Immunomodulation of myeloid-derived suppressor cells by particulate β-glucan in cancer.

Sabrin Husein Albeituni

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IMMUNOMODULATION OF MYELOID- DERIVED SUPPRESSOR CELLS BY PARTICULATE β-GLUCAN IN CANCER

By
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B.S., Birzeit University, 2008
M.S., University of Louisville, 2012

A Dissertation
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in Partial Fulfillment of the Requirements
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Louisville, Kentucky

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June 4, 2015

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DEDICATION

To all cancer patients who fought and are still to the last minute.
To those that believed in us and worked together to win this battle.
To all my teachers who shaped who I am today.
To my friends Ruba, Rawand, Lubna, Laila and Banrida who were always there.
To my dad Husein who taught me how to see the world beyond my eyes.
To my mom Nadra who sacrificed all her life to bring the best in me.
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ABSTRACT

IMMUNOMODULATION OF MYELOID-DERIVED SUPPRESSOR CELLS BY PARTICULATE β-GLUCAN IN CANCER

Sabrin Albeituni

June 4, 2015

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells that promote tumor progression. In this study, we investigated the effect of dectin-1 stimulation by yeast-derived particulate β-glucan in MDSC function and differentiation in cancer. *In vivo* treatment of mice bearing lewis lung carcinoma and mammary cell carcinoma with particulate β-glucan decreased tumor weight and splenomegaly, and reduced the accumulation of polymorphonuclear-MDSC (PMN-MDSC) but not monocyti
c-MDSC (M-MDSC) in the spleen and tumor. In addition, particulate β-glucan differentially modulated the function of different MDSC subsets; it enhanced PMN-MDSC respiratory burst and apoptosis, and induced the differentiation of M-MDSC into F4/80⁺CD11c⁺antigen-presenting cells in a dectin-1 dependent manner. ERK1/2 phosphorylation was also required for the acquisition of APC properties in M-MDSC. Moreover, M-MSDC treated with particulate β-glucan did not promote tumor growth *in vivo* when inoculated with LLC subcutaneously. To evaluate the effect of particulate β-glucan treatment in humans, patients with non-small cell lung cancer (NSCL) were treated with particulate β-glucan for two weeks prior to any other treatment and
surgical excision of the tumor. Strikingly, the frequency of CD14-\text{HLA-DR}^-\text{CD11b}^+\text{CD33}^+ \text{MDSC} decreased in the peripheral blood, and arginase-1 expression significantly decreased in a cohort of 15 patients. This study was the first to assess the effect of particulate β-glucan on MDSC in lung cancer patients, towards a future inclusion of particulate β-glucan in combination therapies in the treatment of lung cancer.
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INTRODUCTION

The battle analogy is commonly used when describing the immune system in action. From DNA restriction, surface exclusion and CRISPs systems (clustered, regularly interspaced short palindromic repeats) in bacteria and archea (1, 2) to a highly specialized immune system in mammals, the immune system has evolved to fulfill one role: elimination of foreign antigens. The immune system in general is described to have two forms: innate and adaptive. Historically, these terms used to suit the idea that an immune cell can either respond to a foreign antigen in a rapid and non-specific fashion (i.e. innate) or adapt to the recognition of a certain antigen, by generating memory, in a highly selective process where these cells respond faster and more robustly after a recall of the same antigen (i.e. adaptive).

---

According to this definition, macrophages, dendritic cells, granulocytes and natural-killer (NK) cells are categorized under ‘innate immunity’, whereas T cells and B cells are considered ‘adaptive’. However, the categorization of immune cells under innate or adaptive immune responses becomes elusive when describing γδ-T cells, B1 cells and invariant NKT (iNKT) cells (3). Moreover, this definition becomes fuzzy after the discovery that NK cells acquire a ‘memory’ phenotype in response to cytomegalovirus (CMV) infection in which ‘memory’ NK cells present a higher cytolytic and cytokine response after the second exposure to the virus, unlike naïve NK cells (4-6).

A twisted mechanism of innate memory emerges when elucidating the recognition of pattern associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) expressed in innate and adaptive immune cells (7-9). The memory-like phenotype in innate cells emerges as a function of epigenetic regulation and chromatin modifications upon the stimulation of macrophages and monocytes with a Toll-like receptor 4 (TLR4) ligand (e.g. lipopolysaccharide LPS) (10, 11) or a dectin-1 ligand (e.g. fungal cell-wall β-glucan) (12-14). Interestingly, repeated exposure of murine macrophages to LPS induce an array of anti-inflammatory genes (non-tolerizable genes, class NT) while silencing the pro-inflammatory genes (tolerizable genes, class T) leading to endotoxin tolerance preventing tissue damage (10, 11). In contrast, pre-stimulation of monocytes with Candida albicans (a source of β-glucan), renders monocytes more responsive upon re-stimulation, inducing what is referred to as ‘trained immunity’ (11-14). Escape from immunosurveillance, is the last ‘E’ of the three ‘Es” in cancer immunoeediting theory that consists of three main phases: Elimination, Equilibrium and Escape (15, 16). It has been well established that the immune system is a key player during tumor development, playing ambivalent roles in tumor
elimination and tumor progression. Immune cells can recognize tumor-associated antigens (17) and eliminate tumor cells through numerous anti-tumor mechanisms. However, during tumor development, tumor factors in the tumor microenvironment modulate immune cells towards a protumorigenic phenotype leading to local and/or systemic immunosuppression, thus inducing tumor growth and establishing a suppressive niche in distant sites facilitating tumor metastasis. This unique interaction between tumor and immune cells in the tumor microenvironment has rendered immunotherapy a daunting task in our battle against cancer.

The tumor microenvironment exclusively promotes the induction and expansion of immune suppressors that ultimately inhibit effector T cell proliferation and the activation of cytotoxic T lymphocytes (CTLs) and anti-tumor NK cells. Earlier studies have described the presence of natural suppressors of lymphoproliferative responses with myeloid-cell characteristics in mice and humans (18-22). The finding that suppressive monocytes expressing CD11b/Mac-1 accumulate in the spleens of tumor-bearing mice (23), has led to the identification of T cell suppressors that express CD11b/Mac-1 and Gr-1 antigens in the spleens of mice immunized with highly immunogenic recombinant anti-cancer vaccines and tumor-bearing mice (24-27). A plethora of following studies in various cancer models, have identified these cells as a heterogeneous population of cells with myeloid origin that has the potential to differentiate into mature granulocytes, macrophages and dendritic cells, but under the influence of tumor factors are hampered in an immature state of differentiation with potent immune suppressive functions. This heterogeneous population of myeloid suppressive cells is collectively known as myeloid-derived suppressor cells (MDSC) (28). Despite the suppressive characteristic attached to its definition, it is now
debatable whether tumor MDSC are suppressive during early stage of tumor development in humans. Eruslanov et al, clearly show that tumor infiltrating neutrophils (TAN) induce T cell responses during early stages of tumor development (29), and propose that induction of suppression can be artificially achieved during tumor preparation in case the enzymatic digestion cocktail sheds the TAN surface costimulatory molecules (30). suggesting that during the equilibrium phase, immune cells may express dual phenotypes, and depending on how far the battle is, the equilibrium may shift towards the escape phase giving tumor cells advantage, or being brought back to the elimination phase, that mainly is rescued upon immunotherapy.

Heterogeneity and T cell suppression are hallmarks of MDSC biology. In mice, MDSC are classified into two main subsets according to their morphology and markers: CD11b+Ly6C<sup>high</sup>Ly6G<sup>-</sup> cells resemble monocytes and are called monocytic MDSC (M-MDSC), and CD11b+Ly6G+Ly6C<sup>low/int</sup> cells with a polymorphonuclear morphology are called granulocytic or polymorphonuclear MDSC (PMN-MDSC/PMN-MDSC) (31, 32). The absence of a Gr-1 homologue in humans led to the inclusion of a broader spectrum of markers in the description of suppressive myeloid cells in cancer patients. In general, M-MDSC express CD14<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>low</sup>CD11b<sup>+</sup>CD15<sup>+</sup>, while PMN-MDSC express CD14<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>low</sup>CD11b<sup>+</sup>CD15<sup>+</sup> and/or CD66b<sup>+</sup> (reviewed in ref (33)). M-MDSC expressing Lin<sup>-</sup>CD11b<sup>+</sup>CD14<sup>+</sup> have also been described in patients with melanoma (34). Another adapted definition by many researchers is by defining M-MDSC in humans as CD14<sup>+</sup>HLA-DR<sup>-low</sup>CD11b<sup>+</sup>CD33<sup>+</sup> (35). It is clearly noted that the expression of a unique marker of immunosuppression in MDSC in mice and humans has been one of the main challenges in the field.
After the identification of MDSC as one of the major suppressors of T cell responses and inducers of T cell tolerance (24, 43), numerous studies have characterized their roles in cancer as suppressors of NK cells (44), inducers of regulatory T cells (Tregs) (45), and precursors of tumor-associated macrophages (39). MDSC-mediated T cell suppression is mainly attributed to the expression of Arginase 1, iNOS, ROS (38) and cystine and cysteine deprivation (46). A main factor responsible for the accumulation of MDSC in cancer is the fact that MDSC are immature and do not subsequently differentiate to anti-tumor macrophages and dendritic cells (DCs) under the influence of tumor-derived factors (33). The fact that MDSC immune suppression can be reversed in vitro and in vivo has led to the development of a multitude of strategies in cancer therapy.

Herein, we will discuss MDSC origin and mechanisms of immune suppression, focusing on the therapeutic strategies targeting MDSC in tumor-bearing mice and cancer patients. In an attempt to study a natural compound that targets MDSC with no side effects, at a low cost and can be easily consumed by oral uptake, we studied the effect of the immunomodulator, particulate β-glucan on MDSC in tumor-bearing animals and non-small cell lung cancer (NSCLC) patients. Whole glucan particles (WGP) are micro-particles of 1,3-β-glucan extracted from the yeast Saccharomyces cerevisiae, that have been shown to activate immune cells through the stimulation of C-type lectin receptor, dectin-1 (47, 48). It is well reported that WGP treatment reduces tumor growth and activates dendritic cells (DC), induces T cell responses and decreases the frequency of Tregs in vivo (49). In addition, a recent report showed that WGP partially induces the differentiation of M-MDSC to F4/80⁺CD11c⁺ cells (50). However, the role of particulate
β-glucan in the modulation of the function of different MDSC subsets in mice and its implementation in the clinic needs further studies.

Herein, we delineated the effect of particulate β-glucan on the function of both PMN-MDSC and M-MDSC in mice. We demonstrated that particulate β-glucan induced a cytotoxic phenotype and subsequent apoptosis in PMN-MDSC, while converting M-MDSC to potent antigen-presenting cells (APCs) that uptake, process and present ovalbumin (OVA) Ag to OVA-specific CD4⁺ T cells and also cross-present Ag to prime OVA-specific CD8⁺ T cells. More importantly, NSCLC patients treated with particulate β-glucan for two weeks had a decreased accumulation of CD14⁻ HLA-DR⁻CD11b⁺CD33⁺ MDSC in their peripheral blood as compared to their frequency in the peripheral blood before treatment, which was correlated with an increased trend in peripheral T cell activation and peripheral monocytes and MDSC Ag presentation function, and a decreased trend in Arginase 1 mRNA expression in peripheral PMN. Overall, these findings provide a further step in our understanding of the mechanisms of how particulate β-glucan enhances anti-tumor immunity in mice and how that findings can be translated in the benefit of cancer patients.

1. Implications of β-glucan treatment on immune cell responses

1. β-glucan nomenclature

β-glucans are polymers of D-glucose linked by β-glycosydic bonds. The nomenclature of glucans varies according to the number or type of carbons that participates in the glycosidic linkage between each two glucose monomers. In the cyclic glucose structure (Figure 1A), the term ‘beta’ is a relative stereodescriptor in Fischer projection used to differentiate it
from ‘alpha’ configuration. In the beta configuration the hydroxyl group (-OH) at the anomeric carbon 1 (C-1) is at a different side relative to the –OH group at C-5, whereas in α-glucose the –OH groups of both carbons are at the same side (51). Glucans are also described by the type of glycosidic linkages and branching in the glucose polymer (52). The linkage designation of the homopolysaccharide refers to the carbons number in the D-glucose that participates in the glycosidic bond, for example, in (1→3)-β-D-glucan the C-1 is linked to C-3 by a beta glycosidic bond (Figure 1B) (51).

2. **β-glucan sources, composition, and antitumor activity**

β-glucans are widely described in nature. A large variety of β-glucans have been reported in the cell walls of different species of mushrooms, yeast, oat, barley, seaweeds, algae and bacteria (53-60). The polymer chains of β-glucans of different sources vary in length, glycosidic linkage configuration, the number and type of branching, the 3-dimensional conformation, and the solubility in water and alkali solutions. Thus, variation in the chemical composition and physical properties of β-glucans render diverse roles, either structural or biological, in such organisms (54). In higher plants, for instance, cellulose fibrils consists of (1,4)-β-glucan chains, whereas linear chains of (1,3)-β-glucan with (1,6)-β-linked branches are the main constituents of callose, which is a polysaccharide
Figure 1. Representation of chemical structures of β-D-glucose (A) and (B) (1,3)-β-linked backbone with β-(1,6) branching.
produced in response to wounding or the synthesis of plant cell plates (59). Interestingly, cellulose synthesis was also reported in prokaryotes such as Agrobacterium xylinum, Agrobacterium tumefaciens, Pseudomonas, Achromobacter, Alcaligenes, Aerobacter, Azotobacter, Rhizobium and Sarcina (61). Similarly, (1,3)-β-glucans provide structural support to the baker’s yeast Saccharomyces cerevisiae by covalently or non-covalently binding to the chitin in the yeast cell wall (62). β-Glucans also give a survival advantage for virulent pathogens, facilitating the formation of cell aggregates and cell adhesion for infection or symbiosis, and providing chemical, biological and mechanical protection (61). Osmoregulated periplasmic glucans (OPGs), such as cyclic (1,2)-β-glucan (CβG), are virulence factors found in the cell walls of gram-negative bacteria (63). Arellano-Reynoso et al, showed that in brucella bacteria, mutants deficient in CβG failed to replicate since they were not able to prevent phagosome-lysosome fusion in host cells, and this function was restored by treating mutants with CβG (63). In the fungal pathogen, Candida albicans, (1,3)-β-glucans with (1,6)-β-branching were shown to have putative roles in biofilm resistance (64). In this study, Nett et al., demonstrated that treatment of C. albicans biofilm with (1,3)-β-glucanase rendered a lower biofilm viability in a dose-dependent manner.

Among a wide variety of polysaccharides, β-glucans have gained a particular attention in biomedical research, due to their wide occurrence in microbes and induction of biological activity in different animal models. Advances in genetic engineering and chemical industry have been applied in the extraction and manufacture of different types of glucans with altered physical properties (65). Early studies reported anti-tumor activity of β-glucans in animal models. Fukuoka group in Japan demonstrated that lentinan extracted from the mushroom Lentinus edodes, a popular mushroom in Japan, have a strong anti-tumor
activity against sarcoma 180 ascites injected subcutaneously in mice (66, 67). In this study, two fractions of lentinan, LC-1 and LC-33, displayed anti-tumor activity when injected intra-peritoneally 24 hours after tumor implantation in mice. The LC-1 fraction was soluble in water and consisted mainly of (1,3) and (1,6)-β-glucan. Whereas LC-33, a linear (1,3)-β-glucan, was less soluble in water and had less anti-tumor activity. Following this study, Fukuoka group reported that chemical modification of the glucan (Pachyman) extracted from *Poria cocos* impacted its anti-tumor properties (67). The linear (1,3)-β-glucan, Pachyman, per se had no anti-tumor activity unless chemically converted, by oxidation-reduction reactions, to a lentinan-like linear (1,3)-β-glucan, with a strong anti-tumor activity in Swiss albino mice implanted with sarcoma 180 ascites. This chemical manipulation of the structure of naturally extracted glucans, and the conversion of non-active compounds to compounds with anti-tumor activity, emphasized the tight link between structure of glucan and its biological functions.

In an attempt to study the anti-tumor activity of a polysaccharide with only one type of linkage, and the effect of the length of the polymer in the tumor activity, Sasaki et al. investigated the anti-tumor activity of a thermally-gelable (1,3)-β-D-glucan on the growth of different tumor cell lines implanted in a Swiss albino model. This gelable glucan, is a curdlan-type extracted from a soil bacterium mutant, Alcaligenes fecalis var. myxogenes (IFO 13140) (68, 69). This glucan is entirely composed of (1,3)-β-linkages, and was administered at different doses, molecular weights and at different routes to optimize the treatment conditions. Interestingly, administration of (1,3)-β-glucan intraperitoneally with a degree of polymerization (DPₙ) >50 at optimal doses showed anti-tumor effects against sarcoma 180 cell line. The fact that the molecular weight of glucan directly impacted its
anti-tumor activity re-emphasizes the effect of glucan supermolecular structure on its anti-tumor activity. Sasaki et al., also assessed the anti-tumor activity of this glucan in Ehrlich carcinoma, NTF reticulum cell sarcoma and CCM adenocarcinoma. Although the glucan treatment was less effective, the differences were still significant. A highly branched (1,3)-β-glucan, SSG, extracted from the fungus *Sclerotinia sclerotiorum* also showed anti-tumor activity on the solid-form of Sarcoma 180, but not ascites, in mice (70).

