DC differentiation, DC-mediated antigen presentation and DC-mediated conversion of T cells to tolerogenic T regulatory cells. Importantly, gangliosides derived from neuroblastoma patients have been reported to reduce the formation of erythroid and myeloid colonies from $CD34^+$ precursors *in vitro* (74). Similarly in melanoma patients, both GM3 and GD3 gangliosides dose-dependently inhibit the phenotypic and functional differentiation of DC, as assessed by down-regulation of CD1a, CD54, CD80, and CD40 antigens and impaired allostimulatory function (73). Furthermore, GM3 and GD3 gangliosides decreased the viable cell numbers and induced DC apoptosis significantly (73). GD3 also impaired differentiating DC maturation and the resulting DC produced low amounts of IL-12 and large amounts of IL-10, a cytokine pattern that might hamper an efficient antitumor immune response (73). All these information suggests that gangliosides impair phenotypic and functional differentiation of DC and induce their apoptosis, which may be an additional mechanism of tumor escape. This apart, toll like receptors (TLRs) are well established as an integral part of the innate immune system (75). The end result of TLR stimulation is induction of NFkappaB and consequent up-regulation of pro-inflammatory cytokine gene expression, including IL-6, IL-12, and TNF-alpha, and up-regulation of costimulatory molecules, including CD80 and CD86 (76). Studies by Weiping *et al.* (76) suggest that exogenous gangliosides potently inhibits ligand-induced activation and pro-inflammatory cytokine production induced by a broad range of TLRs, including TLR2, TLR3, TLR6, and TLR7/8, in addition to a previously identified inhibitory effect on TLR4 and TLR5. Underlying molecular mechanisms suggest that exogenous gangliosides neither inhibit binding of ligands to TLRs nor inhibit the initial activation steps but bypass down-stream activation steps that occur before NFkappaB activation and lead directly to IRAK-M expression, an inhibitor of TLR signaling (76). In addition to inhibition of DC differentiation, studies by Heitger *et al.* (77) suggest that tumor gangliosides may inhibit host anti-tumor cellular immune responses by preventing the effective cellular interactions of the antigen-primed monocyte with the responding T-lymphocyte. To better define the immunomodulatory properties of gangliosides on antigen-specific T-cell activation and development, Jales *et al.* (78) have developed an *in vitro* system using ganglioside-treated murine bone-marrow-derived DCs to prime and activate antigen-specific $CD4^+$ T cells. Using this system, ganglioside treatment promotes the development of a DC population characterized by decreased CD86 (B7-2) expression, and decreased IL-12 and IL-6 production (78). When these cells are used as antigen-presenting cells, $CD4^+$ T cells are primed to proliferate normally, but have a defect in T helper effector cell development (78). This defect in Th effector cell responses is associated with the development of regulatory T-cell activity that can suppress the activation of previously primed Th effector cells in a contact-dependent manner (78).

5.2. Th2 bias: Polluting the environment

A number of studies indicate that a type-1 response (Th1/Tc1) plays a critical role in the rejection of tumors (79,80). Th1-type $CD4^+$ T cells secrete IFNgamma and IL-2 that promote cellular immunity, in part by providing helper signals for the cytotoxic $CD8^+$ T lymphocytes that also have the capacity to produce IFN-gamma in response to antigen. Th2-type cells produce IL-4 and IL-5 and typically promote a humoral immune response, whereas $Th3/T_{reg}$ cells produce immunosuppressive cytokines (IL-10 and TGFbeta) that can dampen both Th1- and Th2-type immune responses (81). With respect to this, several findings show that gangliosides present in the tumor supernatant induce type-2 biased immunity, similar to that promoted by the crude supernatant (81). Studies by Rayman *et al.* (81) demonstrated that a mixture of bovine brain-derived gangliosides inhibited IFN-gamma production but not IL-4

