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Inflammation and Immunity in Cancer

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Preface: From the Knowledge of Immunity and Inflammation to Immunotherapy Against Cancer

Cancer is the first cause of death in advanced nations, and about seven million people die of cancer all over the world every year. In Japan, cancer has been the leading cause of death from 1981, and since 2012, more than 350,000 patients have died from cancer. Although eradication of cancer is one of the earnest wishes of human beings, chemotherapy and radiation therapy in combination with conventional surgery treatment are already reaching a limit. Direct-acting anticancer reagents such as molecular targeting by antibodies or non-toxic chemical drugs (anticancer drugs) appear effective, but a major problem is that cancer cells selectively survive acquiring tolerance to antibodies or chemicals. Development of a theoretical and innovative cure through essential understanding of the disease of cancer is desired immediately. By gaining unrestricted proliferation potency, cancer is the unusual multiplication of a cell, which deviates from the host's control, and expresses illness which spoils the host's health and leads to the host's death as a result of the progression and metastasis that disregarded homeostasis. The main aim of recent cancer research has been on how to elucidate the molecular mechanisms of carcinogenesis at the cell level. Cancer has a feature of "dys-ordered" cell growth, which gains infinite autonomous proliferation potency when hereditary or advanced abnormalities are accumulated in four or more oncogenes, tumor suppressor genes, and repair genes, which exist in the genome of the somatic cells. In spite of having been derived from the host, there is a concept of cancer cells that have non-self cell features in an immunological aspect and drive a host to death at the end of infinite multiplication. Progress of anti-cancer therapies largely has pointed to establishment of the means and the technique of killing and destroying cancer cells as an enemy by molecular targeting of a single point of the process in tumorigenesis. From a different point of view, the immune cells that exist in a cancer tissue are bearing the specific immune response for eliminating cancer cells. The cancer cells with non-self antigen epitopes usually are under the surveillance of host immunity. In fact, it is no longer difficult to experimentally demonstrate that tumors evoke antitumor immunity opposing tumor-specific antigens. Thus, a basic image of cancer is that cells run recklessly transformed, circumventing or against the host immune system. The immune system might be aberrant or silenced due to the

presence of growing tumors. An alternative strategy for anti-cancer therapy is to wake up the immune system from tolerance or dormancy to alarm against the presence of non-self tumor cells during the malignant transformation. Clinical trials using peptides of tumor-associated antigens (TAAs), biological response modifiers, and immunomodulators have been conducted for the last three decades. So far, however, no good therapeutics has been developed to establish cancer immunotherapy for patients. It is notable that tumors contain a large number of myeloid-lineage cells, tumor-associated macrophages, and myeloid-derived suppressor cells. These myeloid cells help tumor progress and invade by assisting in creating a tumor-supportive environment and suppressing the immune system. They appear to harbor unique receptors for sensing tumor-specific pattern molecules (TAMP) and other patterns of host or microbe origin. Their functional properties appear to be reversibly modulated by these extrinsic stimulations. Cells having immunoregulatory properties such as regulatory T cells and tolerogenic dendritic cells may join the control of the immune response to cancer. In fact, a negative co-stimulator CTLA4 and checkpoint inhibitors, PD-1 and PD-L1, are found to be good targets for blocking, to potentiate the immune system and cause the tumor to regress. I feel that the cancer–immune cell ambivalence is going to a paradigm shift. It is being clarified that a "tumor microenvironment" exists in tumor type-specific manners and plays a pivotal role in tumor promotion: indeed, multiple genetic aberrances are a prerequisite for malignant transformation, but additional tumor cell–host factor responses formulate tumorigenesis. How will a host make the microenvironment cancerous, which leads to ruin? I believe the key is inflammation. Some kinds of host cells including immune cells respond to cancer cells (so-called DAMP) or microbial pattern molecules (so-called PAMP), and cause local inflammation to be maintained. It is becoming accepted that as a result, the cancer microenvironment is gradually built as a non-physiological nest. The microenvironment usually consists of variegated cells which are involved in non-parenchymal cells and cells lining the blood vessels. In tumor progression, chronic inflammation has played a positive role. The immune cells that exist in the cancer microenvironment work completely contrary to immunologic surveillance. That is, immune cells switch to a regulatory feature to operate against cell-mediated antitumor immunity. The disease of cancer is a dynamic process nurtured by the interaction of a cancer cell and a host–immune response. Recent advances in understanding the concept of innate immunity suggest that the innate immune system of macrophages and dendritic cells is profoundly involved in regulation of step-by-step tumor promotion.

Innate immune response against smoldering inflammation involves construction of a microenvironment, immune modulation, genomic instability, nuclear reprogramming, and tissue regeneration. Increasing evidence suggests that chronic inflammation facilitates tumorigenesis by promoting cell growth, invasion, and metastasis. On the other hand, inflammation is an essential factor for evoking antitumor immunity. A variety of macrophages are situated in normal tissue/organs as well as malignantly transformed tumors. The tumor-infiltrated macrophages differ in their origin from the resident macrophages living in normal organs. Dendritic cells with a variety of subsets participate in formation of a nest of inflammation and

mature in an inflammation network. Lymphocytes including NK cells check the status of systemic cells under the immune surveillance system. Inflammation takes a two-pronged attack against the fate of tumorigenic cells, only a few of which we believe circumvent the host immune system to develop a visible tumor. It would be important to clarify the molecular mechanism by which the tumor-supporting or tumor-suppressing environment becomes established through innate immune response followed by inflammation. In other words, what is the tumor-supporting or tumor-suppressing environment in the nature of the tumor–immune cell association? Recent progress in the high through-put analysis of genome sequences have enabled us to analyze epigenetic differences between normal and tumor cells. Each tumor has its own profile of transcriptional activation, which will define tumorigenesis in a cell-type-specific fashion. The innate immune system consists of pattern receptors and their signaling, which also are defined by activation of a set of the transcriptional factors involved in dendritic cell maturation and macrophage functional conversion. Hence, both tumor progression and dendritic cell maturation are likely to be rooted in similar, but definitely distinguishable, epigenetic modification occurring in tumor and immune cells secondary to inflammation. Oncogenic bacteria, viruses, and parasites unequivocally induce inflammation with epigenetic conversions in host cells. It would be one good strategy for us to employ the recently developed methods for epigenetic analyses to apply to the analysis of the molecular mechanism of cancer–immune cell inter-association characterized by the establishment of the cancer microenvironment. Although the elucidation of the intermolecular mechanism among inflammation, immunity, and cancer is an important issue, it is very late in the proceeding without being updated. One point in the difficulty of the research in this field is that the diversity of the carcinogenic mechanism in each cancer conceals the basic essentials that connect cancer and inflammation. Studies on the relationship between the epigenetic alteration and activation/regulation of immune cell conversion have only recently been initiated with a tremendous amount of information with SNPs in humans. This book was edited in order to conquer this serious problem that cancer research holds. We hope that the proposals in the book will facilitate prevention and the development of therapeutics for cancer and can be widely adapted for the understanding of the mechanism of general cancers based on inflammation.

We express our deep appreciation to Dr. Kumao Toyoshima, who has encouraged us to continue studying cancer immunotherapy on a scientific basis.

Sapporo, Japan Tsukasa Seya
August 10, 2014

Contents

Chapter 1
Role of Bone Marrow-Derived CCR1^{+} Cells in Colon Cancer Invasion and Metastasis

Makoto Mark Taketo

Abstract Loss of transforming growth factor (TGF)-β family signaling is implicated in colorectal cancer (CRC) progression. Using *cis-Apc*$^{+/}\Delta^{716}$*Smad4*$^{+/-}$ mutant (*cis-Apc/Smad4*) mice, a model of invasive CRC whose TGF-β family signaling is blocked, we have found that immature myeloid cells (iMCs) are recruited from the bone marrow to the tumor invasion front. These iMCs express the matrix metalloproteinases MMP9 and MMP2 and the CC-chemokine receptor 1 (CCR1), and migrate towards the CCR1 ligand CCL9. By deleting *Ccr1* in the background of the *cis-Apc/Smad4* mutant, we have shown an accumulation of iMCs at the invasion front and suppression of tumor invasion. Furthermore, mouse and human CRC cells secrete CCR1 ligands CCL9 and CCL15, respectively, and recruit the CCR1-expressing iMCs. Loss of the *Ccr1, Mmp2, or Mmp9* gene in the host mice dramatically suppresses outgrowths of disseminated tumors in the liver. Consistently, CCR1 antagonist BL5923 blocks the iMC accumulation and metastatic colonization, and significantly prolongs the survival of tumor-bearing mice. These results indicate that loss of TGF-β family signaling in CRC epithelium causes accumulation of iMCs that promote tumor invasion, and that CCR1 antagonists can provide anti-metastatic therapies for patients with disseminated CRC cells in the liver.

Keywords TGF-β • Immature myeloid cells • Matrix metalloproteinase • Chemokine receptor CCR1

1.1 Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths (Jemal et al. 2008). While most primary tumors can be resected surgically, CRC frequently metastasizes to the liver, which is responsible for the high mortality of the disease (Christofori 2006). To achieve metastasis, cancer cells need to invade surrounding

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tissues, penetrate microvessels, survive in circulation, disseminate to distant organs, form micrometastases, and expand into macrometastases. To progress through these steps, tumor cells often acquire the capability of survival and invasion by activating metastatic signaling pathways or inactivating metastasis suppressor genes (Christofori 2006; Smith and Theodorescu 2009). In addition to these cell-autonomous changes, the tumor stroma, including bone marrow-derived myeloid cells, actively participate in early steps of the metastatic cascade in some mouse models (Joyce and Pollard 2009). For example, tumor-associated macrophages (TAMs) promote migration and intravasation of mammary tumor cells (Goswami et al. 2005; Wyckoff et al. 2007). Bone marrow-derived cells that express myeloid cell marker CD11b and granulocyte marker Gr-1 also promote metastasis of breast cancer cells, likely through promotion of intravasation and suppression of immune responses (Yang et al. 2008). These reports suggest that bone marrow-derived myeloid cells can help cancer epithelium in early steps of metastasis. It remains to be determined whether therapeutic targeting of such myeloid cells can prevent cancer metastasis (Gadea and Joyce 2006).

As a model for invasive CRC, we previously constructed *cis-Apc*$^{+/}\Delta^{716}$*Smad4*$^{+/-}$ (*Apc/Smad4*) mice that develop intestinal adenocarcinomas with marked invasions thorough biallelic loss of *Apc* and *Smad4* tumor suppressor genes in the intestinal epithelium (Takaku et al. 1998; Taketo and Edelmann 2009). In the *Apc/Smad4* tumors, we reported that the invading cancer epithelium is associated with immature myeloid cells (iMCs), and that the iMCs promote CRC invasion into the adjacent tissues (Kitamura et al. 2007). Here we have used a transplantation model to determine whether CRC cells can recruit the iMCs in the metastasizing sites. This is an excerpt from the results published recently in (Kitamura et al. 2010).

1.2 Results

1.2.1 Mouse Colorectal Cancer (CRC) Cells Disseminated to the Liver Are Associated with Immature Myeloid Cells (iMCs)

To investigate possible roles of the iMCs in CRC metastasis, we injected CMT93 mouse CRC cells into the spleen of syngeneic C57BL/6 mice, which allowed efficient liver dissemination of the cells. We found massive accumulations of stromal cells that expressed CD11b and CD45 but not vascular endothelial growth factor 1 (VEGFR1) (Fig. 1.1a and data not shown). We further confirmed that they did not express CD31 (a marker for endothelial cells), CD14 (monocytes), B2.20 (B cells), CD3ε (T cells), or αSMA (myofibroblasts). These characteristics fit those of the iMCs of the primary colon cancer in *Apc/Smad4* mice (Kitamura et al. 2010). We also verified their bone marrow origin by transplanting bone marrow cells containing green fluorescent protein (GFP) into irradiated recipients (data not shown). Further analyses showed that the iMCs started to accumulate at day 7 post-injection

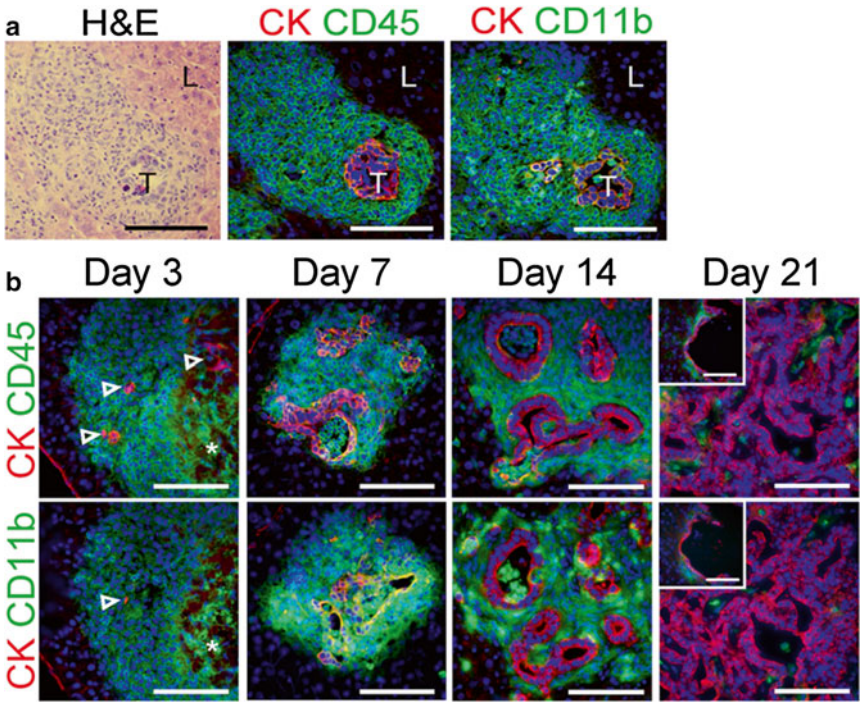

Fig. 1.1 Accumulation of immature myeloid cells (iMCs) around disseminated cancer cells in the liver. (**a**) A metastatic focus in the liver stained with hematoxylin and eosin (H&E) (*left*). Serial sections were immunostained for pan-cytokeratin (CK), CD45, and CD11b. *L* adjacent normal liver, *T* metastasizing tumor. Scale bars 100 μm. (**b**) Liver metastasis foci dissected at days 3, 7, 14, and 21 post-injection. *Open arrowheads* indicate disseminated tumor cells, *filled arrowheads* indicate a cluster of the iMCs, and *asterisks* indicate a necrotic area. *L* adjacent normal liver, *T* tumor glands. *Insets* show a representative carcinoma cyst. Scale bars 100 μm (Reproduced with permission from Kitamura et al. 2010, with minor modification)

when disseminated cancer cells began to form tumor glands. The iMCs accumulated further by day 14 with expansion of the tumor glands in the liver (Fig. 1.1b). By day 21, the iMCs disappeared from the metastatic lesions where the cancer cells formed massive glands or carcinoma cysts, suggesting the possibility that the iMCs contributed to an early phase of metastatic expansion.

1.2.2 Mouse CRC Cells Secrete CC-Chemokine Receptor 1 (CCR1) Ligand CCL9 and Recruit CCR1+ iMCs to the Liver

We next investigated CC-chemokine receptor 1 (CCR1) expression, another important characteristic of the iMCs. Most iMCs at the metastatic foci expressed CCR1, whereas the cancer epithelium did not (data not shown). Because specific ligands

activate CCR1 (Balkwill 2004) and recruit the iMCs (12), we looked for such ligands that were expressed by the CRC cells. Among them, CMT93 cells expressed only *Ccl9* mRNA, but not others (data not shown). Consistently, cultured CMT93 cells secreted the CCR1 ligand CCL9 protein (25 ± 3 pg/10^5 cells) at a similar level to that of intestinal cancer cells from *Apc/Smad4* polyps. Immunostaining data also confirmed that metastasized CMT93 cells expressed CCL9 but the surrounding stromal cells did not (data not shown).

To assess the importance of CCL9 in iMC accumulation in the liver, we prepared CMT93 derivatives that contained short hairpin RNA (shRNA) against *Ccl9* or control scramble RNA. As expected, almost all metastatic foci formed by control cells accumulated iMCs markedly. In contrast, such accumulations were not observed in the majority of foci by the CCL9-reduced CMT93 cells (data not shown). Collectively, these results suggest that activation of CCR1 by cancer-secreted CCL9 is critical for the iMCs to accumulate at the metastatic foci. As an exception, we found several small metastatic lesions where numerous iMCs accumulated despite the lack of CCR1 (data not shown), suggesting a rare alternative mechanism that can recruit iMCs independent of CCR1.

1.2.3 Inactivation of the Mouse CCL9–CCR1 Signaling Blocks Metastatic Expansion of CRC in the Liver, and Prolongs Host Survival

Because the iMCs helped invasion of primary tumors in the intestines (Kitamura et al. 2007), we hypothesized that accumulation of the iMCs could also promote the metastatic expansion of disseminated colon cancer in the liver. We therefore injected luciferase-expressing CMT93 cells into *nu/nu* mice, which enabled monitoring of the liver tumors by bioluminescence over time. In mice injected with the parental CMT93 or CMT93-scramble cells, the intensity of bioluminescence began to increase at day 7 post-injection and increased exponentially thereafter (Fig. 1.2a, b). In contrast, such an increase in luminescence was markedly suppressed in mice injected with CMT93-sh*Ccl9*#1 cells, although they showed essentially the same levels of luminescence as control groups up to day 3. Another shRNA construct (sh*Ccl9*#2) showed a similar effect, though at a weaker level than that of sh*Ccl9*#1 (Fig. 1.2b). Of note, all CMT93 derivatives showed essentially the same luciferase activity and proliferation rate in culture. We then injected luciferase-expressing CMT93 cells into the wild-type and *Ccr1*$^{-/-}$ mice. Although all wild-type mice showed marked increases in the luminescence level at day 14 post-injection, such an increase was not observed in 70 % (7/10) of *Ccr1*$^{-/-}$ mice (Fig. 1.2c). In these hosts, we did not find any macroscopic foci on liver surfaces (Fig. 1.2d). These results indicate that accumulation of the iMCs via the CCL9–CCR1 axis plays a major role in the expansion of liver metastasis foci. Consistently, inactivation of the CCL9–CCR1 signaling prolonged the survival by blocking early metastatic expansion (Fig. 1.2e, f).

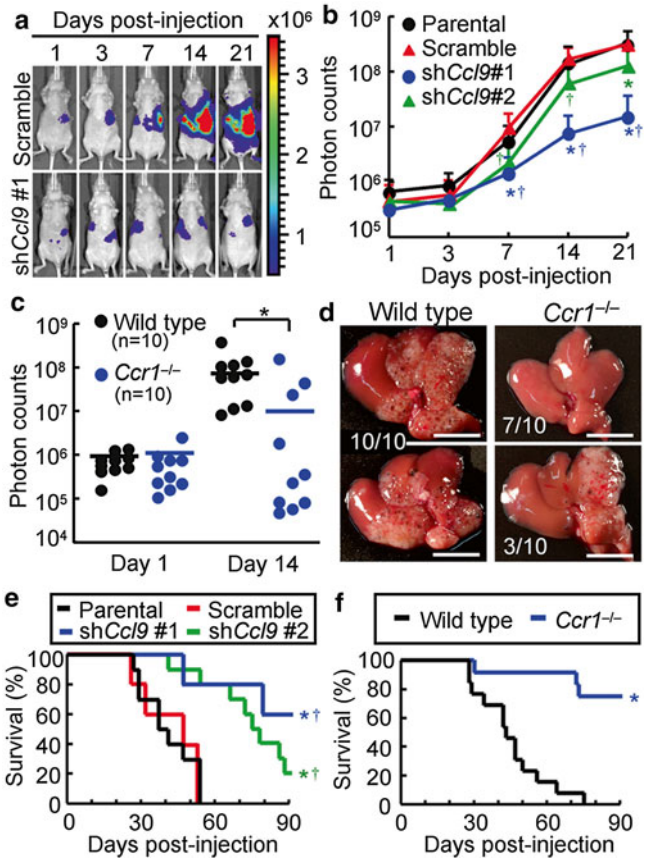

Fig. 1.2 Blockade of CC chemokine receptor 1 (CCR1) ligand 9 (CCL9)–CCR1 signaling suppresses expansion of metastatic lesions and prolongs host survival. (**a**) Representative in vivo bioluminescence images of mice injected with luciferase-expressing CMT93 cells that contained scramble RNA (*top*) or short hairpin RNA (shRNA) against *Ccl9* (*bottom*). The *colored scale bar* represents intensity of bioluminescence in photons/s. (**b**) Quantification of metastatic lesions by bioluminescence (photon counts) from luciferase-expressing CMT93 cells [Parental (Prnt)], or their derivatives that contained scramble RNA [Scramble (Scr)] or shRNA against *Ccl9* (sh*Ccl9*#1 or sh*Ccl9*#2). Results are given as the means ± standard deviation. $*P < 0.02$ and $^{\dagger}P < 0.02$ compared with Parental and Scramble, respectively ($n = 8$–11 mice in each group). (**c**) Quantification of metastatic lesions by bioluminescence from luciferase-expressing CMT93 cells injected into wild-type and *Ccr1*$^{-/-}$ mice. Each *circle* represents an individual mouse. *Horizontal lines* show the means of the respective groups. $*P < 0.01$. (**d**) Representative macroscopic views of the liver dissected from wild-type and *Ccr1*$^{-/-}$ mice injected with CMT93 cells. Scale bars 10 mm. (**e**) Kaplan-Meier plot showing survival of wild-type hosts injected with control CMT93 cells (Prnt and Scr) or those containing shRNA constructs against *Ccl9* (sh*Ccl9*#1 or sh*Ccl9*#2). $*P < 0.01$ and $^{\dagger}P < 0.02$ compared with Prnt and Scr, respectively ($n = 5$–10 mice in each group). (**f**) Survival rates of wild-type and *Ccr1*$^{-/-}$ mice injected with parental CMT93 cells. $*P = 0.0001$ ($n = 12$–13 mice in each group) (Reproduced with permission from Kitamura et al. 2010)

1.2.4 *Lack of Matrix Metalloproteinase (MMP) 2 or MMP9 in the Host Mouse Suppresses Expansion of Metastatic Liver Lesions*

For metastatic expansion in the liver, disseminated cancer cells need to invade the liver parenchyma. We therefore hypothesized that the iMCs promote intrahepatic invasion of disseminated cancer through secretion of proteases. To identify such enzymes, we compared mRNA levels in the metastatic foci between the wild-type and *Ccr1*$^{-/-}$ mice by microarray and RT-PCR. The results showed more than two-fold increases in the levels of matrix metalloproteinase (MMP) genes *Mmp2*, *Mmp7*, *Mmp9*, and *Mmp13* in the iMC-associated foci in wild-type mice (data not shown). In such lesions, MMP2 and MMP9 were expressed in the stromal iMCs, but not in the cancer epithelium (data not shown), whereas MMP7 and MMP13 were found primarily in the epithelium (data not shown). Consistently, MMP2 and MMP9 were absent around the liver foci in *Ccr1*$^{-/-}$ mice where the iMCs were missing, although MMP7 and MMP13 were present (data not shown). We then injected luciferase-expressing CMT93 cells into *Mmp2*$^{-/-}$ mice, and found significant reduction in the liver luminescence at day 14 compared with those in *Mmp2*$^{+/+}$ littermates (data not shown). Likewise, the luciferase luminescence was much lower in *Mmp9*$^{-/-}$ mice than in *Mmp9*$^{+/+}$ littermates, whereas such a reduction was not observed in *Mmp7*$^{-/-}$ mice (data not shown). Notably, almost all metastatic foci in the *Mmp2*$^{-/-}$ and *Mmp9*$^{-/-}$ mice consisted of smaller cancer glands than those in wild-type mice, although they were associated with numerous iMCs (Fig. 1.3). These results suggest that both MMP2 and MMP9 are critical for the iMCs to promote metastatic expansion of CRC cells, but not to accumulate in the liver.

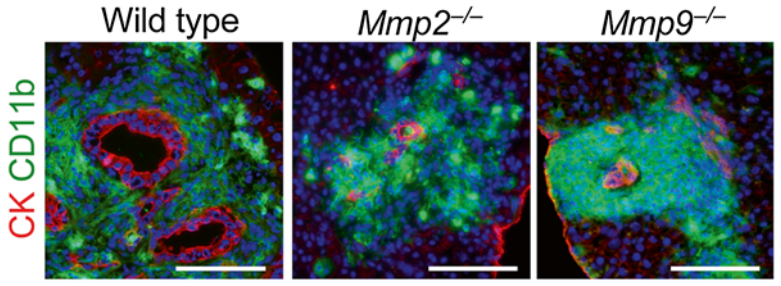

Fig. 1.3 Lack of matrix metalloproteinases MMP2 or MMP9 inhibits expansion of metastatic liver lesions. Liver metastasis foci in wild-type, *Mmp2*$^{-/-}$, and *Mmp9*$^{-/-}$ mice injected with CMT93 cells. Scale bars 100 μm (Reproduced with permission from Kitamura et al. 2010)

1.2.5 Some Human CRC Cells Express CCR1 Ligand CCL15

To test whether human CRC cells could recruit iMCs, we injected four cell lines into the spleen of *nu/nu* mice, and found that HT29 cells were associated with the iMCs in the liver, whereas HCT116, DLD-1, or SW620 cells were not (data not shown). Based on the structural similarity, human orthologs of mouse CCL9 have been suspected to be CCL15 and/or CCL23 (14–16). Thus, we determined the levels of these chemokines in 11 human colon cancer cell lines, and found that six of them, including HT29, expressed *CCL15* mRNA and protein at high levels, but none expressed *CCL23* (data not shown). We could not detect mRNAs for any other CCR1 ligands (CCL3, 4, 5, 7, 14, or 16; data not shown). We further verified by western blotting that 28 % (13/47) of human colon cancer specimens expressed CCL15 protein (Fig. 1.4a). An immunostaining analysis also showed that 29 % (12/41) of primary tumors and 29 % (12/41; a separate combination) of liver metastases expressed CCL15 in the cancer epithelium (Fig. 1.4b). These results demonstrate that a subset of human CRC cases secretes CCL15, and suggest that it may recruit CCR1⁺ human iMCs.