The application of glucans in cancer research, led to the development of new strategies in bioengineering, to design new glucans with more potent applications in biomedical research. A well-known example, is the development of whole-glucan particles (WGP) by Jamas et al, at the Massachusetts Institute of Technology (65). WGP are microparticles (around 2-6 microns) highly purified from the yeast *Saccharomyces cerevisiae*. The method of purification consists mainly of dissolving cell-wall mannans and proteins in an alkali solution at optimal time and temperature, while keeping the cell wall intact with alkali insoluble glucan. The resulting glucan consists mainly of β-(1,3)-linkages with β-(1,6) branches. In this method, these linkages are also chemically altered by acetic acid and laminarase modifying β-(1,3) and β-(1,6)- linkages, respectively. WGP in biomedical research is being applied to study its anti-tumor effects in animal models (71) and as a drug-delivery vehicle and adjuvant (72). WGP has shown to induce the activation of costimulatory molecules in macrophages such as CD80, CD86, MHC class II, CD40 and CD69, the production of proinflammatory cytokines such as IL-6, TNF-α and IL-12, and the chemokine MCP-1 (monocyte-chemotactic protein 1) in a MyD88 and Syk-dependent signaling (73).
WGP application in the clinic has been restricted due its insolubility in water. Therefore, different attempts have been made to solubilize (1,3)-β-glucan. Jamas et al., at Alpha-Beta Technology Inc., also developed new methods to produce water-soluble glucans (74). Water-soluble glucans are referred to as PGG-glucans (poly-(1-6)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranose). Betafectin PGG (Alpha-Beta Technology) is prepared from WGP by sequential treatments with acid and alkaline treatments followed by a series of filtration and ultracentrifugation steps to obtain a non-toxic pharmaceutically applicable, water-soluble compound at neutral pH. The same group also described other methods for the preparation of triple-helical neutral soluble β-glucans (NSG) also derived from WGP (75). Another extract from *S. cerevisiae* vastly described in the literature is Zymosan. This extract is a crude insoluble yeast extract obtained by boiling yeast before and after trypsin treatment, composed of polysaccharides, proteins, lipids and inorganic elements (75, 76). Riggi and Di Luzzio demonstrated that glucan is the biologically active component of Zymosan (76). These initial findings have driven β-glucan research to elucidate the mechanisms by which these biological response modifiers (BRM) act in tumor-bearing animal models to develop new therapeutic strategies in the clinic.

3. **β-glucans as potent immunomodulators**

The fact that β-glucans do not have any direct cytotoxic effects on tumor cells (69) led to the hypothesis that anti-tumor effects of β-glucans are mediated by host cells. The link between β-glucans and the immune system started to be elucidated from early studies using zymosan. Initial studies demonstrated that zymosan induces immune response after administration in mice, such as the increase in the phagocytic activity of macrophages (77),
and activation of the alternative complement pathway by forming a complex with the serum protein Properdin mediating C3 cleavage (78-85). It was not until Riggi and DiLuzio demonstrated that (1,3)-β-glucan is the immunostimulatory agent of zymosan (76), that implication of glucan effects in immune responses started to be investigated. In this study, the glucan preparations from zymosan, but not lipids or mannans, stimulated an increase in the weight of lungs and spleens of treated rats, and enhanced the phagocytic activity of macrophages of the Aschoff’s ‘Reticuloendothelial elements’ in liver and spleen. Yeast hydroglucan was also demonstrated by Diller et al., to cause an increase in the production of phagocytic cells, subsequently inducing significant regression of Sarcoma 37, Sarcoma 80 and Krebs-2 carcinoma when injected intravenously in ICR/Albino mice (86).

Macrophages were the first effector cells to be described in glucan enhanced oncolytic activity. Sherwood et al., demonstrated that glucan enhances the tumoricidal activity of peritoneal macrophages, splenic macrophages and Kupffer cells (87). In this study Sherwood et al., failed to express any direct effects of glucan on NK cells and T cells. However other studies showed that the tumoricidal activity of polymorphonuclear cells (PMN) (88) and NK cells(89) are enhanced by β-glucan. Suzuki et al., proposed that the anti-tumor activity of lentinan, a (1,3)-β-glucan with (1,6)-β-branching, is mediated by CD8+ cytotoxic T lymphocytes (CTL), but not CD4+ T cells or NK1.1 cells (90).

Investigating the effects of β-glucan on DC biology was relatively late compared to macrophages, due to its relatively late discovery by Steinmann in 1973 (91). A series of studies have been conducted to identify the receptors of β-glucans on innate immune cells. Four β-glucan receptors have been identified; Complement Receptor 3 (CR3) also known
as (CD11b/CD18, αMβ2-integrin, Mac-1) (92), Lactosylceramide (LacCer) (93), selected scavenger receptors (SRs) (94) and dectin-1 (95).

4. β-glucan recognition and signaling

a) Complement Receptor 3 (CR3)

Human leukocyte complement receptor type three (CR3) was the first receptor in innate immune cells to be described in β-glucan recognition. Ross et al., showed that unopsonized zymosan directly binds to CR3 in human and mouse macrophages and neutrophils enhancing their phagocytic activity and respiratory burst (96, 97). Two functional domains have been characterized on CR3: The inserted domain (I-domain) containing the opsonic iC3b-binding site, and a cation-independent lectin domain at the C-terminal to the I-domain that binds to β-glucan (98, 99). Simultaneous activation of the lectin site and I-domain is required to mediate CR3-dependent cellular cytotoxicity (CR3-DCC) against iC3b-opsonized target cells (92). Vetvicka et al. demonstrated that priming of CR3 on neutrophils or NK-cells with soluble β-glucan induces a magnesium- and protein tyrosine kinase-dependent conformational change of the I-domain that exposes the CBRM1/5 activation epitope required for mediating phagocytosis and cytotoxic killing of iC3b-coated tumor cells in a way that mimics phagocytosis and killing of iC3b-opsonized yeast (92). The presence of the lectin domain in CR3, acquired this integrin the ability to signal ‘outside-in’ in addition to its ‘inside-out signaling’. This finding was further applied to induce CR3-DCC of iC3b-coated tumor cells through the administration of soluble or particulate (1,3)- β-glucans as adjuvant therapy in combination with anti-tumor monoclonal antibodies (92, 100-103).
Soluble barley and particulate (1,3)-β-glucan (WGP) administered orally were shown to be phagocytosed by macrophages in the gastrointestinal tract and transported to the spleen, lymph nodes and bone marrow of tumor-bearing animals. In the bone marrow, (1,3)-β-glucan undergoes further processing and degradation to smaller β-glucan fragments that are released to bind the CR3 of granulocytes (104). Consistent with these findings, Li et al. demonstrated that intravenously administered soluble β-glucan PGG is captured by macrophages independent of CR3, which process and degrade PGG, releasing biologically active soluble moieties of ~25kDa that subsequently prime CR3 on neutrophils, activate Syk and phosphoinositol 3 kinase (PI3K) and mediate killing of iC3b opsonized tumor cells (105). In this therapeutic strategy, treating tumor-bearing mice with anti-tumor mAb activates complement enhancing the opsonization of tumor cells with iC3b. Blockade of the membrane complement regulatory protein CD55, results in an increase in the production of the chemotactic factor C5a and subsequent neutrophil infiltration in the tumor, increasing the efficacy of anti-tumor mAb treatment combined with β-glucan administration in tumor-bearing mice (106). Amplification of C5a-mediated neutrophil recruitment was also shown to be dependent on the presence of leukotriene B4 (107).

b) **Lactosylceramide (LacCer)**

In an attempt to explore the mechanisms of inflammation induced by the fungal pathogen Pneumocystis, a causative agent of pneumonia in humans, Limper group conducted a series of studies to investigate the role of β-glucan isolated from *Pneumocystis* fungal species (PCBG) (108-112). In human DC, PCBG stimulated the production of IL-23, IL-6 and IL-1β (108). Human DC treated with PCBG also induced CD4+ T cells to secrete IL-17 and
IL-22, which are known to stimulate Th17 responses. In this study, Carmona et al. showed that activation of human DCs by PCBG involved the Dectin-1 receptor leading to Syk activation, Erk phosphorylation and the stimulation of the NF-kB canonical and non-canonical pathways. Interestingly, the secretion of IL-23 and the DNA binding of NF-kB p65 subunit, following PCBG stimulation, also depended on the formation of glycosphingolipid rich rafts (micro-domains) containing lactosylceramide in the DC plasma membrane, implying that β-glucan might induce the mobilization and concentration of lactosylceramide in the DC plasma membrane, leading to the initiation of the subsequent signaling events. The formation of glycosphingolipids microdomains was also shown to be important in alveolar epithelial cells (AECs) challenged with PCBG. Evans et al., demonstrated that glycosphingolipids microdomains are essential for the internalization of fluorescent-labeled PCBG, the localization of protein kinase C (PKC) to AEC microdomains (112), and the production of the macrophage inflammatory protein (MIP-2) and TNF-α (111). Since AEC lack the expression of dectin-1 receptor, these studies highlight the role of lactosylceramide as an alternate β-glucan receptor (108, 109, 111, 112). Lactosylceramide was also shown to be required for the secretion of the neutrophil chemoattractant protein IL-8, mediated by a calcium-dependent MAPK signaling, in a human airway epithelial cell line (1HAEo−) stimulated with PCBG (109).

Limper group, also showed that human DC stimulated with β-glucans, isolated from Pneumocystis species or the yeast S. cerevisiae, upregulated the expression of the costimulatory molecules CD40, CD80 and CD86, the lymph node homing chemokine receptor CCR7, and the expression of Fas and the anti-apoptotic factor FLIP (110). Interestingly, Fas-FasL interaction seems to be important for the induction of Th1
responses mediated by DC treated with β-glucan. Carmona et al., showed that abrogation of Fas-FasL by a FasL inhibitor impaired the production of IL-1β and TNF-α (but not IL-12) by DC, and subsequently IFN-γ production by co-cultured T cells (110). More studies, however, need to be conducted to further identify the mechanisms of glycosphingolipid signaling following β-glucan challenge in DC.

c) Selected scavenger receptors (SRs)

Scavenger receptors (SR) were first identified on macrophages to mediate uptake and degradation of acetylated low density lipoprotein (AcLDL) leading to the deposition of cholesterol (113). This receptor recognized maleylated LDL, maleylated albumin, and the sulfated polysaccharides fucoidin and dextran sulfate (113). Later studies identified carboxymethy-β-1,3-glucan (CMG) and zymosan binding affinity to SRs. Vereschagin et al. (114) showed that the uptake of AcLDL by rat macrophages decreased after co-injection with CMG. Similarly, Dushkin et al demonstrated that SRs were induced after repeated injections of CMG in rats, and that CMG competed with AcLDL for SRs (115). In addition, the uptake of AcLDL by resident peritoneal macrophages in mice decreased by zymosan (116). A biochemical study performed by Rice et al. identified SR as pattern recognition receptors for (1,3)-β-glucans in human U937 monocytes (94). Further elucidating the mechanisms of SR binding to yeast, Wang et al. demonstrated that SR-AI and SR-AII on RAW264.7 macrophages mediate the binding to S. cerevisiae and C. albicans and that transfection of CHO cells with SR-AI or SR-AII induces the internalization of S. cerevisiae compared to non-transfected cells (117). Furthermore, a new scavenger receptor with a collectin-collagen-like domain, called collectin placenta 1 (CL-P1), was identified on
endothelial cells, but not monocyte-macrophage cells, that binds to *S. cerevisiae* (118). Interestingly, the activation state of J774 macrophage cell line impacted the mechanism of yeast recognition by SRs (119).

In macrophages pre-activated with the TLR9 ligand, CpG-ODN, the scavenger receptor SR-A mediates the uptake of zymosan, but not *C. albicans*. In addition, the scavenger receptor MARCO is involved in the uptake of zymosan and *C. albicans* in CpG-ODN treated macrophages (119). However, in macrophages not treated with TLR9 ligand, the uptake of zymosan seems to be mainly mediated by other β-glucan receptors.

d) *Dectin-1*

The anti-tumor effects of β-glucans through the activation of DCs have been only highlighted after the discovery of dectin-1 receptor in DCs. Dectin-1, or DC-associated C-type lectin, is a C-type lectin first described in the mouse DC line XS52 by Ariizumi et al. (95) as a type II membrane-integrated polypeptide with a single carbohydrate recognition domain (CRD) at the COOH-terminal end and with a cytoplasmic domain consisting of a tyrosine-based activation motif (ITAM), or hemITAM in dectin-1 due to the presence of a single tyrosine motif YXXXL (x is an amino acid) instead of the two motifs described in other PRRs (95, 120). The generation of a novel dectin-1 mAb (2A11) allowed the detection of dectin-1 on DC, macrophages, neutrophils and in a subset of T cells (121, 122). Furthermore, immunohistochemical identification of dectin-1 showed its expression on neutrophils, macrophages in the splenic red and white pulp, alveolar macrophages, Kupffer cells, macrophages and DC in the lamina propria of the gut (123). Notably, the extracellular C-type lectin-like fold of dectin-1 was independent on metal ions for zymosan binding,
unlike classic Ca2+- dependent C-type lectins (120). Actin-dependent phagocytosis of zymosan by RAW.2647 macrophages was also shown to be mediated by dectin-1 recognition (120). In an attempt to identify additional dectin-1 ligands, Palma et al. (124) applied the neoglycolipid-based oligosaccharide microarray technology, to screen for possible polysaccharide ligands for dectin-1. It was demonstrated, by this “designer” microarray, that dectin-1 exclusively binds to glucose polymers with (1,3)-linkages.

Brown et al. initially identified the role of dectin-1 as a major β-glucan receptor on macrophages (122). In this study, treating macrophages with specific dectin-1 mAb, but not CR3 mAb (5C6), abrogated the recognition of unopsonized FITC-labeled zymosan particles by thyoglycollate (Tg)-elicited peritoneal macrophages. In addition, soluble β-glucan and particulate β-glucan inhibited the uptake of labeled zymosan particles by macrophages and the uptake of non-opsonized zymosan particles was impaired in dectin-1 knockout macrophages (125). Dectin-1-deficient macrophages also showed an impaired respiratory burst in response to non-opsonized zymosan, which was restored when stimulating macrophages with complement-opsonized zymosan (125).

Characterization of the human homologue of dectin-1 (Gene Bank accession number AY009090) identified two major isoforms (A and B), and 6 minor isoforms (C-H) that originate from alternative splicing (120, 126). Interestingly, human dectin-1 isoforms have different affinities for β-glucan binding, with the two major splice variants (A and B) of human dectin-1 being functional in β-glucan binding (126). Similar to murine dectin-1 human dectin-1 binds to unopsonized zymosan particles, S. cerevisiae and C. albicans in a β-glucan-dependent manner (126). Human dectin-1 was also shown to be widely
distributed in different cell types, including monocytes, monocyte-derived DC, DC derived from peripheral blood or DC differentiated from CD34+ progenitors in the peripheral blood, neutrophils and eosinophils (127, 128), monocyte/macrophage (U937) cell line, HEK293T fibroblast cell line, B-cell (Daudi and Raji) and T-cell (CEM, Molt-4, Hut 78, and Jurkat) lines (126). With human dectin-1 B isoform being significantly expressed on immature DC and mature monocyte-derived macrophages (128).

In addition to its role as a pattern recognition receptor (PRR), human and murine dectin-1 was also shown to directly interact with T cells stimulating their effector functions (95, 122, 126, 128). A recombinant mouse dectin-1 with a soluble extracellular domain induced T cell proliferation, and β-glucan was not able to inhibit the binding of T cells to NIH3T3 cells expressing dectin-1, suggesting the presence of a T cell binding site on dectin-1, apart from the lectin-site (95, 120, 126). Moreover, HeLa cells transfected with human dectin-1b cDNA induced the proliferation and IFN-gamma production of CD4+ and CD8+ T-lymphocytes (127). Human Dectin-1 in immature monocyte-derived DC also mediates the uptake of apoptotic cells and CMV-infected human foreskin fibroblasts (HFF) and subsequent cross-presentation of CMV peptides loaded on MHC-I molecules to antigen-specific CD8+ T lymphocytes (129).

Elucidating the mechanisms of β-glucan activation from different sources has been a main goal to understand the immune evasion strategies evolved in fungal pathogens, and the immunomodulatory effects of β-glucan in cancer models (Table 1). Activation of DC by β-glucan was also shown to be a potent strategy to induce tumor regression in tumor-bearing animals (49, 130). It was reported by Li et al. and Qi et al. that orally administered
particulate β-glucan WGP is effective in inducing tumor regression in mice bearing LLC (Lewis Lung Carcinoma) or RAM-S-MUC1 lymphoma (49, 130). Interestingly, in orally fed mice, WGP trafficked to the spleens and lymph nodes and activated DC in vivo. WGP was also shown to enhance the ability of DC to capture tumor cells and induced the expression of CD80, CD86 and MHC class II, and promote T_{H1} responses by stimulating

<table>
<thead>
<tr>
<th>Glucan</th>
<th>Composition</th>
<th>Cytokines</th>
<th>T cell activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan</td>
<td>Polysaccharides (including (1,3)-β, proteins, lipids and inorganic elements)</td>
<td>TNF-α; IL-2; IL-12; IL-6; IL-10; GM-CSF; IL-23</td>
<td>Th1; Th17; (Tregs?)</td>
</tr>
<tr>
<td>Curdlan</td>
<td>(1,3)-β-glucan</td>
<td>IL-12p40, IL-1β, IL-12p70, IL-6, IL-10; IL-23</td>
<td>Th1; Th17</td>
</tr>
<tr>
<td>WGP</td>
<td>(1,3)-β-glucan with (1,6)-β-branches</td>
<td>IL-12; TNF-α</td>
<td>Th1</td>
</tr>
</tbody>
</table>

Table 1. The main cytokines produced and T cells activated upon bone marrow-derived dendritic cells (BMDCs) stimulation with zymosan, curdlan and WGP.

the production of IL-6, IL-12 and TNF-α, depicted by the stimulation of a higher percentage of IFN-gamma producing CD8+ T cells and the proliferation of antigen-specific CD4+ and CD8+ T cell (49, 130).

The mechanisms of activation of β-glucan in tumor-bearing mice differ depending on the type of physical properties and structure of the β-glucans. For example, WGP activation of DC was shown to be dependent on dectin-1 receptor, whereas yeast-derived soluble β-glucan binding to DC or macrophages was independent on dectin-1 (49). Goodridge et al.
further demonstrated that despite the ability of dectin-1 to bind soluble and particulate β-glucans, signaling events downstream dectin-1 are only triggered by particulate β-glucans (48). This discrepancy in signaling was attributed to the ability of particulate-β-glucan to trigger the formation of ‘phagocytic synapse’ that leads to the exclusion of the tyrosine phosphatases CD45 and CD148 upon engagement of dectin-1 receptor on DC and macrophages with particulate β-glucans on the microbe (48).