after the T-cell stimulation. Herein, they show that bovine-derived GD1a, a ganglioside over-expressed in renal cell carcinoma, could also inhibit the development of type-1 T-cell responses (81). The underlying mechanisms for renal cancer-induced type-2 bias involved apoptosis of Th1 cytokine-producing T cells resulting in predominance of Th2 cytokines (81). It is currently not known which of the gangliosides expressed by renal cell carcinoma and present in supernatants from renal cell carcinoma explants are responsible for the selective suppression of the type-1 response, although this is an active area of ongoing investigations. In another study gangliosides inhibited the production of the Thl-associated cytokines IL-2 and IFN-γ in activated T cells without blocking the expression of IL-4 and IL-10 (82). This could be explained by the ability of gangliosides to impede NFkappaB activation, a transcription factor for IL-2 and IFN-gamma (82). It has also been shown that exposure of mouse splenocytes to gangliosides results in reduced gene transcription of the Th1-associated cytokines, IL-2 and IFNgamma, while leaving gene transcription of the Th2-associated cytokines, IL-4 and IL-10, unaffected (83). In contrast work by Crespo *et al.* indicate that gangliosides have the ability to induce immune deviation in favor of type-2 T cell responses by acting at different levels, including the inhibition of IFN-gamma production by several cell sources and an enhancement of IL-4-dependent differentiation of $CD4^+$ T cells (11). Although the expression and production of IFN-gamma were reduced, and that of IL-4 was enhanced by the presence of gangliosides after T cell stimulation, suggesting a potential modulation of the differentiation of T helper cells, the effects on the production of these two cytokines appeared to be independent of each other (11). Moreover, inhibition of IFN-gamma production took place, not only on $CD4^+$ T cells but also on $CD8^+$ T cells and NK cells (11). It appeared, however, that the inhibitory effect on the production of IFN-gamma after re-stimulation of enriched T cells was attenuated partially by supplementation of the primary cultures with rIFN-gamma, suggesting that gangliosides may antagonize an IFN-gamma-mediated signal or activity (11). Similarly, the effects of gangliosides on the type-2 pathway were not dependent on alterations in endogenous IFN-gamma activity. For example, inhibition of endogenous IFN-gamma was unable to mimic the effect of gangliosides on the production of IL-4, and addition of rIFN-gamma could not prevent their effects. However the enhancing effects of gangliosides on type-2 responses did not take place in cells from CD1d-deficient mice. These mice lack NK T cells, which are responsible for the initial production of IL-4 in several systems. Thus, the absence of NK T cells would have resulted in deficiency of endogenous IL-4 upon initial anti-CD3 stimulation and therefore, in the lack of effect of gangliosides on Th2 differentiation. As for the targets of ganglioside action, several possibilities exist. It is possible that gangliosides may enhance the production of endogenous IL-4 by NK T cells. Although the inability to detect $IL\text{-}4^+$ cells among the NK cells within 24-72h of stimulation would argue against this explanation, limitations in the sensitivity of intracellular staining and low numbers of $IL\text{-}4^+$ cells, particularly in C57Bl/6 mice, preclude discounting this possibility. Nevertheless, measurements of IL-4 levels in 24h cultures (after addition of an anti-IL-4R antibody to prevent absorption to cells) indicated that the presence of gangliosides did not increase early endogenous levels of IL-4 (11). Other explanations include a ganglioside-mediated potentiation of the effects of endogenous IL-4 on Th cell precursors, promoting differentiation toward Th2 effector cells, and as distinct apoptotic pathways operate in Th1 versus Th2 cells, gangliosides may also promote selective apoptosis in Th1 cells, resulting in the preferential survival of Th2 cells (11). Others have reported a ganglioside-induced increase in T cell IL-10 production (46). In addition, Zou *et al.* (84) reported that incubation of peripheral blood mononuclear cells with gangliosides derived from glioma cells results in inhibition of the production of IFN-gamma, IL-12, and TNFalpha and enhances secretion of IL-6 and IL-10.