1.2.6 Human CCL15 Promotes Accumulation of the iMCs and Expansion of Metastatic Foci in Nude Mouse Liver

To assess the role of human CCL15 in iMC accumulation and following metastatic expansion of CRC in the liver, we constructed derivatives of the CMT93-sh*Ccl9*#1 cells in which human *CCL15* was expressed but endogenous mouse CCL9

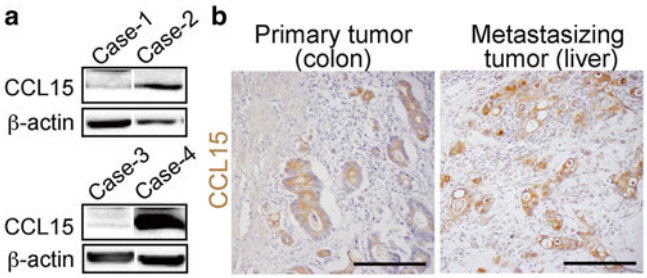

Fig. 1.4 Human CC chemokine receptor 1 ligand 15 (CCL15) recruits the immature myeloid cells (iMCs) and promotes expansion of metastatic lesions in mouse liver. (**a**) Determination of CCL15 protein levels by western blotting. Lysates were prepared from human colon cancer specimens. Cases 2 and 4 expressed CCL15. β-Actin was used as a loading control. (**b**) Human colon cancer specimens immunostained for CCL15 (hematoxylin counterstaining). Tumors were dissected from the primary (colon) and metastatic (liver) sites of the same patients. Scale bars 100 μm (Reproduced with permission from Kitamura et al. 2010)

expression was suppressed by an shRNA. These cells secreted essentially the same level of CCL15 as did HT29 cells. As expected, numerous iMCs accumulated in most tumors of CMT93-sh*Ccl9*:CCL15#1 cells, although iMC accumulation was blocked in the majority of metastatic foci formed by CMT93-sh*Ccl9* control cells (data not shown). These results indicate that human CCL15 can recruit mouse $CCR1^+$ iMCs and promote liver metastasis of CRC cells in mice.

1.2.7 CCR1 Antagonist BL5923 Blocks Metastatic Expansion of CRC in the Mouse Liver and Prolongs Host Survival

Finally, we evaluated a CCR1 antagonist for its suppressive effects on CRC metastasis in the mouse liver dissemination model. We administered BL5923 or vehicle to syngeneic and *nu/nu* mice that were injected with mouse (CMT93) and human (HT29) CRC cells, respectively, as described above. Treatment of the host mice with 50 mg/kg of BL5923, but not with vehicle, markedly reduced the accumulation of iMCs around the metastatic foci of CMT93 as well as of HT29 (Fig. 1.5a). In mice injected with the luciferase-expressing CMT93 cells, BL5923 (at 50 mg/kg) significantly reduced the luminescence levels at day 14 compared with the markedly increased levels in the vehicle-treated mice (data not shown). As anticipated, BL5923 did not affect the proliferation rate of CMT93 cells in culture (data not shown). Likewise, expansion of HT29 cells in the liver was significantly blocked by treatment with 50 mg/kg of BL5923 (data not shown). In contrast, it did not suppress the metastatic expansion of HCT116 cells that could form metastases without iMC accumulation (data not shown), consistent with the mechanism that BL5923 blocks tumor metastasis through CCR1 inhibition in the iMCs. We further found that the BL5923 treatment prolonged the mean survival of the hosts from 37 days post-injection to 62 days and from 84 days to 113 days when CMT93 and HT29 cells were injected, respectively (Fig. 1.5b, c). These results strongly suggest that CCR1 antagonist BL5923 suppresses accumulation of the iMCs and early metastatic expansion of colon cancer, allowing prolonged host survival.

1.3 Discussion

In our CRC liver metastasis model, we have found that the iMCs express MMP2 and MMP9, but the tumor epithelium does not. These results are consistent with previous reports that show stroma-restricted expression of the MMPs in human liver metastases of CRC (Théret et al. 1997; Zeng and Guillem 1995). Abundant expression of MMP2 or MMP9 is associated with poor prognosis and high mortality in colon cancer patients (Cho et al. 2007; Hilska et al. 2007; Sutnar et al. 2007; Langers et al. 2008; Inafuku et al. 2009). Although selective inhibitors of MMP2/9

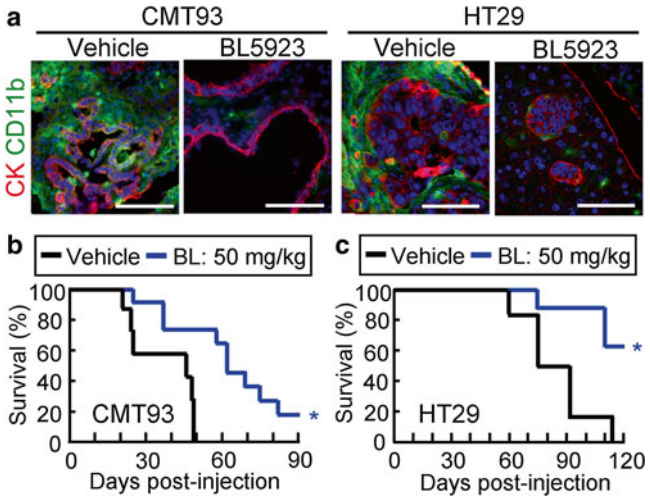

Fig. 1.5 CCR1 antagonist BL5923 blocks expansion of metastatic lesions and prolongs host survival. (**a**) Liver metastasis foci from mice treated with vehicle or BL5923 (50 mg/kg). Mice were injected with CMT93 cells (*left*) or HT29 cells (*right*). Scale bars, 100 μm. (**b** and **c**) Kaplan-Meier plots showing survivals of the host mice treated with the vehicle or BL5923 (BL: 50 mg/kg). Mice were injected with CMT93 (**b**) or HT29 (**c**). $*P = 0.004$ compared with vehicle (n = 7–11 mice in each group) (Reproduced with permission from Kitamura et al. 2010)

significantly block mouse liver metastasis of colon cancer and prolong the survival of tumor-bearing mice (Wagenaar-Miller et al. 2004), clinical trials for MMP inhibitors have failed because of severe side effects such as musculoskeletal pain and inflammation (Coussens et al. 2002; Egeblad and Werb 2002; Overall and Kleifeld 2006). Alternatively, we have demonstrated here that reduced iMC accumulation by inactivation of CCR1 can suppress metastatic expansion of colon cancer. These results provide the rationale for application of CCR1 antagonists to colon cancer treatment that target the MMP-expressing myeloid cells, rather than direct and systemic inhibition of MMPs. Supporting this novel strategy of "cellular target therapy" (Kitamura and Taketo 2007), we have demonstrated here that CCR1 antagonist BL5923 significantly blocks liver metastasis of mouse (CMT93) and human (HT29) colon cancer cells, and prolongs the host survival. Because *Ccr1*$^{-/-}$ mice are healthy unless challenged with specific pathogens (Gao et al. 1997), therapeutic inactivation of CCR1 may exhibit few side effects. Consistently, several CCR1 antagonists were well-tolerated in phase II trials for rheumatoid arthritis and multiple sclerosis (Ribeiro and Horuk 2005). It is therefore possible that administration of CCR1 antagonists as an adjuvant therapy after surgical resection of the primary tumors can improve the survival of patients with CRC who express CCL15.

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Chapter 2
PGE_2-Associated Inflammation and Gastrointestinal Tumorigenesis

Hiroko Oshima and Masanobu Oshima

Abstract Accumulating evidence has indicated that chronic inflammation is associated with a variety of diseases, including cancer, heart attacks, Alzheimer's and other diseases. In the cancer research field, the association of inflammatory infiltration with cancer has been known histologically for a long time. Recent studies have indicated that macrophages and other immune cells infiltrate cancer tissues, expressing cytokines, chemokines and growth factors, thereby constructing an inflammatory microenvironment. In such a microenvironment, nuclear factor (NF)-κB is activated, which contributes to the growth and survival of cancer cells. Moreover, it has also been shown that NF-κB activation is associated with the acquisition of stem cell properties by cancer cells. Using inflammation-associated gastric cancer model mice (*Gan* mice), we have shown that tumor necrosis factor (TNF)-α signaling is activated in the inflammatory microenvironment and plays a tumor-promoting role by inducing Noxo1 in tumor cells. Taken together, these results indicate that regulation of chronic inflammation in tumor tissues would be an effective preventive and/or therapeutic strategy against cancer development and malignant progression.

Keywords Microenvironment • COX-2 • PGE_2 • TNF-α • NF-κB

2.1 Introduction

It has been shown that chronic inflammatory diseases are associated with malignant diseases. For example, reflux esophagitis, inflammatory bowel disease, ulcerative colitis, and chronic pancreatitis are linked to cancers of the esophagus, colon, and pancreas, respectively. Moreover, chronic infections with *Helicobacter pylori*, hepatitis viruses, or papilloma viruses are associated with gastric cancer, hepatocellular carcinoma, and cervical cancer, respectively. These findings indicate that chronic inflammation and infection are important risk factors for cancer development.

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Among the various inflammatory signaling pathways, mouse genetic studies and clinical studies have indicated that prostaglandin E_2 (PGE_2) signaling plays an essential role in inflammation and cancer development. In this chapter, we discuss the role of PGE_2-associated inflammation and the role of the tumor necrosis factor (TNF)-α induced by the PGE_2-related microenvironment in gastric and intestinal cancer development, which have been elucidated by mouse genetic studies.

2.2 Non-steroidal Anti-inflammatory Drugs (NSAIDs) and Cancer Development

Epidemiological studies have indicated that the incidence and mortality rates of colorectal cancer are decreased significantly in a population who takes aspirin, one of the non-steroidal anti-inflammatory drugs (NSAIDs), compared with non-aspirin users (Thun et al. 1993). These results strongly suggested that inflammation is not just associated with cancer, but that it actually promotes cancer development. The target molecules of NSAIDs are cyclo-oxygenase (COX)-1 and COX-2, which are the rate-limiting enzymes for prostaglandin biosynthesis. COX-1 is constitutively expressed in most tissues, and is responsible for the physiological basal level of prostaglandin production, whereas COX-2 expression is induced in inflamed tissues and plays a critical role in inflammatory responses. COX-2 catalyzes the synthesis of prostaglandin H_2 (PGH_2) from arachidonic acid, which is further converted to PGE_2 by microsomal prostaglandin E synthase-1 (mPGES-1). Notably, the expression of COX-2 and mPGES-1 is induced simultaneously in both inflammatory and cancer tissues, including colon cancer and gastric cancer. These results strongly suggested that COX-2 and the downstream product, PGE_2, are important for cancer development through the induction and maintenance of inflammatory responses (Wang and DuBois 2010).

2.3 The Cyclo-oxygenase (COX)-2/Prostaglandin (PG) E_2 Pathway and Intestinal Tumorigenesis

It is well-established that the accumulation of mutations in oncogenes and tumor suppressor genes causes cancer development and malignant progression, which is known as ‘multistep tumorigenesis’. Among these cancer-related genes, an *APC* gene mutation is found in more than 80 % of colon cancer cells, and is responsible for the initial step of tumorigenesis, i.e., the development of adenomatous polyps in the intestine. We have previously constructed *Apc* gene mutant mice, $Apc^{\Delta 716}$ mice, which develop numerous polyps in the entire intestinal tract (Oshima et al. 1995). In the $Apc^{\Delta 716}$ mouse polyp tissues, COX-2 expression is induced in stroma cells, including both macrophages and fibroblasts (Sonoshita et al. 2002). Accordingly,

the tumor microenvironment consisting of COX-2-expressing stromal cells comprises the early stage of tumorigenesis. The expression of mPGES-1 is also induced in the tumor stroma, indicating that PGE_2 is produced in the microenvironment of tumor tissues (Fig. 2.1).

Importantly, disruption of the *COX-2* gene or treatment with COX-2-selective inhibitors resulted in significant suppression of the polyp formation in $Apc^{\Delta 716}$ mice (Oshima et al. 1996, 2001). Consistently, disruption of the *mPGES-1* gene also suppressed intestinal polyposis in other *Apc* gene mutant mice (Nakanishi et al. 2008).

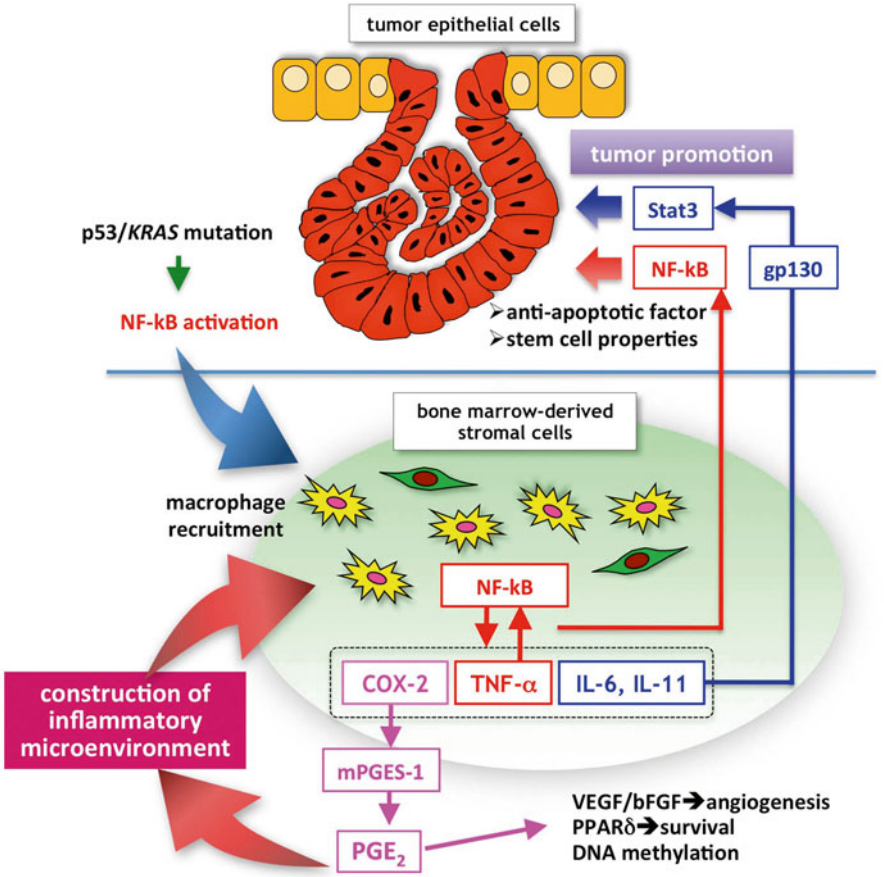

Fig. 2.1 Schematic drawing of the inflammatory microenvironment in gastrointestinal tumors. The cyclo-oxygenase (COX)-2/prostaglandin (PG) E_2 pathway is important for the development and maintenance of the inflammatory microenvironment, including bone marrow-derived cells (BMDCs). PGE_2 signaling also accelerates angiogenesis and cell survival as well as induces DNA methylation. In the activated macrophages, nuclear factor (NF)-κB induces the expression of growth factors and cytokines. The activation of NF-κB and Stat3 in tumor cells suppresses the apoptosis of tumor cells. NF-κB activation also induces the acquisition of stem cell properties (Modified from Oshima and Oshima 2012 with permission from Springer)

Moreover, blocking PGE_2 signaling through the EP2 receptor led to significant suppression of polyp formation in $Apc^{\Delta 716}$ mice (Sonoshita et al. 2001). These results clearly indicate that the induction of COX-2/PGE_2 signaling in the microenvironment is required for tumor development.

Many studies have shown the functions of PGE_2 in tumorigenesis; i.e., angiogenesis through the induction of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Seno et al. 2002), and suppression of apoptosis by the activation of peroxisome proliferator-activated receptor (PPAR)-δ (Wang et al. 2004). Moreover, PGE_2 signaling induces DNA methylation that silences tumor suppressor and DNA repair genes (Xia et al. 2012) (Fig. 2.1). However, the main mechanism by which PGE_2 promotes cancer is still unclear. We have constructed a new mouse model, *K19-C2mE* mice, which express COX-2 and mPGES-1 simultaneously in the stomach, which induces chronic PGE_2-associated inflammation in the gastric mucosa (Oshima et al. 2004) (Fig. 2.2). Therefore, one of the important mechanisms by which the COX-2/PGE_2 pathway is involved in tumorigenesis is by generating an inflammatory microenvironment.

2.4 Nuclear Factor (NF)-κB Signaling and Inflammation-Associated Colon Cancer

In the tissue exposed to chronic inflammation, lipid mediators such as PGE_2, chemokines, and cytokines are induced, resulting in the recruitment of bone marrow-derived cells (BMDCs) such as macrophages, and activation of these cells leads to the development of a cytokine network. Several transcription factors, such as nuclear factor (NF)-κB and Stat3, are activated in the inflammatory lesions. NF-κB is activated by TNF-α signaling and Stat3 is activated by interleukin (IL)-6/IL-11, and these transcription factors both play a role in tumor promotion (Oshima and Oshima 2012). The role of Stat3 induction by IL-6 or IL-11 in intestinal tumorigenesis has been shown in genetic studies (Bollrath et al. 2009; Grivennikov et al. 2009; Putoczki et al. 2013). In this chapter, we discuss the role of NF-κB in the promotion of colon cancer development.

The treatment of mice with chemical mutagen azoxymethane (AOM) induces β-catenin gene mutations, and treatment with dextran sodium sulfate (DSS) in the drinking water induces ulcerative colitis. It is well-established in a mouse model that colitis-associated colon cancer can be induced by treatment with a combination of AOM and DSS. Genetic inactivation of NF-κB in the bone marrow cells of AOM/DSS-treated mice resulted in a decrease in the growth factor expression by stromal cells and suppression of colon tumor development (Greten et al. 2004). Notably, inactivation of NF-κB in intestinal epithelial cells also suppressed colon tumor development by leading to decreased expression of anti-apoptotic factors. These results indicate that inflammatory responses promote tumorigenesis through the activation of NF-κB in both BMDCs as well as tumor epithelial cells by inducing the expression of growth factors and suppressing apoptosis (Fig. 2.1).

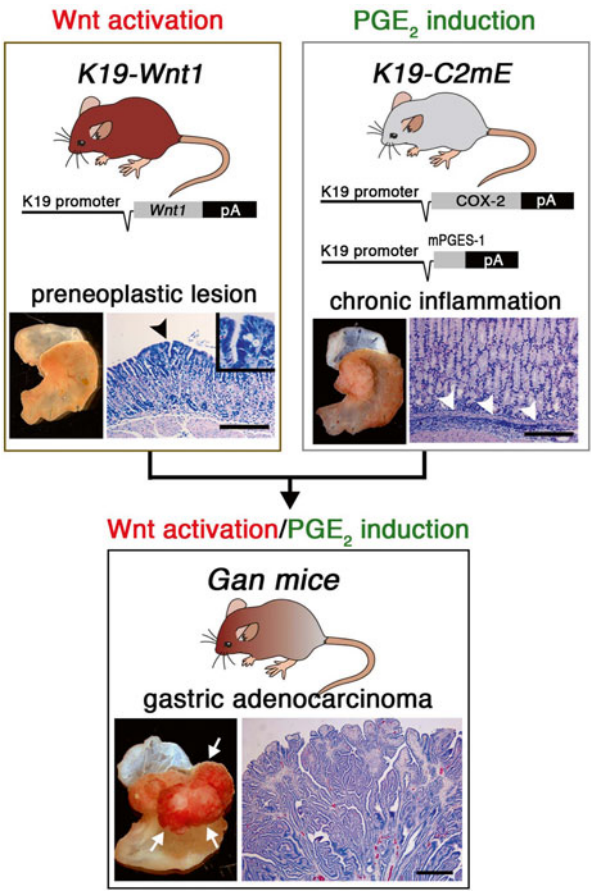

Fig. 2.2 Transgenic mouse models used to examine gastric tumorigenesis. Transgenic vector constructs and representative macroscopic and microscopic photographs of the stomach are shown for each line. *Gan* mice are compound *K19-Wnt1* and *K19-C2mE* transgenic mice. The *arrowhead* in the *K19-Wnt1* mouse stomach indicates a preneoplastic lesion. The *arrowheads* in the *K19-C2mE* mouse stomach indicate inflammatory infiltration. The *arrows* in the *Gan* mouse indicate gastric tumors. *Bars* indicate 100 μm (Modified from Oshima et al. 2009 with permission from Wiley)

Recently, it has been reported that NF-κB is important for the stem cell phenotype. Normal intestinal epithelial cells on villi are terminally differentiated and never proliferate. When Wnt signaling is activated in mouse villous epithelial cells by conditional mutagenesis in the β-catenin gene, the cells do not proliferate, indicating that Wnt activation cannot reset the terminal differentiation. However, if NF-κB is activated together with Wnt signaling, the epithelial cells acquire stem cell properties and start proliferating (Schwitalla et al. 2013a). These results suggest that inflammatory responses contribute to the development or maintenance of an undifferentiated status or stemness of cancer cells through NF-κB activation.

Although infection contributes to the development of an inflammatory microenvironment, it has not been elucidated how chronic inflammation is induced in non-infectious cancer tissues. Recent results have suggested the possibility that there may be oncogene-induced inflammation in tumor tissues. A loss or mutation of the *p53* gene accelerates NF-κB activation in the microenvironment, resulting in an acceleration of colitis-associated tumor development in a mouse model (Schwitalla et al. 2013b; Cooks et al. 2013). Moreover, *KRAS* mutation causes TANK-binding kinase 1 (TBK1)-dependent NF-κB activation, which is required for the survival of cancer cells (Barbie et al. 2009). Accordingly, genetic alterations in oncogenes or tumor suppressor genes promote tumorigenesis not only by its intrinsic oncogenic activations, but also by inducing inflammatory responses.

2.5 *Gan* Mice, a Model of Inflammation-Associated Gastric Cancer

Helicobacter pylori infection is strongly associated with the development of gastric cancer. This infection induces activation of the COX-2/PGE_2 pathway, which plays a role in infection-associated gastritis. We have constructed transgenic *K19-Wnt1* mice that express Wnt1, a ligand for canonical Wnt signaling, specifically in the gastric epithelial cells (Oshima et al. 2006, 2009). These *K19-Wnt1* mice develop small preneoplastic lesions in the gastric mucosa, but they do not develop tumors (Fig. 2.2). We crossed *K19-Wnt1* mice and *K19-C2mE* mice to induce activation of both Wnt signaling and the COX-2/PGE_2 pathway simultaneously in the stomach (Oshima et al. 2006, 2009). Importantly, these double transgenic mice, *Gan* mice, develop large gastric tumors with a 100 % incidence, indicating that the cooperation of Wnt signaling and the COX-2/PGE_2 pathway causes gastric tumor development (Fig. 2.2). Moreover, the gene expression profiles of *Gan* mouse tumor tissues are similar to those of human intestinal-type gastric cancer (Itadani et al. 2009). Accordingly, it can be concluded that *Gan* mice develop gastric cancer by the same mechanism as human gastric cancer—the induction of oncogenic activation and inflammatory responses—and the expression profiles also reflect those of human gastric cancer.

2.6 Tumor Necrosis Factor (TNF)-α Signaling in *Gan* Mouse Gastric Tumorigenesis

In the gastric tumors of *Gan* mice, macrophages infiltrate into the tumor stroma, and they are activated to express proinflammatory cytokines, chemokines, growth factors, and proteases (Oshima et al. 2011a). We found that the expression of epidermal growth factor receptor (EGFR) ligands, including amphiregulin and epiregulin, and

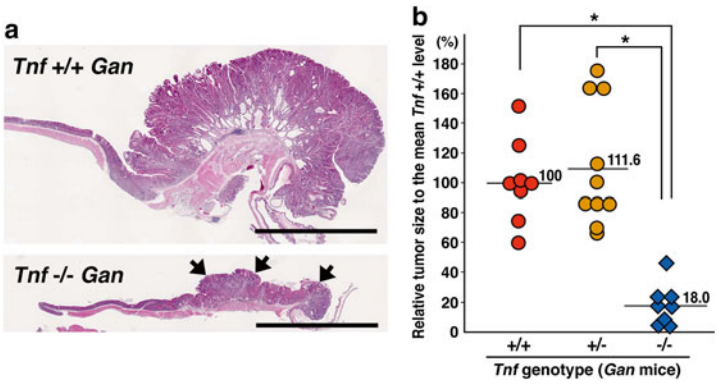

Fig. 2.3 Suppression of gastric tumor development by tumor necrosis factor (TNF)-α gene disruption. (**a**) Representative histological photographs of whole views of *Tnf*$^{+/+}$ *Gan* (*top*) and *Tnf*$^{-/-}$ *Gan* mouse (*bottom*) gastric tumors (hematoxylin and eosin stain). The *arrows* indicate suppressed tumor lesions in *Tnf*$^{-/-}$ *Gan* mice. *Bars* indicate 5 mm. (**b**) The gastric tumor size of *Tnf*$^{+/+}$ *Gan*, *Tnf*$^{+/-}$ *Gan*, and *Tnf*$^{-/-}$ *Gan* mice relative to the mean level of *Tnf*$^{+/+}$ *Gan* mouse tumors (set at 100 %). * $P < 0.05$ (Reproduced from Oshima, 2013 with permission from Nature Publishing Group)

ADAM (A Disintegrin And Metalloproteinase) family proteases, is upregulated in *Gan* mouse tumors by an inflammation-dependent mechanism (Oshima et al. 2011b). The ADAM family proteases activate EGFR ligands by exodomain shedding, resulting in the activation of EGFR signaling, which may be one of the mechanisms by which inflammation leads to tumor promotion.