Signaling events downstream dectin-1 activation were initially reported in bone marrow-derived macrophages and peritoneal macrophages (131). Underhill et al. reported that dectin-1 signals through an ITAM-like, and activation of dectin-1 by zymosan leads to the phosphorylation of spleen tyrosine kinase (Syk) dependent on the presence of Src family kinases, and subsequently enhancing the production of reactive oxygen species (ROS). Phagocytosis of zymosan was independent on Syk activation, or CD11b and FcRγ chain. Interestingly, the response of macrophages to zymosan stimulation was heterogenous with only a subpopulation activating Syk phosphorylation and ROS production. Rosas et al. (132) demonstrated that macrophages irresponsive to β-glucan particles, such as curdlan, can be reprogrammed by priming with GM-CSF to produce a cytokine profile similar to BMDC. Syk activation was also enhanced by cytokines such as IFN-gamma and IL-4 (131). GM-CSF production in human-derived monocytic DC seems also to be enhanced after zymosan treatment, increasing the levels of IL-23 secretion by DC (133). In bone marrow-derived DC (BMDC) upon dectin-1 stimulation (Figure 2), Gross et al. (134) reported the activation of the caspase recruitment domain protein 9 (CARD9) downstream Syk. CARD9 functions as an adaptor protein that mediates the formation of Bcl10-Malt 1 complex activating NF-kB pathway. BMDC deficient in CARD9 or BCl10-Malt-1 showed
impaired production of the cytokines TNF-α, IL-6, IL-12, IL-2 and IL-10 in response to zymosan compared to wild-type BMDC. In contrast, Taylor et al. reported an impaired production of IL-10 or IL-12 by dectin-1 knockout macrophages but not BMDC in response to zymosan (125).

Dectin-1-Syk-CARD 9 signaling in response to curdlan stimulates the maturation of BMDC through the upregulation of CD80, CD86 and CD40, and the increase in the secretion of proinflammatory cytokines such as IL-2, IL-10, IL-6, tumor necrosis factor (TNF), and IL-23 in preference to IL-12 (135, 136). DC stimulated with curdlan induces the differentiation of IL-17 producing CD4+ T-cells in vitro, and promotes the differentiation of Th17 and IFN-gamma producing CD4+ T cells in vivo (135) coupling innate immunity to adaptive immunity. Similarly, C. albicans in the hyphae form stimulates the differentiation of IL-17-producing CD4+ T cells, whereas the yeast form promotes the differentiation of IFN-gamma producing CD4+ T cells (137). In agreement with these findings, dectin-1 activation by the fungal pathogens, Histoplasma capsulatum, Aspergillus and Rhizopus triggers the production of IL-23 by human DC stimulating TH17 responses (138).

A mechanism of negative regulation of Dectin-1-induced activation of DC seems to be mediated by the peroxisome proliferator-activated receptor-gamma (PPAR-γ) ligand troglitazone (TGZ) through the manipulation of CARD9 signaling (139). TGZ inhibits the expression of CARD9 impairing the localization of NF-kB to the nucleus (139). In addition, PPAR-γ activation decreases antigen presentation in DC, impairs cytokine production and DC-mediated T-cell proliferation. Hernanz-Falcon et al., demonstrated that
attenuation of dectin-1 signaling can also be achieved by dectin-1 internalization through phagocytosis (140).

The production of IL-2, IL-10, IL-12p70 and cyclooxygenase-2 (Cox-2) by zymosan-stimulated DC and macrophages was also shown to be regulated by the protein NFAT (141). NFAT activation is triggered by dectin-1 stimulation, and regulates the induction of the early growth response (Egr) family transcription factors Egr2 and Egr3, whereas IL-6 and TNF-α production was not dependent on NFAT activation (141).

Stimulation of Dectin-1 in BMDC with β-glucans such as curdlan or zymosan, induces the activation of mitogen activated protein kinases (MAPK) and NF-kB (134, 135, 142, 143). For example, stimulation of BMDC with curdlan promotes the activation of p38, Erk1/2, Jnk and NF-kB in a Syk-dependent or independent fashion (135, 139). Dectin-1 stimulation on human DC by curdlan and C. albicans, induces the activation of the canonical NF-kB pathway through p65 and c-Rel activation and non-canonical NF-kB pathway through the activation of RelB (143). Dectin-1 activation also induces a Syk-independent Raf-1 kinase mediated pathway. Raf-1 activation converges with Syk-pathway at the NF-kB through the inhibition of Syk-dependent RelB through the formation of RelB-p65 dimers, and promotes p65 acetylation and c-Rel activation. The regulation of NF-kB downstream dectin-1 signaling couples innate to adaptive immune responses activating different cytokine profiles that differentially regulates adaptive immune responses (143). Raf-1 activation induces the expression of IL-12p70, a T_H1 polarizing cytokine, and the production of IL-1β, IL-6 and IL-23 that mediate T_H17 differentiation.
Various studies reported the cooperation or cross-talk between dectin-1 and other PRRs on DC, macrophages and neutrophils. Cross-talk between TLR2 and dectin-1 pathways was reported in DC and macrophages stimulated with zymosan (144, 145). In this model, zymosan particles bind simultaneously to dectin-1 and TLR2/CD14. Dectin-1 activation induces zymosan phagocytosis, the production of ROS, and along with TLR2, the production of proinflammatory cytokines such as IL-12 and TNF-α through the activation of NF-κB. Since zymosan does not consist entirely of β-glucan, the TLR2 ligand on zymosan is yet to be identified. In contrast, Dillon et al., reported the induction of regulatory DC by zymosan. In this study, zymozan activation of dectin-1 and TLR2 in DC and macrophages stimulated the production of ERK-dependent IL-10 and biologically-active TGF-β, respectively (145). The production of IL-10 and TGF-β impaired the production of IL-6, IL-12p70 and TNF-α, favoring the induction of CD4+ regulatory T cells (Tregs). Interestingly, Osorio et al. reported the generation of T regulatory cells with a hybrid phenotype, co-expressing Foxp3 and ROR-γt producing IL-17 upon coculture of Foxp3+ Tregs with DC treated with Curdlan (146). However, treating D2SC/1 dendritic cell line with WGP, not a stimulator of TLR signaling (48), was shown to upregulate the expression of membrane glucocorticoid-induced tumor necrosis factor receptor ligand (mGITRL) decreasing the suppressive activity of Tregs and promoting the proliferation of CD4+ effector T cells (147). Discrepancies in these results with other groups might be due to the use of different cell-lines, mice strains, types of glucans applied, and purify of different glucan preparations (145).

Dectin-1 receptor was also shown to cooperate with the mannan receptor SIGNR1 (dendritic cell-specific ICAM-3 grabbing nonintegrin homolog related-1) in the
nonopsonic internalization of zymosan in RAW264.7 macrophages (148). Kankkunen et al. demonstrated that β-glucan induces the production and secretion of IL-1β through the activation of NLRP3 inflammasome in macrophages (149). Interestingly, in a recent report, Li et al. demonstrated that stimulation of neutrophils by *C. albicans* induce the activation of dectin-1 which subsequently activates CR3 integrin (Mac-1, CD11b/CD18) (150). Binding of dectin-1 to *C. albicans* induces ITAM phosphorylation by Src kinases and recruitment and phosphorylation of Syk, leading to the activation of the Vav/PLCγ complex and Ca^{2+} activating CR3 that subsequently binds to the yeast. Activation of CR3 initiates a series of signaling events leading to the activation of ERK, PAK and Pyk2 through activation of Vav/PLCγ complex initiated by dectin-1 activation. Activation of such targets stimulates cytoskeleton rearrangement required for phagocytosis and the production of ROS in activated neutrophils (150).

Future studies will further delineate the signaling events downstream dectin-1 activation by PAMPs, and its cooperation with other PRRs on signal transduction in immune cells.
Figure 2. A schematic model summarizing the main signaling pathways in bone-marrow derived dendritic cells (BMDCs) upon stimulation of dectin-1 with WGP, Zymosan and Curdlan.
II. Myeloid-derived Suppressor Cells (MDSC)

1. MDSC origin

During differentiation hematopoietic stem cells (HSCs) diverge at a ‘decision-making point’ to common lymphoid progenitors (CLPs) to generate NK cells, T cells or B cells or common myeloid progenitors (CMPs) to generate monocytes, granulocytes, macrophages, dendritic cells (DC), megakaryocytes and erythrocytes in the presence of appropriate factors (151). Since MDSC are a heterogeneous population of monocytic or granulocytic cells with a suppressive property, their origin and factors determining their fate, phenotype and function in cancer is controversial and need to be further elucidated. A common monocyte progenitor (cMoP) from monocyte-macrophage DC progenitor (MDP) that gives rise to monocytes or monocyte-derived macrophages was recently identified (152). Under inflammatory conditions, cMoP differentiate into monocytes which can further differentiate into tissue-resident macrophages (152), consistent with the finding that MDSC in the tumor microenvironment differentiate toward tumor-associated macrophages (TAMs) via HIF-α induced by tumor hypoxic conditions (39). MDSC can be induced from bone marrow (BM) precursors in the presence of the tumor-derived factors granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-6 (153). Inhibition of paired immunoglobulin-like receptor-B (PIR-B) or C/EBPβ promotes MDSC differentiation to macrophages, granulocytes and DC with no suppressive function, suggesting that the suppressive function of MDSC induced by tumor factors is reversible (153, 154).

The origin and fate of Ly6C^{high} and Ly6C^{low} monocytes are subject to further exploration, especially that Ly6C^{high} monocytes with suppressive properties identified as M-MDSC
have been widely studied in inflammation and cancer. It is still controversial whether Ly6C<sup>high</sup> monocytes differentiate to Ly6C<sup>low</sup> monocytes or whether both monocyte subsets arise from independent precursors (155-159). In EL4 mouse thymoma-bearing mice, splenic Ly6C<sup>high</sup> (Gr<sub>1</sub><sup>int</sup> CD11b<sup>+</sup>) M-MDSC transferred to tumor-bearing mice are able to give rise to all CD11b<sup>+</sup> subsets including Gr-1<sup>high</sup> granulocytes (160). Similarly, Youn et al. recently demonstrated that M-MDSC (CD11b<sup>+</sup>Ly6C<sup>high</sup>) in tumor-bearing mice could differentiate into granulocytic MDSC expressing CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>-</sup> (40). This differentiation was mediated by epigenetic silencing of the retinoblastoma gene in M-MDSC mediated by histone deacetylase 2 (HDAC-2). These findings highlight M-MDSC plasticity and its potential to give rise to PMN-MDSC. It is still debatable whether PMN-MDSC consist solely of immature myeloid cells with suppressive function or whether mature neutrophils can acquire suppressive properties in the tumor milieu. A study by Fridlender et al identifies TGF-β as the inducer of suppressive neutrophils in the tumor (N<sub>2</sub>), since blockade of TGF-β enhances the recruitment and activation of neutrophils with anti-tumor functions (N<sub>1</sub>), suggesting that neutrophils can be polarized in tumor-bearing animals or potentially in cancer patients toward a suppressive phenotype (161). Transcriptomic analysis revealed differences between tumor-associated neutrophils (TANs), splenic PMN-MDSC, and neutrophils from naïve mice (162). Compared to neutrophils from naïve mice, TANs display lower expression of cell-cytotoxicity genes, a higher expression of MHC II complex genes and inflammatory cytokines (e.g. TNF-α, IL-1α/β), and upregulation of chemoattractants of T cells, B cells, neutrophils and macrophages. Interestingly, neutrophils from naïve animals are more closely related to splenic PMN-MDSC than to TANs.
Unlike neutrophils from naïve mice, splenic PMN-MDSC are immune suppressive and express higher levels of M-CFSR, CD244, arginase, MPO and ROS, with a lower phagocytic activity (163). A recent study has reported that tumor-promoting neutrophils are recruited to the tumors by tumor-derived oxysterols in a CXCR2-dependent manner, promoting tumor growth and neoangiogenesis (164). Future studies will further unveil the exact origin of MDSC and its relation to other myeloid subsets in cancer.

2. MDSC mechanisms of suppression

Tumor-infiltrating immune cells with a suppressive phenotype include regulatory T cells (Tregs), γδ T cells (165, 166), suppressive TAMs, and MDSC. The detailed mechanisms of MDSC suppression were recently described elsewhere (33, 38, 167). Herein, we briefly highlight the main suppressive mechanisms employed by MDSC in cancer.

Although MDSC subsets share the ability to suppress T cell activation, their different mechanisms of function, recruitment and expansion in tumor-bearing animals are being constantly highlighted. In general, MDSC expansion is induced by tumor factors such as GM-CSF (168), stem-cell factor (SCF-1)(169), prostaglandin E2 (PGE₂) (170), cyclooxigenase-2 (COX-2) (171), vascular endothelial growth factor (VEGF) (172), macrophage colony-stimulating factor (M-CSF) and IL-6 (173). MDSC suppress T cell activity mainly by modulating L-arginine metabolism through the upregulation of arginase-1 (Arg1) and inducible nitric oxide synthase (iNOS) in M-MDSC, and Arg1 and reactive oxygen species (ROS) upregulation in G-MSDC (38). Arg1 catalyzes the conversion of L-arginine to L-ornithine, while iNOS mediates NO production from L-arginine, ultimately leading to the production of urea (38). L-arginine starvation induces the loss of the CD3ζ
chain (174), and inhibition of T cell-cycle progression by restraining the upregulation of cyclin D3 and cyclin-dependent kinase 4 (cdk4) (175). In addition, NO induces Fas-dependent apoptosis (176) and inhibits proteins downstream IL-2 receptor (177). Moreover, reactions between NO and ROS, such as superoxide ions, produce peroxinitrite that impact CD8+ T cell activity by inducing the nitration of CD8+ T-cell receptor (TCR) restraining its recognition by the MHC peptide (167). MDSC also deprive T cell from cystein, an essential amino acid required for T cell activation (46). T cells do not produce cysteine and depends on APCs for cysteine supply after they uptake cystine. However, MDSC limit cystine pool available for APCs depriving T cells from cysteine (46).

M-MDSC and PMN-MDSC suppressive activities are thought to also diverge in the expression and phosphorylation of signal transducers and activators of transcription (STATs). PMN-MDSC suppressive functions are thought to be mainly mediated by STAT3 phosphorylation, while STAT1 seems to play a main role in M-MDSC suppressive biology (167). Current studies show that STAT3 phosphorylation is a key event in regulating PMN-MDSC suppressive activity and inhibition of differentiation through various mechanisms. STAT3 phosphorylation enhances the production of ROS through the activation of calcium-binding proteins S100A9 and S100A8 (178) which are involved in the formation of NAPDH oxidase complex (Nox-2) (179). In addition, upregulation of Nox-2 subunits, such as p47\textsuperscript{phox} and gp91\textsuperscript{phox}, are directly related to the enhanced production of ROS by PMN-MDSC augmenting its suppressive activity (167). STAT3 not only promotes the suppressive activity and expansion of MDSC, but also induces tumorigenesis by mediating the production of myeloid-derived angiogenic factors such as VEGF (180). STAT3 immunosuppressive functions of MDSC can also be mediated by
Hsp72 from tumor-derived exosomes (181). On the other hand, M-MDSC suppressive activities are mediated by factors that regulate Arg1 and iNOS production, such as STAT1. STAT1 activation is mediated by IFN-γ and IL-1β and is thought to play an important role in M-MDSC suppressive activity due to the fact that blocking IFN-γ or disrupting STAT1 partially reduced M-MDSC suppressive functions (31). However, a recent study demonstrated that expression of IFN-γ and IL-4Rα is not required for T-cell suppression by MDSC, since MDSC from IFN-γ−/−, IFN-γR−/− and IL-4Rα−/− tumor-bearing mice suppress CD8+ and CD4+ T cells (182).

MDSC immunosuppression also extends to a vast network of immune cells including Tregs, macrophages, and NK cells. MDSC induce the recruitment and expansion of Tregs in tumor-bearing mice through the production of IL-10 and TGF-β (183), dependent on CD40-CD40L interaction (45). IL-10 production by MDSC also decreases IL-12 production in macrophages enhancing Th2 responses in tumor-bearers (184). However, a controversial report demonstrated that TGF-β mediated generation of induced Tregs (iTregs) (CD4+CD25+Foxp3+) and proliferation of natural Tregs (nTregs) is impaired by PMN-MDSC (185). NK cell activity is also suppressed by MDSC. MDSC from the liver and spleen of tumor-bearing mice inhibit NK cell cytotoxicity, NKG2D expression and IFN-γ through membrane-bound TGF-β in tumor-bearers (44) or NKp30 receptor expression on NK cells in patients with hepatocellular carcinoma (186). Interestingly, a recent report has demonstrated that NK cells can be converted to MDSC in the presence of GM-CSF (187). However, this finding has to be further confirmed since NK cells share a common lymphoid progenitor with T and B cells but not myeloid cells (151).
3. Therapeutic Targeting of MDSC

It is now being greatly highlighted that exploring immunosuppressive regulation by MDSC in the tumor microenvironment will bring a new paradigm in our understanding of cancer as well as for devising novel immunotherapeutic approaches. In recent years, many approaches have been developed with the goal of abolishing their suppressive activity in vivo as a therapeutic intervention in cancer. In the following, we discuss different therapeutic strategies applied in the modulation of MDSC in tumor-bearing mice and cancer patients including inhibition of MDSC suppressive function, expansion, recruitment, and induction of MDSC differentiation (Figure 3).

A. Inhibition of MDSC suppressive function

1- Inhibitor of Reactive Nitrogen Species (RNS) (AT38)

Murine

RNS produced by MDSC and tumor cells such as peroxynitrite anion induces the nitration of the chemokine CCL2 preventing the migration of CTLs to the tumor core. Targeting of RNS with AT38 ([3-(aminocarbonyl) furoxan-4-yl] methyl salicylate) in mice bearing subcutaneous colon carcinoma expressing GM-CSF (C26GM), or thymoma expressing OVA (EG7-OVA), or spontaneous prostate cancer (TRAMP mice), downregulated Arg1,
Figure 3. The main therapeutic compounds targeting MDSC suppression, expansion, recruitment and differentiation in cancer.
iNOS and peroxinitrite in MDSC, enhanced survival and improved the efficacy of adoptive transferred tumor-specific CTLs (188).

2- Nitroaspirin

*Murine*

NO-donating aspirin (NO aspirin) consists of an aspirin molecule covalently linked to a NO donor group. The effects of different NO-donating aspirins (NCX4060, NCX4016) on MDSC suppressive activity in cancer were studied by De Santo et al (189). In BALB/c mice inoculated with a colon adenocarcinoma expressing GM-CSF (C26-GM), treatment with NCX4060 or NCX4016 restored T lymphocyte proliferation in MLR reactions or T cells induced with anti-CD3 and anti-CD28 in the presence of MDSC, increased CTL activity and reduced MDSC Arg1 and NOS activity, *in vitro* and *in vivo*. Despite these effects on MDSC suppressive function, treatment of tumor-bearing mice with NCX4016 orally did not significantly decrease tumor burden or prolonged survival except only when coupled to a recombinant DNA vaccine in two vaccination models:

a) Vaccination of BALB/c mice with a plasmid encoding the full-length *env* gene (pcDNA3-*env*), challenged with colon carcinoma CT26 (gp70 positive) subcutaneously, and orally treated with NXC4016.

b) Immunization of BALB/c mice with a plasmid DNA encoding p186 (extracellular and transmembrane portion of HER-2/neu) prior to challenging with a mammary carcinoma cell line N2C.