5.3. Th17 dysfunction: Misleading the saviors

Th17, a specific type of T helper cell awakens the immune system to the stealthy threat of cancer and triggers an attack of killer T cells custom-made to destroy the tumors. Th17 cells highly express IL-23R which upon binding to IL-23 results in late stage development of Th17 cell and regulation of Th17 functions (85). Consistently, the expression of IL-23 at the tumor site or therapy with DCs expressing IL-23 can induce potent tumor-specific immunity against melanoma and glioma (86). However earlier works have clearly indicated the involvement of tumor gangliosides in improper DC maturation during tumor progression. Also DC dysfunction is positively correlated with the abundance of tumor gangliosides based on which it can be speculated that tumor gangliosides might be responsible for either impairing the anti-tumor functions of Th17 or switching the anti-tumor response to a pro-tumor one since recently there have been a number of studies highlighting the positive and negative aspects of Th17 in context of tumor progression (87,88). Again it is very much appreciated that both T_{reg} and Th17 cells share a common lineage, where terminal differentiation of suppressor versus effector cells at the tumor site may tip the balance between tolerance and tumor rejection (89). Since tumor gangliosides are known to increase the T_{reg} population, it could be possible that by inducing T_{reg} differentiation, these molecules are inhibiting Th17-dependent anti-tumor responses. However an indepth study shall clearly elucidate the role of tumor gangliosides in Th17 function and differentiation.

5.4. T_{reg} induction: Cultivating the terrorists

Apart from inhibiting T cell functions against tumor antigens, gangliosides often increase tolerogenic conditions to mask the leftover of any anti-tumorigenic responses by inducing regulatory T cells (T_{reg}). T_{reg} cells are primarily identified for their role in immunosuppression in different cancers including melanoma, renal cell carcinoma and hepatocarcinoma (90). Recent studies have found an increased number of T_{reg} in both peripheral blood and tissues from patients with hepatocellular carcinoma (91). Tumor culture supernatants from hepatoma-derived cell lines were found to modulate the differentiation of human monocyte-derived DC and/or their ability to

increase T_{reg} (92). Moreover, exposure of DC to tumor supernatants selectively inhibited their capacity to stimulate the proliferation of allogeneic $CD8^+$ T cells, but promoted the generation of $CD4^+CD25^{hi}Foxp3^+$ T_{reg} cells (92). These findings, together with previous clinical studies showing that gangliosides are concentrated within hepatocellular carcinoma tissue (93), suggest that the gangliosides may favor the induction of T_{reg} cells through improper differentiation and maturation of DC. Recently Wang *et al.* (94) have provided the basis for GM1-Gal1 interaction in T_{reg}-mediated immunosuppression. This study has shown cross-linking of GM1 gangliosides in T effector cells by Gal1 expressed by T_{reg} cells to be a crucial element in the suppressive mechanism inhibiting proliferation of the responder cells (94). Interestingly gene expression profile of regulatory *vs.* effector T cells revealed a substantial increase in Lgals1, the gene encoding galectin-1 in natural occurring T_{reg} cells (94). Jales *et al.* (76) found that ganglioside pre-treatment of monocytes before stimulation with TLR agonists promoted the induction of IRAK-M expression paralleled by generation of T_{reg} cells. In another study Xie *et al.* (95) found that in IRAK-M-deficient mice, the immune response to tumors was enhanced, with a concordant decrease in regulatory T cells. IRAK-M is also known to be associated with endotoxin tolerance based on which Jales and colleagues speculated that IRAK-M induced by gangliosides may promote the development of a regulatory T-cell phenotype (76).

6. TUMOR GANGLIOSIDES AND T CELL APOPTOSIS: THE ULTIMATE ENCOUNTER

The above cited literature clearly identifies numerous aspects of tumor gangliosides in defeating the different armed machineries like inhibiting T cell proliferation, inducing Th2 bias, Th17 impairment and T_{reg} induction while winning the war between tumor and immune cells. However since the ultimate defenses of immune system against tumor depends upon the ability of T cells to induce tumor apoptosis it can be predicted that gangliosides by jeopardizing T cell proliferation, function and differentiation cumulatively induce T cell apoptosis. In fact, there are reports demonstrating that inhibition of T cell proliferation by neutralizing IL-2 culminates in T cell apoptosis (96-98). Similarly the presence of Th2 cytokines IL-4, IL-10, etc. has been shown to be involved in the process of T cell apoptosis (99,100). Th17 cells are also important for maintaining T cell viability and stimulating T cells functions (101,102). The effect of T_{reg} cells in inducing T cell apoptosis is also well established (103). These are some of the mechanisms where gangliosides, by regulating accessory machineries, indirectly induce T cell apoptosis. However these molecules are also known to be directly involved in T cell dysfunction and apoptosis. Initial studies by Offner *et al.* (104) demonstrated that gangliosides inhibited the function of T helper cell lines, and simultaneously inhibited the expression of the CD4 molecule. A string of studies later suggested that ganglioside pretreatment induced rapid and selective disappearance of the CD4 molecule from mouse, rat, and human T helper lymphocytes. Preclearance of CD4 by antibody-mediated capping reduced binding of 3H-GM1 to T helper cells (104). These results indicate that gangliosides induce a profound change in the molecular orientation of CD4 within the T helper cell membrane which renders epitopes on the CD4 molecule inaccessible to antibody. The renal line SK-RC-54 that expresses GM2 can induce apoptosis of T cells that can be partially blocked (>50%) by the addition of an antibody to GM2 (105). Similarly gangliosides from leukemic cells FBL-3 were found to inhibit the generation of CTL-specific response for syngeneic FBL-3 tumor cells by inducing CTL apoptosis (106). These however were preliminary studies with respect to ganglioside-induced T cell dysfunction, which have been superseded by recent studies where different mechanisms have been proposed for ganglioside-mediated T cell apoptosis. Therefore identifying mechanisms responsible for ganglioside-mediated T cell apoptosis and its therapeutic intervention may expand the horizon of therapeutic success with respect to immunotherapy.