Among the various proinflammatory cytokines, we have been focusing on TNF-α, because it has been shown that disruption of TNF-α or the TNR-α receptor gene resulted in suppression of chemical carcinogen-induced tumor development in mouse models (Oshima et al. 2013). To examine the role of TNF-α in gastric tumorigenesis, we crossed *Gan* mice with TNF-α gene (*Tnf*) knockout mice to generate *Tnf*$^{-/-}$ *Gan* mice (Oshima et al. 2013). Notably, gastric tumorigenesis was significantly suppressed in *Tnf*$^{-/-}$ *Gan* mice, and the tumor volume decreased to 18 % of the size of the tumors in the *Tnf* wild-type *Gan* mice (Fig. 2.3). Notably, COX-2 and mPGES-1 are constitutively expressed in the *Tnf*$^{-/-}$ *Gan* mouse stomach because they are expressed by exogenous promoters; thus, it is possible that TNF-α signaling is required for tumor promotion even if the PGE_2 pathway is activated.

Of note, bone marrow transplantation into X-ray-irradiated *Tnf*$^{-/-}$ *Gan* mice from wild-type mice rescued the gastric tumor phenotype, indicating that the TNF-α expressed by BMDCs is important for tumor promotion (Oshima et al. 2013). Furthermore, bone marrow transplantation into *Gan* mice from TNF-α receptor *TNFR1* gene knockout mice resulted in significant suppression of the gastric tumor growth. Taken together, these results indicate that macrophage-derived TNF-α promotes gastric tumorigenesis by stimulating BMDCs in the tumor microenvironment in an autocrine or paracrine manner (Fig. 2.4). It has also been reported that TNF-α stimulates cancer cells directly, thus leading to the induction of IL-6 and C-X-C

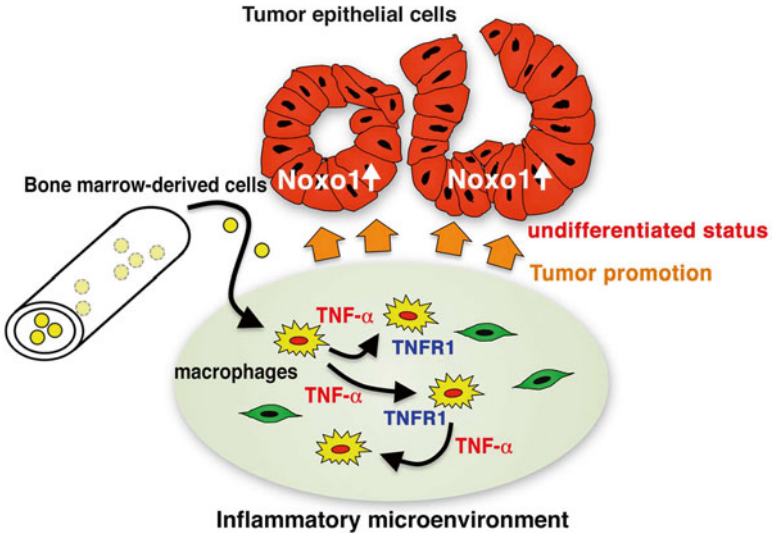

Fig. 2.4 Schematic drawing of the role of tumor necrosis factor (TNF)-α signaling in gastric tumorigenesis. Bone marrow-derived cells (BMDCs), including macrophages, are recruited to the inflammatory microenvironment and express TNF-α, which further activates the TNFR1 receptor on BMDCs, which is important for inducing tumor-promoting factors, including Noxo1, in tumor epithelial cells. Noxo1 expression may induce reactive oxygen species (ROS) production, which is required for the undifferentiated status of tumor cells (Reproduced from Oshima, 2013 with permission from Nature Publishing Group)

motif chemokine 12 (CXCL12), and resulting in an inflammatory network (Kulbe et al. 2012). Therefore, it is conceivable that TNF-α promotes tumorigenesis by stimulating both stromal BMDCs and cancer cells.

2.7 Maintenance of the Undifferentiated Status of Cancer Cells by Inflammation

A microarray analysis using tumor tissues developed in *Tnf*$^{-/-}$ *Gan* mice and *Tnf* wild-type *Gan* mice revealed that more than 150 genes are upregulated in the tumor tissues in a TNF-α-dependent manner (Oshima et al. 2013). Interestingly, the genes specific for intestinal stem cells, such as CD44, CD133 and SOX9, were included in the upregulated genes, suggesting that TNF-α signaling in BMDCs plays a role in the maintenance of the undifferentiated status of tumor cells. Using small-interfering RNA (siRNA) screening, we selected candidate genes that are important for tumorigenesis (Oshima et al. 2013). Among these genes, the inhibition of Noxo1 expression resulted in significant suppression of the tumorigenicity of gastric cancer cell lines. Noxo1 is a component of the nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase NOX1 complex that regulates reactive oxygen species (ROS) production. A recent report indicated that Rac1, another member of the NOX1 complex, is activated by Wnt signaling in the intestinal tumor cells, and that Rac1 activation is required for the undifferentiated status of tumor cells induced by ROS production together with NF-κB activation (Myant et al. 2013). Moreover, Noxo1 expression is also upregulated in the normal gastric epithelial stem cells, suggesting a role of Noxo1 in maintenance of stem cells (Barker et al. 2010). These results strongly suggest that TNF-α signaling promotes tumor development by regulating the maintenance of the undifferentiated status of tumor cells through Noxo1 induction-associated ROS production.

2.8 Conclusion

The COX-2/PGE_2 pathway is induced in most cancer tissues, and it has been established that PGE_2 plays an important role in cancer development. PGE_2 promotes tumor development through a variety of mechanisms, including the development of an inflammatory microenvironment. Accumulating evidence has indicated that activation of NF-κB, an important transcription factor involved in inflammation, is also essential for tumor promotion via its induction of growth factors, protection of tumor cells from apoptosis, and acquisition of stem cell properties. Moreover, the TNF-α induced in the inflammatory microenvironment is required for tumor promotion. TNF-α activation in the BMDCs in the tumor stroma induces Noxo1 expression in tumor cells, which may be important for the maintenance of the undifferentiated status of tumor cells by ROS production. Taken together, these results indicate that the regulation of PGE_2-associated inflammatory responses and TNF-α signaling may be an effective preventive or therapeutic strategy against the development of gastrointestinal cancer.

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Chapter 3
The Role of Innate Immune Signaling in Regulation of Tumor-Associated Myeloid Cells

Hiroaki Shime, Misako Matsumoto, and Tsukasa Seya

Abstract Tumor progression is frequently associated with a profound alteration in myelopoiesis, which results in expansion of tumor-associated myeloid cells represented by tumor-associated macrophages and myeloid-derived suppressor cells. These tumor-associated myeloid cells not only facilitate tumor growth, but also hamper cancer immunotherapy by immune and non-immune mechanisms. However, tumor-associated myeloid cells also have a critical role for tumor growth inhibition in immunotherapy for cancer. Recent evidence indicates that innate immune signaling elicited by Toll-like receptor ligands can induce both differentiation and 're-education' of tumor-associated myeloid cells, which positively and negatively affect tumor development and growth. Therefore, innate immune signaling could be a useful target for cancer treatment by modulating the phenotype of tumor-associated myeloid cells.

Keywords Cancer • Innate immunity • TLR • Tumor-associated macrophages (TAMs) • Myeloid-derived suppressor cells (MDSCs) • Immune suppression

3.1 Introduction

The interaction between tumor cells and tumor-associated stromal cells is critical for the regulation of tumor growth and progression (Coussens and Werb 2002; Hanahan and Weinberg 2010; Hanahan and Coussens 2012). Many types of stromal cells, such as immune cells, fibroblasts, and endothelial cells, infiltrate solid tumors in human and mouse cancer. Myeloid-derived cells such as tumor-associated

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macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) are frequently found in various types of tumors and are associated with cancer-related inflammation and immunosuppression in the tumor microenvironment (Qian and Pollard 2010; Gabrilovich et al. 2012). Several reports have shown that high TAM density in tumors is correlated with poor prognosis of patients and resistance to therapy (DeNardo et al. 2009; Steidl et al. 2010; Mazzieri et al. 2011). Depletion of TAMs and MDSCs from tumor-bearing mice results in augmentation of anti-tumor responses and induces tumor regression (Srivastava et al. 2012; Germano et al. 2013). Thus, TAMs and MDSCs are considered promising targets for cancer therapy (Balkwill and Mantovani 2010; Hanahan and Coussens 2012; De Palma and Lewis 2013). Accumulation of TAMs and MDSCs in a tumor is regulated by tumor cell-derived chemoattractants. TAMs and MDSCs are educated by the tumor microenvironment and are predominantly altered into an immunosuppressive phenotype (Ostrand-Rosenberg and Sinha 2009; Gabrilovich et al. 2012). These myeloid cell subsets suppress anti-tumor immune responses mediated by T cells and natural killer (NK) cells to help tumor cells evade immune cell-mediated elimination. Furthermore, TAMs and MDSCs promote tumor cell proliferation, angiogenesis, vasculogenesis, and metastasis by secreting growth factors, proangiogenic factors, and matrix-degrading enzymes that contribute to tumor growth and development (Gabrilovich and Nagaraj 2009; De Palma and Lewis 2013). Other types of myeloid-derived cells are also implicated in tumor progression. Tumor-associated dendritic cells (TADCs) with an immunosuppressive phenotype dampen anti-tumor immunity and affect therapeutic responses to chemotherapy (Munn and Mellor 2004; Gabrilovich 2004; Jinushi et al. 2013). Mast cells are also involved in the regulation of tumor growth (Khazaie et al. 2011). Tumor-associated neutrophils (TANs) share phenotypic and functional properties with a subpopulation of MDSCs. TANs also show tumor-promoting activities (Gregory and Houghton 2011; Fridlender and Albelda 2012; Galdiero et al. 2013).

Regulation of TAMs and MDSCs and their impact on tumor progression are important issues in the context of the process of cancer development and cancer therapy. Activation of Toll-like receptor (TLR) signaling pathways in tumor-associated myeloid cells influences their development and function, which positively and negatively affects tumor growth. TAMs and MDSCs also respond to the stimulation with cytokines. Thus, both intracellular signals elicited by TLR ligands and TLR-induced cytokines may be involved in regulation of the development, expansion, and function of TAMs and MDSCs. Bystander TLR-expressing cells such as dendritic cells (DCs), tissue-resident macrophages, or stromal cells also affect the development and function of TAMs and MDSCs by producing soluble factors including cytokines and chemokines. Furthermore, recent reports suggest that the ‘re-education’ of tumor-associated myeloid cells into cells with tumor-suppressive function through the activation of TLR signaling could be a useful strategy to treat cancer. In this review, we focus on how TLR signals regulate the development and function of TAMs and MDSCs, and target or exploit them for anticancer therapies.

3.2 Toll-Like Receptors and Cancer

Innate immune responses are triggered by the activation of pattern recognition receptors (PRRs) represented by TLRs (Iwasaki and Medzhitov 2010; Kawai and Akira 2011). TLRs play a central role in the initiation of immune responses against infection of pathogens. Activation of TLRs by the specific ligands is a critical step for triggering immune responses. The TLR family consists of more than ten members in humans and mice (Kawai and Akira 2011). TLRs are widely expressed in hematopoietic cells (monocytes, macrophages, DCs, neutrophils, mast cells, B cells, and T cells) and non-hematopoietic cells (epithelial cells, fibroblasts, and endothelial cells). In addition, some tumor cell lines express several types of TLRs (Huang et al. 2008). Conserved microbial components, which are so-called pathogen-associated molecular patterns (PAMPs), and their related synthetic molecules act as specific ligands for TLRs. TLR2 forms heterodimers with TLR1 or TLR6 and recognizes peptidoglycan derived from Gram-positive bacteria and synthetic lipopeptides. TLR2/6 recognizes diacylated lipopeptides such as macrophage-activating lipopeptide 2 kDa (MALP2) and Pam2CSK4. TLR2/TLR1 recognizes triacylated lipopeptides such as Pam3CSK4. Lipopolysaccharide (LPS) from Gram-negative bacteria and monophosphoryl lipid A (MPLA) are recognized by TLR4. TLR5 recognizes flagellin, which is a component of bacterial flagella. TLR3, TLR7, TLR8, and TLR9 are receptors for nucleic acids. TLR3 recognizes double-stranded RNA (dsRNA) produced during viral infection, the single-stranded RNA genome of poliovirus, and synthetic dsRNA analogs such as poly I:C. TLR7 and TLR8 recognize single-stranded RNA or guanosine-related analogs such as imiquimod. TLR9 recognizes the unmethylated CpG motif of prokaryotic genomes and DNA viruses. TLR ligands are not limited to exogenous molecules but endogenous mammalian cell-derived molecules. TLRs (especially TLR2 and TLR4) recognize a variety of endogenous molecules such as high mobility group box (HMGB) 1, versican, heat-shock protein (Hsp) 72, and necrotic cell-derived RNA. These are known as damage-associated molecular patterns (DAMPs), which are released from necrotic cells in injured tissue. TLRs are localized in distinct subcellular compartments. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are localized on the cell surface, whereas nucleic acid-sensing TLR3, TLR7, TLR8, and TLR9 are located in endosomes (Blasius and Beutler 2010). All TLRs, except for TLR3, transduce signals via myeloid differentiation primary response gene 88 (MyD88), and activate the transcription factor nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines and maturation of antigen-presenting cells (APCs) such as DCs and macrophages. TLR3 and TLR4 recruit Toll/interleukin (IL)-1 receptor (TIR)-containing adaptor molecule-1 [TICAM-1, also known as TIR domain-containing adapter-inducing interferon (IFN)-β (TRIF)] to induce an alternative pathway that leads to activation of IFN-regulatory factor (IRF) 3 and NF-κB (Oshiumi et al. 2003; Yamamoto et al. 2003). The TICAM-1-mediated pathway leads to production of pro-inflammatory cytokines, type-I IFNs, and enhancement of cross-presentation by APCs. Accumulating evidence demonstrates that

TLR activation induces not only inflammation, but also immune suppression, which affects multiple processes of tumor development and progression (Wang et al. 2008; Rakoff-Nahoum and Medzhitov 2009; Lee et al. 2010). MyD88 is demonstrated to be a critical molecule for development of intestinal tumorigenesis (Rakoff-Nahoum and Medzhitov 2007). In contrast, a variety of TLR ligands are reported to induce anti-tumor immunity when administered into tumor-bearing mice. Some TLR ligands have been extensively studied for therapeutic use in cancer patients (Galluzzi et al. 2012; Kaczanowska et al. 2013).

3.3 Supportive Roles of Myeloid-Derived Cells in Tumor Growth and Progression

Immune suppression is frequently observed in most cancer patients. It contributes, in part, to tumor development and progression by subverting immune cell-mediated elimination of tumor cells and hinders success in the immunotherapy for cancer (Zitvogel et al. 2006). Tumor cells successfully evade the host immune system by suppressing innate and adaptive immune responses. Tumor-derived soluble factors, such as immunosuppressive cytokines and metabolic enzymes, which induce proliferation of immunosuppressive regulatory T cells (Tregs), have been identified. In addition, tumor cell-recruited TAMs and MDSCs suppress both innate and adaptive immunity and contribute to the creation of immunosuppression in the tumor microenvironment, which subverts anti-tumor immunity against tumor cells mediated by effector cells such as cytotoxic T lymphocytes (CTLs) and NK cells (Zitvogel et al. 2006; Marigo et al. 2008) (Figs. 3.1 and 3.2). Furthermore, tumor-associated myeloid cells produce proangiogenic factors and matrix-degrading enzymes that are essential for tumor development (Shojaei et al. 2008).

3.3.1 Tumor-Supportive Roles of Tumor-Associated Macrophages (TAMs)

TAMs frequently infiltrate multiple types of solid tumors in humans and mice (Solinas et al. 2009). Development and growth of mammary tumors are greatly reduced in macrophage colony-stimulating factor (M-CSF)-deficient op/op mice, which are deficient in macrophages due to the lack of M-CSF (Lin et al. 2001). Bisphosphonate treatment, which selectively depletes phagocytes including macrophages in vivo, decreases the number of TAMs and induces retardation of tumor growth (Rogers and Holen 2011). These studies suggest that TAMs are essential cells for tumor growth and progression. Both tumor cells and stromal cells in tumor tissue induce the recruitment of circulating monocytes in the peripheral blood by secreting a variety of chemoattractants such as chemokine CC ligand (CCL) 2,

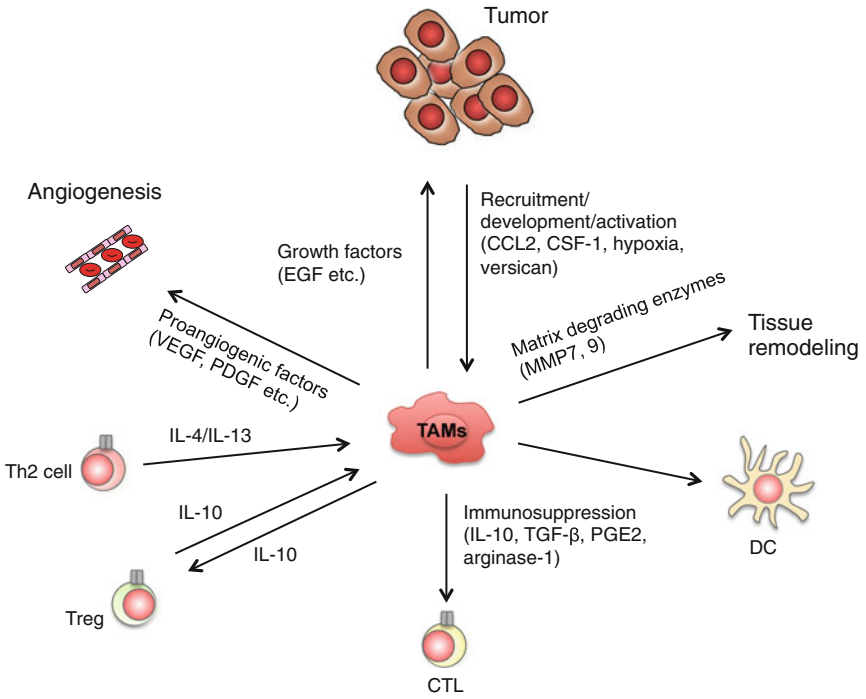

Fig. 3.1 M2 tumor-associated macrophages (TAMs) support tumor growth and progression. Circulating monocytes recruited by tumor-derived chemoattractants develop into TAMs in the tumor microenvironment. TAMs generally show an M2-like phenotype. T helper (Th) 2 cytokines [interleukin (IL)-4 and IL-13] and IL-10 induce M2 polarization of TAMs. M2 TAMs produce tumor-promoting factors including immunosuppressive cytokines, proangiogenic factors, growth factors, and matrix-degrading enzymes [e.g., matrix metalloproteinases (MMPs)]. Stimulation with Toll-like receptor (TLR) ligands or cytokines influence the function of TAMs

CCL5, colony-stimulating factor (CSF)-1 and chemokine CXC ligand (CXCL) 12. Monocytes differentiate into TAMs in response to the tumor microenvironment (Solinas et al. 2009). A recent report demonstrates that the spleen and bone marrow are reservoirs for TAM precursors in tumor-bearing hosts (Cortez-Retamozo et al. 2012). TAMs are also differentiated from tumor-infiltrated monocytic MDSCs (M-MDSCs or Mo-MDSCs) as described below.

Two distinct activation states of macrophages are referred to as classically/alternatively activated or M1/M2-polarized states (Mantovani et al. 2002; Biswas and Mantovani 2010). Classically activated M1 macrophages produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-12, IL-23, IL-6, and IL-1β, leading to induction of T helper (Th) 1-type immune response and high expression of inducible nitric oxide (NO) synthase (iNOS). In vitro experiments have shown that TNF-α and NO are involved in direct killing of certain types of tumor cells

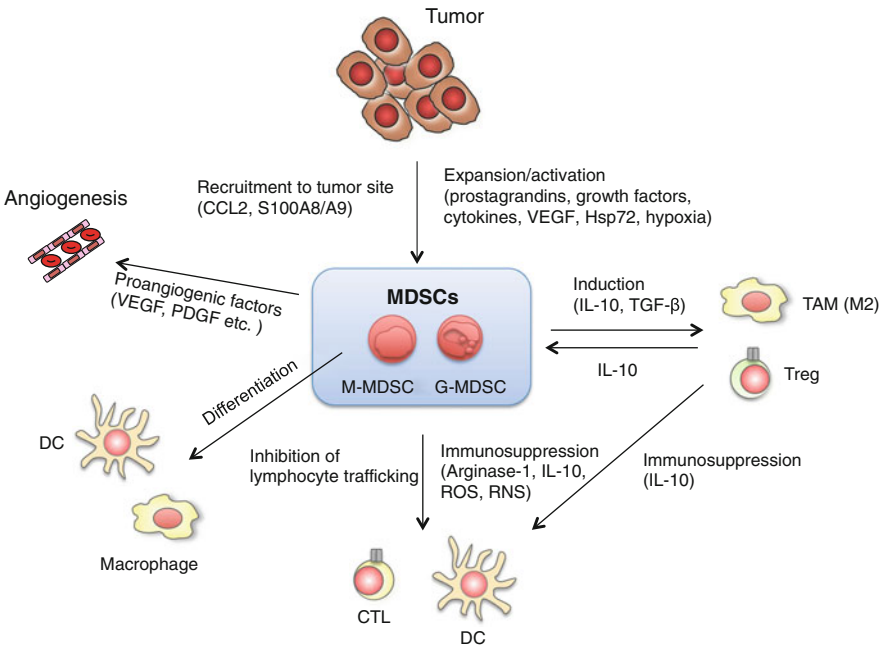

Fig. 3.2 Multiple roles of myeloid-derived suppressor cells (MDSCs) in tumor growth and progression. MDSCs consist of monocytic MDSCs (M-MDSCs or Mo-MDSCs) and granulocytic MDSCs (G-MDSCs), which are distinguished by the expression of Ly6G and Ly6C. M-MDSCs contain precursors of macrophages, dendritic cells (DCs) and G-MDSCs. MDSCs suppress antitumor responses through a variety of mechanisms. MDSCs suppress the activation and proliferation of T cells by production of arginase and reactive oxygen species (ROS), nitration of tyrosine residues of T cell receptors (TCRs), or deprivation of cysteine. MDSCs also produce interleukin (IL)-10 and tumor growth factor (TGF)-β, leading to induction of regulatory T cells (Tregs) and M2 tumor-associated macrophages (TAMs), and suppression of DC function. Proangiogenic factors derived from MDSCs promote tumor growth by regulating angiogenesis. Tumor-derived factors [prostaglandins, growth factors, cytokines, vascular endothelial growth factor (VEGF), and heat-shock protein (Hsp) 72) and hypoxia are involved in the development and the expansion of MDSCs. S100 calcium-binding protein (S100) A8/A9 and chemokine CC ligand (CCL) 2 recruit MDSCs to tumor sites. S100A8/A9 and Hsp72 modulate MDSC expansion and function through the activation of Toll-like receptor (TLR) 4 and TLR2, respectively. TLR signals and TLR signal-induced cytokines regulate MDSC development and expansion through intracellular signaling pathways

by M1 macrophages. In contrast, alternatively activated M2 macrophages are characterized by higher expression levels of IL-10, arginase-1, scavenger receptor (SR), and macrophage mannose receptor (MMR, CD206), and lower expression of proinflammatory cytokines such as IL-12, IL-23, IL-1β, and IL-6. M2 macrophages have poor antigen-presentation capability and immunosuppressive activity by secreting IL-10 and transforming growth factor (TGF)-β. They also produce molecules such as angiogenic factors and matrix metalloproteinases (MMPs), which are involved in tissue remodeling. M1/M2-polarization of macrophages is regulated by

immune signaling (Hu et al. 2007; Lawrence and Natoli 2011). IFN-γ and IFN-β are potent stimulation factors of macrophages and induce M1-like macrophages through Janus kinase/signal transducer and activator of transcription (STAT) 1 activation (Toshchakov et al. 2002). M1 macrophages derived from granulocyte–M-CSF (GM-CSF)-treated human monocytes highly express IRF5 compared with M-CSF-induced M2 macrophages. Over-expression of IRF5 in M-CSF-induced M2 macrophages forces them into macrophages that express M1-specific cytokines, leading to both Th1 and Th17 cell development (Krausgruber et al. 2011). The Notch-recombination signal binding protein for immunoglobulin kappa J (RBPJ) pathway determines M1/M2 polarization by controlling the expression of M1-related genes in macrophages via transcription factor IRF8 (Xu et al. 2012). These results raise the possibility that TLR signaling may directly induce the expression of M1-related genes through IRF5 and IRF8 because these transcription factors participate in TLR signal-induced transcription of cytokine genes (Honda and Taniguchi 2006). In contrast, the jumonji domain-containing 3 (JmjD3)–IRF4 axis regulates M2 macrophage development (Satoh et al. 2010). JmjD3, a H3K27me demethylase, is induced in macrophages in response to the ligands of TLR2, TLR4, and TLR9, and inflammatory cytokines (De Santa et al. 2007). STAT6 and peroxisome proliferator-activated receptor (PPAR)-γ regulate M2 macrophage polarization (Charo 2007; Ishii et al. 2009). Suppressor of cytokine signaling (SOCS) 2 and SOCS3 are involved in M1 and M2 polarization, respectively, by regulating intracellular cytokine signaling (Spence et al. 2013). Furthermore, TLR signals induce chromatin remodeling to control the gene expression through IRF3 and other transcription factors (Foster et al. 2007), which may regulate the expression levels of the transcription factors involved in macrophage polarization. Most importantly, macrophage polarization is not stable (i.e., plasticity), and the manipulation of macrophage function could be achieved by regulating multiple intracellular signaling pathways (Biswas and Mantovani 2010; Lawrence and Natoli 2011; Sica and Mantovani 2012).

Gene expression analysis has demonstrated that TAMs largely display the phenotype that is typical of the M2-polarized macrophages (Mantovani et al. 2008) (Fig. 3.1). However, TAMs polarized into the M1 or M2 phenotype coexist in tumors but localize in different areas of the tumor. It is reported that infiltration of M2-polarized TAMs is correlated with poor prognosis in many types of cancers, including melanoma, colon cancer, and ovarian cancer (Lewis and Pollard 2006; Biswas and Mantovani 2010). Conversely, the density of M1 macrophages, defined as $CD68^{+}HLA\text{-}DR^{+}$, in the tumor is positively associated with the survival time of non-small cell lung cancer patients, whereas $CD68^{+}CD163^{+}$ M2 macrophages are not associated with patient survival (Ma et al. 2010). Thus, the balance of M1 versus M2 population is considered to affect tumor growth.