3- Phosphodiesterase-5 Inhibitors (Sildenafil)
**Murine**

Inhibition of cGMP phosphodiesterase gene family 5 (PDE-5) induces apoptosis of colon cancer cells through the induction of cGMP protein kinase (PKG) in colon tumor cells (190) or apoptosis of B-cell chronic lymphocytic leukemia (B-CLL) cells in a caspase-dependent manner (191). Treatment of mice with Sildenafil, a PDE-5 inhibitor, delayed the progression of tumors in mice inoculated with different tumor cell lines including colon carcinoma (CT26WT), colon carcinoma expressing GM-CSF (C26GM), mammary adenocarcinoma (TS/A), MCA203 fibrosarcoma (192), and a mouse transgenic melanoma model (193). Interestingly, delayed tumor progression was associated with the modulation of T cell immune suppression by MDSC towards a less suppressive phenotype. Treatment of tumor-bearing mice with Sildenafil given in the drinking water, decreased the expression of IL-4Ra, Arg1, NOS2 in tumor infiltrating CD11b+ cells, increased the efficacy of adoptive T cell transfer therapy (ACT), induced the infiltration of tumor infiltrating lymphocytes (TILs) including CD8+ and CD4+ T lymphocytes (192, 193), partially restored the expression of CD3ζ chain in CD8+ and CD4+ T lymphocytes infiltrating skin melanomas and metastatic lymph nodes, increased IL-2 levels and decreased the levels of IL-1β, VEGF, GM-CSF, IL-6, S100A9, and the chemokines CCL2 (MCP-1) and CCL3 (MIP-1α) in metastatic lymph nodes in melanoma-bearing mice (193). The exact mechanism of linking the accumulation of cGMP in MDSC with L-arginine metabolism is not fully elucidated. Although Sildenafil delayed tumor growth in different mice models, tumor eradication was not achieved suggesting the importance of the combined targeting of a network of immune cells with a suppressive phenotype in the tumor microenvironment.
**Human**

PBMCs from patients with multiple myeloma (MM) or head and neck squamous cell carcinoma (NSCLC) stimulated *in vitro* with plate-coated anti-CD3/anti-CD28 Abs in the presence or absence of Sildenafil or Arg1 inhibitor (NorNOHA) or NOS2 inhibitor (L-NMMA) restored the proliferation of CD3 cells. Interestingly, Sildenafil only restored the proliferation of CD8+ T cells in PBMCs from NSCLC cancer patients. CD4+ T cell proliferation in PBMCs from MM patients was still lower than PBMCs from healthy donors (192). More studies need to be performed to demonstrate the significance of these findings in humans, its toxicity, and its efficacy in the clearance of primary or metastatic tumors.

**4- Triterpenoids (CDDO-Me)**

**Murine**

Treatment of mice-bearing colon carcinoma (MC38), Lewis lung carcinoma (LLC) or EL-4 thymoma with a synthetic triterpenoid, CDDO-Me, abrogated MDSC suppressive function through the downregulation of ROS and inhibition of STAT3, and inhibited tumor growth. In addition, CDDO-Me enhanced the efficacy of survivin vaccine *in vivo*. However, CDDO-Me treatment did not affect Arg1 and NO production or the frequency of MDSC in the spleens of tumor-bearing mice (194).

**Human**

Treatment of patients with locally advanced (stage II-III) or metastatic (stage IV) pancreatic cancer with CDDO-Me and gemcitabine in a Phase I clinical trial had no effects
in the frequencies of MDSC in the peripheral blood with an increased response of T cells from treated patients to tetanus toxoid and phytohemagglutinin. However, the effect of CDDO-Me treatment was not assessed in the absence of gemcitabine (194).

5- Very small size proteoliposomes (VSSP)

_Varine_

VSSP is a nanoparticulated adjuvant that promotes DC maturation and enhances CD8^+^ T cell effector function. Treatment of tumor-bearing mice with VSSP increases the accumulation of MDSC in the spleen. However, MDSC from the spleens of VSSP-treated mice are significantly less suppressive than non-treated mice, correlating with enhanced CTL activity in mice treated with VSSP. In addition, VSSP treatment induces the differentiation of MDSC into mature antigen-presenting cells (APC) (195). More studies need to be performed to evaluate VSSP efficacy in the clinic.

6- Inhibition of exosome formation (Amiloride)

_Varine_

Amiloride inhibits exosome formation. It has been reported that membrane associated Hsp72 from tumor-derived exosomes (TDEs) induce MDSC suppressive activity by inducing the STAT3 phosphorylation in a TLR2/MyD88-dependent manner through autocrine IL-6. Treatment of mice-bearing CT26, TS/A, or EL4 tumors with exosome inhibitors such as amiloride, enhanced anti-tumor immune responses when combined with cyclophosphamide(181). However, treatment of mice with exosome inhibitors alone did not reduce tumor growth.
Human

Treatment of patients with colorectal metastatic carcinoma with amiloride for 3 weeks abrogated the suppressive activity of MDSC from peripheral blood *ex vivo*, and decreased the ability of autologous serum to induce STAT3 phosphorylation in MDSC (181).

B. Inhibition of MDSC expansion

1- Gemcitabine and 5-Fluorouracil

Murine

Pyrimidine analogues, such as Gemcitabine (Gem) and 5-Fluorouracil (5-FU), have been currently used in the clinic to induce tumor cell death and hamper tumor growth. Their cytotoxic effects on MDSC in tumor-bearers have currently been described. Administration of Gem and/or 5-FU selectively induced apoptosis of Gr1+CD11b+ MDSC in the spleens and tumors of tumor-bearing mice, with no significant decrease in the levels of CD4+, CD8+, B cells, NK cells or macrophages and enhanced IFN-γ production by tumor-specific CD8+ T cells and NK cells (196-199). The anti-tumor effects of Gem were enhanced when combined with other therapeutic protocols including chemotherapeutic drugs, such as cyclophosphamide (200) and rozigliazone, (201) that target Tregs and PPARγ respectively, or in combination with adenoviral based immunotherapy (202) or IFN-β treatment (196). Despite promising effects in anti-tumor immunity, the ambivalent effects of chemotherapeutic drugs, including Gem and 5-FU, is yet to be further explored. Gem and 5-FU triggered the production of IL-1β in MDSC following the activation of the inflammasome (NOD-like receptor family, pyrin domain containing-3 protein (Nlrp3)-dependent caspase-1 activation complex) in a cathepsin-B dependent manner. Secreted IL-
1β then stimulated the production of IL-17 by CD4+ T cells inducing the expression of angiogenic factors such as *Eng* and *Pecam1* counteracting anti-tumor immunity (203).

2- Cyclooxygenase (COX-2) and Prostaglandin E2 (PGE2) inhibitors

**Murine**

COX-2 and PGE2 are produced by different human and murine cancer cells including LLC, renal carcinoma, colon carcinoma MCA-38 and head and neck tumors (171). Targeting COX-2 and PGE2 with inhibitors, such as indomethacin (204), celecoxib (205), meloxicam (206) and acetylsalicylic acid (ASA) (207), augmented tumor growth in different cancer models. COX-2 inhibitors reduced systemic levels of PGE2, modulating MDSC suppressive function, recruitment and induction. COX-2 inhibitors inhibited Arg1 expression in MDSC induced by tumor released factors such as COX-2 and PGE2, reduced ROS and NO levels, enhanced T cells anti-tumor responses and improved the efficacy of DC-based immunotherapy in tumor-bearing mice (171, 208).

ASA also regulated the recruitment of MDSC to the tumors by decreasing the levels of MDSC-attracting chemokine CCL2 and increasing the expression of CXCL10 and CTL infiltration in glioma (207). COX-2 is a PGE2–forming enzyme that can also be activated through PGE2 receptor EP2 in MDSC by PGE2 (170, 209). Induction of EP2 in MDSC by PGE2 hampered their differentiation to mature APCs from the bone marrow (209), and induced the generation of MDSC from monocytes in vitro by blocking monocyte differentiation to mature CD1a+ DC and enhancing the expression of indoleamine 2,3-dioxygenase (IDO), IL-4Rα, NOS2 and IL-10 (210).

**Human**
CD14⁺ monocytes acquire MDSC phenotype through coculture with human melanoma cells through a COX-2-dependent mechanism (211). In patients with advanced melanoma, inhibition of PGE₂, COX-2, STAT3 and superoxide in MDSC restored T-cell proliferation in culture.

3- **Sunitinib**

**Murine**

Sunitinib, a tyrosine kinase inhibitor, targets a wide range of kinases including, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptors (VEGFR1-VEGFR3), SCF, m-CSF and FMS-like tyrosine kinase 3 (FLT3) (212-214). Sunitib is currently approved by the Food and Drug Administration (FDA) for the treatment of patients with gastrointestinal stromal tumors (GIST) that failed to respond to Imatinib (212, 215) and as a first-line treatment of patients with metastatic RCC (216, 217). In addition to its angiogenic effects and targeting of tumor cells, sunitinib immune modulation of suppressor cells, such as MDSC and Tregs, has been recently described (218-220). Sunitib treatment decreased the percentages of MDSC and Tregs in the spleen of tumor-bearers, reduced the expression of pro-tumorigenic factors in tumor infiltrating lymphocytes (TILs) such as IL-10, TGF-β, Foxp3, CTLA-4 and PDL-1, skewing suppressed T lymphocytes towards an anti-tumor Th1 phenotype, represented by the increased levels of IFN-γ and CTL responses (218). Sunitib combined with IL-12 and 4-1BB activation prolonged survival of mice bearing MCA26 colon tumor, and increased the anti-tumor efficacy of OVA-loaded DC vaccines in mice bearing subcutaneous B16 melanoma expressing OVA (218, 221). However, sunitinib modulation of tumor MDSC seems to be compromised in tumors expressing high levels of GM-CSF, such as 4T1 or
human RCC cells (222, 223). GM-CSF in the tumor microenvironment reprograms MDSC to act independently on STAT3 through the induction of STAT5 phosphorylation, bypassing the inhibition of STAT3 phosphorylation induced by sunitinib (219, 222, 223). Another mechanism of tumor evasion from sunitinib treatment is the increased production of the chemokine stromal cell-derived factor-1 (SDF-1; CXCL12) in the tumor microenvironment following p53 activation induced by sunitinib, leading to increased levels of MDSC in RCC xenografts (224).

**Human**

The clinical outcome of sunitinib treatment in patients with metastatic RCC is limited to its ability to modulate immune suppression in the tumor rather than in the periphery (222, 223). Despite the encouraging findings that sunitinib treatment decreases the levels of MDSC, Tregs and IFN-γ producing T lymphocytes in PBMCs of RCC patients (225, 226), its effects on intratumoral MDSC and proangiogenic factors such as MMP-9, MMP-8 and IL-8 in tumor explants were minimal (222, 223).

4- **Blockade of stem cell factor (SCF; ckit ligand)**

**Murine**

SCF is expressed by tumor cells, with a higher expression in large tumors. SCF production in the tumor microenvironment enhances the accumulation of MDSC in the tumor. SCF siRNA knockdown in tumor cells, and blocking SCFR-SCF binding with anti-ckit reduces MSDC expansion and infiltration in the BM and tumor, reduced angiogenesis, enhanced T cell proliferative responses and decreased Treg development in mice bearing MCA26 colon carcinoma (169).
5- Amino-biphosphonate (Zoledronate, Pamidronate)

*Murine*

Amino-bisphosphonates is a MMP-9 (metalloproteinase-9) inhibitor. The production of MMP-9 by tumor and stromal cells is directly correlated with VEGF production in the tumor microenvironment. Targeting MMP-9 by amino-biphosphate in mice with spontaneous mammary carcinoma reduced the levels of pro-MMP-9 and VEGF in the serum, tumor-enhanced BM hematopoiesis and improved anti-tumor responses induced by DNA vaccination (227).

6- Doxorubicin-cyclophosphamide chemotherapy

*Human*

MDSC (Lin^low^ CD33^+^CD11b^+^ HLA-DR^−^) percentages and absolute numbers in the peripheral blood of breast cancer patients directly correlate with the stage of the disease; patients in stage IV with extensive metastatic tumor having the highest numbers of circulating MDSC and lowest T cell responses. Doxorubicin and cyclophosphamide are common chemotherapeutic drugs included for the treatment of breast cancer. Interestingly, patients treated with doxorubicin-cyclophosphamide displayed increased percentages of MDSC in the peripheral blood but not when followed with paclitaxel treatment, suggesting that treatment with doxorubicin-cyclophosphamide alone may not be favorable in the development of anti-tumor immune responses in patients with breast cancer (228).

C. Inhibition of MDSC recruitment

1- Vemurafenib
Human

Vemurafenib is a specific inhibitor of BRAF$^{V600E}$ (229), a mutation that causes constitutive activation of the MAP Kinase pathway, a common mutation in melanoma patients (230). Treatment of advanced melanoma patients with Vemurafenib decreased the frequencies of M-MDSC (CD14$^+$ HLA-DR$^{-}$low) and PMN-MDSC (CD66b$^+$Arginase1$^+$CD16$^{-}$low) in PBMCs (229). The clinical course melanoma was correlated with an increase in the percentage or M-MDSC and PMN-MDSC, with patients responding more to treatment having lower percentages of M-MDSC, while PMN-MDSC percentages varied among patients. Vemurafenib treatment is thought to modulate the tumor microenvironment impacting MDSC induction since culturing PBMCs from healthy donors in a conditioned media from a primary melanoma cell line in the presence of Vemurafenib failed to induce CD14$^+$HLA-DR$^{-}$low M-MDSC compared to cultures with no Vemurafenib (229). However, it was not shown in this study whether Vemurafenib had direct effects on MDSC suppressive functions.

2- Anti-G-CSF (granulocyte- colony stimulating factor) and anti-Bv8 antibodies

Murine

Bv8 (prokineticin-2) is upregulated in MDSC upon G-CSF receptor activation by G-CSF (231). In tumor-bearing mice Bv8 induced angiogenesis, MDSC mobilization from the BM to the tumor or distant sites establishing a pro-metastatic niche for the colonization of metastatic tumor cells (231, 232). Overall, targeting the G-CSF-Bv8 axis in tumor-bearing animals with specific antibodies decreased MDSC infiltration in the tumor, PMN-MDSC
recruitment to the lungs of tumor-bearing mice, and decreased angiogenesis, tumor growth and metastasis.

3- Anti-CSF-1 receptor (CSF1R) (GW2580)

*Murine*

GW2580 is a kinase inhibitor of CSF1R (233). Targeting CSF1R with GW2580 in LLC-bearing mice decreased M-MDSC infiltration in the tumor with no effect on PMN-MDSC recruitment, decreased the expression of Arg1 and MMP-9 in the tumors, and angiogenesis. Interestingly, treatment of CSF-1 only impaired tumor growth when combined with VEGFR-2 (vascular endothelial growth factor-2 receptor) (234).

4- Anti-CCL2 antibody

*Murine*

Inflammatory monocytes expressing Gr-1 and CCR2 (CCL2 chemokine receptor), in response to CCL2 produced by tumor cells and stroma are preferentially recruited to pulmonary metastases but not to primary tumor. Ablation of CCL2-CCR2 signaling with anti-CCL2 antibody promoted tumor survival and decreased tumor metastasis by blocking the recruitment of inflammatory monocytes to the lungs. Induction of tumor metastasis in the lungs by inflammatory monocytes was dependent on the expression of VEGFA, since inhibition of VEGFA expression in inflammatory monocytes abrogated the index of metastases in the lungs (235).

5- CXCR2 and CXCR4 antagonists

*Murine*

Tumors with Tgfbr2 deletion induced the recruitment of MDSC through the production of CXCL5 and SDF-1, that chemoattractted MDSC expressing CXCR2 and CXCR4. In mice
inoculated with 4T1 mammary carcinoma, blockade of CXCR2 and CXCR4 significantly decreased lung metastasis with no substantial difference in the growth of the primary tumors (236).

D. Induction of MDSC differentiation

1- Vitamin D3

*Human*

Differentiation of suppressive CD34+ myeloid progenitors to DC by the differentiation-inducing hormone 1α, 25-hydroxyvitamin D3, has been reported in mice bearing Lewis Lung Carcinoma (LLC) and patients with NSCLC (237, 238). In a study conducted by Kulbersh et al (238), 17 NSCLC patients were either treated (11 patients) or untreated (6 patients) with 1α, 25-hydroxyvitamin D3 for 3 weeks prior to surgery. Analysis of CD34+ and dendritic cells by immunohistochemistry in the NSCLC tissues revealed reduced infiltration of intratumoral CD34+ cells and immature DC-SIGN+ dendritic cells and increased numbers of intratumoral DC-LAMP+ mature DC. To assess the clinical significance of such findings, a clinical trial was conducted in 32 newly diagnosed patients with NSCLC, with 16 patients left untreated or and the other 16 treated with 1α, 25-hydroxyvitamin D3 for 3 weeks prior to surgical removal of the tumor (239). Immunohistochemical analysis of the tumor revealed a significant increase in the levels of intratumoral CD4+, CD8+ T lymphocytes and cells expressing the activation markers CD69 in patients treated with 1α, 25-hydroxyvitamin D3. To assess the significance of these findings NSCLC patients were monitored for tumor recurrence after surgery, with median recurrence being 181 days in the untreated group compared to 620 days in the treated group,
emphasizing the importance of combining immune therapy by targeting suppressive cells to surgical or chemotherapy procedures in the treatment of cancer. In another study to determine the effects of 1α, 25-hydroxyvitamin D3 in the cytokine profiles in the plasma and tumor tissue of NSCLC patients, it was reported that 1α, 25-hydroxyvitamin D3 differently modulates the cytokine milieu in the plasma compared to tumor tissue, increasing the levels of IL-6, IL-10, IL-2, IFN-γ, TNF-α in the tumor tissue, and increased levels of IL-8, VEGF, IL-1α and IL-1β in the plasma but not tumors of treated patients compared to the levels in untreated patients (240). Although some studies have assessed the effects of 1α, 25-hydroxyvitamin D3 on the cytokine profile of human monocytes and macrophages in vitro (241), more studies need to be performed to link the effects of different cytokine milieu in the tumor and plasma of cancer patients in differentiation of immunosuppressive cells to mature DC.

