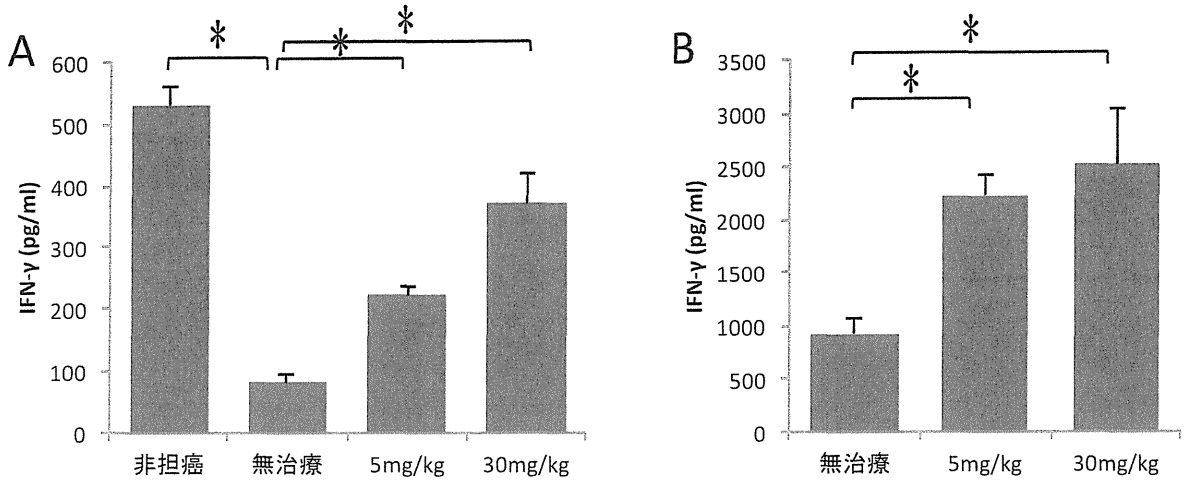

図４１　脾臓(A)、腫瘍組織内(B)より樹状細胞(CD11c 陽性細胞)を分離し、Balb/c 由来のナイーブ T 細胞と CD3 抗体存在下で 3 日間培養し、T 細胞より産生される IFN-γを ELISA で測定した。n=4。*<0.05

以上のことから、漢方成分 No. 19 および漢方成分 No. 19s は、卵巣明細胞性腺がんにおいて、IL-6 産生を抑制し、DC の機能を回復させ、免疫抑制を解除できる可能性が示された。本研究の結果をうけ、現在、卵巣がん患者で、漢方成分 No. 19s を用いた IL-6 の抑制による悪液質の改善を目的とした臨床試験を、企業 A と共同で計画している。

4.3) 漢方成分 No.23

昨年度までの解析において、漢方成分 No. 23 は JHOC5 からの IL-6、888mel、624mel からの IL-10 産生を抑制し、624mel からの VEGF と PK59 からの TGF-βに対しても若干の抑制効果を示した。AhR に関しては、MCF7 では弱いアンタゴニスト活性を示したが、B16F10 では AhR を強く抑制していた（図７）。マウス脾臓細胞からの *in vitro* での iTreg 誘導を抑制するが Th1 誘導は阻害しない特徴を示した。Balb/c マウスに CT26 を移植した担がんマウスへの漢方成分 No. 23 投与により投与開始早期から腫瘍の増殖を抑制する傾向が見られた。腫瘍中で $CD4^+$ T 細胞の頻度の増加、局所リンパ節での NK/NKT 細胞の頻度の増加が見られていた。Treg 細胞頻度はどの臓器においても差が見られなかった。漢方成分 No. 23 投与群の脾臓細胞から誘導した腫瘍抗原特異的な T 細胞では対照群と比較して抗原特異的 IFN-γ産生量が若干増加していた。今年度は、C57BL/6 担がんマウスを用いて昨年度の結果を検証するとともに、細胞分画のより詳細な解析を試みた。

結果

C57BL/6 マウスにがん細胞 MC38 を皮下移植し、腫瘍が生着した 5 日目から 200μg の漢方成分を 2 日に 1 回腹腔内投与した。漢方成分 No. 23 投与群は DMSO 投与群と比較して、有意に腫瘍増殖を抑制することが確認できた（図４２A)。移植後 18 日目の脾臓細胞に腫瘍抗原 gp70 のペプチドをパルスして誘導した抗原特異的 T 細胞は、gp70 発現陰性の腫瘍細胞 EL4 に比べて gp70 発現陽性の腫瘍細胞 MC38 に対して高い IFN-γを産生するが、その産生量は漢方成分 No. 23 治療群において上昇してはいなかった（図４２B)。

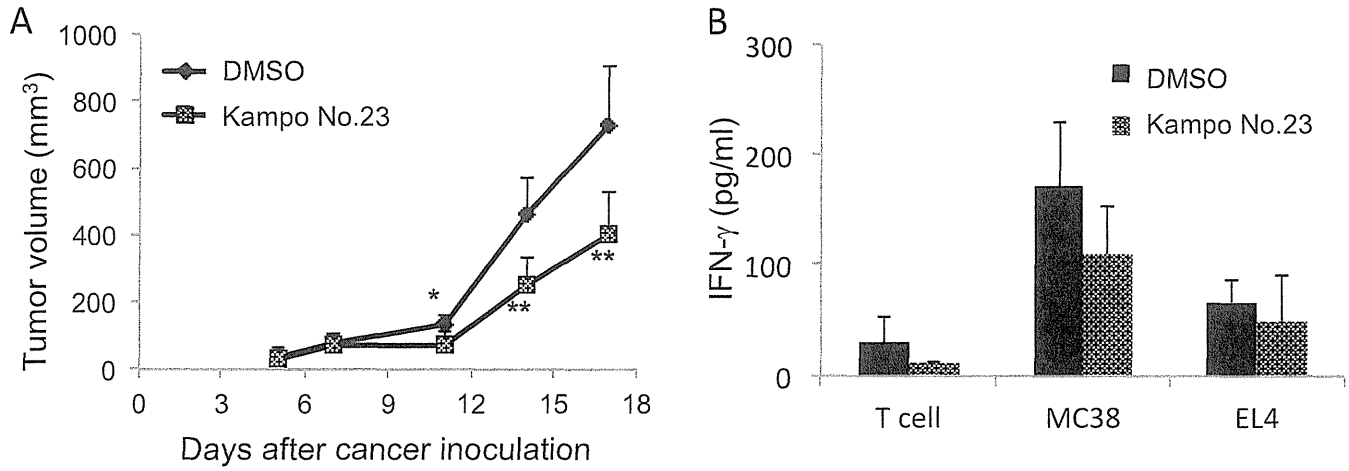

図４２　MC38 担がんマウスモデルに対する漢方成分 No. 23 の治療効果

各群の治療後の脾臓、腫瘍組織における免疫細胞分画を解析したところ、末梢の脾臓細胞では、漢方成分 No. 23 投与群において CD3 陽性 T 細胞の割合の増加傾向、$CD11b^{+}Gr\text{-}1^{+}$の MDSC 細胞の割合の減少、Treg 細胞の減少傾向が見られたが、腫瘍浸潤リンパ球では、漢方成分 No. 23 投与群において、CD3 陽性 T 細胞と CD11c 陽性樹状細胞の割合が減少傾向にあった（図４３）。