Macrophage polarization is affected by several factors in the tumor microenvironment. It has been demonstrated that tumor cell-derived TNF-α, TGF-β, prostaglandin E2 (PGE2), and hypoxia induce M2 polarization of macrophages (Lewis and Pollard 2006; Biswas and Mantovani 2010). Th2 cytokines such as IL-4 drive the development of M2-polarized TAMs (DeNardo et al. 2009). The recently identi-

fied transcription factors that drive macrophage polarization described above may explain a mechanism that regulates the development and polarization of TAMs.

Immune and non-immune mechanisms mediated by TAMs that affect tumor growth have been reported (Mantovani et al. 2008). M2 TAMs directly facilitate tumor growth by secreting a variety of growth factors for tumor cells. M2 TAM-secreted immunosuppressive cytokines such as IL-10 down-regulate the anti-tumor activity of cytotoxic T cells and NK cells. IL-10 may also promote tumor progression by potentiating Treg activity. Furthermore, M2 TAMs produce proangiogenic factors such as vascular endothelial growth factor (VEGF), chemokines such as CCL2, and CXCL8, and growth factors such as platelet-derived growth factor and epidermal growth factor, which stimulate formation of new blood vessels to supply nutrients that are essential for tumor growth (Lin and Pollard 2007). Furthermore, TAMs influence the efficacy of anticancer therapies including chemotherapy, tumor irradiation, vascular-targeted therapies, and antibody therapies (De Palma and Lewis 2013).

Increasing evidence shows that TAMs play critical roles in multiple stages of tumor growth and metastasis in which TLR signaling pathways are involved (Qian and Pollard 2010; Sica 2010). Macrophages are recruited into pre-metastatic organs by tumor-derived factors where TLR4-activated macrophages facilitate invasion and metastasis of tumor cells by secreting proteolytic enzymes such as MMP7 and MMP9 to destroy the extracellular matrix (Hiratsuka et al. 2006, 2008). Versican, an extracellular matrix protein, secreted by tumor cells induces TNF-α production by macrophages through activation of the TLR2 signaling pathway. This promotes tumor metastasis into lungs (Kim et al. 2009). Another study has suggested that activation of TLR4 signaling pathway on M2-polarized TAMs partially induces epithelial–mesenchymal transition (EMT) in pancreatic cancer through increased IL-10 production (Liu et al. 2013).

3.3.2 Expansion and Activation of Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are a heterogeneous cell population that consists of myeloid progenitor cells and immature myeloid cells (iMCs). MDSCs are defined as $CD11b^{+}Gr1^{+}$ cells in mice and $CD14^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{neg/low}$ in humans, and lack maturation markers of macrophages and DCs. MDSCs have potent immunosuppressive activity against both innate and adaptive immunity (Gabrilovich et al. 2012). Although $CD11b^{+}Gr1^{+}$ cells are normally present in healthy mice, they do not have immunosuppressive activity. Myeloid progenitor cells immediately differentiate into neutrophils, macrophages, or DCs in healthy mice. However, in tumor-bearing mice, the differentiation is blocked, which cause MDSC accumulation in spleen, blood, lymph nodes, and primary and metastasized solid tumors. They are also frequently detected in the peripheral blood of human cancer patients (Almand et al. 2001;

Diaz-Montero et al. 2009; Ostrand-Rosenberg and Sinha 2009). In tumor tissues, MDSCs can be distinguished from TAMs by the expression of surface molecules. Gr1 is highly expressed on MDSCs but not TAMs, and F4/80 is expressed on TAMs but less on MDSCs. MDSCs are further characterized into two populations: $Ly6G^{-}Ly6C^{high}$ M-MDSCs and $Ly6G^{+}Ly6C^{low}$ granulocytic MDSCs (G-MDSCs) or polymorphonuclear MDSCs (PMN-MDSCs) (Youn et al. 2008; Movahedi et al. 2008; Peranzoni et al. 2010). Although G-MDSCs are a major subset in tumors and peripheral blood, their suppressive activity is relatively low compared to that of M-MDSCs. Recently, it was revealed that M-MDSCs contain precursors of TAMs and G-MDSC (Youn et al. 2013). MDSCs are distributed throughout the body. Tumor-derived chemokines or chemoattractants such as CCL2, Bv8 (also known as prokineticin-2), and S100 calcium-binding protein (S100)A8/A9 recruit MDSCs from peripheral organs into tumor sites (Huang et al. 2007; Shojaei et al. 2007; Sawanobori et al. 2008; Sinha et al. 2008).

Inflammation-associated factors promote the expansion of MDSCs. Prostaglandins, stem-cell factor (SCF), M-CSF, GM-CSF, TNF-α, IL-1β, IL-6, PGE2, and VEGF promote MDSC expansion (Gabrilovich et al. 2012). Some of these molecules are under the control of TLR signals. In fact, TLR4 activation leads to suppressive activity of MDSCs through a MyD88/NF-κB-dependent mechanism. STAT3, which is activated by stimulation with some of these inflammatory molecules, is one of the key signaling molecules that regulates the expansion of MDSCs. STAT3 is frequently observed to be activated in tumor-infiltrating immune cells (Yu et al. 2007). MDSC expansion is not observed in STAT3 conditional knockout mice or STAT3-specific inhibitor-treated mice under tumor-bearing conditions, which results in an increase of T cell responses (Nefedova et al. 2005; Kortylewski et al. 2005). Constitutive activation of STAT3 leads to the production of S100A8 and S100A9 in iMCs, resulting in inhibition of their differentiation. Increased reactive oxygen species (ROS) concentration driven by S100A8/A9-induced nicotinamide adenosine dinucleotide phosphate (NADPH) activation results in differentiation of iMCs into MDSCs (Cheng et al. 2008). Hsp72-containing tumor-derived exosomes trigger STAT3 activation through TLR2/MyD88-dependent IL-6 production by autocrine mechanisms, which results in the induction of MDSC expansion (Chalmin et al. 2010). Another report shows that STAT3 or STAT5 down-regulates IRF8 to maintain MDSC development (Waight et al. 2013). S100A8 and S100A9 expression is up-regulated in many tumors, including gastric, lung, bladder, mammary, and colon cancer (Srikrishna 2011). Activated neutrophils and macrophages in tumor or necrotic tumor cells release the S100A8/A9 complex, which act as a chemoattractant for MDSCs. The S100A8/A9 complex promotes and amplifies inflammatory responses via direct binding to TLR4 (Ehrchen et al. 2009). Inflammation-induced TNF signaling drives the peripheral accumulation of MDSCs through TNF receptor (TNFR)-2, but not TNFR-1. TNF-α inhibits differentiation and enhances suppressive activity of iMCs during chronic inflammation, resulting in generation of MDSCs. TNF-α-induced S100A8 and S100A9 proteins and their corresponding receptor, receptor for advanced glycan endproducts (RAGE), augment MDSC-suppressive activity (Sade-Feldman et al. 2013). Activation of

complement cascades accompanied by TLR-induced inflammation also regulates tumor growth by modulating MDSC function. Complement component C5a, a cleaved product of C5, is generated by inflammation in the tumor microenvironment and recruits MDSCs and enhances their suppressive function against $CD8^+$ T cell proliferation, which contributes to tumor growth. Enhanced suppression is achieved by increased ROS and reactive nitrogen species (RNS) in M-MDSCs but not G-MDSCs, which results in reduced T cell responses against tumor cells (Markiewski et al. 2008). Another report shows that the signaling balance of paired immunoglobulin-like receptor (PIR) family members PIR-A and PIR-B are expressed on MDSCs and are important for the regulation of MDSC differentiation. MDSCs isolated from PIR-B-deficient $Lilrb3^{-/-}$ mice preferentially differentiate into an M1-like rather than M2-like immunosuppressive phenotype in Lewis lung cancer (LLC; also known as 3LL) tumor-bearing mice. LPS and IFN-γ stimulation enhances M1 polarization by suppressing STAT3 activation in the absence of PIR-B (Ma et al. 2011). Expansion of MDSCs are also regulated by other transcription factors (Condamine and Gabrilovich 2011; Sonda et al. 2011). Collectively, TLR signals are involved in MDSC expansion directly and indirectly.

3.3.3 Regulation of Immune Responses by MDSCs

MDSCs suppress anti-tumor T cell responses by several mechanisms (Fig. 3.2). The immunosuppressive activities of MDSCs are divided into four categories. First, inhibition of T cell proliferation is mediated by depleting nutrients in the microenvironment. MDSCs highly express arginase-1, which rapidly decreases the concentration of L-arginine in the microenvironment. Reduced level of L-arginine concentration causes a profound inhibition of T cell proliferation by the inability to up-regulate cyclin D3 and cyclin-dependent kinase 4 upon antigen stimulation, cytokine production, and expression of the CD3ζ chain of the T cell receptor (TCR) (Zea et al. 2005). MDSCs also inhibit T cell proliferation by sequestering cystine and limiting the availability of cysteine (Srivastava et al. 2010). Second, ROS and RNS produced by MDSCs modulate immune responses. MDSC-derived peroxynitrate inhibits T cell responses by inducing nitration of tyrosine residues in TCRs, resulting in an altered TCR/major histocompatibility complex (MHC) peptide recognition (Nagaraj et al. 2007). In parallel, peroxynitrite induces tumor cell resistance to CTLs by modifying MHC class I–antigen complex (Lu et al. 2011). Among two subsets, G-MDSCs suppress antigen-specific $CD8^+$ T cells predominantly by producing ROS (Youn et al. 2008). Third, MDSCs modulate lymphocyte trafficking. CD62L (L-selectin) expression on naive $CD4^+$ and $CD8^+$ T cells are decreased by a disintegrin and metalloprotease 17 (ADAM17) on MDSCs, leading to the inhibition of recruitment to lymph nodes (Hanson et al. 2009). Fourth, MDSCs indirectly affect T cell activation by inducing immunomodulatory cells such as $Foxp3^+$ Tregs and M2 TAMs. MDSC-derived TGF-β and IL-10 are required for Treg induction (Huang et al. 2006; Serafini et al. 2008). In addition to the differentiation of

MDSCs into M2 macrophages in tumors, MDSC-derived IL-10 and cell–cell interaction promote M2 polarization of macrophages, as well as impair cytokine production and antigen presentation by DCs.

How MDSCs are implicated in the regulation of NK cell function is controversial. MDSCs inhibit NK cell cytotoxicity against tumor cells and IFN-γ production through direct cell–cell interaction. Membrane-bound TGF-β1 expressed on MDSCs inhibits NK cell cytotoxicity and IFN-γ production, and induces anergy of NK cells in a liver transplant model (Li et al. 2009). MDSCs also inhibit NK cell activation by blocking the expression of NK group 2D (NKG2D). However, another report suggests that the $F4/80^+$ population of MDSCs express retinoic acid early inducible 1 (RAE-1), the ligand for NKG2D, activate NK cell cytotoxicity (Nausch et al. 2008).

These immunoregulatory functions of MDSCs are regulated by TLR and TLR-induced cytokine production. IFN-γ stimulation induces suppression of antigen-specific T cell responses by M-MDSCs, which requires STAT1 activation (Movahedi et al. 2008). STAT3 signaling is potentially activated by pro-inflammatory cytokines such as IL-6 and is implicated in arginase-1 expression in $CD14^+HLADR^{-/low}$ MDSCs from head and neck cancer patients (Vasquez-Dunddel et al. 2013). Hypoxia-inducible factor (HIF)-1α activation enhances immunosuppressive function and differentiation of MDSCs in the tumor microenvironment (Corzo et al. 2010). LPS-induced TLR4 signal activates HIF-1α (Frede et al. 2006), suggesting that TLR4-triggered MDSC accumulation and development may be induced via HIF-1α-mediated transcriptional regulation. Activation of TLR as well as IL-1 receptor and receptor tyrosine kinases activate $Gr1^+CD11b^+$ MDSC-like cells to promote tumor inflammation and progression through phosphoinositide 3-kinase γ (Schmid et al. 2011).

3.4 Tumor-Associated Myeloid Cells as a Therapeutic Target of Cancer

Stromal cells in the tumor microenvironment are considered to be promising targets for cancer treatment (Quail and Joyce 2013). Recent reports suggest that regulation of immunosuppressive activity of tumor-associated myeloid cells could be useful for improving the efficacy of cancer immunotherapy. Therefore, strategies for elimination of tumor-associated myeloid cells or modulation of their function in tumor-bearing hosts are currently being investigated (Ugel et al. 2009; Talmadge and Gabrilovich 2013). Molecules that are responsible for the accumulation and immunosuppressive activity of MDSCs and TAMs could become therapeutic targets. There are several classes of inhibitors or reagents that can control the population or modulate the function of tumor-associated myeloid cells as described in other review (Ugel et al. 2009).

3.4.1 *Re-education of Immunosuppressive Myeloid-Derived Cells Expanded in Cancer*

Re-education of immunosuppressive cells toward cells with anti-tumor activity is an emerging concept of cancer therapy (Allavena and Mantovani 2012; Yang et al. 2013; Quail and Joyce 2013). It has been demonstrated that the conversion of TAMs and MDSCs into tumoricidal effector cells or ablation of their immunosuppressive activity results in tumor regression. Innate immune signaling induced by endogenous TLR ligands predominantly leads to expansion of TAMs and MDSCs with tumor-promoting activity, whereas manipulation of tumor-associated myeloid cell function could be achieved by using purified exogenous TLR ligands. Innate immune signaling on tumor-associated myeloid cells could be an attractive target for overcoming immune suppression induced by tumor and tumor-associated myeloid cells.

Purified TLR ligands have been studied in clinical trials for cancer immunotherapy (Galluzzi et al. 2012; Kaczanowska et al. 2013). It has been demonstrated that ligands of TLR2 [Pam3CSK4, Pam3CSK4, and bacillus Calmette-Guerin (BCG) and BCG cell wall skeleton (BCG-CWS)], TLR3 (poly I:C, poly I:C-LC, Ampligen, poly A:U), TLR4 (MPLA), TLR5 (flagellin), TLR7 and TLR8 (imiquimod), and TLR9 [CpG oligodeoxynucleotides (ODNs)] are capable of inhibiting tumor growth by modulating DC function in mouse models (Ahonen et al. 2004; Sfondrini et al. 2006; Huang et al. 2008; Seya et al. 2010). In spite of the presence of tumor-associated myeloid cells with immunosuppressive activity, these TLR ligands elicit innate immune responses against cancer. Therefore, innate immune signals seem to overcome the suppression by making an alteration of tumor-associated myeloid cell function. Recent reports have suggested that immunosuppressive TAMs and MDSCs could be converted into cells that have anti-tumor activity by manipulating signaling pathway induced by innate immune signaling (Figs. 3.3 and 3.4)

3.4.2 *Modification of TAM Function by Innate Immune Signaling*

M1 polarization of TAMs and tumor destruction are achieved by activating innate immune signaling. Bacteriophages induce secretion of M1-related pro-inflammatory cytokines and increased expression of molecules involved in antigen presentation and co-stimulation in TAMs through the TLR/MyD88 pathway (Eriksson et al. 2009). Another report shows that Notch signaling determines M1/M2 polarization in tumor-infiltrating macrophages (Wang et al. 2010). Forced activation of Notch in macrophages promotes M1 polarization while repressing M2 polarization through RBPJ. TLR4 signal-induced NF-κB and the IL-1 receptor-associated kinase 2 (IRAK2)/ MAPK-interacting kinase 1 (Mnk1) pathway cooperatively activate RBPJ

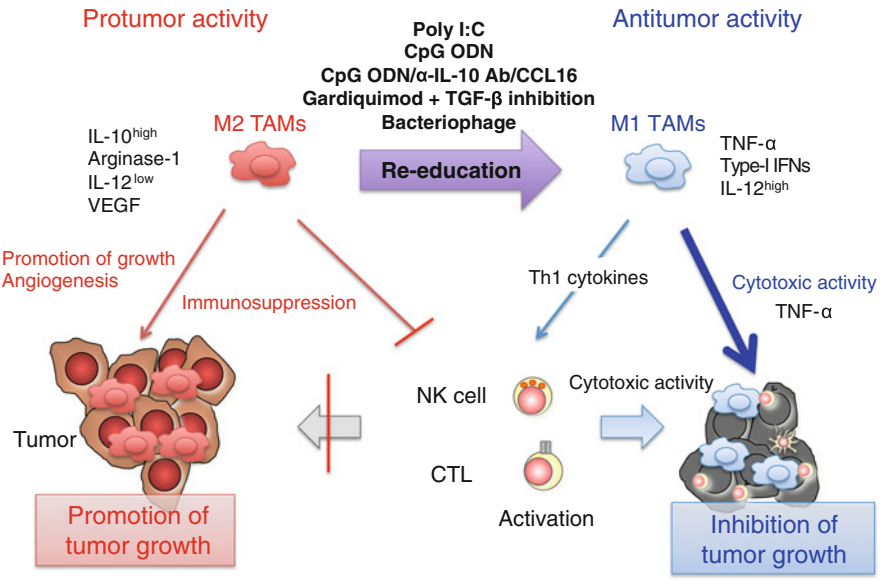

Fig. 3.3 Induction of M1-like tumor-associated macrophages (TAMs) by Toll-like receptor (TLR) activation. Poly I:C (TLR3), CpG oligodeoxynucleotide (ODN) (TLR9), or combined use of CpG ODNs, anti-interleukin (IL)-10 antibody, and chemokine CC ligand (CCL) 16-expressing vector, gardiquimod (TLR7), or bacteriophage alter the phenotype of TAMs from immunosuppressive M2 to anti-tumor M1 through activation of the intracellular TLR signaling pathways in TAMs. M1-like TAMs produce interferon (IFN)-β and pro-inflammatory cytokines including IL-12 and tumor necrosis factor (TNF)-α. TNF-α is responsible for direct killing of a certain type of tumor cells by M1-like TAMs. Other T helper (Th) 1 cytokines contribute to the activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells

signaling. Notch/RBPJ augments TLR4-induced M1-related genes via IRF8 expression in macrophages (Xu et al. 2012). Therefore, TLR4 ligands may affect macrophage polarization in tumors by regulating the Notch/RBPJ signaling pathway. TLR signals mostly activate NF-κB to promote expression of pro-inflammatory cytokines. However, NF-κB activation induced by IL-1β/MyD88 signaling is involved in M2 polarization of TAMs and targeting of NF-κB signaling can re-educate TAMs to become M1 macrophages (Hagemann et al. 2008). When NF-κB signaling is inhibited by using dominant negative form of IκB kinase β (IKKβ), TAMs show an M1-polarized phenotype represented by IL-12^{high} and MHC I^{high}, but IL-10^{low} and arginase-1^{low} and anti-tumor activity. We have recently demonstrated that poly I:C administration into tumor-bearing mice leads to re-education of TAMs, resulting in retardation of tumor growth (Shime et al. 2012). In mice implanted with 3LL lung cancer cells, poly I:C injection rapidly (within 1 h) up-regulates TNF-α production in tumor by F4/80^{+} TAMs but not CD11b^{+}Gr1^{+} MDSCs. It results in retardation of tumor growth by TNF-α-induced direct killing of 3LL tumor cells and hemorrhagic necrosis of the tumor. Poly I:C treatment up-regulates expression of M1-related genes such as *I FN-β*, *IL-12*, *IL-1β*, *IL-6*, and *Cxcll1*, while the expression of

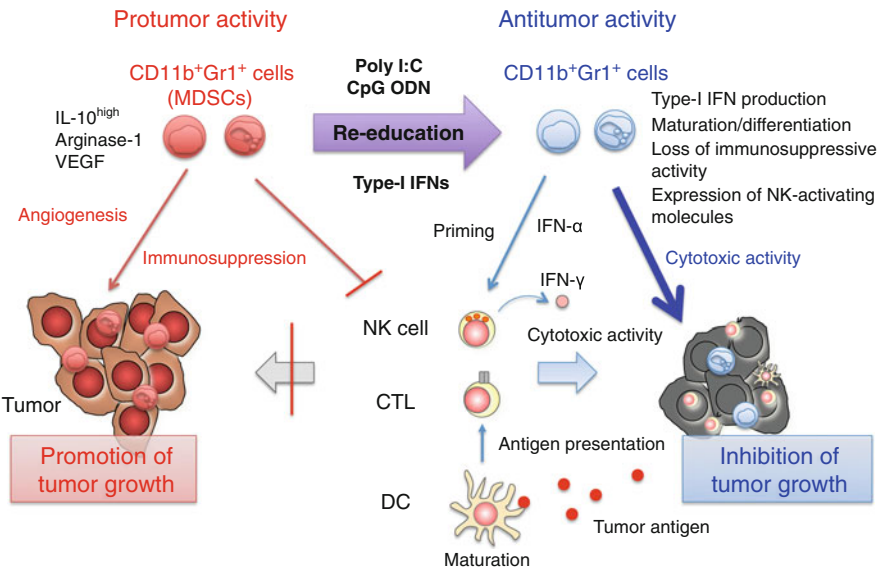

Fig. 3.4 Modification of myeloid-derived suppressor cell (MDSC) function by innate immune signaling. Poly I:C [Toll-like receptor (TLR) 3 and melanoma differentiation-associated protein-5 (MDA5)], CpG oligodeoxynucleotide (ODN) (TLR9), and interferon (IFN)-α produced by CpG ODN-activated plasmacytoid dendritic cells (pDCs) induces maturation and activation of MDSCs. As a result, immunosuppression is abrogated and tumor growth is inhibited by restoring cytotoxic T lymphocyte (CTL) responses. Furthermore, MDSCs produce IFN-α and express natural killer (NK)-activating molecules in response to poly I:C, leading to IFN-γ production by NK cells

M2-related genes such as *Arg1* is not affected. These responses require the activation of the TLR3/TICAM-1 (TRIF) signaling pathway, which is mostly essential for poly I:C-induced $CD8^+$DC activation to induce cytotoxicity of CTLs and NK cells (Akazawa et al. 2007; Azuma et al. 2012; Seya et al. 2012). Thus, the TLR3/TICAM-1 signaling pathway induces re-education of TAMs as well as DC activation. A recent study demonstrated that macrophages stimulated with tumor-derived versican produce TNF-α, which promotes tumor metastasis to the lungs (Kim et al. 2009). Besides, MDSC accumulation in tumor-bearing mice is induced by TNF-α signaling (Zhao et al. 2012). These study suggest that TNF-α has both positive and negative effect on tumor growth and metastasis by regulating TAMs and MDSCs. These divergent effects of TNF-α in tumor regulation may be explained by the difference in the local concentration of TNF-α in the tumor between tumor development and treatment with TLR ligand. TAMs accumulate in 3LL tumors, where local TNF-α concentration transiently increases in response to poly I:C stimulation. Antitumor activity of TNF-α has been demonstrated by using a high dose of exogenous TNF-α or forced expression of TNF-α in tumor cell lines (Blankenstein et al. 1991; Zhao et al. 2007). Therefore, concentration of TNF-α derived from accumulated TAMs seems to be a critical determinant for growth of certain types of tumor cells.

Combined use of TLR ligand and other reagents modifying intracellular signaling in TAMs is demonstrated to be effective for inhibiting tumor growth mediated by tumor-associated myeloid cells. Local injection of CpG ODN and adenovirus encoding CCL16 chemokine into tumor, combined with systemic IL-10 receptor (IL-10R) antibody, effectively induces tumor eradication. In this case, resident tumor-infiltrating macrophages with M2-like phenotype are switched to macrophages with M1-like phenotype. Consequently, tumor-infiltrating DCs are matured and secrete pro-inflammatory cytokines to induce adaptive immune responses for tumor rejection (Guiducci et al. 2005). TLR7 ligand in combination with blocking reagent for TGF-β signaling induces the conversion of TAMs in their phenotype from M2 to M1. As a result, tumor apoptosis is increased and the number of $CD4^+$, $CD8^+$, and $CD19^+$ cells as well as neutrophils infiltrated into tumor is elevated (Peng et al. 2013).

3.4.3 Modification of MDSC Function by Innate Immune Signaling

TLR ligands alter MDSCs into cells with anti-tumor activity directly and indirectly. CpG ODN directly modifies MDSC function. Shirota et al. have reported that intratumoral injection of CpG ODN stimulates MDSCs to lose their suppressive activity on T cell proliferation, produce Th1 cytokines, and differentiate into macrophages with tumoricidal capability (Shirota et al. 2012). Other reports suggest that type-I IFNs produced in response to TLR stimulation modify MDSC function. Type-I IFNs are critical cytokines required for efficient anti-tumor immune responses elicited by TLR3, TLR7, and TLR9 signaling pathways. It has been demonstrated that type-I IFNs modulate the function of not only tissue-resident DCs and macrophages but also tumor-associated myeloid cells. Zoglmeier et al. have reported that CpG ODN treatment decreases the suppressive activity of MDSCs in mice bearing C26 tumors and CEA424-Tag mice bearing autochthonous gastric tumors (Zoglmeier et al. 2011). CpG induces maturation of MDSCs through plasmacytoid DC (pDC)-mediated type-I IFN production, which results in reduction of suppressive activity of MDSCs on T cell proliferation. Poly I:C also has similar activity on MDSCs via type-I IFN production, probably by hematopoietic and non-hematopoietic cells that express TLR3 and/or melanoma differentiation-associated protein-5 (MDA5) (McCartney et al. 2009). MDSCs activated by in vivo poly I:C treatment also produce IFN-α, implying the existence of an autocrine mechanism for MDSC activation (Shime et al. 2014). We have revealed that poly I:C treatment also induces maturation and activation of $CD11b^+Gr1^+$ MDSC-like cells to induce partial activation of NK cells (i.e., up-regulation of IFN-γ production, but not cytotoxicity) (Shime et al. 2014). Poly I:C treatment induces activation of MDSCs to produce IFN-α and express several surface molecules that are known to be involved in matured DC-mediated NK cell activation, such as RAE-1, IL-15, CD70, CD155

[poliovirus receptor (PVR)], CD150 [signaling lymphocyte-activation molecule (SLAM)] and IRF-3-dependent NK-activating molecule (INAM) (Newman and Riley 2007; Ebihara et al. 2010). In this pathway, IFN-α/-β receptor (IFNAR) signaling triggered by IFN-α on NK cells is critical for NK cell IFN-γ production induced by MDSCs from poly I:C-treated tumor-bearing mice. IFN-α produced through activation of the Mitochondrial Antiviral Signaling Protein (MAVS) (also known as IPS-1, VISA, and Cardif) pathway is responsible for both MDSC activation and NK cell priming by MDSCs. Therefore, MDSCs could be re-educated to have anti-tumor activity through the activation of the poly I:C-triggered MAVS signaling pathway. These reports suggest that type-I IFNs are critical factors for re-education of MDSCs.