In a recent report, fibrocytes expressing CD45+CD34+HLA-DR+ with a suppressive phenotype were found to expand in patients with metastatic pediatric sarcomas, demonstrating both features of neutrophilic and monocytic cells, suppressed anti-CD3 induced T cell proliferation through IDO, and its expansion correlated with an increased Th2 phenotype in patients (242). Since 1α, 25-hydroxyvitamin D3 is believed to skew CD34+ differentiation to mature DC, their effect in other types of cancers on suppressive CD34+ cells is still to be elucidated.

2- Taxanes (Docetaxel and Paclitaxel)

Docetaxel and Paclitaxel are semi-synthetic taxanes with anti-tumor properties. These drugs target tubulin in rapidly dividing cells, stabilizing microtubules during cell division
leading to cell arrest and cell death. It has been reported in several studies that these taxanes modulate immune responses in cancer patients and tumor-bearing mice (243, 244). Herein, we summarize the main effects of Docetaxel and Paclitaxel on MDSC in murine tumor models and cancer patients.

**Murine**

In a murine squamous cell carcinoma model, SCC VII/SF, docetaxel in conjunction with vitamin D₃ increased the numbers of intratumoral active T cells compared to vitamin D₃ alone. However, docetaxel had no effect on the levels of CD34⁺ cells in the spleens and lymph nodes, and no difference in tumor weight was found between untreated mice or mice treated with vitamin D₃ and/or docetaxel. It is important to note that the efficacy of vitamin D₃ treatment in clinical trials on the recurrence of NSCLC in patients was assessed post surgery (240), suggesting that additional procedures are required along with immune therapy in the treatment of cancer.

In a mammary carcinoma model, 4T1-Neu, intraperitoneal treatment of tumor-bearing mice significantly decreased tumor growth through the modulation of MDSC (245). Docetaxel treatment decreased the percentage of splenic MDSC, decreased its suppressive activity, increased T cells CTL activity, upregulated the expression of CCR7 (M1 marker), MHC II, CD11c, CD86 in MDSC, and preferentially induced cell death of Mannose Receptor (MR⁺; M2 marker) MDSC. Incubation of MDSC for 6 hours decreased STAT3 phosphorylation, suggesting that docetaxel directly modulates MDSC signaling. Consistent with these findings, treatment of B16-melanoma-bearing mice with docetaxel following total body irradiation (TBI), improved T cell transfer and dendritic cell therapy, improving
CTL function in vaccinated mice, by targeting highly suppressive MDSC and blocking their rapid reconstitution following TBI (246). However, the mechanisms of docetaxel effects on MDSC differentiation and the presence of possible receptors have not been identified.

Combination of ultra low-doses of Paclitaxel with a peptide vaccine derived from the melanoma antigen Tyrosine related protein 2 (TRP2) enhanced vaccine efficiency in healthy mice. The vaccine efficiency was associated with an increase in the levels of TRP-2 specific T cells in the spleen, and correlated with decreased levels of Tregs and immature myeloid cells, increased levels of effector CD8+ and CD4+ T lymphocytes in the bone marrow and spleen, IFNγ-producing NK cells in the bone marrow and NKT cells in the lymph nodes (247). Stimulation of MDSC in vitro with Paclitaxel in ultra-low doses induced the expression of CD11c, CD86 and CD40 in a TLR4-independent manner (248), suggesting that the decrease in the levels of MDSC in Paclitaxel-treated mice was due to its differentiation to DC, however the levels of DC in tumor-bearing mice treated with Paclitaxel was not speculated.

**Human**

The levels of circulating MDSC was assessed in a clinical trial involving forty-one women diagnosed with HER-2 neu negative breast cancer in stages II-IIIa and received three chemotherapeutic drugs: doxorubicin-cyclophosphamide followed by docetaxel every 3 weeks followed by NOV-002, a disodium glutathione disulfide (249). It was found that patients who achieved pathologic complete response (pCR) (defined as no metastatic tumor in the axillary lymph nodes, no invasive tumor in the breast or and invasive tumor ≤ 10mm
in dimension) had lower levels of circulating MDSC (Lin+HLA-DR−CD11b+CD33+) in the blood compared to patients who did not achieve pCR. In this study 15 out of 39 patients achieved a pCR. Suggesting that MDSC targeting may increase the efficacy of chemotherapy regimens currently used in the clinic.

3- All-trans-Retinoic s (ATRA)

Murine

Retinoic acid was previously shown to induce granulocytic differentiation of cells from patients with acute promyelocytic leukemia in vitro (250). In mice bearing subcutaneous C3 fibrosarcoma or DA3-HA mammary adenocarcinoma, treatment with subcutaneously implanted ATRA pellets induced the differentiation of adoptively transferred MDSC to DC (CD11c+ IA^b^), macrophages (F4/80^+) and granulocytes (Gr1^+CD11b^), and improved CD4^+ T cell responses (251). Although treatment of tumor-bearing mice with ATRA decreased the percentages of MDSC (Gr1^+CD11b^) in the spleens and bone marrow of tumor-bearing mice, it did not decrease tumor growth except only when combined with a DC cell vaccine transduced with Ad-p53 (251). ATRA-induced differentiation of MDSC from tumor-bearing mice and patients with renal carcinoma was shown to be through the activation of glutathione synthase (GSS) and accumulation of glutathione (GSH) leading to a neutralization of reactive oxygen species (ROS) in an ERK1/2-dependent mechanism (252). In addition, ATRA enhanced the immunogenicity, and differentiation of MDSC loaded with NKT cell ligand α-GalCer in a mechanism dependent on GSH activation in MDSC and IFN-γ production by NKT cells (253).

Human
ATRA induction of MDSC differentiation in murine models was extended to clinical trials in patients with metastatic renal cell carcinoma (RCC) and small cell lung cancer (SCLC) (254, 255). In RCC patients treated with ATRA prior to IL-2 treatment had reduced numbers of MDSC, a higher myeloid/lymphoid dendritic cell ratio, and decreased the suppressive activity of mononuclear cells (254). Interestingly, IL-2 treatment abrogated ATRA effect. In vitro culture of MDSC from patients with metastatic RCC with ATRA induced its differentiation to functional APCs, and abrogated its immune suppression (256). Similarly, in patients with SCLC, ATRA treatment increased DC vaccine efficacy decreasing MDSC levels in the peripheral blood and improving antigen-specific CD8+ T cell responses (255).

4- TLR9 activation by CpG

Murine

Activation of TLR9 receptor in MDSC by CpG induced MDSC maturation and differentiation, and abrogated MDSC suppressive function, especially PMN-MDSC, in mice-bearing subcutaneous C26 tumors and in CEA424-Tag mice autochthonous gastric tumors. MDSC differentiation and inhibition of suppressive function were promoted by IFN-α produced by plasmacytoid DC (pDC) in vitro. However, the effect of IFN-α on MDSC differentiation and function needs to be further elucidated (257).

5- Curcumin

Murine

In a human gastric cancer xenograft model and a mouse colon cancer allograft model, treatment with curcumin in the diet or intraperitoneally decreased the percentage of MDSC in the tumor, spleen and blood. PMN-MDSC percentage was decreased upon curcumin
treatment, while M-MDSC differentiated to an M1-like phenotype with an increased expression in CXCR7. Curcumin treatment of MDSC cocultured with cancer cells or myofibroblast-conditioned medium in the presence of IL-1β inhibited p-Stat3 and IL-6 production by MDSC. However, it was not determined in this study whether curcumin impacted MDSC suppressive function (258).

6- Whole-glucan particles (WGP)

Murine

Treatment of M-MDSC from LLC-bearing mice in vitro with WGP induced a population of cells expressing CD11c<sup>+</sup> F4/80<sup>+</sup> Ly6C<sub>low</sub>. M-MDSC suppressive function was also decreased upon incubation with WGP. Oral treatment of LLC-bearing mice with WGP significantly reduced the percentage of Gr-1<sup>+</sup> CD11b<sup>+</sup> cells and Tregs, and increased the percentage of macrophages, DC and effector CD8<sup>+</sup> T cells in vivo, with a significant decrease in tumor burden (50). The effects of WGP on M-MDSC were dependent on Syk and NF-κB p65 signaling.

The main therapeutic approaches applied up to date in the clinic to target MDSC in cancer patients are summarized in Table 2.
<table>
<thead>
<tr>
<th>Therapeutic drugs</th>
<th>Effect on MDSC</th>
<th>Overall Results</th>
<th>Tissue/ type of cancer</th>
<th>Method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphodiesterase-5 inhibitor</td>
<td>Inhibit MDSC suppression</td>
<td>Restore T cell proliferation</td>
<td>PBMCs; MM and NSCLC</td>
<td>Sildenafil added in vitro to PBMCs cultures</td>
<td>(192)</td>
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<td>(Sildenafil)</td>
<td></td>
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<tr>
<td>Triterpenoid (CDDO-Me) + Gemcitabine</td>
<td>Inhibit MDSC suppression</td>
<td>-No effect on MDSC frequency in peripheral blood - Induces T cell proliferation in vitro</td>
<td>PBMCs; Stage II-IV pancreatic cancer</td>
<td>Phase I trial T cell activation assessed ex vivo</td>
<td>(194)</td>
</tr>
<tr>
<td>Inhibitor of exosome formation</td>
<td>Inhibit MDSC suppression</td>
<td>Decrease pStat3 in MDSC</td>
<td>PBMCs; colorectal metastatic carcinoma</td>
<td>Phase I trial -MDSC suppressive function assessed ex vivo - Serum from treated patients have decreased ability to induce pStat3 in MDSC</td>
<td>(181)</td>
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<tr>
<td>(Amiloride)</td>
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</tr>
<tr>
<td>COX-2 and PGE-2 inhibitors</td>
<td>Inhibit MDSC induction, expansion and suppression</td>
<td>Restores CD3 proliferation</td>
<td>PBMCs; melanoma</td>
<td>COX-2 and PGE-2 inhibitors added in vitro to CD14$^+$ and T cell cocultures</td>
<td>(211)</td>
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<tr>
<td>(Celecoxib)</td>
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<tr>
<td><strong>Dexorubicin-cyclophosphamide</strong></td>
<td>Killing tumor cells and Treg modulation</td>
<td>Increase MDSC percentages in peripheral blood; MDSC retained suppressive activity - Patients treated with after paclitaxel have less increase in MDSC percentages</td>
<td>PBMCs; Stage I/II, stage III and stage IV breast cancer</td>
<td>Percentages of MDSC and suppressive assays assessed ex vivo after each cycle of chemotherapy</td>
<td>(228)</td>
</tr>
<tr>
<td><strong>Vemurafenib (BRAF V600E inhibitor)</strong></td>
<td>Inhibit MAP kinase pathway in tumor cells</td>
<td>Decreases frequency of M-MDSC (CD14⁺HLA-DR&lt;sup&gt;low&lt;/sup&gt;) and PMN-MDSC (CD66b⁺Arginase1⁺CD16&lt;sup&gt;−&lt;/sup&gt;/low) in PBMCs</td>
<td>PBMCs; Advanced melanoma</td>
<td>Percentages of MDSC and suppressive assays assessed ex vivo in patients treated with Vemurafenib</td>
<td>(229)</td>
</tr>
<tr>
<td><strong>Vitamin D3 (1α,25-hydroxyvitamin D3)</strong></td>
<td>Differentiation of suppressive CD34⁺ myeloid progenitors to DC</td>
<td>Increases the numbers of TILs and cells expressing CD69 - Delays tumor recurrence post surgery - Increases the levels of IL-6, IL-10, IL-2, IFN-γ, TNF-α in the tumor - Increases levels of IL-8, VEGF, IL-1α and IL-1β in the plasma</td>
<td>Tumor tissue; NSCLC</td>
<td>Immunohistochemical analysis of the tumor of patients treated 3 weeks prior surgery</td>
<td>(237-241)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Targeting of tumor cells and immune cells (T cells, MDSC)</td>
<td>Decrease levels of circulating MDSC (Lin⁻ HLA-DR⁺CD11b⁺CD33⁺)</td>
<td>PBMCs; HER-2 neu negative breast cancer in stages II-IIIa</td>
<td>Percentages of MDSC in PBMCs assessed after treatment</td>
<td>References</td>
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<tr>
<td>doxorubicin-cyclophosphamide + docetaxel</td>
<td>Targeting of tumor cells and immune cells (T cells, MDSC)</td>
<td>Decrease levels of circulating MDSC (Lin⁻ HLA-DR⁺CD11b⁺CD33⁺)</td>
<td>PBMCs; HER-2 neu negative breast cancer in stages II-IIIa</td>
<td>Percentages of MDSC in PBMCs assessed after treatment</td>
<td>(243, 244)</td>
</tr>
<tr>
<td>every 3 weeks followed by NOV-002, a disodium glutathione disulfide</td>
<td>Prior to IL-2 treatment ATRA Induces MDSC differentiation to functional APC and decreases MDSC suppressive activity</td>
<td>-Reduce numbers of MDSC -Increase myeloid/lymphoid dendritic cell ratio - No direct effect on tumor growth - Increased efficacy of DC vaccine -IL-2 treatment abrogated ATRA effect</td>
<td>PBMCs; metastatic renal cell carcinoma, SCLC</td>
<td>Clinical trial Among 18 patients, there were 1 complete response, no partial responses, 11 stable diseases and 3 progression. 3 patients had IL-2 treatment discontinued.</td>
<td>(254, 255)</td>
</tr>
<tr>
<td>ATRA (Vesanoid) + IL2</td>
<td>Inhibition of MDSC expansion</td>
<td>Decreases the levels of MDSC, Tregs and IFN-γ producing T lymphocytes in PBMCs</td>
<td>PBMCs; RCC</td>
<td>-Effect of Sunitib assessed in vitro on MDSC from peripheral blood</td>
<td>(222, 223, 225, 226)</td>
</tr>
<tr>
<td>Sunitinib (tyrosine kinase inhibitor)</td>
<td>Inhibition of MDSC expansion</td>
<td>Decreases the levels of MDSC, Tregs and IFN-γ producing T lymphocytes in PBMCs</td>
<td>PBMCs; RCC</td>
<td>-Effect of Sunitib assessed in vitro on MDSC from peripheral blood</td>
<td>(222, 223, 225, 226)</td>
</tr>
</tbody>
</table>
RATIONALE, SIGNIFICANCE AND MAIN FINDINGS

Chemotherapeutic protocols already established in the clinic mainly include drugs that target tumor cell proliferation and induce tumor cell death. The success of current chemotherapeutic protocols in the clinic has been limited due to the ability of cancer cells to develop resistance and evade immunesurveillance. MDSC have been widely described in the literature and it is currently accepted that targeting MDSC in the clinic is vital for the development of effective anti-tumor immunity. One of the main challenges in MDSC research is its heterogeneity and the absence of a universal marker for immunosuppression. The availability of a wide spectrum of markers to describe different MDSC subsets in mice and the absence of a Gr-1 homologue in humans have limited the success of MDSC targeting in the clinic. The absence of unique markers has led to the lack of consensus in the literature, especially in human subject studies. In addition, due to limitations in the acquisition of tumor tissue from cancer patients, most studies describing MDSC phenotype, suppressive activity and response to therapeutic treatment in human cancer patients are restricted to the peripheral blood. Current data from tumor-bearing mice and clinical trials have revealed MDSC plasticity, with discrepancies in the responses to therapy between tumor-infiltrating MDSC and peripheral MDSC (e.g. spleens of tumor-bearing mice, peripheral blood of cancer patients). The presence of tumor-infiltrating MDSC in the tumor microenvironment has rendered it more resistant to therapy in the clinic.
The introduction of immunomodulatory agents with no side-effects and at a low cost have been a challenge in the clinic. In this study, we provide evidence that particulate β-glucan has immunomodulatory functions on MDSC. Particulate β-glucan is a natural compound, can be provided at a low-cost, ingested orally and with no reported side-effects. We herein demonstrate that particulate β-glucan differentially modulate the function of M-MDSC and PMN-MDSC subsets. First, we show that particulate β-glucan induce a cytotoxic phenotype in PMN-MDSC and subsequent apoptosis through dectin-1 signaling. Second, we demonstrate that particulate β-glucan induces M-MDSC differentiation into antigen-presenting cells in a dectin-1 dependent manner. Third, we are the first to highlight the effect of particulate β-glucan treatment in patients with NSCLC, by demonstrating that the frequency of MDSC in the peripheral blood of these patients decreased in two weeks after treatment. These findings support the administration of particulate β-glucan as a form of adjuvant therapy in the clinic.
Figure 4. Schematic model showing how particulate β-glucan modulates MDSC function. Particulate β-glucan binds to dectin-1 receptor on M-MDSC inducing its differentiation to F4/80⁺CD11c⁺ cells with upregulated expression of CD86, MHCI, MHCII and CD40 enhancing the synaptic potential between M-MDSC and T cells for antigen-presentation. M-MDSC upon particulate β-glucan engagement convert to potent APC that cross-present antigen to antigen-specific CD8⁺ T cells and present antigen to CD4⁺ T cells, which produce Granzyme B and IFN-γ, leading to a cytolytic response loop against tumor cells. On the other hand, particulate β-glucan activates PMN-MDSC
respiratory burst and subsequent apoptosis in a dectin-1 dependent manner. Therefore, providing a pool of ROS upon PMN-MDSC priming, that may negatively impact tumor cell growth.
MATERIALS AND METHODS

Mice and tumor models

Wildtype (WT) C57BL/6 mice were purchased from the National Cancer Institute (NCI). Dectin-1 KO mice were described previously (120, 259). OT-I Rag1<sup>−/−</sup> and OT-II CD4 ovalbumin TCR-Tg mice were purchased from Taconic.

Tumor models and particulate β-glucan therapeutic protocol were performed as described previously (49). Briefly, C57BL/6 WT or dectin-1 KO mice were implanted subcutaneously (s.c.) with Lewis Lung Carcinoma (LLC) (2x10<sup>5</sup>/mouse) or mammary-cell carcinoma (E0771) cell lines (6x10<sup>5</sup>/mouse). On day 8 post-implantation, mice with palpable tumors were orally administered daily by gavage with 100 µl particulate β-glucan (Biothera, 800 µg/mouse in PBS suspension) or with 100µl PBS. Tumor diameters were measured with a caliper every three-four days, and mice were killed when the tumor diameter reached 15mm. The tumor volume was calculated based on the formula=length x width<sup>2</sup>/2. The protocols on murine tumor models were performed according to the institutional guidelines and laws, and were approved by the Institutional Animal Care and Use Committee at the University of Louisville.
**Single-cell suspensions from tumors**

Mouse tumor tissues (12-15 mm in diameter) were excised and minced into small pieces. Additional mechanical digestion was performed with gentleMACS dissociator (Milteneyi Biotec). Tumors were enzymatically digested in RPMI 1640 medium containing 10% FBS, type IV collagenase (1 μg/ml) and hyaluronidase (10 ng/ml) for 45 minutes at 37°C on a rotator. A second step of mechanical dissociation was performed after enzymatic digestion. The digested cells were then filtered, pelleted and re-suspended in complete RPMI.