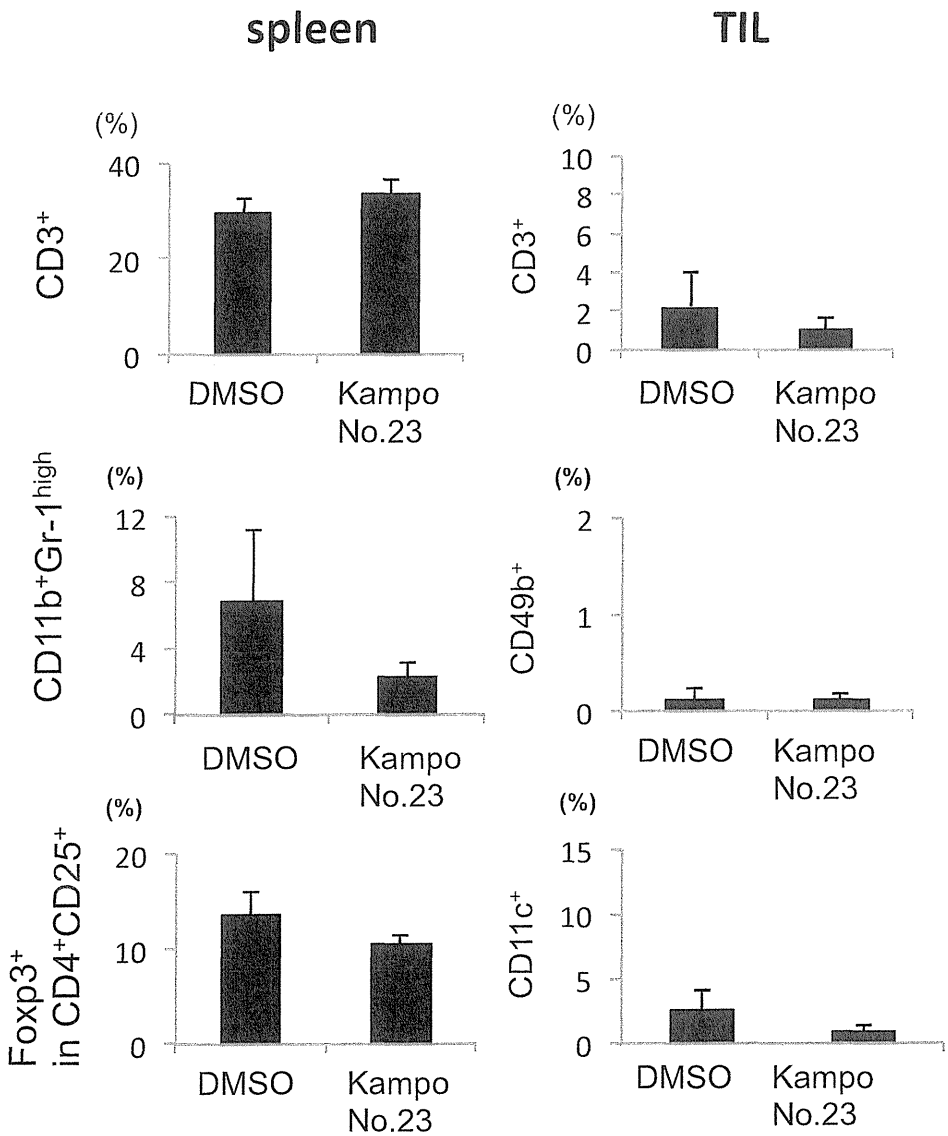

図４３　漢方成分 No. 23 投与による脾臓、腫瘍組織における免疫細胞分画の変化

考察

これまでの解析から漢方成分 No. 23 は JHOC5 からの IL-6、888mel、624mel からの IL-10 産生を抑制し、AhR に対してもアンタゴニスト活性を示した。888mel を漢方成分 No. 23 存在下で培養すると STAT3 と ERK1/2 のリン酸化が減少することも昨年度示した。マウス脾臓細胞からの iTreg 誘導を抑制するが Th1 誘導は阻害しないという抗腫瘍免疫に有利な特徴も示した。これらの *in vitro* の結果に基づき、担がんマウスへの投与による治療効果の解析を進めてきた結果、Balb/c マウスに CT26 を移植した場合と C57BL/6 マウスに MC38 を移植した場合の両方において、漢方成分 No. 23 投与により腫瘍の増殖は抑制された。脾臓細胞から誘導した腫瘍抗原特異的な T 細胞の解析では、Balb/c マウスに CT26 を移植した系では漢方成分 No. 23 投与により抗原特異的 IFN-γ産生量が若干増加していたが、C57BL/6 マウスに MC38 を移植した系では抗原特異的 IFN-γ産生量の増加は見られなかった。今年度の解析では、末梢において、CD3 陽性 T 細胞の割合の増加傾向、$CD11b^{+}Gr\text{-}1^{+}$ の MDSC 細胞の割合の減少、Treg 細胞の減少傾向がみられたものの、腫瘍組織では T 細胞や樹状細胞の浸潤が減少傾向にあり抗腫瘍免疫応答の増強にはつながっていないと考えられた。漢方成分 No. 23 は、がん細胞の STAT3 や ERK といったシグナル経路を阻害する働きがあることが示唆され、腫瘍の増殖にかかわるこれらの因子に影響を及ぼした結果、腫瘍増殖を抑制したのではないかと考えられた。

4.4) 漢方成分 No.24

漢方成分 No. 24 は JHOC5 からの IL-6、888mel、624mel からの IL-10 産生を抑制し、624mel からの VEGF と PK59 からの TGF-βに対しても若干の抑制効果を示した。このように、サイトカインに対する効果は漢方成分 No. 23 と類似した挙動を示した。また、MCF7 と B16F10 の両方において、AhR に対する強いアンタゴニスト活性を示した。マウス脾臓細胞からの *in vitro* での iTreg 誘導および Th1 誘導に対しては顕著な作用は認められなかった。Balb/c マウスに CT26 を移植した担がんマウスへの漢方成分 No. 24 投与により腫瘍の増殖を顕著に抑制する傾向が見られた。このとき、脾臓中で $CD8^{+}$T 細胞と NKT 細胞の頻度が増加する傾向、腫瘍中の Treg 細胞頻度が減少する傾向が見られた。漢方成分 No. 24 投与群の脾臓細胞から誘導した腫瘍抗原 AH-1 特異的な T 細胞では対照群と比較して抗原特異的 IFN-γ産生量が顕著に増加していた。今年度は、C57BL/6 担がんマウスを用いて昨年度の結果を検証するとともに、細胞分画のより詳細な解析を試みた。