Therapeutic use of TLR ligands combined with other agents has been proposed (Vanneman and Dranoff 2012). However, it should be noted that inappropriate use of TLR ligand as an adjuvant may induce undesirable effects on tumor growth. Imiquimod, a TLR7 ligand, significantly increases the levels of MDSCs and Tregs in mice immunized with self tumor antigen such as insulin-like growth factor-binding protein-2 (IGFBP-2) (Dang et al. 2012). Moreover, imiquimod reduces anti-tumor immunity induced by GM-CSF treatment through the expansion of MDSCs and Tregs. Although TLR ligands effectively induce anti-tumor immunity and tumor regression, it should be taken into consideration that they may have undesirable effects when used in combination therapy with other reagents.

3.5 Concluding Remarks

TAMs and MDSCs are proposed to be a target of cancer immunotherapy because they frequently accumulate in solid tumors and have critical roles in tumor growth and progression. Furthermore, they display a high degree of plasticity in their phenotype. Recent results have highlighted that TLR signaling pathways have important roles for switching between immunosuppressive phenotype and anti-tumor phenotype of TAMs and MDSCs.

Adjuvant immunotherapy using purified TLR ligands or TLR agonists seems to be a promising treatment for cancer by inducing DC-mediated anti-tumor responses. Recent reports suggest that TAMs and MDSCs play important roles in adjuvant therapy. However, it is true that TLR signals such as TLR2 and TLR4 contribute to promote tumor growth by inducing immunosuppressive activity of TAMs and MDSCs. Timing of administration and selection of TLR ligands may determine the outcome of adjuvant immunotherapy for cancer because the tumor microenvironment continuously changes during the course of cancer progression where the population and the function of TAMs and MDSCs are varied. Recent studies show that poly I:C is capable of inducing not only DC-mediated anticancer immune responses that lead to the activation of NK cells and CTLs, but also anti-tumor activity of TAMs and MDSCs. Therefore, poly I:C-induced TLR3/TICAM-1 and MDA5/MAVS pathways are promising targets of cancer treatment. Further basic studies to

clarify the mechanisms of anti-tumor and pro-tumor effects induced by innate immune signaling on tumor-associated myeloid cells are still required to establish adjuvant therapy for cancer.

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Chapter 4
The Role of PIKfyve in Toll-Like Receptor-Mediated Responses

Kaoru Hazeki, Kiyomi Nigorikawa, and Osamu Hazeki

Abstract PIKfyve is the only kinase that phosphorylates the 5′-position of phosphatidylinositol (PtdIns) 3-phosphate to produce PtdIns (3,5)-bisphosphate, which is indispensable for intracellular trafficking. Thus, this kinase is a potential regulator of various cellular events, such as autophagy, stress-mediated responses, and membrane and ion transport. However, little is known about the physiological function of PIKfyve in innate immune responses. A chemical that has been subjected to clinical tests for autoimmune diseases for the last decade was recently identified as a PIKfyve inhibitor. Additionally, in 2013, a few studies reported some roles of PIKfyve in Toll-like receptor-mediated responses. Although the reported findings are convincing and interesting, the interpretation of the function of PIKfyve based on these reports remains contradictory. In this review, we survey these reports together with data from the clinical tests of the PIKfyve inhibitor and present our perspective on the role of PIKfyve in innate immunity.

Keywords PIKfyve • Phosphoinositides • Toll-like receptor

4.1 Introduction

Phosphoinositides (PIs), the phosphorylated derivatives of phosphatidylinositol (PtdIns), play a central role in cytoskeletal dynamics and membrane trafficking (Lindmo and Stenmark 2006). At present, we know that 19 PI kinases and 28 PI phosphatases are involved in the PI interconversion reactions that generate seven PIs in mice (Sasaki et al. 2009). The details of these reactions and enzymes are described in other reviews (Krauss and Haucke 2007; Mayinger 2012; Sasaki et al. 2009). The various PIs found on the membrane are markers for the recruitment of a specific and distinct set of proteins that determine the membrane identity. One of the most well-documented PIs associated with membrane trafficking is PtdIns(3)P,

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which is mainly produced by the class III PI3-kinase Vps34 (Krauss and Haucke 2007). PtdIns(3)P and Vps34 are enriched in subcellular compartments, such as the phagosome, autophagosome, and early endosome (Krauss and Haucke 2007; Mayinger 2012). The fate of newly synthesized PtdIns(3)P on the endosome is not well-documented, but it is known that this is hydrolyzed to form PtdIns and also phosphorylated to form PtdIns(3,5)P_2 and PtdIns(3,4)P_2. Among these, PtdIns(3,5)P_2 is enriched in the acidic late endosome or endolysosome (Ho et al. 2012).

Mammalian PIKfyve, so named after its function and domain structure [PI Kinase for five position containing a Fyve finger; also known as type III PtdIns phosphate kinase (PIPKIII)] is the only lipid kinase that phosphorylates the 5′-positions of PtdIns and PtdIns(3)P to yield PtdIns(5)P and PtdIns(3,5)P_2, respectively, in vitro (McCartney et al. 2014). However, in intact cells, it remains unclear whether PIKfyve directly generates most of the cellular PtdIns(5)P and whether PtdIns(3,5)P_2 serves as a precursor for PtdIns(5)P (Ho et al. 2012; McCartney et al. 2014). PIKfyve is hypothesized to form a large complex with Vac14 and Sac3 (Ho et al. 2012; McCartney et al. 2014), two components that are necessary for its full activation. General information on PIKfyve, such as its structure, functional domains, associated proteins, and regulation of its activity, is described in some excellent previously published reviews (McCartney et al. 2014; Shisheva 2008; Takasuga and Sasaki 2013).

PIKfyve knockout mouse models have not been available because of embryonic lethality (Ikonomov et al. 2011). In the past years, most of the related findings were deduced from the function of the yeast ortholog, Fab1. However, YM201636 was reported as a specific inhibitor of PIKfyve in 2008 (Jefferies et al. 2008). Since then, this inhibitor, together with small-interfering RNA (siRNA), has been used as a good probe for various experimental approaches. Last year, STA-5326/apilimod was identified as another inhibitor of PIKfyve (Cai et al. 2013). Surprisingly, this new inhibitor has been subjected to a phase II clinical test for autoimmune diseases for a decade. There are many clinical data on the effect of the inhibitor on interleukin (IL)-12 production. In this review, we first trace the history of apilimod and then review the current studies on PIKfyve in terms of immune responses. We also present our perspective on this issue.

4.2 History of Apilimod

4.2.1 Clinical Tests

Apilimod first appeared in 2004 as a candidate novel drug for the treatment of inflammatory and autoimmune diseases, such as Crohn's disease, psoriasis, rheumatoid arthritis, and multiple sclerosis (Borchardt 2004). These diseases are characterized by a T helper (Th) 1 cell response and elevated levels of IL-12 (Wada et al. 2007). IL-23, which shares the p40 protein subunit with IL-12, is also increased. IL-23 causes a Th17 response, which plays a significant role in inflammatory

responses (Wada et al. 2007). Thus, antibodies against IL-12/IL-23 may provide a significant medical benefit. However, a small-molecule IL-12 inhibitor that can be administrated orally remains highly desirable. Thus, apilimod has been subjected to clinical experiments as an oral inhibitor for the treatment of autoimmune diseases. The first test we identified in the PubMed database was performed with 73 patients with active Crohn's disease (Burakoff et al. 2006). Clinical activity was observed in most patients (>70 %), with mild-to-moderate side effects. Preclinical studies demonstrated the successful inhibition of IL-12 and IL-23 production by the drug. Thus, apilimod appeared to be a promising treatment for Crohn's disease (Billich 2007; Burakoff et al. 2006). The next test was performed as a multicenter, phase II, randomized, double-blinded, placebo-controlled study to evaluate the efficacy of apilimod in the treatment of 220 adult patients with Crohn's disease (Sands et al. 2010). Unexpectedly, apilimod did not demonstrate efficacy over the placebo in patients with active Crohn's disease (Sands et al. 2010). A similar phase II study of apilimod was performed in 29 patients with active rheumatoid arthritis (Krausz et al. 2012). Although there was a small but significant reduction in the DAS28 (28-joint Disease Activity Score of rheumatoid arthritis), the researchers did not observe the effect of apilimod on IL-12 and IL-23 expression in CD68+ (macrophage-like) synovial cells. As a conclusion, the authors did not confirm the hypothesis that IL-12/IL-23 inhibition by apilimod is able to induce clinical improvement in rheumatoid arthritis (Krausz et al. 2012). In contrast, an open-label clinical study of oral administration in patients with psoriasis reported a positive conclusion (Wada et al. 2012). Substantial improvements in histology were observed in patients receiving apilimod. The expression of IL-23p19 and IL-12p40 in skin lesions was reduced, whereas that of IL-10 was increased in the apilimod-treated group. Although a statistical analysis revealed a significant effect of apilimod, there were non-responders in terms of a histological response (Wada et al. 2012). Interestingly, the epidermal CD11c+ cells in the lesions were almost completely cleared in the responder group but not in the non-responders. Hence, the authors described that the dramatic reduction of Th1/Th17 responses in the apilimod-treated group is the consequence of the clearance of IL-12-/IL-23-producing CD11c+ cells (Wada et al. 2007). The clinical studies that are found in PubMed are summarized in Table 4.1. In most cases, apilimod is more or less effective, but there were differences among individuals even when the final decision is positive (Wada et al. 2007). The effect of apilimod on IL-12 production was also varied: it was decreased in most people, but extremely increased in a few people (Krausz et al. 2012). These reports led us to hypothesize that the effect of apilimod on IL-12 production varies depending on the substantial cell types involved.

The therapeutic effect of apilimod was also tested in a mouse model (experimental autoimmune uveoretinitis) by oral administration. The researchers who studied this mouse model reported a decreased level of serum IL-12p40 and a suppression of inflammation (Keino et al. 2008). Additionally, in vivo studies using a mouse inflammatory bowel disease model demonstrated that the oral administration of apilimod markedly reduced the inflammatory histological changes in the colon (Wada et al. 2007).

Table 4.1 The results of clinical tests on apilimod

Diseases	Number of patients	Results	Effect of apilimod on cytokine production	Ref.
Crohn's disease	73	Significant		Burakoff et al. (2006)
Crohn's disease		Significant		Billich (2007)
Crohn's disease	220	Not significant		Sands et al. (2010)
Rheumatoid Arthritis	29	A small but significant	IL-12/IL-23p40→ (synovial CD68+ cell)	Krausz et al. (2012)
Psoriasis		Significant	IL-12/IL-23p40↓, IL-10↑ (skin lesions CD11c+ cells)	Wada et al. (2012)
Mouse (in vivo)				
Mouse model (experimental autoimmune uveoretinitis)		Significant	IL-12↓ (serum)	Keino et al. (2008)
Mouse inflammatory bowel disease model		Significant	IFN-γ↓ (lamina propia mononuclear cells)	Wada et al. (2007)

IL interleukin, *IFN* interferon

4.2.2 Apilimod, an Interleukin-12 Inhibitor, Is a PIKfyve Inhibitor

In 2007, apilimod was discovered by a biological strategy independent of the clinical tests. Wada et al. discovered this drug among an 80,000-compound library in a cell-based screen aimed at identifying an inhibitor of IL-12 production using interferon (IFN)-γ/lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs) (Wada et al. 2007). The IL-12 production induced by IFN-γ/*Staphylococcus aureus* Cowan I (SAC) was also susceptible to apilimod in PBMCs (Wada et al. 2007). The cytokine inhibitory spectrum of apilimod that was described by the paper published by Wada et al. is seen in Table 4.2.

When the above-referenced work was published, the inhibitory target of apilimod was not known. The target molecule, PIKfyve, was identified in 2013 through a chemical proteomics approach (Cai et al. 2013). A bioactive analog of apilimod, APA10, was immobilized on gel matrices and incubated with a THP-1 cell extract premixed with either dimethyl sulfoxide or excess apilimod. The specifically bound proteins were eluted and identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS). As a result, only three proteins were identified: PIKfyve, Vac14, and Sac3. As mentioned above, PIKfyve displays its full activation as a large complex with Vac14 and Sac3. These researchers demonstrated that apilimod does

Table 4.2 Cytokine inhibitory spectrum of PIKfyve inhibitors

Cell	Stimuli	Cytokine	Apilimod IC_{50} (nM)	TLR	Ref
Human PBMC	IFN-γ/SAC	IL-12p70	1	2	Wada et al. (2007)
Human PBMC	SAC	IL-12p70	5	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-23	10	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-12/IL-23p40	20	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IFN-γ	20	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-6	100	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-10	200	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	TNF-α	1,000	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-1β	>1,000	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-8	>1,000	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-18	>1,000	2	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-12p70	10	4	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-6	>1,000	4	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-10	>1,000	4	Wada et al. (2007)
Human PBMC	IFN-γ/SAC	IL-12p40	2.4	4	Cai et al. (2013)
Human PBMC	R837	IL-12p40	3	7	Cai et al. (2013)
Human PBMC	ssRNA	IL-12p40	2.8	7	Cai et al. (2013)
Human PBMC	ConA	IL-5	100		Wada et al. (2007)
Human PBMC	ConA	IL-4	20		Wada et al. (2007)
Human PBMC	αCD3/ αCD28Ab	IFN-γ	400		Wada et al. (2007)
Human PBMC	αCD3/ αCD29Ab	IL-4	40		Wada et al. (2007)
Human PBMC	αCD3/ αCD30Ab	IL-2	800		Wada et al. (2007)
Human THP-1	IFN-γ/SAC	IL-12p70	10	2	Wada et al. (2007)
Human THP-1	IFN-γ/SAC	IL-6	>1,000	2	Wada et al. (2007)
Human THP-1	IFN-γ/LPS	IL-12p40	23	4	Cai et al. (2013)
Human THP-1	R848	IL-12p40	31	8	Cai et al. (2013)
Human THP-1	IFN-γ/LPS	IL-8	>1,000	4	Cai et al. (2013)
Human dendritic cells	IFN-γ/SAC	IL-12p70	1	2	Wada et al. (2007)
Human monocytes	IFN-γ/SAC	IL-12p70	5	2	Wada et al. (2007)
Mouse spleen cells	IFN-γ/SAC	IL-12p70	2	2	Wada et al. (2007)
Mouse PBMC	IFN-γ/SAC	IL-12p70	20	2	Wada et al. (2007)
Cell	Stimuli	Cytokine	Apilimod	TLR	Ref
Mouse BMDC	IFN-γ/LPS	IL-12p40	↓	4	Cai et al. (2013)
Mouse BMDC	IFN-γ/LPS	CXCL2	→	4	Cai et al. (2013)
Mouse BMDC	R848	IL-12p40	↓	7	Cai et al. (2013)
Mouse BMDC	R848	CXCL2	→	7	Cai et al. (2013)

(continued)

Table 4.2 (continued)

Cell	Stimuli	Cytokine	YM201636	TLR	Ref
MEF	LPS	IFN-β (mRNA)	↓	4	Kawasaki et al. (2013)
MEF	polyI:C	IFN-β (mRNA)	↓	3	Kawasaki et al. (2013)
HEK293	LPS	ISRE (reporter assay)	↓	4	Kawasaki et al. (2013)
HEK293	polyI:C	ISRE (reporter assay)	↓	3	Kawasaki et al. (2013)
HEK293	LPS	NF-κB (reporter assay)	→	4	Kawasaki et al. (2013)
HEK293	polyI:C	NF-κB (reporter assay)	→	3	Kawasaki et al. (2013)
Mouse macrophage	CpG	IL-12p40	↓	9	Hazeki et al. (2013)
Mouse macrophage	CpG	IL-10	↓	9	Hazeki et al. (2013)
Mouse macrophage	CpG	TNF-α	↓	9	Hazeki et al. (2013)
Mouse macrophage	CpG	IL-1β	↓	9	Hazeki et al. (2013)
Mouse macrophage	LPS	IL-12p40	→	4	Hazeki et al. (2013)
Mouse macrophage	LPS	IL-10	↑	4	Hazeki et al. (2013)
Mouse macrophage	LPS	TNF-α	→	4	Hazeki et al. (2013)
Mouse macrophage	LPS	IL-1β	↓	4	Hazeki et al. (2013)

BMDC bone marrow-derived cell, *CXCL* C-X-C motif chemokine, *HEK* human embryonic kidney, IC_{50} half-maximal inhibitory concentration, *IFN* interferon, *IL* interleukin, *ISRE* IFN-stimulated response element, *LPS* lipopolysaccharide, *NF* nuclear factor, *PBMC* peripheral blood mononuclear cell, *SAC Staphylococcus aureus* Cowan I, *ssRNA* single-stranded RNA, *TLR* Toll-like receptor, *TNF* tumor necrosis factor

not inhibit other lipid kinases or protein kinases (Cai et al. 2013). The specificity is better than YM201636, which inhibits class I PI 3-kinase at higher concentrations (Ikonomov et al. 2009). These researchers mainly used human PBMCs, THP-1 cells and mouse bone marrow-derived cells (BMDCs) to determine the cytokine inhibitory spectrum of apilimod (Table 4.2). As a conclusion, these researchers reported that apilimod is a specific inhibitor of IL-12/IL-23 production induced by Toll-like receptor (TLR) ligation (Cai et al. 2013).

4.3 The Role of PIKfyve in the Intestinal Immune System

PIKfyve knockout mouse embryos die by embryonic day 8.5 (Takasuga et al. 2013). However, intestine-specific knockout mice were recently generated (Takasuga et al. 2013). Interestingly, the mice exhibit diarrhea and bloody stool, and their gut epithelial layers show inflammation and fibrosis, as observed in inflammatory bowel diseases (Takasuga et al. 2013). In the intestinal mucosa of these mice, the mRNA expression levels of inflammatory cytokines, namely tumor necrosis factor (TNF)-α, IL-12p40, C-C chemokine ligand 2 (CCL2), IL-6, C-X-C motif chemokine 5 (CXCL5), IL-1β and transforming growth factor (TGF)-β1, are increased (Takasuga et al. 2013). This effect appears contradictory to the effect of the PIKfyve inhibitor on IL-12 production. However, as mentioned above, the effect of the inhibitor varies depending on the cell type. Most of the works demonstrating the inhibitory effect of apilimod on IL-12 production were performed with human PBMCs or mouse BMDCs. Because intestinal cells are always exposed to resident intestinal bacteria, it is important for them to maintain a balance between protective immunity and tolerance to commensal bacteria. The failure in maintaining this balance results in inflammatory bowel diseases. Regulatory T cells (Tregs) play a substantial role in maintaining immune tolerance (Rouse and Sehrawat 2010). Two different subsets of dendritic cells (DCs), namely CD103+/CD11c+ and CD103+/CD11b+ DCs, are reported to be involved in the differentiation of Tregs in the intestine (Lewis et al. 2011; Persson et al. 2013; Schlitzer et al. 2013). Macrophages also play a role in maintaining immune tolerance through the production of an anti-inflammatory cytokine, namely IL-10 (Murai et al. 2009). It is likely that PIKfyve plays a role in the regulation of the function of these cells to maintain the homeostasis of the intestinal immune system.

4.4 The Role of PIKfyve in *Salmonella* Replication

A defect in PIKfyve function induced by siRNA-mediated knockdown or YM201636 treatment inhibits the intracellular replication of *Salmonella* in epithelial cells (Kerr et al. 2010). PtdIns(3,5)P_2 is indispensable for macropinosome–late endosome/lysosome fusion. This PtdIns(3,5)P_2-dependent step is required for the proper maturation of the *Salmonella*-containing vacuole. Although the inhibition of PIKfyve in macrophages inhibits *Salmonella* replication, it also disrupts the macrophage's bactericidal response (Kerr et al. 2010).

4.5 The Role of PIKfyve in Pathogen-Associated Molecular Patterns Signals

Innate immune responses are triggered by pathogen-associated molecular patterns (PAMPs), which are recognized by pattern-recognition receptors (PRRs), including TLRs, retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), C-type lectin

receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and intracellular sensors for DNA (Kawai and Akira 2011; Sancho and Reis e Sousa 2012; Strowig et al. 2012). CLRs comprise a large family of receptors that bind to carbohydrates (Sancho and Reis e Sousa 2012). Most of the family members are trans-membrane receptors (Sancho and Reis e Sousa 2012). RLRs, NLRs, and the DNA sensors reside in the cytosol and recognize double-stranded RNA (dsRNA), cytoplasmic PAMPs, and double-stranded DNA (dsDNA), respectively (Kawai and Akira 2011; Sancho and Reis e Sousa 2012; Strowig et al. 2012). TLRs can be divided into two groups based on their subcellular localization: TLRs 1, 2, 4, 5, and 6 are cell surface receptors that recognize surface structures of pathogens, whereas TLRs 3, 7, 8, and 9 reside in intracellular compartments and detect microbial nucleic acids (McGettrick and O'Neill 2010). Thus, it is likely that the localization and trafficking of the receptors and their ligands are important factors in the regulation of innate immunity (McGettrick and O'Neill 2010). Hence, PIKfyve may play a role in the appropriate arrangement of these receptors and their ligands.

4.5.1 Toll-Like Receptor (TLR) 9-Mediated Responses

TLR9 is a receptor for oligodeoxynucleotides that contain unmethylated CpG motifs (CpG). Because TLR9 possesses retention signals that maintain it in the endoplasmic reticulum, TLR9 cleavage by proteases is necessary for its recruitment to the endosomes, where it meets its ligand (Latz et al. 2004; Leifer et al. 2006; Park et al. 2008). CpG has been shown to associate with TLR9 after internalization (Fig. 4.1). Thus, the intracellular trafficking of both the receptor and the ligand is essential in TLR9 signaling (Kawai and Akira 2010; Takeshita et al. 2004). Class III

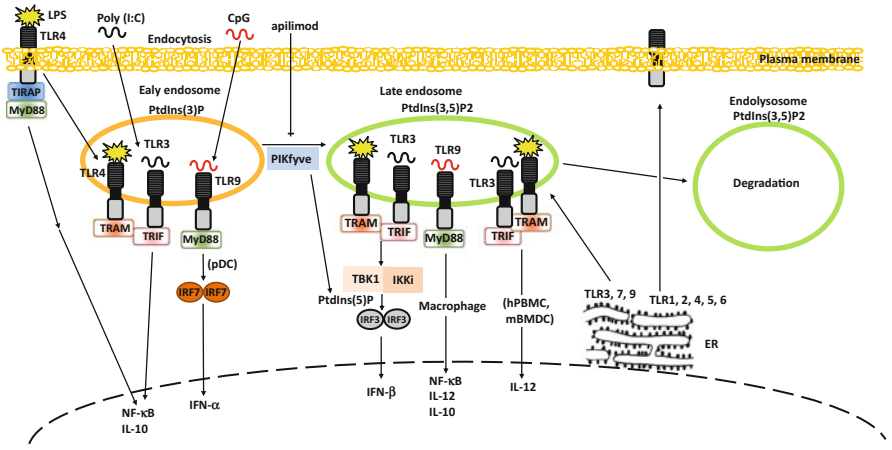

Fig. 4.1 Role of PIKfyve in Toll-like receptor (TLR)-mediated responses (perspective)

PtdIns-3 kinase, which generates endosomal PtdIns(3)P, is involved in TLR9 signaling by regulating CpG uptake (Kuo et al. 2006). We monitored the localization of Rox-CpG and PtdIns(3)P through time-lapse imaging; the latter was visualized with an enhanced green fluorescent protein (EGFP)-fused protein probe. In a normal macrophage, PtdIns(3)P is first co-localized with CpG but rapidly disappears from the CpG-containing endosomes (unpublished data). In contrast, in a macrophage with deficient PIKfyve function, PtdIns(3)P stayed in the CpG-containing endosomes for longer periods (unpublished data). Because TLR9 is waiting for CpG in an acidic late endosome, CpG could not bind to TLR9 in cells with deficient PIKfyve. As a result, the TLR9-mediated signaling events and cytokine production were attenuated in PIKfyve-deficient cells (Hazeki et al. 2013) (Fig. 4.1).

In plasmacytoid DCs (pDCs), the CpG in the early endosome causes type I IFN production, whereas CpG in the lysosome causes nuclear factor (NF)-κB activation (Guiducci et al. 2006; Honda et al. 2005; Kerkmann et al. 2005) (Fig. 4.1). It is speculated that PIKfyve disruption may increase type I IFN production and decrease NF-κB activation in pDCs. We are now attempting to demonstrate this hypothesis.

4.5.2 TLR4-Mediated Responses

TLR4 is a well-characterized cell surface TLR that recognizes LPS with the help of some other proteins, such as LPS binding protein (LBP), CD14, and MD2 (Lu et al. 2008). As expected from the effect of apilimod, the siRNA-mediated knockdown of PIKfyve in THP-1 cells results in the inhibition of IL-12p40 production induced by IFN-γ/LPS (Cai et al. 2013). BMDCs from spontaneous mutant mouse *ingls*, which carries a missense mutation in Vac14, were found to exhibit swollen vacuole formation similar to that observed in apilimod-treated cells (Cai et al. 2013). The TLR7- or TLR4-mediated production of IL-12p40, but not that of CXCL2, was decreased in these mutant cells (Cai et al. 2013). Unexpectedly, however, the PIKfyve inhibitor YM201636 did not inhibit LPS-induced IL-12 production in thioglycollate-induced mouse peritoneal macrophages (Hazeki et al. 2013). Different from apilimod, YM201636 inhibits class I PI 3-kinase and Akt at higher concentrations (Hazeki et al. 2013; Ikonomov et al. 2009). Since class I PI 3-kinase negatively regulates the TLR-mediated IL-12 production (Fukao et al. 2002; Hazeki et al. 2006; Tsukamoto et al. 2008), it is likely that YM201636 increased the IL-12 production by the inhibition of class I PI 3-kinase while decreased it by the inhibition of PIKfyve.