**Flow Cytometry and Cell Sorting**

Single-cell suspensions were treated with Fc-blocker for 10 min on ice and stained with the relevant fluorochrome-labeled mAbs for 30 min on ice. Cells were washed 2X with PBS or staining buffer (PBS+0.1%FBS) (for FACS analysis) or running buffer (Milteney) (for cell sorting). For FACS analysis, cells were acquired using FACS Calibur or FACS Canto II (BD biosciences). MDSC were sorted using BD FACSARia III cell sorter or MoFlo XDP (Beckman Coulter). The purity of sorted cells was >98% assessed by flow cytometry. For intracellular staining of IFN-γ and granzyme B, cells were first stained with either anti-CD4 or anti-CD8 Abs, fixed, permeabilized with Fixation/permeabilization buffer (Biolegend), washed with 1X permeabilization/washing buffer (Biolegend) and stained with the relevant cytokine Abs. Cells were then washed 2X with 1X perm/washing buffer and analyzed by flow cytometry. Data analysis was performed using FlowJo software (Tree Star). The following fluorochrome-labeled monoclonal antibodies were used: anti-mouse Gr-1, anti-mouse Ly6G, anti-mouse Ly6C,
anti-mouse CD11b, anti-mouse CD45, anti-mouse F4/80, anti-mouse CD11c, anti-mouse CD40, anti-mouse CD86, anti-mouse CD80, anti-mouse IA/IE, anti-mouse H2-K^b, anti-CD8, anti-CD4, anti-IFN-γ, anti-GranzymeB and their corresponding isotype controls were purchased from Biolegend. The following anti-human monoclonal antibodies: anti-CD11b, anti-CD14, anti-CD33, anti-HLA-DR, anti-CD3 anti-IFN-γ with their corresponding isotype controls were also purchased from Biolegend.

**Apoptosis assays**

Splenocytes from LLC-bearing C57BL/6 or Dectin-1^-/-^ mice were stimulated with WGP for 3, 6, 14, or 18 hours and then stained with anti-Gr-1 and anti-CD11b Abs (Biolegend) and 7AAD and Annexin V dyes (BD biosciences). Cells were analyzed by flow cytometry.

**Respiratory Burst assays**

Sorted PMN-MDSC from WT or Dectin-1^-/-^ mice were stained with dihydrorhodamine (DHR) 123 as described previously (260, 261). Briefly, cells were incubated with 1μM DHR and catalase for 5 min at 37°C. Particulate β-glucan was then added for 30 min, 60 min and 120 min. Rhodamine (RHO) was then detected on FL-1 channel on a FACS Calibur or FACS Canto II.

**Western Blot analysis**

Sorted M-MDSC or PMN-MDSC stimulated with or without particulate β-glucan (100 μg/ml) for indicated times were lysed in Triton X-100 lysis buffer in the presence of protease and phosphatase inhibitor. The whole cell extracts were subjected to SDS-PAGE
and electro-transferred to PDVF membrane. The membranes were blocked and probed overnight at 4°C with the relevant primary and then incubated for 1 hour at room temperature with the secondary Abs. The blots were developed with ECL Plus Western Blotting Detection Reagents (GE Healthcare). The primary Abs included: p-Erk1/2 (Thr202/Tyr204, Cell Signaling), Erk1/2 (MK1, Santa Cruz), p-Stat3 (Tyr705, Cell Signaling), p-AKT (Ser473, Cell Signaling), p-p38 (Thr180/Tyr182, Cell Signaling), p-Zap/Syk (Tyr319/Tyr352, Cell Signaling), STAT3 (C-20, Santa Cruz), p-SAPK/JNK (Thr183/Tyr185, Cell Signaling) and β-actin (Sigma-Aldrich).

**Monocytic-MDSC (M-MDSC) differentiation assay**

M-MDSC (CD11b⁺Ly6C<sup>high</sup>Ly6G⁻) were sorted from the spleens of LLC-bearing WT or dectin-1 KO mice and cultured for 7 days with 50µg/ml particulate β-glucan in 48-well plates (corning).

**T cell proliferation and Ag-presentation assays**

For T cell proliferation assay, M-MDSC and PMN-MDSC sorted from the spleens or Gr-1<sup>−</sup>CD11b<sup>+</sup> MDSC from tumors of LLC-bearing mice, were co-cultured with 1μM carboxyfluorescin dye (CFSE)-labeled splenocytes from OT-II or OT-I mice in the presence of OVA (100 µg/ml in OT-II cultures, 50µg/ml in OT-I cultures, and 10 µg/ml in some splenic PMN-MDSC suppression experiments) and particulate β-glucan (50 µg/ml). Three days later, cells were harvested and stained. In addition, some T cell proliferation assays were performed by co-culturing sorted MDSC with CFSE-labeled splenocytes from C57BL/6 mice stimulated with plate-bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (2 µg/ml).
For Ag-presentation assay, sorted M-MDSC from the spleens of LLC-bearing WT or dectin-1 KO mice were cultured in the presence or absence of particulate β-glucan (50μg/ml) for 7 days. In some experiments, MEK1/2 inhibitor (PD98059) (30 ng/ml) or DMSO was added to cultures during differentiation. Cells were washed and co-cultured with sorted and CFSE-labeled CD8+ or CD4+ T cells from OT-I and OT-II mice, respectively, in the presence or absence of whole OVA-Ag (50 μg/ml). T cell proliferation and IFN-γ or granzyme B production were assessed 4-5 days later by flow cytometry.

**RNA extraction and Quantitative Real-Time PCR (qRT-PCR)**

RNA was extracted with Trizol reagent (Invitrogen) as described in the manufacturer protocols. Extracted RNA was transcribed to cDNA with a Reverse Transcription Kit (Bio-Rad). qRT-PCR reaction was performed using SYBR Green Supermix (Bio-Rad) with the relevant primers (Table 3) and the reaction was detected on MyiQ single color RT-PCR detection system (Bio-Rad). The change in gene expression was quantified by measuring the change in threshold (ΔΔCT), where ΔCt= Ct target gene- Ct House keeping gene and ΔΔCt= ΔCt induced- ΔCt reference.

**In vivo M-MDSC/LLC admixture experiments**

Sorted M-MDSC were treated with or without particulate β-glucan for 18 hours and mixed with LLC cells (1x10^5) at a 1:1 ratio. Cells were mixed with Matrigel® Matrix Basement membrane (Corning) and implanted subcutaneously in the flanks of C57BL/6 mice. Tumor diameter was measured every two days and mice were sacrificed on day 25.
**Human subjects**

NSCLC patients that were newly diagnosed, received particulate β-glucan treatment at the James Graham Brown Cancer Center, University of Louisville. These patients did not receive any other treatment prior or during β-glucan treatment. The study was approved by the Institutional Ethical Board and blood samples were collected upon a written informed consent. Each NSCLC patient received 500 mg of particulate β-glucan for 10-14 days. Peripheral blood was collected before and after treatment. Whole blood cells were treated with ACK lysis buffer, washed 2X with PBS and stained with human anti-CD14, anti-HLA-DR, anti-CD11b and anti-CD33 Abs (Biolegend) for 30 min on ice. Samples were then washed 2X prior to acquisition on FACS Calibur. Neutrophils were purified with a histopaque density gradient and stored into Trizol at -80°C for RNA extraction (105).

**Mixed lymphocyte reaction (MLR)**

CD14+ HLA-DR+ CD3- or CD14+HLA-DR+CD11b+CD33+CD3- cells were sorted from the PBMCs of NSCLC patients and co-cultured with sorted and CFSE-labeled CD3+ cells from the PBMCs of allogeneic donor at 1:1 ratio for 5 days. Cells were then harvested and stained with human anti-CD3 and human anti-IFN-γ mAbs for subsequent flow cytometry analysis.

**Statistical analysis**
Data were analyzed using GraphPad Prism (5.0) software. Unpaired student t-test was used to calculate significance. Significance was assumed to be reached at p<0.05. All graph bars are expressed as mean ± SEM.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| Mouse *TNF-α*         | Forward: 5'- ACCCACGGCTCCACCCTCTC -3’  
                         | Reverse: 5'- CCCTCTGGGGGGCGATCAGCTC -3’                                         |
| Mouse *IL-12 p35*     | Forward: 5'- CAGAATACAACCACATCAGCAG -3’  
                         | Reverse: 5'- CCACCTGTGATGGGTCAGGAC -3’                                         |
| Mouse *iNOS*          | Forward: 5'- AATAGAGGAACATCTGGCCAGG -3’  
                         | Reverse: 5'- ATGGCGGACCTGATGTTGC -3’                                           |
| Mouse *IL-6*          | Forward: 5'- TCCCTTCCAGAGGCAGCAGCTA -3’  
                         | Reverse: 5'- CAGGGGTAGATGGCCGTCGAG -3’                                         |
| Mouse *TGF-β1*        | Forward: 5'- TGCTAATGGTGGACCAGGCA -3’  
                         | Reverse: 5'- CACTGCTTCCCAATGCTGTA -3’                                          |
| Mouse *β-MG*          | Forward: 5'- CTTTTCTGGTCTTGTCTC -3’  
                         | Reverse: 5'- TCAGTATGTTGGCTCTCC -3’                                            |
| Human *B2M* (beta-2 microglobulin) | Forward: 5'- GCCTTAGCTG TGCTCGGCTC -3’  
                                      | Reverse: 5'- AGTCGACCAG TCCTTGCTGA -3’                                         |
| Human *Arginase 1*    | Forward: 5'- CAGAGCATGAGCGGCCCAAGT -3’  
                         | Reverse: 5'- ATCACAACCTTTGTTTGAAGTTCTCA -3’                                   |

Table 3. Primer sequences used in qRT-PCR analysis of different mRNA expression used in this study.
RESULTS

PMN-MDSC preferentially expand in the spleens and tumors during tumor progression

In order to characterize the abundance of MDSC populations during tumor progression before β-glucan treatment, we subcutaneously implanted mice with Lewis Lung Carcinoma (LLC), mammary carcinoma (E0771) and B16 F10 melanoma. And then assessed the frequency of different MDSC subsets in the spleen and tumor of tumor-bearing mice at different tumor sizes. The groups are described as follows: naïve mice, small tumors (5-8mm), medium tumors (9-12mm) and large tumors (12-15mm). Despite many studies describing the expansion of MDSC during tumor progression (40), we sought to ensure first that MDSC expansion positively correlates with tumor progression in the three animal models tested, to then study the effect of particulate β-glucan on MDSC frequencies. Indeed during tumor progression, PMN-MDSC preferentially expand in the spleens (Figure 5) and tumors (Figure 6) of tumor-bearing mice. PMN-MDSC are CD45^+CD11b^+Ly6G^+Ly6C^{int} and M-MDSC are CD45^+CD11b^+Ly6G^+Ly6C^{high} (Figure 7).
Figure 5. Kinetics of MDSC frequencies in the spleens of tumor-bearing mice during tumor progression. Pie-graph demonstration of PMN-MDSC and M-MDSC frequencies in the spleens of tumor-bearing mice implanted with B16 F10 melanoma (n=8, 2 mice per group), E0771 (n=18, 5 mice per group except the large tumor group n=3), and LLC (n=19, 5 mice per group except the large tumor group n=4). Naïve mice were mice not implanted with tumors. The tumor sizes are: small: 5-8 mm; medium: 9-12 mm; large: 12-15 mm. Mice of different groups were injected at different days to ensure that mice implanted with one tumor cell line be killed in the same day.
Figure 6. Kinetics of MDSC frequencies in the tumors of tumor-bearing mice during tumor progression. Pie-graph demonstration of PMN-MDSC and M-MDSC frequencies in the spleens of tumor-bearing mice implanted with B16 F10 melanoma (n=4, 1 mouse representing the large tumor, 1 mouse the medium tumor and 2 mice the small tumors), E0771 (n=13, 5 mice per group except the large tumor group n=3), and LLC (n=14, 5 mice per group except the large tumor group n=4). Naïve mice were mice not implanted with tumors. The tumor sizes are: small: 5-8 mm; medium: 9-12 mm; large: 12-15 mm. Mice of different groups were injected at different days to ensure that mice implanted with one tumor cell line be killed in the same day.
Figure 7. Representative dot plots of MDSC in tumor during tumor progression in LLC, E0771 and B16-bearing mice. MDSC in tumor-bearing mice consist of mainly two heterogenous subsets: polymorphonuclear (PMN-MDSC) and monocytic (M-MDSC). PMN-MDSC are CD45+CD11b+Ly6G+Ly6C\text{int} and M-MDSC are CD45+CD11b+Ly6G−Ly6C\text{high}. 
Particulate β-glucan oral treatment diminishes tumor growth and differentially impacts the frequency of MDSC subsets in spleens and tumors

To delineate the effect of particulate β-glucan treatment on the composition of different MDSC subsets in spleens and tumors, C57BL/6 mice were challenged with LLC and E0771 cell lines subcutaneously. Mice with palpable tumors were administered orally through gavage with PBS or particulate β-glucan (WGP) as previously established (49, 130). β-Glucan treatment significantly reduced tumor volumes (Figure 8A), splenomegaly (Figure 8B), and tumor weight (Figure 8C) in both models. When analyzing the frequencies of MDSC subsets in the spleens and tumors, particulate β-glucan-treated mice had a significant decrease in PMN-MDSC frequencies in the spleen, but not M-MDSC, in both models (Figure 9). In addition, particulate β-glucan treatment caused a significant decrease in the frequencies of PMN-MDSC and increase in the frequencies of M-MDSC in the tumors of E0771 mice, but only trending in LLC-bearing mice (Figure 10).
Figure 8. Particulate β-glucan treatment in vivo reduces tumor burden and splenomegaly. (A) C57BL/6 WT mice (n=7, 8) were injected subcutaneously (s.c) with LLC or E0771 tumor cell lines. Once palpable tumors were formed (day 8), mice were orally administered with particulate β-glucan (800 μg, daily) or PBS with a gavage needle at indicated time. Tumor diameters were measured every three days and tumor volumes were then calculated. (B) On day 32 (LLC model) or day 35 (E0771 model), mice were killed and spleens were excised and weighed. Each point in the data plot represents the spleen weight of each mouse in grams. PBS-treated group was compared to particulate β-glucan treated group (WGP) in both models. (C) Tumor tissues were excised and weighted from WGP or PBS-treated mice. *p<0.05, ** p<0.01, *** p<0.001.
Figure 9. Particulate β-glucan treatment in vivo impacts the frequency of MDSC in spleens and tumors of LLC and E0771-bearing mice. Flow cytometry analysis of the frequencies of M-MDSC (Ly6G-Ly6Chigh) and PMN-MDSC (Ly6G+Ly6Cint) in the spleens of LLC and E0771-bearing mice treated with PBS or particulate β-glucan (WGP). Cells were gated on CD11b+ cells. *p<0.05, ** p<0.01, *** p<0.001.
Figure 10. Particulate β-glucan treatment in vivo reduces PMN-MDSC but not M-MDSC frequency in tumor-bearing mice. Frequencies of M-MDSC and PMN-MDSC in the tumors of LLC and E0771-bearing mice treated with PBS or particulate β-glucan (WGP). Cells were gated on CD45+CD11b+ cells. *p<0.05, ** p<0.01, *** p<0.001.
Particulate β-glucan treatment abolishes splenic and tumor MDSC-mediated T cell suppression

Since suppression of CD8+ effector T cells in tumor-bearing mice is largely attributed to MDSC (262, 263), we thought to determine whether particulate β-glucan impacts T cell suppression mediated by splenic and tumor MDSC. To this end, we studied the effect of particulate β-glucan treatment on MDSC-mediated inhibition of OVA-specific CD4+ and CD8+ T cells or Ag non-specific T cell proliferation induced by anti-CD3/CD28 stimulation. Suppression of OVA-specific CD4+ T cells mediated by splenic M-MDSC or PMN-MDSC was partially or completely obliterated upon particulate β-glucan treatment (Figure 11A). Similarly, particulate β-glucan treatment significantly increased the proliferation of OVA-specific CD8+ T cells in the presence of suppressive M-MDSC or PMN-MDSC (Figure 11B). Particulate β-glucan treatment also diminished the MDSC-mediated suppression of IFN-γ produced by OVA-specific CD4+ (Figure 12A) and CD8+ T cells (Figure 12B). In addition, particulate β-glucan treatment decreased the ability of M-MDSC to suppress CD4+ T cells and CD8+ T cells stimulated with anti-CD3/CD28 (Fig 13). However, splenic PMN-MDSC did not suppress the proliferation of T cells stimulated with anti-CD3/CD28, as previously reported (32). To generalize the efficacy of particulate β-glucan on the modulation of MDSC suppression, tumor Gr-1+CD11b+ cells were also sorted from LLC tumors and co-cultured with OVA-specific CD4+ or CD8+ T cells in the presence of OVA at different ratios. While Gr-1+CD11b+ MDSC suppressed the proliferation and IFN-γ production of CD4+ T cells (Figure 14A) and CD8+ T cells (Figure 15), particulate β-glucan treatment significantly enhanced T cell proliferation and IFN-γ
production on CD4\(^+\) and CD8\(^+\) T cells (Figure 14,15). Taken together, these data emphasize the ability of particulate β-glucan to reverse MDSC-mediated T cell suppression.