結果

C57BL/6 マウスにがん細胞 MC38 を皮下移植し、腫瘍が生着した 5 日目から 200μg の漢方成分を 2 日に 1 回腹腔内投与した。漢方成分 No. 24 投与群は DMSO 投与群と比較して、有意に腫瘍増殖を抑制することが確認できた（図４４A)。移植後 18 日目の脾臓細胞に腫瘍抗原 gp70 のペプチドをパルスして誘導した抗原特異的 T 細胞は、gp70 発現陰性の腫瘍細胞 EL4 に比べて gp70 発現陽性の腫瘍細胞 MC38 に対して高い IFN-γを産生するが、その産生量は漢方成分 No. 24 治療群において上昇していた（図４４B)。

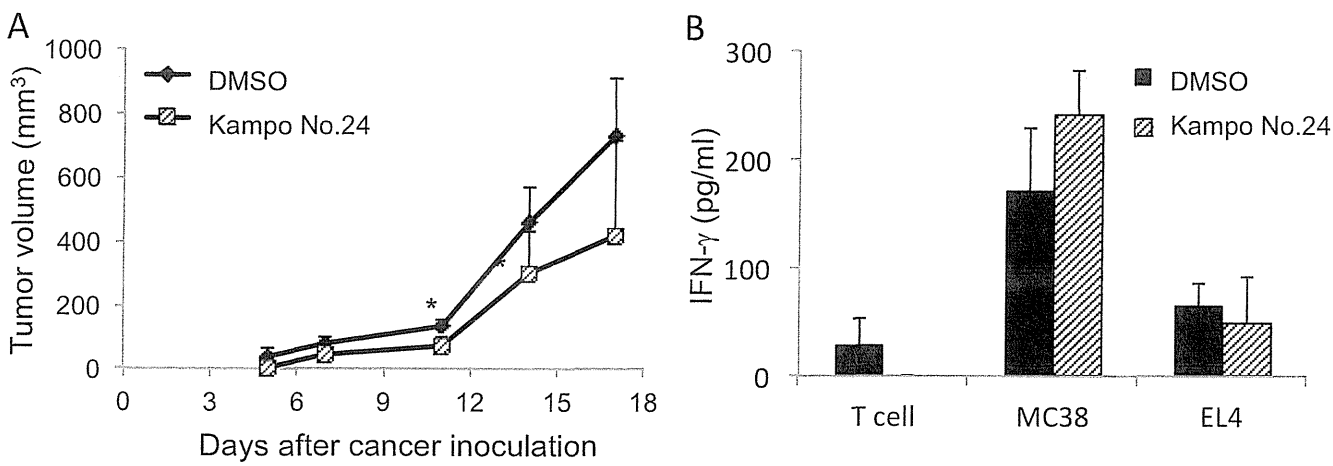

図４４　MC38 担がんマウスモデルに対する漢方成分 No. 24 の治療効果

各群の治療後の脾臓、腫瘍組織における免疫細胞分画を解析したところ、末梢の脾臓細胞では、漢方成分 No. 24 投与群において CD3 陽性 T 細胞の割合の増加傾向、$CD11b^{+}Gr\text{-}1^{+}$の MDSC 細胞の割合の減少、Treg 細胞の

減少傾向が見られ、さらに、腫瘍浸潤リンパ球では、漢方成分 No. 24 投与群において、CD3 陽性 T 細胞、NK/NKT 細胞、樹状細胞の割合の増加が見られた（図４５）。