Interestingly, LPS-induced IL-10 production was increased in cells deficient in PIKfyve function (Hazeki et al. 2013). A similar increase in IL-10 production was observed in apilimod-treated human blood stimulated with SAC (TLR2), as described in the Sect. 4.2.1 (Wada et al. 2012).

Because inhibitors of dynamin increase LPS-induce NF-κB activation, the translocation of TLR4/LPS to the endosome was regarded as a negative regulatory mechanism of TLR4 signaling (Husebye et al. 2006). Recently, however, a group of

researchers revealed that the inhibition of TLR4 internalization in macrophages results in a loss of LPS-induced IFN regulatory factor 3 (IRF3) phosphorylation without affecting NF-κB activation (Kagan et al. 2008). Thus, although TLR4/LPS translocates to the endosome to be subjected to degradation, TLR4/LPS activates the TIR-domain-containing adapter-inducing IFN-β (TRIF)-dependent pathway to produce type I IFN before its degradation. Thus, it is speculated that the inhibition of PIKfyve may increase NF-κB-mediated cytokine production through the inhibition of receptor degradation, but also attenuates IRF3-dependent type I IFN production (Fig. 4.1).

Gram-negative bacteria-induced IL-1β production is another TRIF-dependent response. The activation of the NLRP3 inflammasome via TRIF ligation is important for the lysosomal degradation of pro-IL-1β (Rathinam et al. 2012). In addition, LPS-induced IL-1β mRNA expression is severely downregulated by either PIKfyve knockdown or YM201636 treatment (Hazeki et al. 2013), which indicates that the maturation of the TLR4/LPS-containing endosome is indispensable for IL-1β mRNA expression. Chloroquine, an inhibitor of endosome acidification, also decreases LPS-induced IL-1β mRNA expression in human PBMCs and macrophages/monocytes (Jang et al. 2006). Hence, it is likely that both the mRNA expression and the processing of pro-IL-1β depend on endosome maturation and acidification in these cells.

4.5.3 Viral Infection-Mediated Responses

Another role of PIKfyve in the antiviral response was discovered by an in vitro screening system. During bacterial infection, IRF3 controls the expression of the *IFN-β* gene. In the quiescent state, IRF3 is located in the cytoplasm. Upon stimulation with viral infection or LPS challenge, it is phosphorylated on serine and threonine residues to form a dimer and moves to the nucleus (Fitzgerald et al. 2003). TANK-binding kinase 1 (TBK1) and inducible IκB-kinase (IKK-i) are responsible for IRF3 phosphorylation (Fitzgerald et al. 2003). When recombinant TBK1 and IRF3 were mixed and subjected to in vitro kinase reaction, the addition of the lipid fraction from human embryonic kidney (HEK) 293 T cells increased the TBK1-mediated IRF3 activation. The screening of the lipids identified PtdIns(5)P as the factor required for the phosphorylation of IRF3 by TBK1 (Kawasaki et al. 2013). In mouse embryonic fibroblast (MEF) cells, the PIKfyve inhibitor YM201636 inhibited the LPS- or poly(I:C) (TLR3)-induced production of IFN-β (Kawasaki et al. 2013). A reporter assay in HEK293 cells revealed that the overexpression of PIKfyve increases the promoter activity of the IFN-stimulated response element (ISRE) induced by LPS or poly(I:C) without influencing NF-κB promoter activity (Kawasaki et al. 2013). In addition, siRNA targeting PIKfyve was found to inhibit the IFN-β mRNA expression induced by LPS or poly(I:C) in MEF cells. Intriguingly, synthetic-C8-PtdIns(5)P promotes the production of IFN-β. As a conclusion, PtdIns(5)P directly binds to IRF3, and this binding is necessary for phosphorylation by TBK1 (Kawasaki et al. 2013) (Fig. 4.1).

4.6 Concluding Remarks

The effect of PIKfyve inhibitors in vivo and in vitro is summarized in Tables 4.1 and 4.2, respectively. The inhibitor apilimod has been regarded exclusively as an IL-12 inhibitor, but a detailed analysis of the data from the clinical tests showed that the inhibitory effect of apilimod on IL-12 production appears to vary among individuals (Wada et al. 2012). Furthermore, it does not inhibit IL-12 production at least in macrophage-like synoviocytes of human origin (Krausz et al. 2012). Additionally, the IL-12p40 production in the mouse intestinal mucosa deficient in PIKfyve is rather increased (Takasuga et al. 2013). It is known that the polarized cells, such as the cells in the intestinal mucosa, and professional phagocytic cells, such as macrophages, have many more different types of endosomes than other cell types. The function of PIKfyve may also vary in these cells. We have generated CD11b- or CD11c-specific PIKfyve knockout mice to unravel the role of PIKfyve in IL-12 production. The results may contribute to the design of new therapeutic strategies.

Both CpG-containing endosomes and *Salmonella*-containing vacuoles do not mature in the absence of PIKfyve (Hazeki et al. 2013; Kerr et al. 2010). The fact that TLR9-dependent signals are attenuated by PIKfyve inhibition suggests that TLR3- or TLR7/8-mediated events are also susceptible to PIKfyve inhibition. However, because the CpG signal in pDCs emerges from the early endosome and not from the late endosome (Guiducci et al. 2006; Honda et al. 2005; Kerkmann et al. 2005), it is likely that PIKfyve-dependent endosome maturation negatively regulates the TLR9-dependent type I IFN production. Thus, not all of the responses induced by endosomal TLRs may be inhibited by PIKfyve inhibition. Some viruses and bacteria are known to translocate to the late endosome via the autophagosome. Because PtdIns(3,5)P_2 controls autophagosome maturation, PIKfyve may also play a role in these autophagosome-mediated events.

TLR4 is a cell surface receptor, but LPS-mediated IRF3 phosphorylation to induce *IFN-β* gene expression is initiated by TRIF-dependent signals in the endosome. Thus, it is possible that PIKfyve contributes to the LPS-induced activation of IRF3 via endosome maturation. In addition, PIKfyve produces PtdIns(5)P, which accelerates the phosphorylation and dimerization of IRF3. Both mechanisms may be involved in the PIKfyve-mediated regulation of IFN-β production. There is another aspect in the maturation of cell surface receptor containing endosomes. It is generally accepted that cell surface receptors are downregulated by internalization, and PIKfyve-mediated endosome maturation may be involved in this negative regulation. Thus, it is likely that PIKfyve is involved in cell surface receptor signaling either negatively or positively.

The possible role of PIKfyve in innate immunity is summarized in Fig. 4.1. The currently available knowledge is too limited to provide a rational interpretation for the function of PIKfyve. To clarify the tangled effect of PIKfyve inhibition, a cell type-specific analysis will prove informative.

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Chapter 5
Emerging Roles of an Innate Immune Regulator TAPE in the Toll-Like Receptor and RIG-I-Like Receptor Pathways

Kuan-Ru Chen and Pin Ling

Abstract Inflammation alerts the mammalian immune system to defend pathogen invasion or to resolve tissue damage. Dysregulation of inflammation contributes to the development of infectious diseases or inflammation-mediated chronic diseases such as obesity, diabetes, atherosclerosis, and cancer. Pattern-recognition receptors (PRRs) and their downstream regulators in the innate immune system function to trigger inflammation upon sensing molecular components from invading pathogens or damaged host cells. Better understanding of the regulation of PRR-mediated inflammation provides critical insights toward developing the treatment of infectious diseases and chronic inflammatory diseases. However, signaling networks underlying these PRR pathways still remain complicated and have much to be explored. Emerging evidence indicates that subcellular compartments or lipid organelles function as signaling platforms for conveying PRR signaling. Several cellular mediators of these subcellular organelles and vesicle trafficking have emerged to regulate the PPR pathways. In this review, we first highlight recent advances in the cell biology aspects of the Toll-like receptor (TLR) and cytosolic retinoic acid-inducible gene (RIG)-I-like receptor (RLR) pathways. We then focus on discussing a recently identified innate immune regulator called TBK1-associated protein in the endolysosomes (TAPE), also known as CC2D1A/Freud-1/Aki-1.

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TAPE is an endolysosomal adaptor shown to regulate the endosomal TLR3 and TLR4 pathways and the cytosolic RIG-I and melanoma differentiation-associated gene (MDA)-5 pathways at an early stage.

Keywords Innate immunity • TLR • RIG-I • MDA5 • TAPE

5.1 Introduction

The innate immune system is an evolutionarily primitive defense system existing in all living organisms from microbes, plants, and invertebrates to vertebrates. The adaptive immune system emerges from vertebrates to provide antigen-specific immune protection and long-term memory. In light of the "Pattern Recognition Theory" proposed by late Charles A. Janeway (Janeway 1989), scientists then started to appreciate the operations of the innate immune system and its cooperation with the adaptive immune system (Iwasaki and Medzhitov 2010). Several families of pattern-recognition receptors (PRRs) have been identified in the mammalian innate immune system, including Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and cytosolic DNA sensors (Takeuchi and Akira 2010; Paludan and Bowie 2013). PRRs function to trigger inflammation upon sensing pathogen-associated molecular patterns (PAMPs) from microbes or damage-associated molecular patterns (DAMPs) from host cell damage. These PRRs are distributed at discrete subcellular compartments, such as the cell surface, endolysosome and cytoplasm, for surveying PAMPs derived from the different stages of pathogen infection (Kagan 2012; Barton and Kagan 2009). Cytosolic innate immune regulators help relay the PRR signals to the common downstream pathways, including nuclear factor (NF)-κB, mitogen-activated protein kinase (MAPK), and/or interferon (IFN) regulatory factor (IRF) 3/7, which in turn induce the production of inflammatory cytokines and/or type I IFNs for mounting innate immune responses. Although substantial progress has been made in understanding the operations of the innate immune system, continued efforts in identification and characterization of novel regulators in the innate immune system are still warranted. Moreover, recent studies on the post-translational regulation and the spatial control of the innate sensing pathways have also revealed critical insights into understanding how these pathways function to detect pathogen infection at the subcellular context. In this review, we first highlight the recent progress in the TLR and RLR pathways, and then focus on emerging roles of a recently identified innate immune regulator, called TAPE (**TBK1-Associated P**rotein in the **E**ndolysosomes), in the TLR and RLR pathways.

5.1.1 RIG-I-Like Receptor (RLR) Sensing and Signaling

The RLR family consists of three members, including RIG-I, melanoma differentiation-associated gene (MDA) 5 and LGP2. Genetic and biochemical studies demonstrate that RIG-I and MDA5 serve as cytosolic RNA sensors for differentially detecting RNA virus infection while the function of LGP2 still remains uncertain (Loo and Gale 2011; Goubau et al. 2013). RIG-I and MDA5 are shown to recognize distinct viral RNA species. RIG-I preferentially recognizes 5′ triphosphate viral RNA while MDA5 selectively detects long viral double-stranded RNA (dsRNA) or non-*2′-O*-methylated mRNA (Goubau et al. 2013). A prevailing idea about RLR signaling suggests that after detection of RNA ligands, activated RIG-I and MDA5 translocate onto mitochondria to engage with a mitochondrial adaptor called MAVS (mitochondrial antiviral-signaling protein), also known as IPS-1/VISA/Cardiff, for triggering downstream pathways (Goubau et al. 2013). Ubiquitination of RIG-I by the E3 ligases tripartite motif (TRIM) 25 and Riplet is essential for the activation of RIG-I signaling (Oshiumi et al. 2010; Gack et al. 2007). Furthermore, K63-linked polyubiquitin chains act to trigger the oligomerization of RIG-I and MDA5 for MAV activation (Zeng et al. 2010; Jiang et al. 2012). Two mitochondrial fusion protein, mitofusion 1 (MFN1) and MFN2, have been shown to regulate the mitochondrial–membrane potential for the optimal MAVS activation (Koshiba et al. 2011). Focal adhesion kinase and a microtubule-associated guanine nucleotide exchange factor (GEF)-H1 were recently shown to participate in RLR signaling, suggesting a key role for the microtubule network in this regulation (Chiang et al. 2014; Bozym et al. 2012). These newly emerged RLR regulators underscore the intricate regulation of RLR signaling. However, some issues with regard to RLR signaling still remain elusive. First, although RIG-I and MDA5 are shown to form a complex with MAVS in mammalian cells, evidence demonstrating their direct interactions is still lacking. Furthermore, there is no microscopy evidence showing the translocation of RLRs onto mitochondria after RLR ligand stimulation or viral infection. Further studies are needed to elucidate the spatial regulation of RLR signaling.

5.1.2 Cell Biology Aspects of Toll-Like Receptor (TLR) Signaling

TLRs represent the prototype family of PRRs and detect a variety of PAMPs on the cell surface or within the endolysosomal compartments. TLRs trigger downstream signaling events mainly through TIR domain-containing adaptors, such as MyD88 and Trif/Ticam-1 (O'Neill and Bowie 2007). Several excellent reviews have summarized the function and regulation of TLRs (Takeuchi and Akira 2010; Broz and

Monack 2013). Here we focus more on recent advances regarding the cell biology aspects of TLR signaling. Several cellular factors implicated in vesicle trafficking and the secretory pathway have appeared to regulate TLR trafficking and localization. For example, Unc93b1 is an endoplasmic reticulum adaptor acting to convey multiple nucleic acid-sensing TLRs to endosomes (Kim et al. 2008). Further evidence suggests that Unc93b1 differentially regulates endosomal TLR trafficking (Lee et al. 2013). Notably, Unc93b1 mutations which cause aberrant TLR trafficking confer to the development of infectious or autoimmune diseases. Like TLR3 mutation, Unc93b1 mutation leads to herpes simplex virus (HSV)-1-mediated encephalitis in children (Casrouge et al. 2006), supporting a key role for the TLR3–Unc93b1 axis in antiviral responses against HSV-1. Mice harboring Unc93b1 mutation (D34A) develop an exacerbated inflammation due to imbalance of TLR7 and TLR9 signaling (Fukui et al. 2011). Several studies have revealed another adaptor protein, AP-3, as a sorting adaptor escorting TLR7 and TLR9 to lysosome-related organelles for type I IFN activation (Blasius et al. 2010; Sasai et al. 2010). Further research in understanding the spatial control of TLR trafficking and signaling might provide critical insights into the differential manipulation of endosomal TLR functions in the future.

5.2 TAPE is a TBK1-Interacting Adaptor Implicated in TLR and RLR Signaling

TBK1 is an IKK (IκB kinase)-related protein kinase linking multiple PRRs to IFN-β production, including TLR3, TLR4, cytosolic RLRs, and DNA sensors (Paludan and Bowie 2013; Hacker and Karin 2006). Several adaptors have been shown to interact with and regulate the activity of TBK1, including TANK, NAP1, SINTBAD, SIKE and TAPE (Huang et al. 2005; Sasai et al. 2006; Kawagoe et al. 2009; Ryzhakov and Randow 2007; Chen et al. 2012; Chang et al. 2011). Among these adaptors, TAPE represents a new type of innate immune regulator and plays functional roles in the endosomal TLR3, TLR4, and cytosolic RLR pathways at the early stage. Here we discuss our current understanding of TAPE in innate immune signaling.

5.2.1 TAPE Structure and Its Role in Endocytosis

TAPE is also known as CC2D1A (coiled-coil and C2 domain-containing 1A)/Freud-1 (five repressor element under dual repression binding protein-1)/Aki1 (Akt kinase-interacting protein 1), and it contains four DM14 (*Drosophila* melanogaster 14) domains in the N-terminal region, coiled-coil motifs, and a C2 domain in the distal C-terminus (Fig. 5.1) (Rogaeva and Albert 2007; Nakamura et al. 2008). The DM14 domain was first found in *Drosophila* but its function is still poorly defined

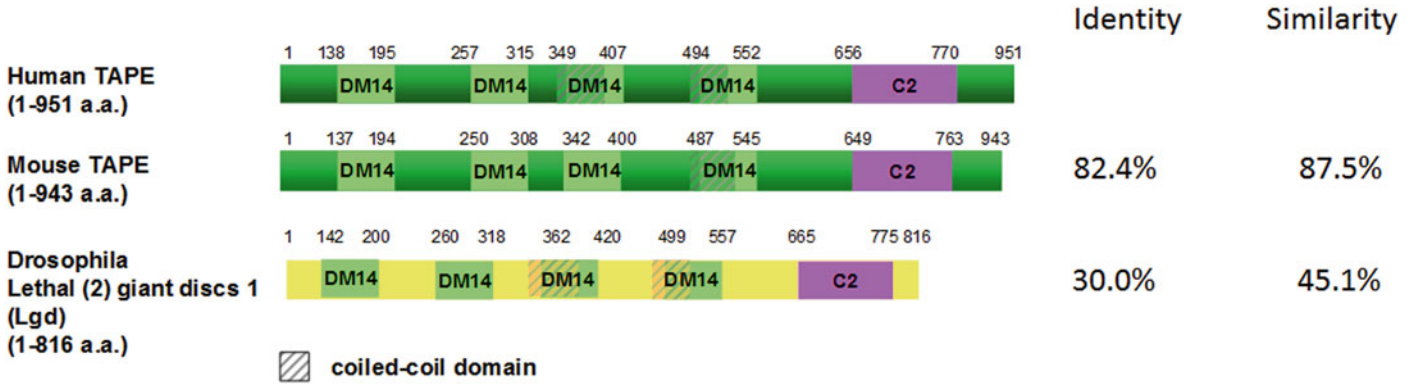

Fig. 5.1 Schematic structures of human TBK1-associated protein in the endolysosomes (TAPE), mouse TAPE and *Drosophila* Lethal (2) giant discs (Lgd). The identity and similarity of the amino acid sequence between human TAPE and mouse TAPE or *Drosophila* Lgd is analyzed. The structures of TAPE and Lgd contain four *Drosophila* melanogaster 14 (DM14) domains in the N-terminal region, coiled-coil motifs, and a C2 domain in the C-terminus

(Rogaeva and Albert 2007). Recent findings that reveal that the DM14 domain of TAPE binds to a key component of the ESCRT (endosomal sorting complexes required for transport) machinery are discussed below. The coiled-coil motifs are commonly found in endosomal and Golgi proteins to mediate the oligomerization of proteins (Gillingham and Munro 2003). The C2 domain is one of phospholipid-binding domains involved in membrane binding (Lemmon 2008). If and how these protein domains function in TAPE still remains to be determined.

TAPE has been shown to be located in several subcellular compartments, including the nucleus, endolysosomes, and centrosomes, to perform its different biological roles. Lethal (2) giant discs (Lgd), a *Drosophila* ortholog of TAPE, is shown to regulate endosomal trafficking (Childress et al. 2006; Gallagher and Knoblich 2006; Jaekel and Klein 2006). Notch and other transmembrane proteins are accumulated in enlarged early endosomes in Lgd mutant cells. The C2 domain of Lgd is possibly involved in binding phospholipid. A genome-wide screening by small interference RNA (siRNA) indicates that TAPE is one of cellular factors involved in endocytosis (Collinet et al. 2010). Evidence from confocal microscopy reveals that TAPE is co-localized with an endosomal marker Rab5 and a lysosomal marker Lamp1 in mammalian cells (Chang et al. 2011). Furthermore, ectopic expression of TAPE mutants results in aberrant distribution of Rab5 and Lamp1 (Chang et al. 2011). Our unpublished results showed that recombinant TAPE, like other membrane-binding proteins, was able to bind several phospholipid species. These findings suggest a regulatory role for TAPE in endosomal trafficking. The ESCRT machinery is required for multivesicular body biogenesis, cytokinesis, virus budding, and autophagy (Hurley and Hanson 2010). Recent findings noted that both TAPE and Lgd utilize their DM14 domains to interact with CHMP4B, a component of the ESCRT-III complex in mammals and flies (Usami et al. 2012; Troost et al. 2012; Martinelli et al. 2012). Furthermore, TAPE negatively regulates CHMP4B function during HIV-1 budding (Usami et al. 2012). Together, these lines of genetic and biochemical evidence reinforce the importance of TAPE in regulating endosomal trafficking and membrane modification.

5.2.2 Roles of TAPE in the TLR3 and TLR4 Pathways

Early studies first demonstrated that TAPE is an activator of the NF-κB pathway (Matsuda et al. 2003; Zhao et al. 2010). Proteomics research indicates that TBK1 and TAPE are present in a signaling complex containing the nuclear hormone receptor co-activator SRC-2 (Jung et al. 2005). Subsequent biochemical analyses further confirmed that TAPE interacts with TBK1 and IKKε via its N-terminal region in mammalian cells (Chang et al. 2011). Ectopic expression of TAPE in mammalian cells leads to the activation of the IFN-β, NF-κB and Erk pathways, and the decrease of viral replication (Chang et al. 2011), implying a key role for TAPE in antiviral immunity. As a TBK1-interacting protein and its endosomal location, TAPE was then demonstrated to be involved in the endosomal TLR3 and TLR4 pathways but

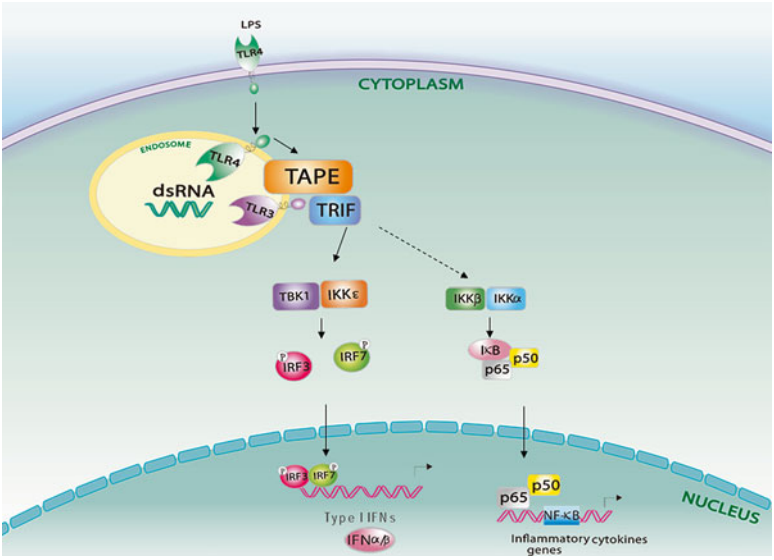

Fig. 5.2 Model for TBK1-associated protein in the endolysosomes (TAPE) in endosomal Toll-like receptor (TLR) 3 and TLR4 signaling. TAPE bridges activated TLR3 and endocytic TLR4 to Trif-mediated downstream signaling

not other surface TLR pathways (Chang et al. 2011). TAPE is able to interact with Trif and potently enhance Trif-mediated IFN-β activation. Of interest, silencing of TAPE by siRNA impairs TLR3-mediated IFN-β activation but not Trif-mediated IFN-β activation, suggesting that TAPE functions upstream of Trif in the TLR3 pathway (Fig. 5.2). Likewise, silencing of TAPE blocks the TLR4–Trif pathway to IFN-β activation. Conversely, TAPE knockdown has no obvious blocking effect on MyD88-mediated or surface TLR-mediated NF-κB activation. These findings link the TAPE endosomal location to its regulatory role in the TLR3 and TLR4 pathways. Currently, the mechanistic mechanism by which TAPE links TLR3 and TLR4 to Trif-mediated downstream pathways has yet to be explored. Further research to confirm the in vivo function of TAPE in the TLR3 and TLR4 pathways is warranted.

5.2.3 Roles of TAPE in the RLR Pathways

Since TBK1 is a key protein kinase implicated in multiple PRR signaling mentioned above, it is tempting to speculate that TAPE might participate in other PRR pathways in addition to TLR3 and TLR4. A recent study provides biochemical and genetic evidence to reveal a functional role for TAPE in the RIG-I and MDA5 pathways (Chen et al. 2012). TAPE forms a complex with MAVS and RIG-I or MDA5 in mammalian cells. Silencing of TAPE impairs RIG-I and MDA5-mediated IFN-β

activation but not MAVS-mediated IFN-β activation, suggesting that TAPE functions upstream of MAVS during RLR signaling. This kind of regulation was also confirmed by the genetic knockout approach. TAPE-deficient mouse embryonic fibroblasts and macrophages are impaired in type I IFN and inflammatory cytokine production upon RLR ligand stimulation. Furthermore, TAPE deficiency or knockdown diminishes cytokine production and antiviral immunity against RNA virus infection, including influenza A virus and vesicular stomatitis virus. These data reveal a vital role for TAPE in linking the RLR pathways to antiviral immunity. Further investigation using genetic knockout mice is essential to validate the in vivo role of TAPE in this kind of regulation. Studies from independent groups show that disruption of TAPE leads to a neonatal lethality in newborn mice (Chen et al. 2012; Zhao et al. 2011; Al-Tawashi et al. 2012). The conditional knockout approach will be an alternative to study the in vivo role of TAPE. Another key issue is the underlying mechanism of how an endolysosomal adaptor such as TAPE might participate in cytosolic RLR signaling. Two hypothetical models are proposed to explain this role of TAPE in RLR signaling (Fig. 5.3). One possibility is that upon signaling transduction, TAPE translocate onto mitochondria to couple RLRs to MAVS. The other possibility is that activated RLRs first engage with TAPE at endolysosomes and then form a complex with MAVS on mitochondria. Further research on this regulation is key to understanding the spatial and temporal control of RLR signaling.