Figure 11. Particulate β-glucan in vitro treatment restores IFN-γ production by antigen-specific T cells co-cultured with MDSC (A) OT-II splenocytes co-cultured with sorted M-MDSC or PMN-MDSC from the spleens of LLC-bearing mice in the presence of OVA (100 μg/ml) with or without particulate β-glucan (50 μg/ml) for 3-4 days at 1:1 ratio and stimulated with PMA/Ionomycin for intracellular IFN-γ staining. Data represent the percentage of IFN-γ+ cells gated on CD4+ T cells. The experiment was repeated 3 times.
with similar results. (B) CFSE-labeled OT-I splenocytes co-cultured with sorted M-MDSC or PMN-MDSC from the spleens of LLC-bearing mice in the presence of OVA (50 μg/ml in M-MDSC cultures and 10 μg/ml in PMN-MDSC cultures) and particulate β-glucan (50 μg/ml) for 3 days at 1:1 ratio and then stimulated with PMA/Ionomycin for intracellular IFN-γ staining. Data represent the percentage of IFN-γ+ CFSE diluted cells gated on CD8+ T cells. Results are representative of three independent experiments. * p<0.05, **p<0.01, ***p<0.001.
Figure 12. Particulate β-glucan treatment in vitro subverts splenic MDSC-mediated T cell suppression. (A) CFSE-labeled OT-II splenocytes co-cultured with sorted M-MDSC or PMN-MDSC from the spleens of LLC-bearing mice in the presence or absence of particulate β-glucan (100 μg/ml in the M-MDSC cultures and 50 μg/ml in the PMN-MDSC cultures) and OVA (100 μg/ml) for 3 days at 1:1 ratio. Data represent the percentage of CFSE diluted cells gated on CD4+ T cells. The experiment was repeated two times with similar results. (B) CFSE-labeled OT-I splenocytes were co-cultured with sorted M-MDSC or PMN-MDSC from the spleens of LLC-bearing mice in the presence of OVA (50 μg/ml) and particulate β-glucan (50 μg/ml) for 3 days at 1:1 ratio. Data represent the percentage of CFSE diluted cells gated on CD8+ T cells. Data is representative of three independent experiments. * p<0.05, **p<0.01, ***p<0.001.
Figure 13. Particulate β-glucan in vitro treatment reverses antigen non-specific suppression in T cells co-cultured with M-MDSC but not PMN-MDSC. CFSE-labeled splenocytes from C57BL/6 mice stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) (white bar) and co-cultured with sorted splenic M-MDSC or PMN-MDSC from LLC-bearing mice for 3 days with (black bar) or without particulate β-glucan (50 μg/ml) (grey bar). Data represent the frequency of CFSE diluted cells gated on CD4+ or CD8+ T cells. The experiment was repeated twice with similar results. * p<0.05, **p<0.01, ***p<0.001.
Figure 14. Particulate β-glucan reduces tumor Gr-1+CD11b+ MDSC-mediated suppression of IFN-γ production by OVA-specific CD4+ T cells. (A) Tumor Gr-1+CD11b+CD45+ MDSC sorted from LLC-bearing mice were co-cultured with CFSE-labeled OT-II splenocytes at indicated ratios, in the presence of OVA (100 μg/ml) with or without particulate β-glucan (50 μg/ml) for 3-4 days. Data represent the frequency of CFSE diluted cells gated on CD4+ T cells. The experiment was repeated twice with similar results. (B) Same cell cultures as (A) were further stimulated with PMA/Ionomycin for intracellular IFN-γ staining. The experiment was repeated twice with similar results. * p<0.05, **p<0.01, ***p<0.001.
Figure 15. Particulate β-glucan restores IFN-γ production by OVA-specific CD8+ T cells co-cultured with tumor Gr-1+CD11b+ MDSC. Splenocytes of OT-I mice were co-cultured with sorted Gr-1+CD11b+CD45+ tumor MDSC from LLC-bearing mice at indicated ratios, in the presence of OVA (50 μg/ml in M-MDSC cultures and 10 μg/ml in PMN-MDSC cultures) with or without particulate β-glucan (50 μg/ml). Data represent the percentage of IFN-γ+ cells and CFSE diluted cells gated on CD8+ T cells. Results are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001.
**Dectin-1 stimulation with particulate β-glucan induces PMN-MDSC respiratory burst and apoptosis via the dectin-1 signaling**

Given that WGP treatment reduced PMN-MDSC-mediated T cell suppression and decreased PMN-MDSC frequency in the spleens and tumors of tumor-bearing mice, we tested whether particulate β-glucan treatment has a further impact on PMN-MDSC viability. Particulate β-glucan treatment enhanced PMN-MDSC apoptosis at 3 hours, 6 hours and more drastically at 14 and 18 hours post-stimulation (Figure 16A). PMN-MDSC apoptosis was mediated by dectin-1 receptor since particulate β-glucan did not induce PMN-MDSC apoptosis in PMN-MDSC sorted from the spleens of LLC-bearing dectin-1 knockout mice (Figure 16B). Since particulate β-glucan-induced apoptosis is more drastic at late-time points, we asked whether particulate β-glucan treatment enhances PMN-MDSC respiratory burst, a hallmark of neutrophil-mediated cytotoxicity. We detected PMN-MDSC respiratory burst by dihydrorhodamine 123 (DHR) dye that oxidizes to fluorescent rhodamine 123 (RHO) in the presence of intracellular ROS (261). Upon particulate β-glucan stimulation, PMN-MDSC respiratory burst was significantly enhanced upon 1 hour and 2 hours post-stimulation (Figure 17). To exclude the effect of dectin-1 independent phagocytosis on the induction of PMN-MDSC respiratory burst, we stimulated PMN-MSDC from dectin-1 knockout mice with particulate β-glucan. As expected, particulate β-glucan stimulation did not induce respiratory burst in dectin-1 knockout PMN-MDSC (Figure 17).

Previous studies have highlighted the importance of STAT3 phosphorylation in the cascade events mediating PMN-MDSC survival and suppression (38). Since particulate β-
glucan treatment abrogated PMN-MDSC mediated T cells suppression and induced PMN-MDSC apoptosis, we measured STAT3 phosphorylation at 15, 30 and 60 min post-stimulation. STAT3 phosphorylation was inhibited after 15 min and completely abrogated after 30 min upon β-glucan stimulation (Figure 18). This effect was mediated by dectin-1 signaling, since particulate β-glucan stimulation enhanced Syk phosphorylation. Moreover, particulate β-glucan stimulation enhanced phosphorylation of molecules downstream of the dectin-1 receptor such as Akt, JNK and Erk1/2 kinases but not p38 (Figure 18).
Figure 16. Particulate β-glucan induces apoptosis in PMN-MDSC in a dectin-1 dependent manner. (A) Representative dot plots showing the frequency of annexin V+ cells gated on Gr-1{high}CD11b+ PMN-MDSC in splenocytes of LLC-bearing mice cultured with media only or with particulate β-glucan (100 μg/ml) for indicated time. Summarized data are also shown (n=8). (B) Frequencies of annexin V+ cells gated on Gr-1{high}CD11b+ PMN-MDSC from WT or dectin-1 KO LLC-bearing mice cultured for 18 hours with or without particulate β-glucan (100 μg/ml) (n=2). *p<0.05, **p<0.01, ***p<0.001.
Figure 17. Particulate β-glucan induces respiratory burst in PMN-MDSC in a *dectin-1 dependent manner*. Respiratory burst in PMN-MDSC sorted from spleens of LLC-bearing WT or dectin-1 KO mice stimulated with particulate β-glucan (100 μg/ml) for indicated time. Reduction of dihydorhodamine 123 (DHR) to fluorescent rhodamine 123 (RHO) was assessed by flow cytometry. Data is representative of 6 independent experiments (WT) and two independent experiments (KO).
**Figure 18.** Particulate β-glucan inhibits p-STAT3 phosphorylation and enhances MAPK signaling in PMN-MDSC. Western blot analysis of p-STAT3, p-Zap/Syk, p-Akt, p-SAPK/JNK, p-Erk1/2, p-p38, STAT3 and β-actin in PMN-MDSC sorted from the spleens of LLC-bearing mice and treated with particulate β-glucan (100 μg/ml) for 0, 15, 30 and 60 minutes. Results are representative of at least three independent experiments.
Particulate β-glucan treatment fully converts M-MDSC to potent APC

Previous studies have demonstrated the ability of particulate β-glucan to enhance dendritic cell Ag-presenting capability (130), and that 8-13% of M-MDSC cultured with particulate β-glucan and GM-CSF are F4/80−CD11c+ (50). To further determine the effect of particulate β-glucan on M-MDSC, we solely treated M-MDSC with particulate β-glucan in the absence of GM-CSF for 5 days or 7 days and assessed the expression of F4/80, CD11c, CD11b, Gr-1, CD80, CD86, MHC class I, MHC class II and CD40. The majority of M-MDSC cultured with particulate β-glucan for 7 days differentiated to F4/80lowCD11c−CD11b+Gr1− cells and expressed CD86, CD80, MHC class II, MHC class I and CD40 (Figure 19A and B), cells were viable (trypan-blue exclusion) and 7AAD− (data not shown).

It has been proposed that MDSC can uptake, process and present Ag, establishing a stable synapse with T cells required for Ag-specific T cell suppression (264). Freshly isolated splenic M-MDSC cultured with sorted OVA-specific CD4+ T cells in the presence of OVA did not induce CD4+ T cell proliferation or IFN-γ production (Figure 20). To test whether M-MDSC co-cultured with particulate β-glucan for 7 days differentiated to potent APC, we co-cultured M-MDSC with particulate β-glucan for 7 days and then cultured with sorted and CFSE-labeled OVA-specific CD4+ T cells in the presence of whole OVA Ag for 4-5 days. Differentiated M-MDSC induced CD4+ T cell proliferation and IFN-γ production (Figure 21). Moreover, differentiated M-MDSC cross-presented Ag to OVA-specific CD8+ T cells and immensely induced CD8+ T cell proliferation and cytokines associated with effector functions such as IFN-γ and Granzyme B (Figure 22). Taken together, these data
clearly demonstrate that particulate β-glucan converts suppressive M-MDSC to potent APC that can promote Th1 differentiation and Ag cross-presentation to CD8\(^+\) effector T cells.

**Dectin-1 receptor signaling is required for the conversion of M-MDSC to potent APC**

To exclude the possibility of any artifacts in M-MSDC differentiation that might be promoted by particulate β-glucan phagocytosis alone and to test whether it is directed by dectin-1 receptor, we performed Ag presentation assays with wild-type or dectin-1 KO M-MDSC pretreated with particulate β-glucan for 7 days. WT M-MDSC but not dectin-1 KO M-MDSC induced OVA-specific CD4\(^+\) T and CD8\(^+\) T cell proliferation and IFN-γ production (Figure 23). In addition, particulate β-glucan stimulation enhanced the phosphorylation of Syk, Akt, JNK and Erk1/2 but not p38 (Figure 24). Surprisingly, STAT3 phosphorylation was enhanced after particulate β-glucan stimulation, opposite from PMN-MDSC. Pretreatment of M-MDSC with WGP in the presence of MEK1/2 inhibitor (PD98059) abrogated the acquired Ag-presenting capability of differentiated M-MDSC (Figure 25). In addition, M-MDSC treated with particulate β-glucan for 7 days increased the expression of TNF-α, IL-12, iNOS and IL-6, and decreased TGF-β compared to freshly sorted M-MDSC (Figure 26). Enhancement of TNF-α and IL-12 mRNA expression was completely abrogated in dectin-1 KO M-MDSC upon particulate β-glucan stimulation (data not shown).
Figure 19. Particulate β-glucan induces M-MDSC differentiation and the expression of costimulatory molecules. (A) Expression of CD11c, F4/80 and CD11b surface markers on M-MDSC cultured with particulate β-glucan (50 μg/ml) for 0, 5 and 7 days. Results were repeated at least three times with similar results. (B) Expression of Gr-1, CD80, CD86, MHC class II, MHC class I, and CD40 surface markers on M-MDSC cultured with particulate β-glucan (50 μg/ml) for 7 days. Histograms represent the results of three independent experiments.
Figure 20. M-MDSC do not present Ag to CD4+ T cells. Frequency of IFN-γ+ CFSE-gated on CD4+ T cells in cultures where freshly sorted M-MDSC from the spleens of LLC-bearing mice were incubated for 5 days with CFSE-labeled CD4+ T cells in the presence of OVA (100μg/ml). Splenocytes were used as APC positive control.
Figure 21. Differentiated M-MDSC with particulate β-glucan present OVA Ag to OVA-specific CD4+ T cells. Sorted and CFSE-labeled CD4+ OT-II T cells were co-cultured with splenic M-MDSC pre-cultured with particulate β-glucan for 7 days in the presence of OVA (50 μg/ml) for 4-5 days. The frequencies of CFSE diluted cells and IFN-γ+ CD4+ T cells were shown. Splenocytes were used as APC positive control. The results are representative of at least four independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 22. Differentiated M-MDSC with particulate β-glucan present OVA Ag to OVA-specific CD8+ T cells. Sorted and CFSE-labeled CD8+ OT-I T cells were co-cultured for 4-5 days with OVA (50 μg/ml) and splenic M-MDSC pre-cultured with particulate β-glucan for 7 days. The frequencies of CFSE diluted cells, IFN-γ+ cells and Granzyme B+ cells gated on CD8+ T cells are demonstrated. Results are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 23. Particulate β-glucan-induced M-MDSC antigen-presenting function is dectin-1 dependent. (A) CD4+ and CD8+ T cells were sorted from OT-II and OT-I mice, respectively, CFSE-labeled and co-cultured for 4-5 days with M-MDSC sorted from the spleens of WT or dectin-1 KO LLC-bearing mice. M-MDSC were pre-treated with particulate β-glucan for 7 days prior to co-culture with CD4+ or CD8+ T cells. The frequency of CFSE-IFN-γ+, gated on CD4+ T cells (upper panel) or CD8+ T cells (lower panel), are represented in the dot plots. Results are representative of two-independent experiments. *p<0.05, **p<0.01.
Figure 24. Particulate β-glucan induces p-STAT3 and Syk downstream dectin-1 in M-MDSC. Western blot analysis of p-STAT3, p-Zap/Syk, p-Akt, p-SAPK/JNK, p-Erk1/2, p-p38, STAT3 and β-actin in M-MDSC sorted from the spleens of LLC-bearing mice and stimulated with particulate β-glucan (100 μg/ml) for 0, 15, 30 and 60 minutes.
Figure 25. Particulate β-glucan induces M-MDSC differentiation to APC in an Erk1/2-dependent manner. CD4+ T cells sorted from OT-II mice were CFSE-labeled and co-cultured with OVA and M-MDSC pre-treated with particulate β-glucan in the presence of MEK1/2 inhibitor (PD98059) (30 ng/ml) or DMSO for 7 days. Data demonstrate the frequencies of CFSE diluted cells and IFN-γ+ cells gated on CD4+ T cells. Results are representative of two independent experiments.
Figure 26. Differentiated M-MDSC express pro-inflammatory cytokines upon particulate β-glucan treatment. The relative mRNA expression of TNF-α, IL-12, iNOS, IL-6, and TGF-β in MDSC, sorted from the spleens of LLC-bearing mice, and incubated with particulate β-glucan for 7 days compared to its expression in freshly sorted M-MDSC. *p<0.05, **p<0.01, ***p<0.001.
Particulate β-glucan-treated M-MDSC do not enhance tumor growth and development *in vivo*

To evaluate the effect of particulate β-glucan-treated M-MDSC on tumor development in *in vivo*, we performed admixture experiments with M-MDSC/tumor cells. LLC cells were mixed with M-MDSC or M-MDSC treated with particulate β-glucan for 18 hours at a 1:1 ratio, and injected subcutaneously into C57BL/6 mice. M-MDSC but not particulate β-glucan-treated M-MDSC induced tumor progression and growth compared to LLC alone (Figure 27A). In addition, LLC/M-MDSC tumors had a significantly higher weight compared to tumors implanted with LLC/ particulate β-glucan-treated M-MDSC (Figure 27B).

**Particulate β-glucan treatment reduces the frequency of HLA-DR·CD14− CD11b+CD33+ MDSC in the peripheral blood of NSCLC patients**

To imply the significance of the current findings into human patients, we conducted a particulate β-glucan clinical trial in patients with non-small cell lung cancer (NSCLC) that were newly diagnosed and had not received any treatment. Particulate β-glucan was administered orally for two weeks at 500 mg dose and blood was withdrawn before and after treatment. Consistent with previous studies (40, 41), the frequency of HLA·CD14− CD33+CD11b+ MDSC was substantially increased in the peripheral blood of patients with NSCLC compared to those in age and sex matched healthy donors (Figure 28). Particulate β-glucan treatment significantly reduced the percentage of HLA-DR·CD14·CD33·CD11b+ MDSC in the peripheral blood of NSCLC patients when compared to its frequency in the peripheral blood before treatment (Figure 29).
Next, we sought to determine the Ag-presenting capability of CD14^+HLA-DR^+ monocytes and CD14^+HLA-DR^+CD11b^+CD33^+ MDSC in a mixed lymphocyte reaction as previously described (265). CD14^+ HLA-DR^+ monocytes significantly induced the proliferation and IFN-γ production of allogeneic T cells, whereas CD14^+ HLA-DR^-CD11b^-CD33^+ MDSC induced minimal response (Figure 30). Next, we sought to assess the effect of particulate β-glucan treatment on the Ag-presenting capability of CD14^+HLA-DR^+ monocytes and CD14^+HLA-DR^-CD11b^-CD33^+ MDSC from NSCLC patients. Particulate β-glucan treatment induced an enhanced trend in proliferation and IFN-γ production by allogeneic T cells in both populations (Figure 31). In addition, IFN-γ production by T cells from patients after treatment were also trending enhanced although it did not reach statistical significance (data not shown).

In NSCLC, Arginase 1 expression was reported to be expressed by CD14^-CD11b^-CD33^+ SSC^high PMN-MDSC (41, 266). To assess the effect of particulate β-glucan treatment on the function of PMN-MDSC, we compared the expression of Arginase 1 mRNA in the PMN-isolated from the peripheral blood of NSCLC patients before and after particulate β-glucan treatment. The expression of Arginase 1 mRNA was significantly decreased in a cohort of 15 patients, and became comparable with Arginase 1 mRNA expression in healthy controls (Figure 32 left panel), whereas it did not significantly change in the other cohort of 20 patients (Figure 32 right panel). Overall, NSCLC patients have a decreased frequency of CD14^+HLA-DR^-CD11b^-CD33^+ MDSC with improved allogeneic effector T cell responses after particulate β-glucan treatment.
Overall we delineated the effect of particulate β-glucan treatment on MDSC from tumor-bearing mice (Figure 33) and assessed the *in vivo* effect of particulate β-glucan treatment in NSCLC patients.