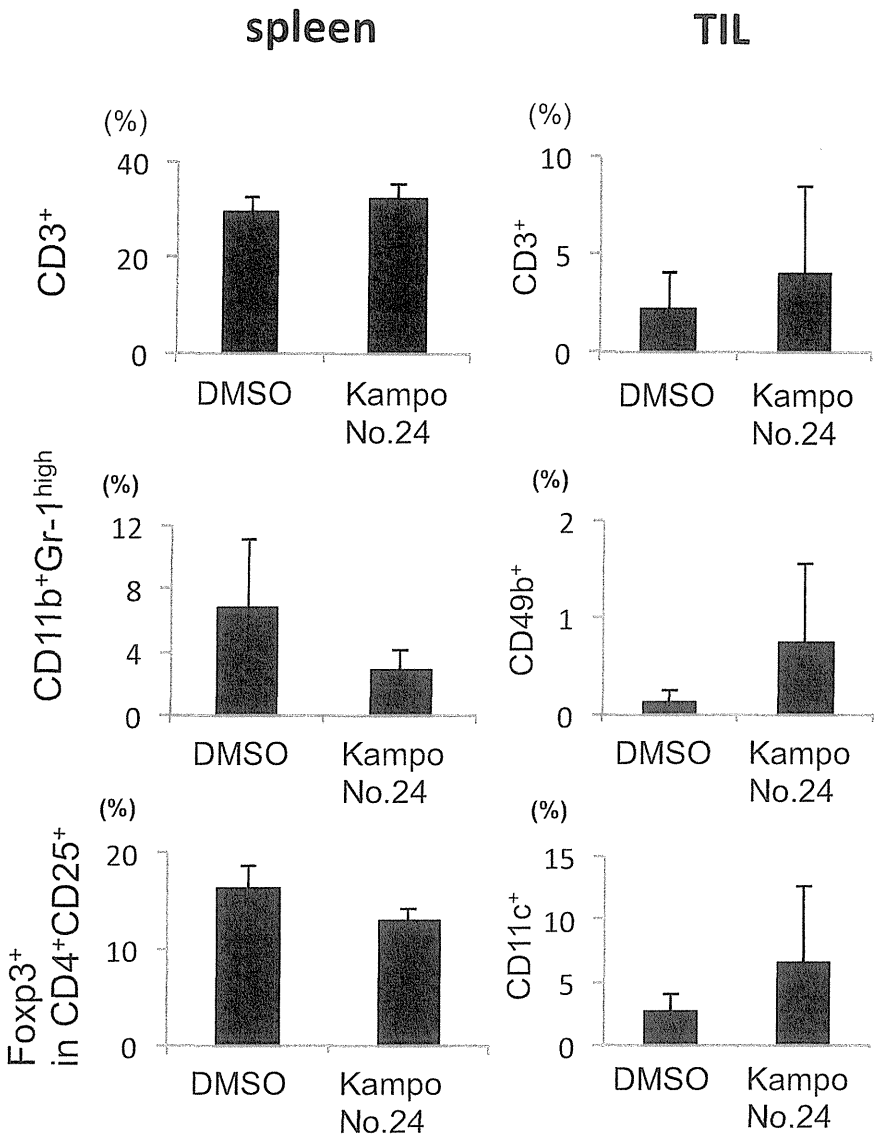

図４５　漢方成分 No. 24 投与による脾臓、腫瘍組織における免疫細胞分画の変化

考察

漢方成分 No. 24 は漢方成分 No. 23 と非常に類似した化学構造をもつ化合物である。これまでの解析から漢方成分 No. 24 は JHOC5 からの IL-6、888mel、624mel からの IL-10 産生を抑制し、サイトカインに対する効果は漢方成分 No. 23 と類似した挙動を示した。また、AhR に対して強いアンタゴニスト活性を示した。888mel を漢方成分 No. 24 存在下で培養した際の STAT3 と ERK1/2 のリン酸化は漢方成分 No. 23 よりも少ない程度ながら減少させた。ただし、マウス脾臓細胞からの *in vitro* での iTreg 誘導および Th1 誘導に対しては顕著な作用は認められなかった。これらの *in vitro* の結果に基づき、担がんマウスへの投与による治療効果の解析を進めてきた結果、Balb/c マウスに CT26 を移植した場合と C57BL/6 マウスに MC38 を移植した場合の両方において、漢方成分 No. 24 投与により腫瘍の増殖は顕著に抑制された。脾臓細胞から誘導した腫瘍抗原特異的な T 細胞の解析では、Balb/c マウスに CT26 を移植した系で漢方成分 No. 24 投与により抗原特異的 IFN-γ産生量が顕著に増加し、C57BL/6 マウスに MC38 を移植した系においても抗原特異的 IFN-γ産生量の増加が見られた。今年度の解析では、末梢において、CD3 陽性 T 細胞の割合の増加傾向、$CD11b^{+}Gr\text{-}1^{+}$の MDSC 細胞の割合の減少、Treg 細胞の減少傾向がみられた点は漢方成分 No. 23 と共通していた。漢方成分 No. 23 投与マウスの腫瘍組織では T 細胞や樹状細胞の浸潤が減少傾向にあり抗腫瘍免疫応答の増強にはつながっていないと考えられたのに対し、漢方成分 No. 24 投与マウスの腫瘍組織では CD3 陽性 T 細胞、NK/NKT 細胞、樹状細胞の割合の増加が見られ、抗腫瘍免疫応答の増強が起きている可能性が示唆された。漢方成分 No. 23 が、がん細胞の

STAT3 や ERK の阻害を介してがん細胞自体の増殖に影響を及ぼして腫瘍増殖を抑制したと考えられたのに対し、漢方成分 No. 24 はがん細胞自体の増殖に対する影響に加えて、抗腫瘍免疫応答の増強をも介して腫瘍の増殖を抑制させたのではないかと考えられる。

本研究では、「担がん生体の免疫抑制環境の改善」と「抗腫瘍免疫の増強」に有効な漢方成分を同定し、その作用機構を解明するとともに、それをリード化合物としてがん治療に応用可能な創薬につなげることを目的として研究を進めてきた。担がん生体のがん微小環境が免疫抑制的になる一つの要因は、がん細胞内でのシグナル伝達の異常に起因して起こるがん細胞からの免疫抑制性因子の放出や、それに付随した免疫抑制性細胞群の誘導と動員であり、がん細胞のシグナル伝達異常を解除することが免疫抑制環境の改善につながることを我々は示してきた。昨年度までの *in vitro* および *in vivo* での様々な解析結果をもとに、この目的に有効であろうと考えられた漢方成分について、本年度は担がんモデルマウスの治療実験を中心に進めた。

本年度の解析に用いた成分の内、漢方成分 No. 21、漢方成分 No. 35、漢方成分 No. 16、漢方成分 No. 19、漢方成分 No. 23、漢方成分 No. 24 で担がんマウスでの腫瘍増殖を抑制する効果がみられた。また、漢方成分 No. 25、漢方成分 No. 35、漢方成分 No. 16、漢方成分 No. 19、漢方成分 No. 24 で抗腫瘍免疫の増強効果がみられた。

本年度解析した漢方成分 No. 13、漢方成分 No. 22、漢方成分 No. 25、漢方成分 No. 19 は NF-κB 阻害作用をもっている。漢方成分 No. 16 は顕著に STAT3 と ERK を阻害する活性をもち、漢方成分 No. 16、漢方成分 No. 24、漢方成分 No. 15、漢方成分 No. 19、漢方成分 No. 22、漢方成分 No. 35 は AhR アンタゴニストとしての作用をもっている。NF-κB は正常な免疫応答においても重要な働きを担う転写因子であるため、その阻害が抗腫瘍免疫応答を弱める危険性も秘めている。漢方成分 No. 13、漢方成分 No. 22 でみられた腫瘍抗原特異的 T 細胞応答の減弱は免疫細胞の NF-κB に作用した弊害の可能性がある。しかしながら、漢方成分 No. 25 は腫瘍抗原特異的 T 細胞応答を有意に増強しており、現時点でその作用機序の違いは明らかでないが、漢方成分によっては抗腫瘍免疫応答の増強につながる可能性を示している。漢方成分 No. 25 は腫瘍組織への T 細胞の動員を促進する処置との併用が有効であると考えられる。漢方成分 No. 35、漢方成分 No. 16、漢方成分 No. 19、漢方成分 No. 24 はいずれも AhR アンタゴニストであり、AhR の阻害が腫瘍増殖の抑制と抗腫瘍免疫の増強の両方に効果的であることが示唆される。

本研究の結果、漢方成分 No. 16、漢方成分 No. 19、漢方成分 No. 24（および類似化合物である漢方成分 No. 23）が、がん細胞や免疫細胞のシグナル伝達分子や転写因子の阻害作用などを介して抗腫瘍免疫応答を制御できることを明らかにした。次のステップとして、これら 4 化合物をリード化合物として、より有効な化合物の開発することを検討中である。また漢方成分 No. 16 は、すでに既存薬としてがん以外の疾患に使用されており、漢方成分 No. 16 を、がん免疫病態制御作用の観点から、がん患者に対して臨床試験を実施することを検討している。漢方成分 No. 23 は、将来の臨床試験の可能性を検討するために、A 社から濃縮製剤を入手し検討中である。漢方成分 No. 19 は、世界的に様々な疾患で臨床試験が行われていたが、体内吸収性に問題があった。上記のように高吸収性製剤を開発した A 社と共同で、A 社の製剤がヒトがん細胞やマウス腫瘍モデルで抗腫瘍免疫制御作用をもつことを明らかにし、すでに臨床教室と共同でがんに対する臨床試験プロトコールの作成を進めている。臨床試験においては、他のがん治療との併用による治療効果の増強、また、NF-κB 抑制による悪液質誘導作用をもつ IL-6 などの炎症性サイトカインの産生低下による緩和治療目的での利用を検討している。

III. 研究成果

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IV. 研究成果の刊行に関する一覧表

書籍

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V. 研究成果の刊行物・別刷

Michael R. Shurin · Viktor Umansky
Anatoli Malyguine *Editors*

The Tumor Immunoenvironment

Springer

Chapter 12
Roles of Signaling Pathways in Cancer Cells and Immune Cells in Generation of Immunosuppressive Tumor-Associated Microenvironments

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Abstract Cancer cells trigger multiple immunosuppressive cascades and generate immunosuppressive tumor-associated microenvironments including tumor and sentinel lymph nodes. Constitutive activation of various signaling pathways (e.g., MAPK, STAT3, NF-κB, β-catenin) in human cancer cells was found to trigger the multiple immunosuppressive cascades through the production of immunosuppressive cytokines, such as TGF-β, IL-10, IL-6, and VEGF, and induction of immunosuppressive immune cells, such as regulatory T cells, tolerogenic dendritic cells, and myeloid derived suppressor cells. Some of these cancer-derived cytokines impair various immune cells through activation of their signaling molecules such as STAT3 and NF-κB. Inhibitors for these activated signals could inhibit the multiple immunosuppressive cascades by acting on both cancer cells and immune cells. Since common signaling mechanisms are often utilized for some of the hallmarks of cancer (e.g., cell proliferation/survival, invasion/metastasis, and immunosuppression), targeting these common signaling pathways may be an attractive strategy for cancer therapy, including immunotherapy.