7. MOLECULAR MECHANISMS: BLUE PRINT OF THE 'WAR PLAN'

It is acknowledged that NFkappaB/p65RelA is critical for the control of a variety of cellular processes including maintenance, activation, proliferation and cytokine production in T cells (107-110). Defective activation of NFkappaB has been reported in T cells from tumor-bearing mice and cancer patients (1,111-113). Interestingly, gangliosides isolated from supernatants of renal cell carcinoma (RCC) explants suppress NFkappaB activation in peripheral blood T cells (114). The deficiency stems from impaired nuclear localization of the p65/p50 NFkappaB heterodimer, a problem that has been noted in both tumor-infiltrating lymphocytes (TIL) and peripheral T cells isolated from patients with RCC (115). Coincident with impaired NFkappaB is the demonstrated enhanced susceptibility of RCC patient T cells, particularly TILs, to apoptosis (116). A mixture of bovine brain-derived gangliosides (GM1, GD1a, and GD3), when co-cultured with T cells, results in the loss of RelA protein expression and cell death (115). The exact identity of the gangliosides expressed by RCC that are responsible for suppressing NFkappaB activation and inducing apoptosis are not yet known. However, bovine brain-derived gangliosides GM1, GD1a, and GD3 are reported to inhibit kappaB-binding activity in both T cells and hepatocytes (114,117,118). Given that renal tumors show increased expression of GM1 and GD1a, whereas melanoma over-expressed GD3, suggest that these tumor-derived gangliosides may promote NFkappaB suppression. The fact that GM3 does not inhibit NFkappaB-binding activity indicates that only selected gangliosides are involved in this process (114,117,118). This notion is supported by recent ganglioside fractionation studies showing that only some ganglioside peaks suppressed NFkappaB and induced apoptosis (119). Given that gangliosides are heterogeneous in their ability to suppress immune cells, the composition of gangliosides expressed by a given tumor may dictate the type and severity of immune alterations that may occur. Thornton *et al.* (115) on the other hand suggest that gangliosides expressed by RCC lines can induce degradation of p65 and p50 protein levels within both Jurkat T cells and peripheral blood T lymphocytes that coincided with the onset of apoptosis. These gangliosides activate the mitochondrial pathway of apoptosis by initiation of the caspase cascade *via* caspase-9 (115). Activated caspases in turn stimulates a non-caspase