5.3 Concluding Remarks and Perspectives

Identification of novel PRRs and regulators in the innate immune system has advanced our understanding of the activation of innate immunity over the past decade. In addition to plasma membrane, subcellular compartments such as endolysosomes, peroxisomes, and mitochondria have also emerged as signaling platforms for relaying innate immune signals. It is, however, less clear how PRRs and innate immune regulators interact with these subcellular compartments to enact their roles in innate immune sensing and signaling during pathogen infection. Pathogens frequently invade host cells to utilize different subcellular compartments or organelles to facilitate their propagation. Thus, it is also important to understand how pathogens develop strategies to target innate immune regulators or subcellular compartments to intercept innate immune signaling. TAPE is an endolysosomal adaptor recently shown to involve in the endosomal TLR (TLR3 and TLR4) and cytosolic RLR pathways. Several features regarding this TAPE-mediated innate immune regulation are of interest. First, it is noted that TAPE acts upstream of Trif/Ticam-1 or MAVS to regulate TLRs or RLRs, respectively. To the best of our knowledge, TAPE is the first regulator implicated in both the endosomal TLR and cytosolic RLR pathways at such an early step. The second feature is that TAPE interacts with

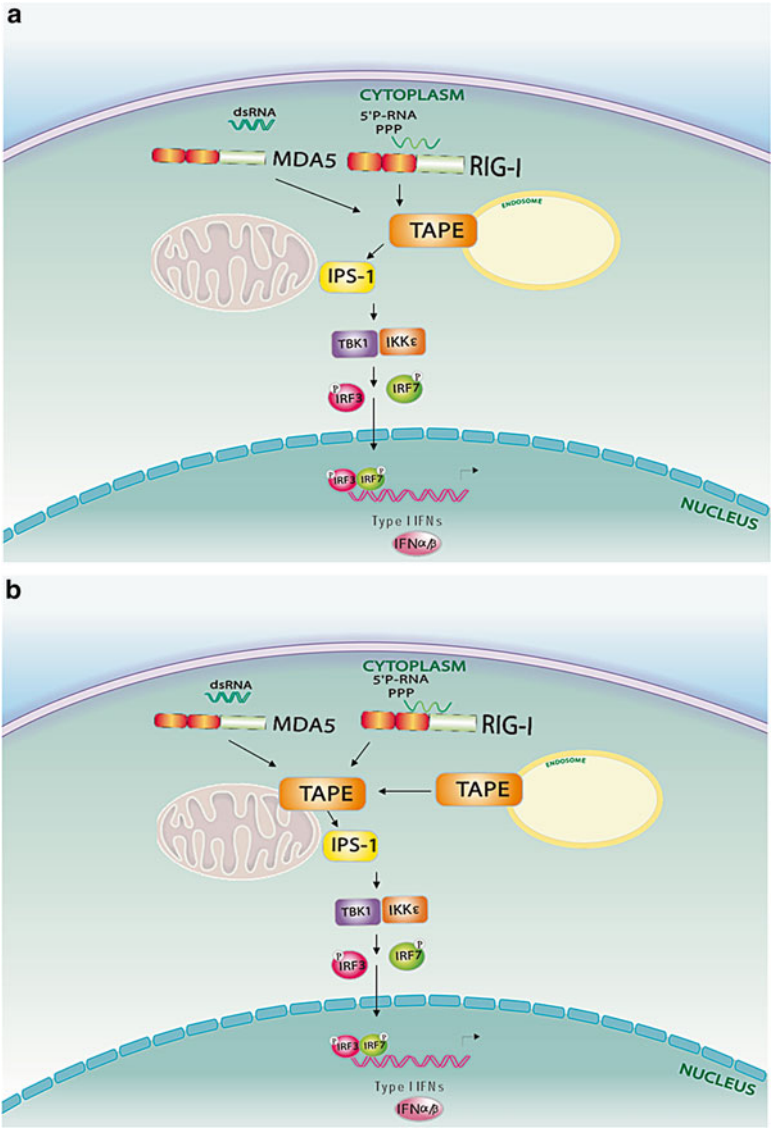

Fig. 5.3 Two proposed signalling models for TBK1-associated protein in the endolysosomes (TAPE) in retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA)5 signaling. (**a**) TAPE engages with RIG-I and MDA5 at endosomes first, and then links them to MAVS-mediated downstream signaling at mitochondria. (**b**) Both TAPE and activated RIG-I-like receptors (RLRs) are recruited onto mitochondria to engage with MAVS for downstream signaling

the different innate immune sensors and regulators mentioned above (Chang et al. 2011; Chen et al. 2012) and other host and viral factors (our unpublished results). These proteins share very little similarity in structural features. Thus, it is unlikely that TAPE uses its limited protein domains to directly interact with diverse host and viral proteins; instead, it may interact with them through a common subcellular compartment such as endolysosomes. The third feature is that although TAPE is a relatively weaker IFN-β activator, it potently enhances the activation of IFN-β by other innate immune regulators, including Trif/Ticam-1, MAVS, and TBK1. Taken together, these features suggest that TAPE represents a new type of innate immune regulator, which controls a key cellular mechanism essential for both endosomal TLR signaling and cytosolic RLR signaling. Several outstanding questions need to be addressed to appreciate the importance of TAPE in innate immunity. First of all, in vivo functions of TAPE in the TLR3, TLR4, and RLR pathways have to be further confirmed by the conditional knockout approach. Secondly, it is critical to explore the mechanistic mechanisms by which TAPE regulates these TLR and RLR pathways. Particular attention is needed to reconcile the current idea of RLR signaling, centered on MAVS-located mitochondria, with the emerging RLR signaling model (Fig. 5.3), which is involved in TAPE-containing endolysosomes. Lastly, it will be interesting to explore the potential roles of TAPE in other innate immune pathways, such as endosomal TLR7/8, TLR9, and cytosolic DNA sensors. Better understanding of TAPE-mediated innate immune signaling may reveal novel regulatory mechanisms in innate immunity, and also provide insights into targeting the TLR and RLR pathways when they are deregulated in infectious or inflammatory diseases.

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Chapter 6
Anchorage-Dependent Multicellular Aggregate Formation Induces CD44 High Cancer Stem Cell-Like Phenotypes in Adult T Cell Leukemia/Lymphoma Cells

Yukiko Miyatake and Masanori Kasahara

Abstract Adult T cell leukemia/lymphoma (ATL) is a highly invasive and intractable T cell malignancy caused by human T cell leukemia virus-1 infection. Leukemia/lymphoma cells that have invaded the tissues exhibit a propensity for strong resistance to chemotherapy, presenting a major obstacle to the treatment of ATL patients. Therefore, understanding how tissue-infiltrating leukemia/lymphoma cells acquire intractable features is important for developing effective treatments for ATL patients. We have recently found that, when co-cultured with epithelial-like feeder cells, ATL cells form anchorage-dependent multicellular aggregates and that a fraction of aggregate-forming ATL cells acquire quiescent CD44 high cancer stem cell-like phenotypes. This observation suggests that the intractability of tissue-infiltrating ATL cells may be partly accounted for by the acquisition of cancer stem cell-like properties.

Keywords Adult T-cell leukemia • Cancer stem cells • CD44 • Coculture • Multicellular aggregates

6.1 Introduction

Adult T cell leukemia/lymphoma (ATL) is an intractable and fatal T cell malignancy caused by human T cell leukemia virus type 1 (HTLV-1) infection (Poiesz et al. 1980). After more than two decades of long-term latency, approximately 4 % of HTLV-1 carriers develop ATL (Uchiyama et al. 1977; Yoshida et al. 1982). A striking feature of ATL is an aggressive invasion of leukemia/lymphoma cells into the skin and epithelial linings of the gastrointestinal tract and lung (Bittencourt and de

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Oliveira 2010; Ohshima 2007). Leukemia/lymphoma cells that have invaded the tissues become more resistant to chemotherapy, presenting a major obstacle to the treatment of ATL (Nasr et al. 2011). Therefore, understanding how tissue-infiltrating leukemia/lymphoma cells acquire resistance to chemotherapy is important for developing effective treatments for ATL patients.

Cancer stem cells (CSCs) are defined as a very small fraction of tumor cells that display properties of self-renewal and high tumorigenicity, resulting in cancer metastasis and recurrence (Huntly and Gilliland 2005; Mani et al. 2008). CD44, an important maker for CSCs, is a cell surface glycoprotein involved in cell–cell and cell–extracellular matrix adhesion as well as cell migration, differentiation, and survival (Zöller 2011). HTLV-1 Tax-transgenic mice, a mouse model of ATL, exhibit strong infiltration of CD44+ leukemic cells into various epithelial tissues (Hasegawa et al. 2006). Recent work has shown that CD44 is expressed on skin-infiltrating tumor cells in ATL patients (Chagan-Yasutan et al. 2011). Furthermore, plasma concentrations of soluble CD44 are elevated in ATL patients and correlated with disease severity (Chagan-Yasutan et al. 2011), suggesting the existence of CD44+ CSCs in ATL patients.

We have recently found that ATL cells form anchorage-dependent multicellular aggregates (MCAs) when co-cultured with epithelial-like feeder cells and that a fraction of MCA-forming ATL cells acquire quiescent CD44 high CSC-like phenotypes (Miyatake et al. 2013). We suggest that the intractability of tissue-infiltrating ATL cells may be partly accounted for by the acquisition of CSC-like properties. We advance our idea that feeder cells play a critical role in conferring ‘stemness’ upon tumor cells.

6.2 Recent Advances in Cancer Stem Cell (CSC) Research

CSCs share many biological properties with normal stem cells. Thus, CSCs were originally defined as tumor cells occupying a very small proportion of heterogeneous tumor cells, with stemness properties such as self-renewal and multilineage differentiation. On the basis of this definition, the majority of studies have focused on the isolation and identification of very rare CSCs from a large population of heterogeneous daughter tumor cells, with the aim of developing new promising drugs targeting CSCs. Thus far, various markers for CSCs have been identified in a variety of tumors. However, there are no specific markers for CSCs. Therefore, the use of CSC markers has considerably broadened the definition of CSCs (Medema 2013). Indeed, ‘CSCs’ are now widely accepted as a principal driving force for tumorigenesis, metastasis, and drug resistance. Thus, the current meaning of ‘stemness’ in CSCs seems to imply intractable phenotypes in cancer cells.

Similar to normal stem cells, the majority of CSCs are in a quiescent state of cell cycle. Therefore, CSCs are resistant to a number of conventional chemotherapeutic agents that impair mitosis (Dean et al. 2005; Li and Bhatia 2011). Thus, a clinically important feature of CSCs is cellular quiescence that leads to resistance to chemo-

therapies. However, the relationship between self-renewal and cellular quiescence is not fully understood. Endospores produced by certain bacteria are dormant, highly resistant structures formed under unfavorable environmental conditions (McKenney et al. 2013). In a sense somewhat analogous to the formation of endospores, many of the CSC properties might be considered as a highly developed cellular stress response that enables tumor cells to endure and survive under unfavorable conditions.

Of the many CSC markers identified thus far, CD44 appears to be of particular biologic importance. CD44 can directly reprogram stem cell properties in colon cancer cells (Su et al. 2011). Furthermore, CD44 interacts directly with Nanog (Bourguignon et al. 2008), a key transcription factor regulating cellular pluripotency in stem cells and CSCs (Ben-Porath et al. 2008; Chambers and Tomlinson 2009; Wang et al. 2012). Also, a recent study showed that expression of CD44 variant exons 8–10 contributes to cellular defenses against reactive oxygen species by upregulating the synthesis of a major antioxidant, glutathione (Ishimoto et al. 2011). More recently, it was shown that CD44 splicing isoform switches from variant isoforms (CD44v) to the standard isoform (CD44s) when breast cancer cells undergo epithelial–mesenchymal transition (Brown et al. 2011). Therefore, enhanced expression of CD44 appears to have the potential to confer both stemness and intractable properties upon cancer cells.

6.3 Modeling Tumor Microenvironment In Vitro: The Potential of Feeder Cells in Conferring ‘Stemness’ upon Tumor Cells

CSC properties are regulated and maintained by the surrounding microenvironment (niche), composed of cellular structures such as epithelium, vasculature, mesenchymal cells, and extracellular matrix (Borovski et al. 2011; Li and Neaves 2006; Solis et al. 2012). Thus, the CSC niche is of crucial importance not only for primary tumor growth but also for metastasis formation. However, the mechanisms of how CSCs emerge and are maintained in the niche are poorly understood, mainly because it is difficult to expand CSCs in vitro.

Feeder cells have been used to support the growth and stemness potential of stem cells in vitro (Takahashi et al. 2007). Mesenchymal cells such as mouse embryonic fibroblasts and human mesenchymal stem cells have been employed as feeder cells to support the growth of human embryonic and induced pluripotent stem cells (Havasi et al. 2013; Hovatta et al. 2003; Takahashi et al. 2009). Although human embryonic cells can be maintained under feeder-free conditions, absence of feeder cells often results in karyotype abnormalities due to chromosomal instability (Catalina et al. 2008; Draper et al. 2004).

The importance of direct contact with feeder layers for maintaining stemness has been also well-demonstrated in the field of hematopoietic stem cell research.

Long-term culture-initiating cells are the primitive human hematopoietic cells, the growth of which is dependent on mesenchymal feeder cells (van Os et al. 2008). Also, marrow repopulating ability is evaluated by counting the frequency of cobblestone area-forming cells using bone marrow cell suspensions in the presence of mesenchymal feeder layers (van Os et al. 2008; Wagner et al. 2007, 2008). Therefore, in vitro co-culture models have successfully filled the gap present between basic in vitro monocultures and in vivo animal models in the field of stem cell research. Given the basic similarities of CSCs and normal stem cells, it is reasonable to assume that feeder cells play a similarly important role in the emergence of CSCs and the formation of CSC niches.

6.4 New Insights into the Intractability of Tissue-Infiltrating Adult T Cell Leukemia/Lymphoma (ATL) Cells

We have recently found that co-culture with feeder cells induces anchorage-dependent MCA formation by ATL cells and confers quiescent CD44 high CSC-like phenotypes upon a fraction of MCA-forming ATL cells (Miyatake et al. 2013).

6.4.1 Co-culture of ATL Cells with Epithelial-Like Feeder Cells Induces Anchorage-Dependent Multicellular Aggregate (MCA) Formation by ATL Cells

When ATL cells were co-cultured overnight with feeder cells such as HEK293T cells, a normal tissue-derived epithelial-like cell line, and normal tissue-derived primary epithelial cells, the majority of ATL cells adhered to the monolayer of feeder cells and formed anchorage-dependent MCAs (Fig. 6.1). Interestingly, not all feeder cells supported MCA formation by ATL cells. Thus, ATL cells co-cultured with mesenchymal feeder cells such as primary human dermal fibroblasts did not form MCAs.

6.4.2 Emergence of Quiescent CD44 High CSC-Like ATL Cells Through Anchorage-Dependent MCA Formation

Co-culture with epithelial-like feeder cells greatly increased the proportion of ATL cells in the resting G0/G1 phase and decreased the proportion of Ki-67+ proliferating cells, suggesting that the majority of MCA-forming ATL cells entered a

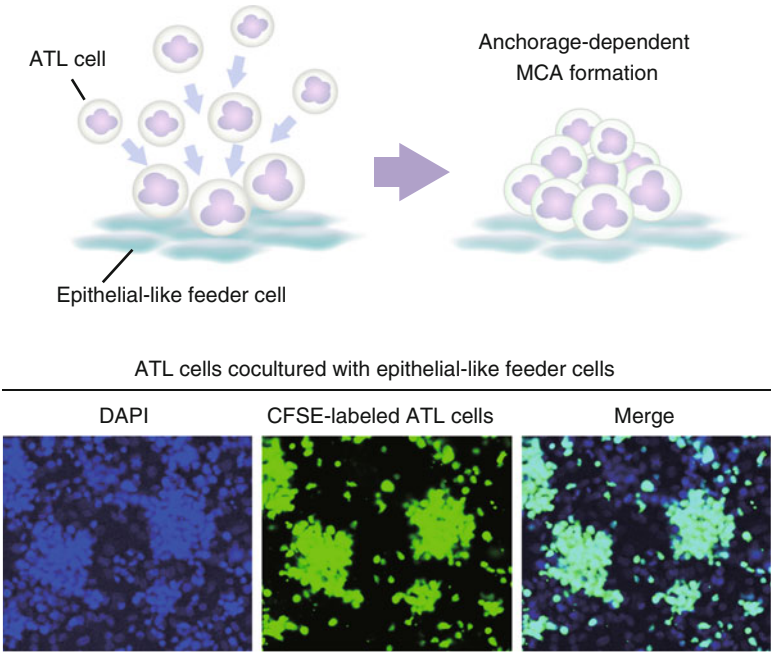

Fig. 6.1 Anchorage-dependent multicellular aggregate (MCA) formation. When adult T cell leukemia/lymphoma (ATL) cells are directly co-cultured with epithelial-like feeder cells overnight, the majority of ATL cells adhere to the feeder layer and form MCAs in an anchorage-dependent manner. ATL cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) prior to coculture

quiescent state. At the same time, ATL cells forming MCAs began to express CD44 strongly. In particular, ATL cells located at the top of MCAs were strongly positive for intracellular CD44 (Fig. 6.2). When co-cultured with feeder cells, primary leukemia cells isolated from ATL patients showed similar MCA formation and CD44 staining patterns regardless of whether the patients were suffering from chronic-type or acute-type ATL. By contrast, when ATL cells were co-cultured with mesenchymal feeder cells, they neither formed MCAs nor increased CD44 expression. Similar results were obtained when HUT78 cells, an epidermotropic HTLV-1-negative T cell lymphoma cell line established from a patient with Sezary syndrome, were co-cultured with HEK293T cells. Interestingly, when certain pancreatic cancer cells were co-cultured with HEK293T cells, they also formed MCAs and increased CD44 expression (unpublished data). Therefore, co-culture with epithelial-like cells may induce anchorage-dependent MCA formation and the emergence of CD44 high CSC-like cells in a broad range of tumor cells.

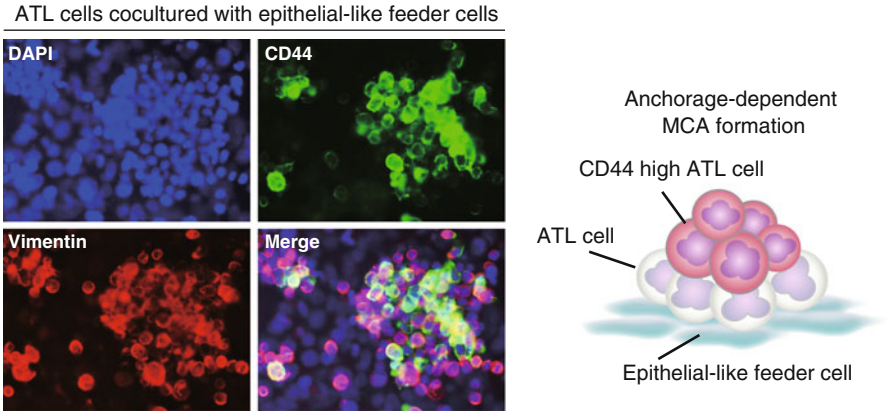

Fig. 6.2 Multicellular aggregate (MCA)-forming adult T cell leukemia/lymphoma (ATL) cells are quiescent and express high levels of CD44. Anchorage-dependent MCA formation induces CD44 high ATL cells. CD44 high cells are located primarily at the upper and central regions of aggregates

6.4.3 Aggregate-Forming ATL Cells Acquire Resistance to Histone Deacetylase Inhibitor-Induced Apoptosis

When ATL cells were cultured alone in the presence of histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA), valproic acid sodium salt and sodium butyrate, they showed a dose-dependent increase in the percentage of apoptotic cells. By contrast, ATL cells co-cultured with HEK293T cells escaped from HDAC inhibitor-induced apoptosis, suggesting that anchorage-dependent MCA formation induces resistance to HDAC inhibitor-induced apoptosis in ATL cells (Fig. 6.3). HDAC inhibitors have emerged as a new class of promising chemotherapeutic agents against cancer (Marks et al. 2000; West and Johnstone. 2014). Vorinostat, also known as suberanilohydroxamic acid, has been approved by the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma (Mann et al. 2007). However, monotherapeutic and combined-therapeutic clinical trials with HDAC inhibitors have had only limited success in most types of cancers (Olsen et al. 2007; Piekarz et al. 2011). Our study suggests that the therapeutic efficacy of HDAC inhibitors will be reduced against leukemia/lymphoma cells that have invaded into epithelial tissues.

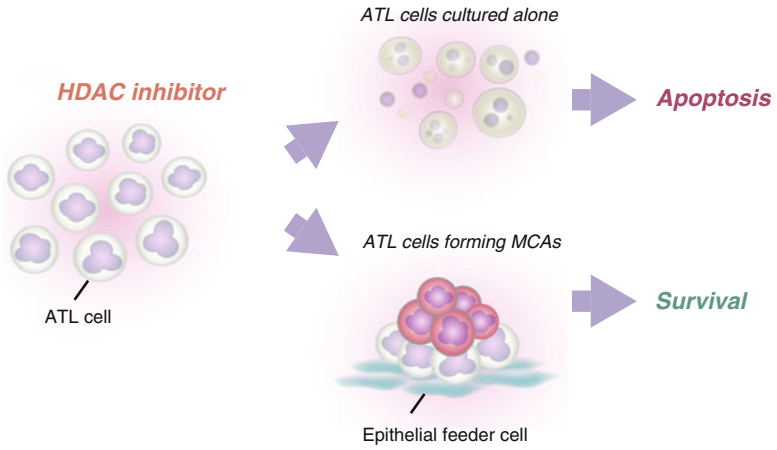

Fig. 6.3 Multicellular aggregate (MCA)-forming adult T cell leukemia/lymphoma (ATL) cells are resistant to histone deacetylase (HDAC) inhibitor-induced apoptosis. When ATL cells are exposed to HDAC inhibitors, they undergo apoptosis in a dose-dependent manner. However, when they are exposed to HDAC inhibitors in the presence of epithelial-like feeder cells, they do not undergo apoptosis

6.4.4 Anchorage-Dependent MCA Formation Suppresses Genomic Instabilities in ATL Cells

HTLV-1 proteins are generally undetectable in HTLV-1-infected cells isolated from HTLV-1 carriers because of viral gene silencing. Such silencing is observed not only in asymptomatic carriers but also in ATL patients (Furukawa et al. 1995; Taniguchi et al. 2005). Recent work has shown that type I interferon-induced HTLV-1 gag expression is suppressed in an ATL cell line when it is co-cultured with stromal cells (Kinpara et al. 2009). Epigenetic regulations are also involved in viral gene silencing in ATL cells (Ego et al. 2002; Michael et al. 2006; Mosley et al. 2006). Treatment with HDAC inhibitors can activate viral genes in ATL cells in single cell culture in vitro (Ego et al. 2002; Mosley et al. 2006). However, when ATL cells were co-cultured with HEK293T cells, treatment with TSA failed to increase the transcriptional activity of the HTLV-1 long terminal repeat, indicating that co-culture with epithelial-like feeder cells can block TSA-induced viral gene reactivation. Taken together, viral gene expression in ATL cells may be suppressed by the dual action of the microenvironment of the host–tumor interface and epigenetic regulation in host cells. HDAC inhibitors induce transcriptional activation of viral and host genes as well as genomic instability by a variety of mechanisms. Thus, our data suggest that epithelial-like feeder cells play a key role in protecting ATL cells from genomic instability, including the reactivation of viral genes.

6.5 Concluding Remarks

Metastatic cancer cells occur as both single cells and MCAs in several solid tumors (Hudson et al. 2008), pointing to the importance of MCA formation. However, the mechanism underlying MCA formation is poorly understood. Recent work indicates that MCA formation determines gene expression, proliferation, drug resistance, and immune escape in vitro in follicular lymphoma (Gravelle et al. 2014), suggesting that it affects the malignant potential of the tumor. Anchorage-dependent MCA formation discussed in this chapter involves both cell–cell interactions between ATL cells and cell–cell interactions between MCA-forming ATL cells and epithelial-like feeder cells. Both types of cell–cell interactions are presumably important for ATL cells to acquire intractable CSC-like phenotypes.

In summary, we suggest that the intractability of tissue-infiltrating ATL cells may be partly accounted for by the acquisition of CSC-like phenotypes. The simple co-culture system we have described here should be useful for dissecting the tumor microenvironment in vitro. It may also provide insights into the nature of CSCs and the CSC niche.

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Competing Financial Interests The authors declare no competing financial interests.

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Chapter 7
Innate Immune Receptor Signaling and IRF Family of Transcription Factors: Good Deeds and Misdeeds in Oncogenesis

Hiroaki Ikushima, Hideyuki Yanai, and Tadatsugu Taniguchi

Abstract Harnessing the immune system to control and eradicate cancer has been a major objective for immunologists. Despite much aspiration and effort, decades of frustration have passed following many unsuccessful approaches to induce productive immune responses to "originally self-derived" cancer cells. In recent years, however, significant progress has been made, exemplified by the antibody-mediated blockade of the so-called immune checkpoints. In terms of our understanding of the immune system's regulation of oncogenesis, there have been advances in how innate immune receptor signaling pathways are involved in the regulation of oncogenesis. The many classes of innate immune receptors and their downstream signaling pathways have been well studied in the context of their role against invading pathogens. The interferon regulatory factor (IRF) family of transcription factors in particular has been described as key downstream regulators of innate receptors. Here, we summarize the yin-yang nature of the innate immune receptors and IRFs in the regulation of oncogenesis.

Keywords Innate immunity • Pattern recognition receptor • C-type lectin receptor • Interferon regulatory factor • Dectin-1 • IRF5

Abbreviations

CLR	C-type lectin receptor
DAMP	Damage-associated molecular pattern molecule
IFN	Interferon
IRF	Interferon regulatory factor
NLR	NOD-like receptor

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PAMP Pathogen-associated molecular pattern
PRR Pattern recognition receptor
RLR RIG-I-like receptor
TAMP Tumor-associated molecular pattern
TLR Toll-like receptor

7.1 Pattern Recognition Receptors (PRRs) in Antitumor Immunity

7.1.1 Toll-Like Receptors and Cytosolic Receptors

PRRs are critical for the evocation of innate immune responses, but unlike antigen receptors of the adaptive immune system, they show broad specificities to conserved and invariant structures associated with pathogens (Janeway and Medzhitov 2002). As such, a cardinal feature of PRRs is their relatively haphazard recognition of structures, known as pathogen-associated molecular patterns (PAMPs) that are associated with these microorganisms. While this tenet holds true, some PRRs also recognize self-derived molecules which can be released under abnormal conditions such as tissue damage. These molecules are generally referred to as damage-associated molecular pattern molecules (DAMPs) (Rubartelli and Lotze 2007). As such, the question as to whether and/or how such DAMP-PRR interactions could contribute to the control of tumor immunity has been raised.