Keywords Immunosuppression · BRAF · STAT3 · β-catenin · NF-κB · TGF-β · IL-10 · MAPK · MDSC · Regulatory T cells

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12.1 Introduction

We have previously identified various human tumor antigens recognized by T cells, and developed various antigen specific immunotherapies (Kawakami et al. 2004; Kawakami et al. 1994; Kawakami et al. 1994; Rosenberg et al. 1998). The vaccine using gp100 melanoma antigenic peptide plus IL-2 resulted in 16 % objective response with 9 % CR in the recent multicenter randomized trial (Schwartzentruber et al. 2011), while the recent adoptive immunotherapy using cultured melanoma specific T cells after the myeloablative treatment, which depletes various immunosuppressive cells, resulted in more than 70 % objective response with 21 % durable CR for advanced melanoma patients with multiple metastases (Rosenberg et al. 2011). Immunological analysis of the clinical trials indicated that immunosuppression in cancer patients is one of the major obstacles for development of effective immunotherapy. Thus, understanding of the mechanisms for the immunosuppression in cancer patients and development of strategy to overcome it is important for improvement of cancer therapy.

12.2 Immunopathology of Cancer-Associated Microenvironments

Roles of the immune system in cancer have recently been extensively exploited. The murine studies analyzing the interaction between cancer cells and immune system (and other stromal cells) during cancer development revealed that innate cells such as macrophages and mast cells rather have tumor promoting activity though increase of cancer cell proliferation and invasion ability, as well as induction of angiogenesis. In contrast, T cells and NK cells have the ability to eliminate cancer cells (Immunosurveillance). However, cancer cells having intrinsic genetic instability subsequently evade the immune defense system by losing highly immunogenic tumor antigens and acquiring various immunoresistant and immunosuppressive mechanisms (Immune evasion). This process is also known as Immunoediting (Schreiber et al. 2011). In fact, cancer cells developed from immunocompromised hosts are sensitive to immune cell attack, and cancer cells obtained from patients have a variety of immune-suppressive and resistant features (Zou 2005; Gajewski et al. 2006; Yaguchi et al. 2011). Therefore, the immunological characteristics of cancer cells are defined by both cancer cells' intrinsic nature and immune reactivity of patients.

When investigating the mechanisms of the immunosuppression in cancer patients, it is important to consider tumor-associated microenvironments, including tumor tissues where effector immune cells should eliminate cancer cells, sentinel lymph nodes (SLNs) where tumor specific T cells should be primed, and bone marrow where is a source of various immunosuppressive cells including myeloid-derived suppressor cells (MDSCs) and mesenchymal stem cells (MSCs), as well as

a reservoir for tumor specific memory T cells. Analysis of tumor tissues obtained from patients revealed that tumor appears to be under immunosuppressive conditions suggested by the expression of various immunosuppressive molecules in cancer cells (e.g., soluble molecules such as TGF-β, IL-10, IL-6, VEGF, GM-CSF, IL-13, PGE2, sMICA, membrane molecules such as PD-L1, FasL, ILT7L, intracellular molecules such as IDO, COX2), and by accumulation of various immunosuppressive cells (e.g., Treg, MDSCs, M2-like macrophages, tolerogenic dendritic cells (DCs), plasmacytoid DCs, cancer-associated fibroblasts (CAFs), MSCs). Similarly, in the sentinel lymph nodes of cancer patients, accumulation of such immunosuppressive cells was also observed (Kim et al. 2006; Swartz and Lund 2012; Fridman et al. 2011). However, the comprehensive analysis of the molecular and cellular mechanisms for the immunosuppression in the tumor-associated microenvironments remains to be performed.

It has recently been reported that levels of spontaneous $CD8^+$ T cell responses (infiltration of memory $CD8^+$ T cells in the tumor tissue prior to the cancer treatment) are different among patients with various cancers, including colon cancer, ovarian cancer, and melanoma. High infiltration of memory $CD8^+$ T cells in tumor significantly correlated with better prognosis, and its prognostic predictive value appeared to be better than TNM staging (Fridman et al. 2011; Mlecnik et al. 2011). It was also correlated with response to immunotherapy in melanoma and even chemotherapy in colon cancer (Gajewski et al. 2011). Therefore, international collaborative study "Immunoscore validation task force" is currently in progress to confirm the diagnostic value of infiltration of $CD8^+$ T cells in colon cancer (Galon et al. 2012). However, it has not yet been understood what makes the difference of spontaneous $CD8^+$ T cell response among patients. It may be regulated by both cancer cell characteristics and immunological constitution of hosts.

One important point is that immune condition in cancer patients is regulated by complex immune networks and it is first triggered by cancer cells, more specifically genetic or epigenetic alterations in cancer cells. Cancer cells trigger multiple immunosuppressive cascades in which various immunosuppressive molecules such as TGF-β, IL-10, IL-6, VEGF, PD-L1, COX2, and IDO, and immunosuppressive cells, such as tolerogenic DCs, MDSCs and Treg cells, are involved, and finally immunosuppressive conditions are established in the tumor-associate microenvironments.

12.3 Immunosuppressive Cascades Triggered by Gene Alterations in Cancer Cells

To understand the immunosuppressive cascades triggered by cancer cells, we have evaluated the role of TGF-β, which is produced by most human cancer cells, infiltrated immune cells and stromal cells, in the regulation of immunological conditions in tumor and SLN. In our mouse tumor model, increase of TGF-β in the

tumor microenvironment by implantation of the TGF-β gene-transduced tumor cells resulted in increased accumulation of $CD11b^+$ $Gr\text{-}1^+$ MDSCs and $FoxP3^+CD4^+$Treg cells in both tumor and SLN. Numbers of DCs infiltrated the tumor tissue were decreased. Interestingly, numbers of DCs were increased in SLN compared to non-SLN in mice implanted with either TGF-β-transduced tumor cells or control tumor cells, but function of DCs from mice with abundant TGF-β expression in the tumor microenvironment was significantly impaired as assessed by their T cell stimulatory activity. Implantation of TGF-β-producing tumor cells also induced M2-like macrophages, which produced abundant CCL22 in SLN; CCL22 appeared to recruit $CCR4^+$ Tregs into SLN (Tsujikawa et al. 2012). Consequently, induction of tumor antigen specific T cells from SLN was significantly reduced, and, finally, infiltration of $CD8^+$ T cells in tumor appeared to be reduced in the mice with abundant TGF-β expression. It has been reported that inhibition of TGF-β signaling by injection of plasmid DNA encoding TGF-β type II receptor near the tumor sites was reported to enhance tumor antigen specific T cells accompanied by decrease of Treg cells (Fujita et al. 2009). Therefore, these mouse models recapitulate the observations in the analysis of clinical samples and indicate that immunosuppressive molecules, such as TGF-β, may be one of the factors to define the immune status in tumor and SLN, including spontaneous $CD8^+$ T cell response.