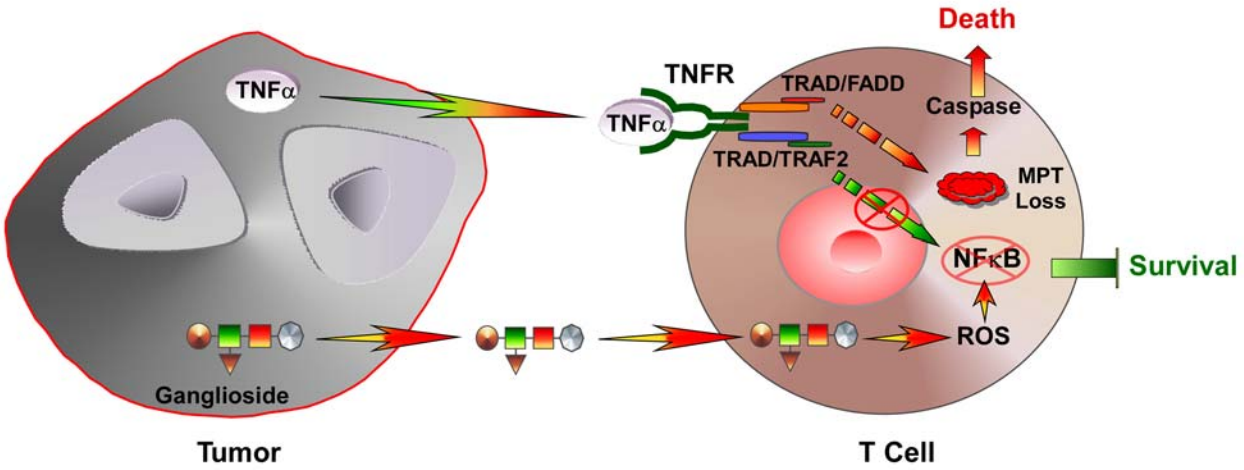

Figure 6. Molecular mechanism of ganglioside-mediated T cell apoptosis. Gangliosides alone or in combination with TNFα inhibit the NFκB-dependent survival pathway and trigger both intrinsic and extrinsic apoptotic cascade.

protease to degrade the NFkappaB transactivating complex, p65/p50. The loss of this NFkappaB complex further promotes apoptosis through decreased expression of select anti-apoptotic genes, including Bcl-xL and Bcl-2, which are controlled by NFkappaB (115). Accompanying RCC-induced T-cell apoptosis was a tumor-mediated reduction in lymphocyte Bcl-2 expression levels, which also was abrogated by blocking ganglioside expression on the tumor line (120). Bcl-2 is involved in protecting mitochondria from reactive oxygen species (ROS) accumulation, disruption of transmembrane potential, and cytochrome c release (121). Sa G and colleagues (122) have demonstrated that ganglioside is internalized by activated T cells, initiating a series of pro-apoptotic events, including the induction of ROS, an enhancement of p53 and Bax accumulation, an increase in mitochondrial permeability, cytochrome c release, and the activation of caspase-9. There is now accumulating evidence that cell soluble forms of ceramide and some gangliosides may induce apoptosis of various cell types by directly modulating mitochondrial permeability (123,124). These molecules cause the accumulation of ROS and the initiation of the mitochondrial permeability transition, leading to cytochrome *c* release, caspase 9 activation and apoptosis. Collel *et al* (118) have shown that hepatocytes treated with a sublethal dose of GD3 produced both mitochondrial ROS and blocked the activation of NFkappaB and subsequent kappaB-dependent gene expression induced by TNFalpha, which sensitized hepatocytes to TNF-alpha-induced apoptosis suggesting that gangliosides are efficient death effectors by a dual mechanism that involves mitochondrial recruitment and suppression of the NFkappaB-dependent survival pathway. Concurrently, studies by Das *et al.* (125) have described the significance of both Fas receptor-dependent and receptor-independent pathways during ganglioside-mediated T cell apoptosis in RCC patients. Although previous studies demonstrated that the apoptosis of T cells mediated by squamous cell carcinoma of the head and neck is initiated by Fas receptor-dependent signals but significantly amplified by a mitochondrial loop, Das *et al.* extended these studies by demonstrating the ability of tumor-gangliosides to stimulate apoptosis in a strictly death receptor-independent fashion by directly inducing loss of mitochondrial transmembrane potential (125). The same group further extends their previous studies by demonstrating that tumor-derived TNFalpha enhances RCC apoptogenicity not only by inducing ganglioside synthesis but also by initiating receptor-dependent apoptosis in T cells in which the NFkappaB activation pathway has been inhibited by GM1 (126). All these information suggest that tumor-shed gangliosides internalized into T cells to produce ROS that in turn perturb NFkappaB. In these NFkappaB-disturbed cells tumor-derived TNFalpha induces apoptosis *via* mitochondrial death cascade (Figure 6). Though there have been several studies describing the mechanisms of ganglioside-mediated T cell apoptosis, a lot more awaits to be unveiled for further implications in anti-ganglioside-mediated immunotherapeutic strategies .