**Figure 27.** Particulate β-glucan-treated M-MDSC do not promote tumor progression when co-implanted with tumor cells *in vivo*. (A) Splenic M-MDSC sorted from LLC-bearing mice treated with or without particulate β-glucan (100 μg/ml) for 18 hours were mixed with LLC cells at 1:1 ratio and co-implanted s.c. in C57BL/6 mice in matrigel (n=6). Tumors were measured every two days and mice were sacrificed on day 25 post-inoculation. Tumor volumes are shown. (B) Tumor weight (grams) from mice injected with LLC or LLC+M-MDSC or LLC+M-MDSC treated with WGP. *p<0.05, **p<0.01,** **p<0.001.
Figure 28. MDSC frequency is enhanced in the peripheral blood of patients with NSCLC compared to healthy donors. Frequency of CD33+CD11b+ MDSC gated on CD14-HLA-DR- cells in the peripheral blood of NSCLC patients (n=21) compared to age and sex matched healthy donors (n=13). **** p<0.0001.
Figure 29. Particulate β-glucan treatment decreases the frequency of MDSC in the peripheral blood of NSCLC patients. Frequency of CD33+CD11b+ MDSC gated on CD14-HLA-DR- cells in the peripheral blood of NSCLC patients 28 before and after particulate β-glucan treatment for 14 days (n=22). *p<0.05.
Figure 30. CD14+ HLA-DR+ but not CD14+HLA-DR-CD11b+CD33+ induce IFN-γ production and proliferation of allogeneic T cells. IFN-γ production and proliferation (CFSE diluted cells) of allogeneic T cells (CD3+) cultured at 1:1 ratio with CD14+HLA-DR+CD11b+CD33+ or CD14-HLA-DR-CD11b+CD33+ sorted from the peripheral blood of NSCLC patients.
Figure 31. Particulate β-glucan induces and enhances trend of allogeneic T cell responses upon co-culture with CD14+ or CD14- cells. IFN-γ production and proliferation (CFSE diluted cells) of allogeneic T cells (CD3+) cultured at 1:1 ratio with CD14+HLA-DR-CD11b-CD33+ or CD14-HLA-DR-CD11b+CD33+ isolated from the peripheral blood of NSCLC patients before and after particulate β-glucan treatment (n=11).
Figure 32. Particulate β-glucan treatment decreases the mRNA expression of arginase 1 in PMN from the peripheral blood of 15 patients. Relative Arginase1 mRNA expression levels in neutrophils (PMN) isolated from the peripheral blood of healthy controls, and patients with NSCLC before and after particulate β-glucan treatment. Arginase 1 relative mRNA expression significantly decreased in a cohort of 15 patients, whereas no significant change was reported in the other 20 patients (total n=35). *p<0.05.
Figure 33. Dual effect of particulate β-glucan (WGP) on MDSC subsets. Particulate β-glucan treatment induces PMN-MDSC cytotoxic phenotype with an enhanced respiratory burst and subsequent apoptosis. Whereas M-MDSC differentiated to potent APC upon particulate β-glucan treatment. In both subsets, particulate β-glucan reduces MDSC-mediated T cell suppression.
DISCUSSION

Despite the significant advances in our understanding of how the immune system is ‘programmed’ to fight disease, the generic mechanisms of immunity are still elusive. The obscurity of the detailed mechanisms by which the immune system functions does not necessarily imply that our knowledge of how it really works is minimal, but that there is still many facts to be revealed. An important aspect to be mentioned in this line vis à vis our study, is the role that the immune system plays in cancer. It was not until 2011 that Hanahan and Weinberg added ‘evasion of immunological destruction’ by tumor cells as an ‘emerging’ hallmark of cancer (267).

The reductionist view of the tumor as a whole, as an autonomous, uniform and homogenous entity, is part of the past. It is now well appreciated that alongside with hyperproliferating and heterogeneous cancer cells, cancer stem cells, endothelial cells, pericytes and fibroblasts lie the tumor-infiltrating immune cells, and altogether constitute the tumor microenvironment (267, 268).

Our increased understanding of the mechanisms governing tumor progression has revealed that tumor-derived factors create a suppressive milieu within the immune cell pool in the tumor microenvironment driving immune suppression. The idea that immune cells can have a pro-inflammatory or anti-inflammatory phenotype describes the bipolarity of immune cells and their plasticity (269-271). However, the generalization of the concept of
the existence of two extreme phenotypes seems imprecise when considering the various factors present in the tumor microenvironment during different stages of progression that have the potential to generate a spectrum of activation states in the immune cells (272).

Almost every immune cell has been associated with a regulatory phenotype, among them are: TAMs (269, 271), regulatory T cells (Treg) (273, 274), tolerogenic DCs (275, 276), NK cells tolerant (277), regulatory B cells (278), regulatory γδ-T cells (165, 166), TANs (161) and MDSC (33). The notorious phenotype associated with many immune cells in the fight against tumor maybe challenged by the view that the acquisition of an anti-inflammatory phenotype in the persistence of Ag or inflammation, is essential for the persistence of the immune cells for a longer fight. That is, exhaustion might be the shortest metabolic path for an immune cell to survive in such situation (279), meaning that a continuous evolving and adapting immune system will pay a price by entering a state of exhaustion in order to stay alive and in the battle. This radical view implies that the interference with immunotherapy, in order to succeed, has to be applied at the right time and at the right phase during disease progression.

Numerous therapeutic agents to target the tumor environment are present in the clinic. Many of which have not been applied in humans or have been reported to have off target effects. Our notion of the importance of the usage of natural compounds as potent biological response modifiers have been increasing with our understanding of the importance of targeting immune cells that infiltrate the tumor microenvironment. In this study, the therapeutic agent particulate β-glucan was applied in two animal models of subcutaneous tumor, mouse lung carcinoma and mammary carcinoma, and in newly
diagnosed patients with NSCLC. Particulate β-glucan is a natural compound, of low cost and can be orally ingested.

Previously, we delineated the potent adjuvanticity of natural compound particulate β-glucan on T cells through the modulation of DCs (49, 130) and macrophages (unpublished data). Recently, it was also reported that particulate β-glucan modulates immunosuppression by partially inducing the differentiation of M-MDSC in tumor-bearing mice (50). However, in the latter study the attribution of the decreased in tumor growth to M-MDSC differentiation was not well-reflected in the *in vitro* differentiation assays in which only 8-13% of the cells acquired F480<sup>+</sup>CD11c<sup>+</sup> expression. In addition, the acquisition of these surface markers does not reflect MDSC functionality, since the only functional assays performed in the study where in an antigen non-specific system, in which purified CD4<sup>+</sup> T cells where cultured with M-MDSC pre-treated with particulate β-glucan. An enhancement in CD4<sup>+</sup> T cell proliferation in these assays can only be attributed to the effect of particulate β-glucan on soluble factors that mediate MDSC suppression but not due to the acquisition of APC capability.

Herein, we demonstrate that treatment with yeast-derived particulate β-glucan, reduces tumor growth and differentially modulates PMN-MDSC and M-MDSC frequencies in tumor-bearing mice. Especially that we also showed that PMN-MDSC are the main cells expanding during tumor progression as reported by others (40). Interestingly, particulate β-glucan had a higher impact on PMN-MDSC frequency compared to M-MDSC. In LLC and E0771 models, PMN-MDSC frequency significantly decreased in the spleens of treated animals. Meanwhile it had no effect on M-MDSC frequency. In the same line,
particulate β-glucan decreased PMN-MDSC while enhanced M-MDSC frequency in the tumors, suggesting that impacting PMN-MDSC in the spleen alone can cause a systemic shift towards an anti-tumor immune response (152, 160, 280). Moreover, M-MDSC enhanced percentage is also associated with an enhanced frequency of tumor-infiltrating macrophages after particulate β-glucan treatment. From this observation, and the fact that M-MDSC upon particulate β-glucan treatment lose their suppressive function and convert to APCs, implying that particulate β-glucan converts M-MDSC to anti-tumor immune cells despite of their enhanced frequency. That is also reflected by a significant decrease in tumor growth. In addition, total Gr-1⁺ CD11b⁺ cells failed to suppress antigen-specific CD4⁺ and CD8⁺ T cells when co-cultured with particulate β-glucan.

The differential modulation on both MDSC subsets induced by particulate β-glucan was also shown in its effect on the functions of both subsets. Firstly, we demonstrated that particulate β-glucan reverses PMN-MDSC suppression, induces respiratory burst and enhances apoptosis. On the second axis, particulate β-glucan skews the immature suppressive phenotype of M-MDSC towards a potent APC phenotype that drives the differentiation of Th1 CD4⁺ T cells and induces the differentiation of cytotoxic CD8⁺ T cells. On a third and most prevalent axis, particulate β-glucan was tested in patients newly diagnosed with NSCLC as an adjuvant therapy in cancer. This is the first clinical trial with particulate β-glucan in patients newly diagnosed with NSCLC that have not yet been subjected to any other treatment. It is important to note that particulate β-glucan is a natural compound with no reported off target effects, with a low cost and can be administered orally.
Particulate β-glucan induced PMN-MDSC respiratory burst early after activation and largely enhanced PMN-MDSC apoptosis, which positively correlates with the decreased frequency of PMN-MDSC in tumors and spleens upon particulate β-glucan treatment. Although ROS have been implied as one of the main mechanisms for T cell suppression (281) which is regulated by STAT3 activation (179), other reports suggest that PMN-MDSC can acquire a tumor cytotoxic phenotype in the absence of TGF-β (161). That goes in line with what we previously showed that TGF-β mRNA expression is reduced in tumors of mice treated with particulate β-glucan (49). In addition, activation of neutrophils with β-glucan, enhanced neutrophil cytotoxicity against Candida albicans (150). Interestingly, STAT3 phosphorylation levels in PMN-MDSC decreased after dectin-1/Syk activation with particulate β-glucan that might have led to PMN-MDSC apoptosis, and correlates with the findings that treatment of splenic Gr-1+CD11b+ from tumor-bearing mice with the JAK2/STAT3 inhibitor JSI-I24 in vitro for 7 days in the presence of GM-CSF and tumor-conditioned medium enhanced cell death compared to untreated cells (282).

Despite several studies reporting the anti-tumor effect of particulate β-glucan through the activation of innate immune cells and induction of Th1 T cell responses (49, 104, 130), little is known on the role of particulate β-glucan in the modulation of tumor-associated macrophages (TAMs), MDSC and regulatory T cells. It is reported that particulate β-glucan induces the differentiation of M-MDSC to F4/80+ CD11c+ cells with a decreased suppressive phenotype (50). However, this study showed that the differentiated F4/80+ CD11c+ cells represent only 8-13% of the gated M-MDSC population cultured for 48 hours in the presence of GM-CSF. Whereas, Youn et al showed that GM-CSF alone could induce the expression of CD11c to around 35% and F4/80 to around 60% in splenic M-MDSC at
day 3 (32). In our study, the differentiation of M-MDSC to potent APC was only induced when cultured with a pharmaceutical grade particulate β-glucan (Biothera) that contains >99% β-glucan rather than a less pure particulate β-glucan. It is important to consider that different compositions of β-glucan particles could highly impact immune cell function (104). In addition, expression of F4/80 or CD11c surface markers does not necessarily imply a non-suppressive phenotype, since it has been shown that MDSC do differentiate to suppressive F4/80+ cells in the tumor microenvironment(283) (39, 154). The increased frequency of M-MDSC in the tumors of particulate β-glucan treated mice correlates with a previous report that particulate β-glucan treatment increases the frequencies of F4/80+ and CD11c+ cells in the tumors of treated mice (49) and since particulate β-glucan reduced tumor growth it implies that the function of these cells would also be skewed towards an anti-tumor phenotype.

We also demonstrated that dectin-1 receptor signaling is required for the acquisition of Ag-presenting function, since M-MDSC from dectin-1 KO mice treated with particulate β-glucan for 7 days did not acquire such capability. Although STAT3 phosphorylation has been shown to be a key mechanism for MDSC-mediated suppression (284), we found that activation of STAT3 in M-MDSC did not impact the reversal of suppression and acquisition of Ag-presenting phenotype, maybe largely due to the shift in the cytokine profile towards an ‘M1-like’ phenotype with enhanced expression of IL-12, TNF-α, iNOS and inhibition of TGF-β, in addition to the upregulation of MHC class II, MHC class I and the costimulatory markers such as CD86 and CD40. However, the Erk inhibitor completely abrogated the conversion of M-MDSC to APC mediated by particulate β-glucan.
It has been well documented that MDSC accumulate in different human cancers such as brain (285, 286), head and neck (287), breast (228), lung cancer (255, 288) and others (reviewed in (33, 42, 289)). In many of these studies, the accumulation of MDSC in patients was correlated with poor prognosis. In the current study, newly diagnosed patients with NSCLC were given particulate β-glucan orally for two weeks. Treated patients had a significant decrease in the percentage of CD14+ HLA-DR−CD11b+CD33+ MDSC in the peripheral blood. Importantly, these patients were not exposed to any therapy during the administration of particulate β-glucan, which allowed the sole assessment of particulate β-glucan efficacy in patients. In addition, an enhanced trend in IFN-γ production by CD3+ cells and T cell allogeneic responses to CD14+HLA-DR+ and CD14+HLA-DR−CD11b+CD33+ cells was observed after treatment. The administration of particulate β-glucan adjuvant for only two weeks may not have been enough to modulate APC function in vivo, however it was sufficient to reduce the percentages of CD14+HLA-DR−CD11b+CD33+ MDSC in the peripheral blood.

The expression of arginase I in PMN-MDSC has been reported in NSCLC patients and correlated with poor prognosis and CD8+ T cell suppression (41, 266). Interestingly, a cohort of 15 patients had a significant decreased expression of arginase I in PMN from the peripheral blood while no significant change was reported in the other 20 patients subjected to particulate β-glucan treatment. The patients with decreased arginase I after particulate β-glucan had a higher trend in arginase I expression compared to healthy controls before the treatment. Unlike the other patients, the level of arginase I was comparable to healthy controls, suggesting that patients’ variable responses to the treatment might have been due to different stages, tumor sizes, and progression. Nevertheless, this study provides a first
insight towards introducing particulate β-glucan as an adjuvant therapy against solid tumors in humans.
Since their discovery in 1999, MDSC became a great focus of researchers. More than 3500 studies have been published containing the key words ‘myeloid suppressor cells’. However, the definition of MDSC is still elusive. Amidst the many components in the tumor microenvironment, immune cell functions are being constantly reshaped by tumor-derived factors that mainly switch a large portion of myeloid cells to immune suppressors. Despite numerous studies describing myeloid cells as key players during tumor development, their origin, markers of suppression and biology in different cancers is prompted to vast exploration, especially in humans. Our definition of TAM and MDSC does not reflect their heterogeneity. In the microenvironment pool, the presence of a spectrum of phenotypes varying between highly suppressive and least suppressive makes it difficult to specifically target or re-educate these cells. In this line, it becomes of great importance to identify a specific marker of suppression that distinguishes inflammatory monocytes/granulocytes from MDSC. In addition, it is still questionable whether all suppressive cells are immature and whether a signature for immaturity and its relation to suppression can be identified in the future. In addition, MDSC in humans is still elusive, especially that humans lack the Gr-1 homolog. The use of generic myeloid markers such as CD11b and CD33 does not really differentiate myeloid cells with a suppressive
phenotype from the ones with an anti-tumor phenotype. Therefore, it becomes of great importance to define master transcriptional factors that regulate immune cell suppression.

The need to redefine the transcriptional factors that regulate immune cell fate has led to the establishment of large-scale projects such as the Immunological Genome Consortium and the Human Immunology project consortium. High-throughput data analysis in such projects started to reshape our understanding of the complex systems governing immune cell functions in steady-state and disease onset in mice and humans. It would be of great significance to establish large-scale studies to define the phenotype of the myeloid cell compartments in the tumor tissue and peripheral blood in a large cohort of patients with different types of cancer to elucidate the role of myeloid cells and further define the transcriptional factors that regulate their suppressive phenotype. More studies using the yeast-one hybrid system approach and a large-set screening of different cDNA libraries might be needed to further narrow down key transcriptional factors regulating MDSC suppression.

More than a century after Coley’s vaccine, cancer immunotherapy has reached its golden age. The list of immunotherapies approved by the FDA has significantly increased over the last decade. Monoclonal antibodies such as anti-CTLA-4, anti-PD1 and anti-CD20 have been widely introduced in cancer therapy, and CAR (chimeric antigen receptor) T cell therapy with its different versions has also made its way to the clinic. Emphasizing that targeting tumor cells with chemotherapy is not the only key to eradicate tumors, but re-educating immune cells, and blocking suppressive checkpoints is proven to be a better way to fight tumor. The formulation of cancer vaccines with adjuvants is well described to help
providing ‘signal 3’ in antigen-specific adaptive immune responses. The study of better vehicles for cancer vaccines is being extensively studied. An ‘ideal adjuvant’ is generally described as having no off-target effects, promotes priming of antigen-specific immune cell responses and does not cause any associated immune cell suppression. In this study, we demonstrated that particulate β-glucan, a natural compound with no reported off-target effects, has the ability to reverse MDSC suppression in mice, and reduce their frequency in the peripheral blood in patients with NSCLC, suggesting that particulate β-glucan might be a strong candidate to be used in combination with other immunotherapies. Overall this study, with its different dimensions, provides evidence that MDSC can be re-educated to acquire an anti-tumor phenotype.

This study has answered many questions but also opened the doors to the establishment of many projects in the future to further delineate the transcriptional regulators that define the acquisition of antigen-presenting function in M-MDSC, the metabolomic enzymes and metabolites that maybe activated upon dectin-1 activation in MDSC subsets, the effect of dectin-1 activation on MDSC-mediated tumor cell metastasis, the effect of particulate β-glucan on the exosome profile from MDSC and how that might shape tumor cell responses, and many other questions of great importance that still need to be elucidated to allow the development of new therapeutic strategies².

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PUBLICATIONS
Published articles

Articles under review

Book Chapters

ABSTRACTS AND PRESENTATIONS
Oral Presentations
1- Dectin-1 activation subverts the suppression of myeloid-derived suppressor cells by inducing PMN-MDSC apoptosis and monocytic MDSC differentiation to potent


Poster presentations


LABORATORY SKILLS

In vivo main skills:

- Mice: Maintaining mice colonies, breeding, genotyping, handling tumor-bearing mice, intraperitoneal, intravenous, footpad and subcutaneous injection, blood and organ collection
- *In vivo* T cell proliferation assays
- *In vivo* imaging

Expert at:

- Flow cytometry
- Cell sorting from tissues and cell lines: FACS sorting, magnetic beads cell sorting
- Cell culture: culture of primary cells, tumor cell lines
- Processing of human blood and human tumors for FACS analysis
- DNA and RNA isolation: PCR and quantitative RT-PCR
• ELISA (enzyme-linked immunosorbent assay)
• Lymphocyte proliferation assays, mixed-lymphocyte reaction assays, antigen proliferation assays, antigen-presentation assays

*Competent at:*

• Western blot
• Confocal microscopy

*Instruments used:*

• Flow cytometry: FACS Calibur, FACS Canto II
• Cell sorting: FACS Aria III, AutoMacs Cell Sorter
• *In vivo* Imaging: Advanced Molecular Imaging (AMI) Systems