We have previously reported that TGF-β-induced-Snail stimulated not only epithelial-to-mesenchymal transition (EMT) of cancer cells, but also production of immunosuppressive cytokines and chemokines, including TGF-β, IL-10, CCL2, and TSP-1, which caused DC impairment and Treg induction. The impaired DCs could also induce Tregs. CCL2 impairs DCs and recruits immunosuppressive MDSCs into tumor. The blockade of Snail in the tumor microenvironment by intratumoral administration of Snail-specific siRNA restored immunocompetence of mice having Snail-transduced tumor and resulted in enhanced induction of tumor antigen specific T cells in vivo (Kudo-Saito et al. 2009). These results illustrate that TGF-β production in the tumor microenvironment by either cancer cells or infiltrated stromal cells, including various immune cells, triggers multiple immunosuppressive cascades involving various immunosuppressive cytokines/chemokines and cells. This reemphasizes that the TGF-β cascade is an attractive target for reversal of cancer-induced immunosuppression (Fig. 12.1).

The molecular mechanisms of the increased production of TGF-β by human cancer cells have not been well understood. In human melanoma, production of TGF-β was not mainly regulated by MAPK and STAT3 pathways as described below. We have recently found that one of the intracellular kinase, which is frequently phoshphorylated in various cancer cells, is involved in TGF-β production by human melanoma cells. This was assessed by screening signaling molecules in melanoma cells involving suppression of DC function by using kinase siRNA library (manuscript in preparation). Therefore, this activated kinase in cancer cells can be an upstream target to inhibit the TGF-β-triggered immunosuppressive cascades. We are currently searching for small molecular drugs which efficiently inhibit this kinase. This is one example of immunosuppressive

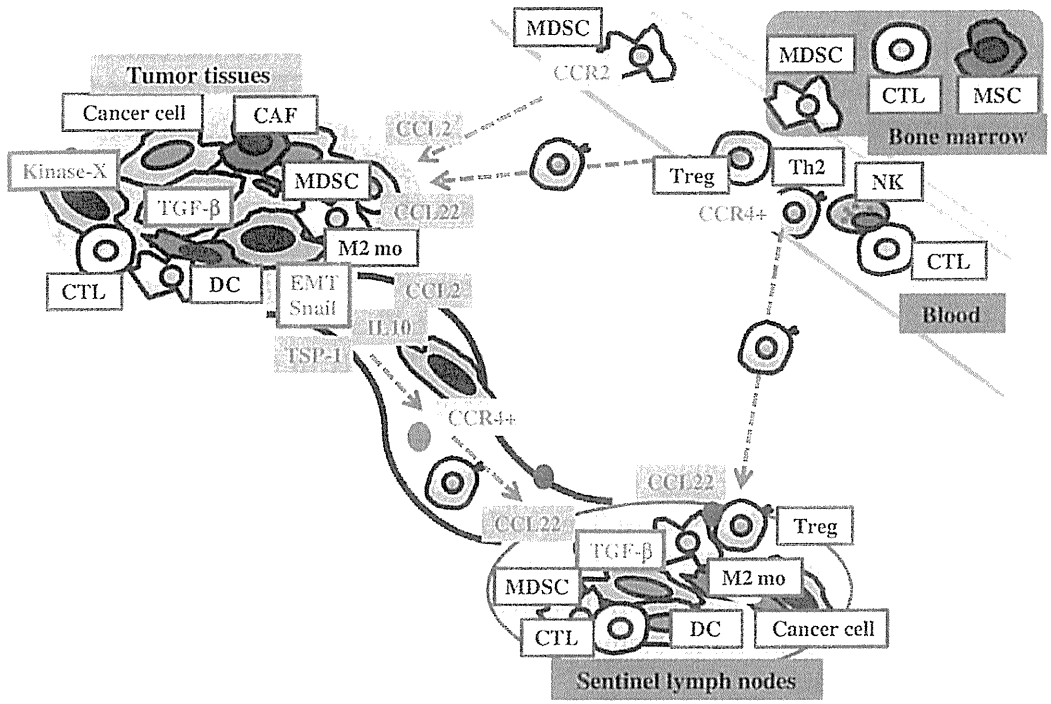

Fig. 12.1 Cancer cell triggered immunosuppressive cascades: TGF-β cascade. TGF-β produced by cancer cells and infiltrated stromal cells including various immune cells triggers immunosuppressive cascade (production of CCL2, IL-10, TGF-β , etc., and subsequent recruitment and induction of various immunosuppressive cells such as MDSC, M2 macropages, Treg cells via CCL2, CCL22, IL-10, TGF-β , etc.), and generates immunosuppressive condition in the tumor and sentinel lymph nodes. TGF-β-Snail axis induces not only epithelial-to-mesenchymal transition (EMT) which enhances invasion ability of cancer cells, but also immunosuppression which may further enhance metastasis of cancer cells

cascades triggered by gene alterations in cancer cells. Since gene and signal alterations in human cancer cells vary among cancer types, even among patients with the same type of cancer, there are multiple immunosuppressive cascades to be investigated.

12.4 Alterations of Gene and Signal Pathways Involved in the Immunosuppression in Human Cancer

Human cancer cells have various genetic and epigenetic alterations. Thus, understanding of immunosuppressive cascades triggered by each gene/signal alteration is important. Here, we describe some of our recent observations on human cancer cells.