8. CONCLUSION

The T cell-mediated immune response developed against tumor is complex. T cells are critical for host immunity, and at the same time interact with tumors to facilitate their extinction. However tumor gangliosides, *via* numerous mechanisms, contribute to the defects seen in $CD4^+$ T cells beginning early from T cell activation to T cell differentiation. In spite of extensive basic and applied research in tumor biology over the last decade, we are still in the early stages of understanding all aspects of tumor gangliosides interaction with, and inhibition of, T cells. Several evidences show that tumor gangliosides have immune modulatory effects on different T cell subsets, including impairment of maturation, altered cytokine profile, defective T cell stimulatory function and T cell apoptosis. The mechanism of the diverse cross-talk between tumor gangliosides and T cells is now explained by the formation of interrelated pathways. Further insight into the role of tumor gangliosides in this *tug-of-war* with T cells, may lead to new measures to develop vaccines that can inactivate or inhibit ganglioside-mediated T cell dysfunction in one hand, and induce cancer regression on the other.

In fact, there has been a long fascination with developing vaccine against cancer. Recently, attention has been focused on the development of molecularly defined vaccines. Aberrant glycosylation is a universal feature of a cancer cell. The examples of such differences from normal cells are the observed quantitative and qualitative changes in the expression of gangliosides of the tumors (127). The presence of large amounts of these tumor-specific immunosuppressive carbohydrate antigens on cancer cells, as compared to normal cells, opens the possibilities to target them not only to inhibit tumor progression but also to protect the immune system and thereby making the latter available for immunotherapy. Ganglioside-vaccine, therefore, may act as a double sword that, in one hand, regresses tumor directly and on the other *via* improved immunomodulatory circuit. In this context it may also be mentioned that although besides gangliosides, tumors shed various immunosuppressive molecules (Table 1), gangliosides are superior candidates for vaccine development since (a) there is no significant qualitative difference in the structure of other molecules irrespective of their cells of origin, thereby raising the possibility of non-specificity, and (b) besides being apoptogenic themselves, gangliosides also mind the immune cell microenvironment in favor of death so that the other tumor-shed immunosuppressive molecules can now induce apoptosis (126). Therefore, instead of inhibiting each of those molecules, most of which are also essential for the normal cell development and survival, targeting of gangliosides will be more specific as cancer vaccine that will also restrict other immunosuppressive molecules from inducing apoptosis in immune cells although they will remain available for normal cellular functions, if any. Direct evidence of the importance of gangliosides as potential targets for active immunotherapy has been suggested by the observation that human monoclonal antibodies against these glycolipids induce shrinkage of human melanomas (128). Such discussion on the current wealth of clinical and preclinical information is important in understanding the future scenario in which by manipulating the tumor-gangliosides, the synchronized blockade of immunosuppressive mechanisms with simultaneous inhibition of tumor growth might be effective in combination with other conventional strategies to significantly alter clinical outcomes.

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Abbreviations: APC: antigen presenting cell; CTL: cytotoxic T lymphocytes; DC: dendritic cells; ER: endoplasmic reticulum; FoxP3: forkhead box P3; GBM: glioblastoma; GM-CSF: Granulocyte-macrophage colony stimulating factor; GSL: glycosphingolipids; IFN-γ: interferon-γ; IL: interleukin; MDSC: myeloid derived suppressor cell; MHC: major histocompatibility complex; MoDC: monocyte-derived dendritic Cell; NFκB: nuclear factor kappa B; NK cell: natural killer cell; PGE2: Prostaglandin E2; RCC: renal cell carcinoma; ROS: reactive oxygen species; TCR: T cell receptor; TGFβ: transforming growth factor beta; Th1: T helper type-1; Th2: T helper type-2; TIL: tumor-infiltrating lymphocytes; TNF-α: tumor necrosis factor-α; T_{reg}: regulatory T cell; VEGF: Vascular endothelial growth factor

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Send correspondence to: Gaurisankar Sa, Division of Molecular Medicine, Bose Institute, P-1/12 CIT Scheme VII M, Kolkata 700 054, India, Tel: 91-33-2569-3258, Fax: 91-33-2355-3886, E-mail: gauri@boseinst.ernet.in

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