12.4.1 RAS/BRAF/MEK/MAPK Signaling Pathway

When common mutation of BRAF (V600E), a molecule in MAPK signal pathway, was discovered by sequencing signaling molecules in human melanoma cells (Davies et al. 2002), we evaluated the role of the mutant BRAF (V600E) for malignant characteristics of human melanoma cells by using BRAF (V600E)-specific lentiviral shRNA. We found that the BRAF mutation was involved in the cell proliferation and invasion ability of melanoma cells (Sumimoto et al. 2004). We have also found that production of multiple cytokines, IL-6, IL-10, and VEGF, which have the ability to suppress function of DCs, were significantly decreased by BRAF(V600E) shRNA without affecting cell survival of the some melanoma cell lines (Sumimoto et al. 2006). These cytokines suppress DC activity to stimulate T cells mainly through the inhibition of IL-12 and TNF-α production, and augmentation of IL-10 production. Treatment of melanoma cells with BRAF (V600E)-specific shRNA or MEK inhibitors resulted in decrease of immunosuppressive activity of melanoma cells, indicating the MAPK pathway is essential for DC impairment by melanoma cells. MEK inhibitors were also reported to increase susceptibility of melanoma cells to CTL lysis partly due to increased expression of melanoma antigens, such as MART-1/melan-A and gp100 (Kono et al. 2006; Boni et al. 2010). "Avoiding immune destruction" resulting from the loss of highly immunogenic tumor antigens and acquiring immunoresistant and immunosuppressive mechanisms is now generally recognized as one of "the hallmarks of cancer" (Hanahan and Weinberg 2011). These results indicate that the BRAF-MAPK axis is commonly involved in the cancer cell proliferation, invasion, and immunosuppression (Fig. 12.2).

These observations indicate that blockade of the BRAF-MAPK axis may not only suppress proliferation and invasion of cancer cells, but also inhibit immunosuppressive activity and increase susceptibility of melanoma cells to T cells. This suggests that it is a common attractive target for melanoma treatment, particularly in combination with various immunotherapies. Since MAPK signal is also important for T cell proliferation, administration of MAPK pathway inhibitors may also suppress anti-tumor T cell response. However, two BRAF inhibitors, which preferentially inhibit mutant BRAF, have recently been developed, and their administration has already been shown to be effective in patients with melanoma (Chapman et al. 2011; Hauschild et al. 2012). These selective mutant BRAF inhibitors actually cause melanoma cell death in vivo resulting in reduction of tumor sizes in some patients. Therefore, the selective mutant BRAF inhibitors may be useful for combination with immunotherapies through the following mechanisms:

1) Tumor destruction causes release of endogenous tumor antigens which include multiple patient's unique mutated antigens, leading to induction of multiple autologous tumor specific T cells,
2) Reduction of tumor burden via inhibition of cancer cell proliferation and cancer cell death results in reduction of immunosuppressive condition,

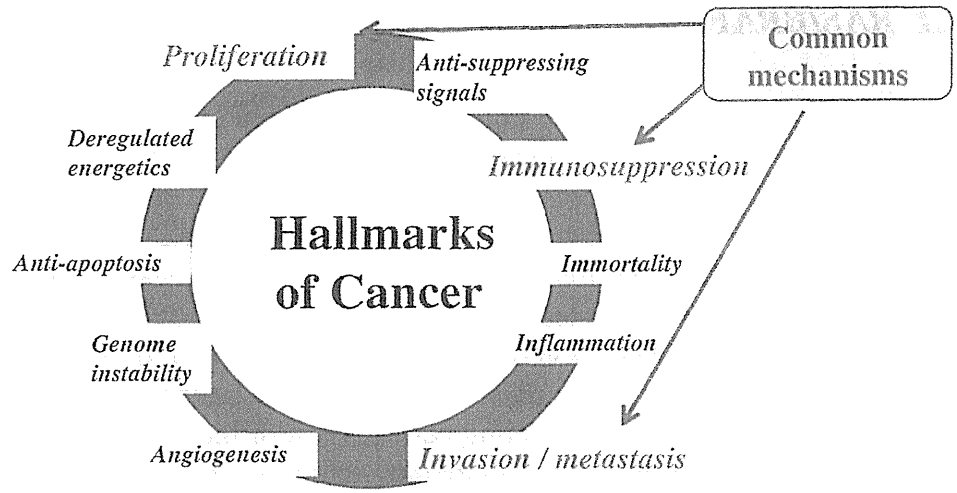

Fig. 12.2 Common mechanisms are sometimes used for hallmarks of cancer such as proliferation, metastasis and immunosuppression. Common signaling pathways such as MAPK signaling are sometimes utilized for some of the hallmarks of cancer including cancer cell proliferation, invasion, and immunosuppression. Therefore, inhibitors against the common pathways may be useful for cancer treatment through simultaneous inhibition of multiple hallmarks of cancer. Combination of molecular targeted drugs and immunotherapy may be an attractive strategy for cancer treatment

3) Decrease of production of multiple immunosuppressive cytokines results in simultaneous inhibition of multiple immunosuppressive cascades,
4) Susceptibility of cancer cells to cytotoxic T cells (CTLs) is increased partly via increased expression of tumor antigens,
5) Mutant BRAF selective inhibitors are less inhibitory for proliferation of anti-tumor T cells, and
6) Invasion and metastatic ability of cancer cells is decreased.

It has recently been reported that administration of mutant BRAF selective inhibitors did not suppress immune response in general (Hong et al. 2012), and actually increased infiltration of T cells, particularly granzyme positive $CD8^+$ T cells, in tumors, which was correlated with tumor reduction and necrosis (Wilmott et al. 2011). In vivo immunological effects of a MEK inhibitor which is also effective for patients with melanoma having mutation of either NRAS or BRAF remain to be investigated (Flaherty et al. 2012). The same strategy may also be applied for other cancers with BRAF mutations, including colon cancer and thyroid cancer.

12.4.2 JAK/STAT3 Signaling Pathway

In human melanoma, in addition to RAS/BRAF mutation, activation of STAT3 is frequently observed. Depletion of STAT3 by lentiviral shRNA in STAT3 active melanoma also resulted in the inhibition of multiple immunosuppressive

cytokines, including IL-6, IL-10, and VEGF (Sumimoto et al. 2006). Interestingly, these tumor-derived cytokines activate STAT3 in various immune cells including DCs, MDSCs, and Tregs, and affect their functions. The cytokine-induced STAT3 activation resulted in the generation of low IL-12 and high IL-10 producing human DCs with decreased T cell stimulatory activity.

In the mouse tumor model, STAT3-depleted DCs obtained from myeloid-specific STAT3-conditional knockout mice, were resistant to these tumor-derived immunosuppressive cytokines, and also had strong T cell stimulatory activity along with sustained high IL-12 production. Injection of the STAT3-depleted DCs into tumor, which is under the immunosuppressive condition, showed strong anti-tumor effects accompanied by induction of higher IFN-γ producing tumor antigen specific Th1 cells compared to the injection of control DCs (Iwata-Kajihara et al. 2011). Similarly, STAT3-depleted macrophages were resistant to tumor-derived immunosuppressive cytokines, and induction of immunosuppressive macrophages and MDSCs were partially inhibited by STAT3 depletion. STAT3 was also reported to be involved in expansion of MDSCs (Wu et al. 2011). STAT3 activation was actually observed in $CD14^{+}HLA\text{-}DR^{negative/low}$ MDSCs in blood of cancer patients (Poschke et al. 2010). STAT3 is also important for Treg cells (Pallandre et al. 2007). STAT3 inhibition of anti-tumor $CD8^{+}$T cells was reported to enhance their effects when adoptively transferred into tumor-bearing mice (Kujawski et al. 2010). These results indicate that constitutive activation of STAT3 in cancer cells triggers induction of various immunosuppressive immune cells, including tolerogenic DCs, MDSCs, and Tregs, partly through activation of STAT3 in these immune cells (Kortylewski et al. 2005; Yu et al. 2007). Therefore, STAT3 inhibitors may be useful for reversal of cancer-induced immunosuppression through not only acting on cancer cells, but also acting on various immune cells.

Recently, molecular targeted therapies acting on various signaling molecules in cancer cells have been used for cancer treatment. STAT3 inhibitors are being evaluated in clinical trials. In murine tumor model, various STAT3 inhibitors have been shown to augment anti-tumor immunity (Lee et al. 2011). In addition to STAT3 inhibitors, inhibitors to molecules present at upstream of STAT3, including inhibitors for direct upstream molecule JAK and further upstream molecules EGF-R/VEGF-R (which have already been available for clinical use), may also be useful for reversal of immunosuppression and combination with immunotherapy. JAK inhibitors have been shown to augment anti-tumor immunity and enhance anti-tumor effects in combination with immunotherapies, such as IL-12 administration (Burdelya et al. 2002). We have observed that EGF-R inhibitors suppress production of some of the immunosuppressive cytokines, such as IL-6 and VEGF, from human lung cancer cells with EGF-R mutations. In the murine tumor model, administration of the EGF-R inhibitors along with cancer vaccines showed synergistic anti-tumor effects through indirect (via decrease of immunosuppressive cytokines from cancer cells) and direct enhancement of DC ability to stimulate T cells. Administration of multikinase inhibitor Sunitinib, which also suppresses downstream STAT3 signaling, to RCC patients was

reported to result in decrease of MDSCs and Tregs along with increase of IFN-γ producing T cells (Xin et al. 2009; Ozao-Choy et al. 2009; Ko et al. 2009). Another multikinase inhibitor Dasatinib was reported to increase response rate in about half of patients with $Ph1^+$CML and ALL accompanied by LGL lymphocytosis and autoimmune like syndrome, such as pleuritis and colitis; it was reported to inhibit STAT3 signaling in immune cells after administration (Mustjoki et al. 2009; Jalkanen et al. 2010). Therefore, there are various ways of STAT3 signal inhibition for reversal of immunosuppression in cancer patients in clinic. We have recently screened natural compounds contained in the Japanese traditional Kampo medicines, and found that some of the compounds are able to inhibit STAT3 and MAPK pathways, possibly by targeting upstream signaling molecules. Their systemic administration augmented tumor specific T cells accompanied by decrease of Tregs in the tumor in tumor-bearing mice (manuscript in preparation).

12.4.3 NF-κB Signaling Pathway

We have also observed similar phenomenon—involvement of the same signaling pathway in both cancer cells and immune cells for generation of immunosuppressive condition, in human ovarian cancers with constitutively activated NF-κB, which causes high production of IL-6, IL-8, and CCL2. High levels of plasma IL-6 and IL-8 were found to correlate with poor prognosis of cancer patients and poor response to various immunotherapies, including vaccinations with cancer antigen peptides and DCs (manuscript in preparation). NF-κB inhibitor inhibited not only production of these immunosuppressive cytokines and chemokines by cancer cells, but also had direct effects on monocytes: they inhibit their differentiation to immunosuppressive macrophages in the presence of cancer cell-derived factors. Although the cross-talk, such as positive feedback loop between IL-6, STAT3 and NF-κB was previously reported to be involved in chronic inflammation (Yu and Pardoll 2009; Murakami and Hirano 2011), significant role of such cross-talk was not observed in these ovarian cancers. Systemic administration of appropriate dose of a NF-κB inhibitor augmented anti-tumor T cell responses possibly through reversal of immunosuppressive condition in a murine tumor model, although NF-κB signal is also essential for induction of anti-tumor T cells.

NF-κB was found to be involved in the intrinsic expression of ILT7 ligand (ILT7L) in some of human renal cell cancers (RCC), although ILT7L can also be up-regulated by IFN-γ from infiltrated T cells. ILT7L inhibits IFN-α production by plasmacytoid DCs and is possibly involved in immunosuppression in the tumor microenvironments, since type-I IFN was reported to be critical for induction of spontaneous anti-tumor T cell response (Fuertes et al. 2005; Gajewski et al. 2012). NF-κB inhibitor suppressed the intrinsic expression of ILT7L on RCC cells (Tsukamoto et al. 2009). It has recently been reported that expression of PD-L1 on

cancer cells was mainly induced by IFN-γ produced by tumor-infiltrating T cells, and the PD-L1 expression on cancer cells and $CD8^+$T cell infiltration correlated with significantly better response to anti-PD-1 antibody treatment (Taube et al. 2012). However, some cancer cells intrinsically express PD-L1 partly due to activation of AKT pathway via PTEN deletion (human glioma) (Parsa et al. 2007) or activated MAPK pathway in some other cancers. We have found new inhibitors, which suppresses intrinsic expression of PD-L1. These observations indicate that signal inhibitors may also be useful for inhibition of these immunosuppressive membrane molecules (e.g., ILT7L and PD-L1) intrinsically expressed through altered signaling in human cancer cells.

12.4.4 Wnt/β-Catenin Signal Pathway

Activation of β-catenin pathway (nuclear staining of β-catenin) was observed in about 30 % of human melanoma, and correlated with expression of IL-10 by immunohistochemical analysis. We found that β-catenin directly activated IL-10 transcription in human melanoma (Yaguchi et al. 2012). Supernatant from cultured β-catenin-accumulating melanoma cells induced high IL-10-, low IL-12-producing DCs with low T cell stimulatory activity in vitro, which was IL-10-dependent; these DCs also had the ability to induce FOXP3-positive immunosuppressive Treg cells. Pretreatment of melanoma cells with shRNA for β-catenin reduced their immunosuppressive activities. Interestingly, supernatant from cultured melanoma also inhibited the effector function of melanoma specific cytotoxic T cells in a β-catenin-dependent, but IL-10-independent manner, indicating that other immunosuppressive molecules are also involved in the β-catenin induced immunosuppression.

When β-catenin-activated human melanoma cell lines were implanted in immunodeficient SCID mice, the level of human IL-10 in blood was increased, and mouse DCs in the spleen and tumor were impaired for T cell stimulatory activity. This was likely because human IL10 can also act on mouse cells and suppress mouse DCs. Systemic administration of a β-catenin inhibitor restored mouse splenic DC activity to stimulate T cells along with decrease of human IL-10 in the serum. Interestingly, a β-catenin inhibitor also had the ability to directly enhance T cell stimulatory activity of human DCs partly due to decreased IL-10 production by DCs. β-catenin was also reported to be involved in generation of regulatory DCs (Fu and Jiang 2010; Manicassamy et al. 2010) and survival of Tregs (Ding et al. 2008). These results indicate again that signal inhibitors may be useful for reversal of cancer-induced immunosuppression by acting on both cancer and immune cells.