There are several functionally distinct families of signal-transducing PRRs. The Toll-like receptor (TLR) family was the first to be identified and are perhaps the most well studied. TLRs are transmembrane receptors expressed either on the cell surface or on the endosomal membrane. Each can recognize various microbial components (Blasius and Beutler 2010; Kawai and Akira 2011) and upon binding to their respective ligands initiates signals to activate transcription factors such as interferon (IFN) regulatory factors (IRFs) and nuclear factor-κB (NF-κB) for the evocation of innate immune responses. Following the well-established tenet of Janeway and Medzhitov which states that innate immunity instructs adaptive immunity (Janeway and Medzhitov 2002), data indicate that TLRs boost adaptive immune responses against cancers (Apetoh et al. 2007; Krieg 2007; Rakoff-Nahoum and Medzhitov 2009).

On the other hand, many studies indicate a procarcinogenic association of TLRs (Rakoff-Nahoum and Medzhitov 2009; Pradere et al. 2014). For instance, the activation of TLR2 and TLR6 in macrophages enhances metastasis of tumor cells, wherein receptor activation is mediated by tumor cell-derived versican, an extracellular matrix proteoglycan that is upregulated in many tumor cells (Kim et al. 2009). In addition, increased rates of proliferation and decreased rates of apoptosis are evident in metastatic tumors that formed after the injection of a colon adenocarcinoma cell line followed by an intraperitoneal injection of a TLR4 ligand, lipopolysaccharide (LPS) (Luo et al. 2004). The suggested mechanisms for these

results include a host-dependent increase in circulating levels of tumor necrosis factor-α (TNF-α), which leads to the upregulation of antiapoptotic factors, such as B-cell lymphoma-extra large (BCL-xL), cellular inhibitor of apoptosis protein-1 (cIAP1), and cIAP2, in the cancer cells (Luo et al. 2004). The procarcinogenic involvement of TLRs has also been demonstrated using spontaneous mouse cancer models using *ApcMin/+* mice. These mice are heterozygous for a mutant allele of the tumor suppressor adenomatous polyposis coli (APC) and develop intestinal tumors. It has been shown that *ApcMin/+* mice deficient in myeloid differentiation primary response 88 (*Myd88*), the common adaptor molecule for most TLRs, have both decreased incidence and size of tumors as compared with *Myd88*-sufficient *ApcMin/+* mice (Rakoff-Nahoum and Medzhitov 2007). In this model, MyD88 positively regulates the expression of matrix metalloproteinase 7 (MMP7), cyclo-oxygenase-2 (COX2), and cytosolic phospholipase A2 (cPLA2), which promote both inflammation-induced carcinogenesis and cancer progression.

In addition to transmembrane innate receptors such as TLRs, numerous cytosolic innate receptors exist which include NOD-like receptors (NLRs). Although the role of other classes of cytosolic receptors in cancer progression is still enigmatic, NLRs have been reported to play dual roles in cancers: tumor-promoting and tumor-suppressive effects (Janowski et al. 2013; Zitvogel et al. 2012). The NLR family is comprised of three distinct subfamilies: the NODs (NOD1–2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, and CIITA), the NLRPs (NLRP1–14, also called NALPs), and the IPAF subfamily (Schroder and Tschopp 2010). NLRPs and IPAF subfamily proteins are involved in the formation of the inflammasome. Inflammasomes are composed of NLRs, adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the cysteine protease caspase-1. Upon recognition of PAMPs or DAMPs by NLRs, activated caspase-1 within the inflammasome cleaves pro-interleukin-1β (pro-IL-1β) and pro-IL-18 into their mature forms which can then be secreted from the cell. Recent reports have also demonstrated important roles of caspase-11 and caspase-1 in inflammasome activity (Kayagaki et al. 2011). In the context of cancer, IL-1β is a potent proinflammatory cytokine associated with tumor growth and angiogenesis (Janowski et al. 2013; Zitvogel et al. 2012). Breast cancer induced by the transgenic expression of fibroblast growth factor receptor 1 (FGFR1) in mammary cells is associated with local IL-1β production and is inhibited by systemic treatment with a neutralizing IL-1β-specific antibody (Reed et al. 2009). Moreover, *Il1b*-deficient mice had remarkably reduced subcutaneously transplanted B16 melanoma size and lung metastasis as compared to wild-type mice (Voronov et al. 2003). Cell-autonomous effects of IL-1β also contribute to tumor growth and angiogenesis (Krelin et al. 2007; Saijo et al. 2002). In contrast to these effects of IL-1β, NLRP3 and NLRP6 are important for prevention of colitis-associated colorectal cancer development in the azoxymethane and dextran sodium sulfate (AOM-DSS) model (Allen et al. 2010; Zaki et al. 2010; Elinav et al. 2011; Normand et al. 2011; Chen et al. 2011). *Nlrp3−/−* or *Nlrp6−/−* mice have increased polyp numbers and sizes as compared to wild-type mice. This phenotype was also reported in *Asc−/−* or *caspase-1−/−* mice, suggesting that the NLRP3 and NLRP6 inflammasomes are important in suppressing inflammation-induced tumor development.

Another class of PRRs is C-type lectin receptors (CLRs). Some CLRs detect the molecular patterns of microbes, whereas others recognize molecules derived from damaged cells (Sancho and Reis e Sousa 2012). In the following sections, we will introduce the basics of CLRs and also their involvement in tumor immunity.

7.1.2 CLRs

CLRs comprise a large family of receptors which recognize carbohydrates through one or more carbohydrate recognition domains (CRDs) or possess structurally similar C-type lectin-like domains (CTLDs) which do not necessarily bind to carbohydrates (Hoving et al. 2014). Based on their signaling potential, CLRs can be grouped into one of the following three categories (Sancho and Reis e Sousa 2012) (Fig. 7.1):

1. Activation Syk-coupled CLRs: Spleen tyrosine kinase (Syk) is a tyrosine kinase involved in the signaling induced by a subset of CLRs. The coupling of CLRs to Syk can be direct via a single immunoreceptor tyrosine-based activation motif

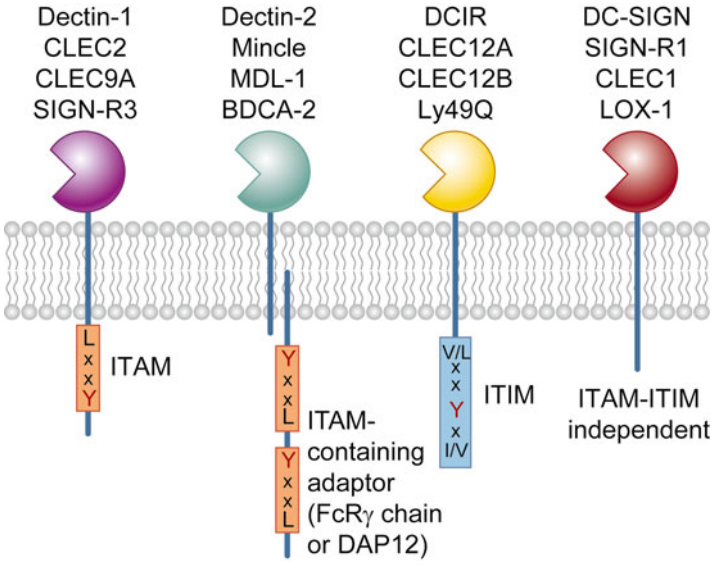

Fig. 7.1 Classification of CLRs by intracellular signaling motifs. Representative CLRs are classified based on their intracellular signaling motifs: ITAM-containing receptors (such as Dectin-1, CLEC2, CLEC9A, and SIGN-R3), adaptor-recruiting receptors (such as Dectin-2, Mincle, MDL-1, and BDCA-2), ITIM-containing receptors (such as DCIR, CLEC12A, CLEC12B, and Ly49Q), and ITAM-ITIM-independent receptors (such as DC-SIGN, SIGN-R1, CLEC1, and LOX-1). See related review articles for more details (Geijtenbeek and Gringhuis 2009; Sancho and Reis e Sousa 2012). Abbreviations: SIGN-R3, DC-SIGN-related protein 3; MDL-1, myeloid DAP12-associating lectin 1; BDCA-2, blood dendritic cell antigen 2; DCIR, dendritic cell immunoreceptor; SIGN-R1, DC-SIGN-related protein 1; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1

(ITAM) domain found in the cytoplasmic domain of some CLRs or indirect through the adaptors Fc receptor γ chain (FcRγ) or DNAX-activating protein of 12 kDa (DAP12), which bear classical Syk-recruiting ITAM motifs. Active Syk binds directly to several substrates, which in turn activate many downstream signaling pathways (Mocsai et al. 2010).
2. Inhibitory CLRs with immunoreceptor tyrosine-based inhibition motif (ITIM) domains: Some CLRs express ITIM motifs that recruit phosphatases and thereby negatively regulate signaling through kinase-associated receptors, notably the Syk-coupled CLRs.
3. CLRs without clear ITAM or ITIM domains: Although these CLRs can engage the endocytic machinery, the signaling pathways induced by these CLRs remain unclear.

Many pathogens possess atypical glycans that serve as ligands for CLRs, and some CLRs signal to potentiate microbicidal activity of myeloid cells and contribute to the activation of adaptive immunity (Geijtenbeek and Gringhuis 2009). The best example of this comes from the study of fungal infection. Fungal pathogens such as *Candida albicans* are recognized by multiple myeloid CLRs, including Dectin-1 (also known as C-type lectin domain family 7 member A, or CLEC7A), Dectin-2 (CLEC6A in human, CLEC4N in mouse), and Mincle (CLEC4E). Another example is *Mycobacterium tuberculosis*, which can be recognized by mannose receptor (MR) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Mocsai et al. 2010; Tailleux et al. 2003; Kang et al. 2005). CLRs also detect molecules released from dead cells or exposed by cell corpses to induce or suppress inflammation. Such "self" ligands for CLRs include uric acids, F-actin, and a histone deacetylase complex subunit SAP130 (Shi et al. 2003; Neumann et al. 2014; Zhang et al. 2012; Ahrens et al. 2012; Yamasaki et al. 2008).

Although, as just described, the critical contribution of CLRs to detect pathogens has been extensively studied, it has been enigmatic as to whether and how CLRs are involved in tumor immunity. Very recently, it was found that Dectin-1 recognizes live tumor cells and triggers antitumor immune responses (Chiba et al. 2014). We therefore focus on Dectin-1 in the next section and discuss its roles in antitumor immunity.

7.1.3 Dectin-1

Dectin-1 is expressed in myeloid cells including dendritic cells and macrophages (Taylor et al. 2002) and works as a PRR for β-1,3-linked glucans present in the cell wall of fungi, bacteria, and plants (Brown 2006). Dectin-1 has an ITAM domain in the cytoplasmic tail and thus can directly activate Syk upon binding to agonistic ligands (Rogers et al. 2005; Underhill et al. 2005). Activated Syk, directly or indirectly, triggers the recruitment of the adaptor CARD9 to the membrane, resulting in activation of the IκB kinase (IKK) complex for canonical NF-κB signaling. Through activation of canonical NF-κB signaling, Dectin-1-Syk signaling induces

secretion of cytokines, including IL-2, IL-6, IL-10, IL-23, and TNF-α (Sancho and Reis e Sousa 2012; Geijtenbeek and Gringhuis 2009). In addition, Dectin-1 can activate the noncanonical NF-κB pathway in CARD9-independent manner (Gringhuis et al. 2009). The Dectin-1-Syk axis also induces mitogen-activated protein kinase (MAPK) cascades and nuclear factor of activated T-cell (NFAT) signaling (LeibundGut-Landmann et al. 2007; Slack et al. 2007; Goodridge et al. 2007).

In addition to Syk activation, Dectin-1 induces a second signaling pathway mediated by the serine-threonine kinase Raf-1. Although Raf-1 does not depend on Syk signaling for its activation, Dectin-1-Raf-1 pathway converges with Syk-coupled pathways at the level of NF-κB activation. Dectin-1-induced phosphorylation of p65 at Ser276 subsequently leads to its acetylation. Acetylated p65 becomes transcriptionally active and then induces secretion of IL-6, IL-10, and IL-12 (Gringhuis et al. 2007, 2009).

7.2 The IRF Family of Transcription Factors as Regulators of PRR Signaling and Oncogenesis

The discovery of mammalian IRF family of transcription factors dates back to 1988, when a cDNA clone encoding a mouse protein that binds to a virus-inducible enhancer element of the *IFNB* gene was identified (Miyamoto et al. 1988). Since then, the IRF family has been shown to include nine members, IRF1–9, and these family members share significant homology within the conserved amino (N)-terminal DNA-binding domain (DBD) (Tamura et al. 2008; Yanai et al. 2012; Honda and Taniguchi 2006).

With the discovery of signal-transducing PRRs, IRFs have gained attention as essential regulators for the activation of immune cells (Honda and Taniguchi 2006; Kawai and Akira 2011; Geijtenbeek and Gringhuis 2009; Holm et al. 2013; Ikushima et al. 2013). It has been shown that certain microbial components, such as double-stranded RNA, LPS, and oligodeoxynucleotides containing unmethylated CpG motifs, activate the IRF-mediated induction of type I IFN genes through TLRs (Kawai and Akira 2011). In addition, the recent identification and characterization of receptors for cytosolic nucleic acids, such as cyclic GMP-AMP synthase (cGAS), stimulator of IFN genes (STING), IFN-γ-inducible gene 16 (IFI16), DNA-dependent activator of IRF (DAI), high-mobility group box proteins (HMGBs), retinoic acid-inducible protein I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5), has revealed that these receptors also activate IRF3- and IRF7-dependent pathways for type I IFN induction (Ikushima et al. 2013; Sun et al. 2013; Burdette et al. 2011; Yin et al. 2012; Unterholzner et al. 2010; Takaoka et al. 2007; Yanai et al. 2009; Yoneyama et al. 2004; Kang et al. 2002; Holm et al. 2013).

Although IRFs were originally identified as transcriptional regulators of type I IFN and IFN-inducible genes, recent studies have revealed that IRFs are involved in other facets of PRR signaling. For example, IRF5 associates with MyD88 and

regulates the induction of proinflammatory cytokine genes, such as TNF-α, IL-6, and IL-12p40 (Takaoka et al. 2005). Interestingly, recent findings have shown that this IRF5 function is inhibited by RIG-I-like receptor (RLR)-activated IRF3 (Negishi et al. 2012). IRF1, which is induced by IFN-γ, is another mediator that is activated by TLR-MyD88 pathway for the induction of specific genes such as IFN-β, inducible NO synthase (iNOS), and IL-12p35 (Kamijo et al. 1994; Liu et al. 2003; Ikushima et al. 2013). Further, IRF4 and IRF8 participate in TLR-mediated signaling in dendritic cells (Negishi et al. 2005; Tsujimura et al. 2004). Thus, many of the IRF members are essential regulators in PRR-mediated signaling. In addition, accumulating evidence indicates the oncogenic or anti-oncogenic function of IRFs in the regulation of cellular responses linked to oncogenesis (Table 7.1), for which

Table 7.1 A summary of oncogenic and anti-oncogenic roles of IRF

	IRF	Function associated with oncogenesis	References
Oncogenic IRFs	IRF2	Impairs p53 function	Pettersson et al. (2009)
		Promotes oncogenesis by antagonizing IRF1 (see below)	Harada et al. (1993)
			Nguyen et al. (1995)
	IRF4	Promotes oncogenesis in multiple myeloma	Iida et al. (1997)
			Heintel et al. (2008)
			Shaffer et al. (2008)
Anti-oncogenic IRFs	IRF1	Suppresses oncogene-induced transformation	Harada et al. (1993)
		Required for DNA damage-induced cell cycle arrest	Tanaka et al. (1996)
		Required for DNA damage-induced apoptosis	Pamment et al. (2002)
			Frontini et al. (2009)
			Tanaka et al. (1994)
			Tamura et al. (1995)
			Kano et al. (1999)
	IRF3	Promotes DNA damage-induced apoptosis	Kim et al. (1999)
	IRF5	Suppresses oncogene-induced transformation	Yanai et al. (2007)
		Required for DNA damage-induced apoptosis	Yanai et al. (2007)
			Hu et al. (2005)
		Required for Fas-induced apoptosis	Couzinet et al. (2008)
		Induces antitumor immune responses	Chiba et al. (2014)
	IRF6	Promotes cell cycle arrest	Bailey et al. (2008)
	IRF7	Suppresses bone metastasis	Bidwell et al. (2012)
	IRF8	Inhibits myeloid cell growth	Hao and Ren (2000)
			Tamura et al. (2003)
			Burchert et al. (2004)
			Dror et al. (2007)
		Promotes FAS-induced apoptosis	Hu et al. (2011)
	IRF9	Stimulates p53 pathway	Takaoka et al. (2003)
		Promotes DNA damage-induced responses	Weihua et al. (1997)

detailed reviews are now available (Yanai et al. 2012). On the other hand, evidence is still limited about how IRFs, activated downstream of PRR signaling, contribute to the regulation of oncogenesis. Here, we focus below on IRF5, because of the recent finding for its involvement in Dectin-1 signaling in antitumor innate immune responses.

Indeed, IRF5 has emerged as an interesting IRF family member in that it exerts tumor-suppressive function in two ways; first, it functions as an intrinsic tumor suppressor and mediator of antitumor innate immunity. It has been reported that IRF5 expression is suppressed in human leukemia and human ductal carcinoma and the attenuated expression of IRF5 correlates with disease stage (Barnes et al. 2003; Bi et al. 2011). In addition, a single point missense mutation (G202C), termed as IRF-5P68, was identified in peripheral blood cells from patients with adult T-cell leukemia/lymphoma (ATL) and chronic lymphocytic leukemia (CLL) (Yang et al. 2009). IRF-5P68 acts as a dominant negative regulator that interferes with IRF5 activity. These reports indicate that IRF5 inactivation relates to the development of human cancer. Ha-Ras-expressing *Irf5−/−* MEFs show resistance to DNA damage-induced apoptosis and undergo transformation to form tumors in nude mice (Yanai et al. 2007). Interestingly, *Irf5* mRNA is induced upon DNA damage by the tumor suppressor p53 (Yanai et al. 2007; Mori et al. 2002). However, since several p53 target genes, such as those encoding proapoptotic Puma and Noxa, are induced even in *Irf5−/−* MEFs, IRF5 may act on an apoptotic pathway that is distinct from that of p53 (Yanai et al. 2007). Indeed, overexpression of IRF5 inhibits in vitro and in vivo B-cell lymphoma tumor growth in the absence of wild-type p53 (Barnes et al. 2003). Furthermore, ectopic expression of IRF5 sensitizes p53-proficient and p53-deficient colon cancer cells to DNA damage-induced apoptosis (Hu et al. 2005). Another antitumor function of IRF5 has been demonstrated; IRF5 activated by Dectin-1 signaling in dendritic cells and macrophages is critical to the enhancement of tumor-killing activity of natural killer (NK) cells, and this aspect is further elaborated below.

7.3 Antitumor Innate Immune Responses Through Dectin-1-IRF5 Axis

It has been well established that NK cells are essential effector cells of the innate arm of the immune system to control tumor progression by exerting their cytotoxicity (Yokoyama and Plougastel 2003). However, little has been known about whether or how other immune cells recognize tumor cells to assist NK cells for their tumoricidal activities.

During the course of the study on the role of IRF family members in antitumor immunity, it was found that B16F1 melanoma cells potently metastasize to lungs in *Irf5−/−*, but not in *Irf3−/−* or *Irf7−/−* mice (Chiba et al. 2014). Bone marrow

transplantation experiments revealed that IRF5 is required in cells of the hematopoietic origin for the control of tumor metastasis. It was found that dendritic cells and macrophages from splenocytes enhance tumor-killing activity of NK cells, wherein IRF5 in these cells, but not in NK cells, is required for the enhancement. Further, evidence was provided that these cells potentiate tumoricidal activity of NK cells via cell-to-cell contact, an observation consistent with the previous report showing the importance of direct cell contact between dendritic cells and NK cells in the enhancement of NK cell-mediated tumoricidal activity (Fernandez et al. 1999).

As such, these observations raise the interesting issue of whether and how the PRRs known to activate IRF5 are involved in oncogenesis. Of the pathways described for IRF5 activation, the TLR-MyD88 pathway is the best known (Takaoka et al. 2005; Ikushima et al. 2013). Nevertheless, the antitumor orchestration of dendritic cells, macrophages, and NK cells remains unaffected by MyD88 deficiency, suggesting that TLRs are not involved. Dectin-1 expressed by dendritic cells and macrophages activates IRF5 upon stimulation by curdlan, a fungi-derived β-1,3 polysaccharide, which leads to the induction of type I IFNs for antifungal immunity (del Fresno et al. 2013). When Dectin-1-mediated IRF5 activation was examined in splenocytes by B16F1 melanoma cells, nuclear translocation of IRF5, a hallmark of its activation (Takaoka et al. 2005; Ikushima et al. 2013), was observed in wild-type splenocytes but not in splenocytes from Dectin-1-deficient mice (*Clec7a−/−* mice). Thus, live tumor cells can activate Dectin-1-dependent IRF5 activation (Chiba et al. 2014). Furthermore, enhancement of NK cell-mediated tumoricidal activity by dendritic cells and macrophages was attenuated when splenocytes from *Clec7a−/−* mice were used in lieu of those from wild-type mice. Expectedly, a marked enhancement of metastasis of B16F1 cells was seen in the lungs of *Clec7a−/−* mice, which was even more pronounced as compared to *Irf5−/−* mice; the enhanced tumor growth was also observed when mutant mice were inoculated subcutaneously with B16F1 cells.

These results revealed that Dectin-1 in innate immune cells directly recognizes tumor cells to activate IRF5 and other signaling pathways, which mediates antitumor activity by NK cells. Indeed, in addition to B16F1 cells, Dectin-1 binds strongly to several mouse and human cancer cell lines but weakly to non-transformed cells (Chiba et al. 2014) (Fig. 7.2). Since the Dectin-1 binding to B16F1 cells is markedly reduced upon N-glycosidase treatment, there is a major requirement for N-glycan structures to Dectin-1 binding. Consistently, the tumoricidal activity of splenocytes was markedly reduced for the N-glycosidase-treated B16F1 cells (Chiba et al. 2014). These data strongly indicate that Dectin-1 recognizes N-glycan structures overexpressed in those tumor cells, leading to the activation of IRF5 and other pathways. However, the precise recognition structure needs to be clarified further. The critical target genes of the Dectin-1 pathway in dendritic cells and macrophages, which contribute to the enhancement of NK activity by these cells, also need to be identified.

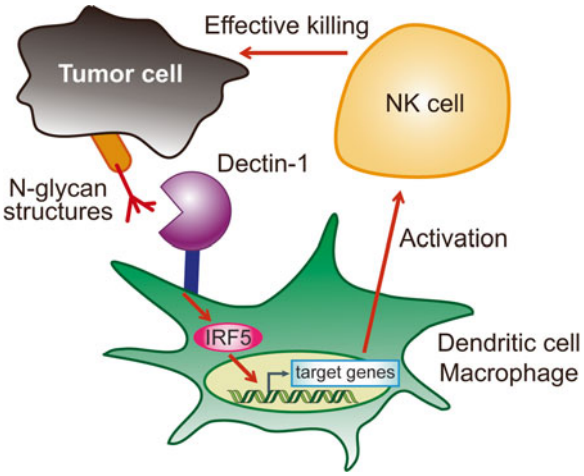

Fig. 7.2 Activation of NK cell-mediated tumor killing through recognition of tumor cells by Dectin-1. Dectin-1 expressed on dendritic cells and macrophages critically contributes to the enhancement of NK cell-mediated killing of tumor cells. IRF5 is activated by Dectin-1 signaling in these immune cells, and this Dectin-1-IRF5 pathway is required for effective tumoricidal activity of NK cells. Tumor cell-mediated Dectin-1 signaling is instigated by receptor recognition of N-glycan structures on the surface of some but not all tumor cells, which we propose to term tumor-associated molecular patterns (TAMPs)

7.4 Implications and Future Perspectives

Although the recognition of tumor-specific antigens by lymphocytes of the adaptive immune system has been studied extensively (Restifo et al. 2012), data for the role of innate PRRs in antitumor innate responses was scarce. The critical role of Dectin-1 (and its downstream activation of IRF5) in the orchestration of antitumor innate immune responses is the first demonstration that an innate immune receptor contributes to antitumor recognition and signaling, offering new insights into the NK cell-mediated antitumor activity of the innate immune system. Although yet to be fully investigated, it is possible that recognition of N-glycan structures by Dectin-1 is contingent on the absolute expression levels of N-glycans and/or their associated proteins on the cell; that is, tumor cells with "increased self" molecules are targets for innate immune recognition for the activation of the immune system. On the other hand, it is also plausible that recognition by Dectin-1 requires particular tumor-specific N-glycan-containing structures, which may belong to "altered self" (Medzhitov and Janeway 2002). Whichever the case, Dectin-1-binding structures may fit into the category of "tumor-associated molecular patterns (TAMPs)" vis-à-vis PAMPs for invading pathogens and DAMPs for normal cells subjected to stress or death (Rubartelli and Lotze 2007). This issue obviously merits more advanced study. In this regard, an interesting future issue is whether other members of the CLR family may also contribute to the antitumor innate immune system by the recognition of other TAMPs.

Since tumor cells are phenotypically and functionally heterogeneous within the tumor mass (Meacham and Morrison 2013), tumor cells may show a differential expression profile for Dectin-1 ligands in vivo. If this is the case, one may envisage that the in vivo progression of a tumor is controlled via direct and indirect Dectin-1 signaling in that NK cells activated by dendritic cells and macrophages via the receptor signaling may exert tumoricidal activities on tumor cells regardless of their Dectin-1 ligand expression. In light of the well-accepted tenet that innate immunity instructs adaptive immunity (Janeway and Medzhitov 2002), an interesting future issue is whether Dectin-1 signaling by tumor recognition also affects antitumor adaptive immune responses and also may provide new means for the efficient immune responses for cancers such as Dectin-1 agonistic antibodies. Thus, the study on the innate receptor signaling and downstream signaling mechanisms in the regulation of tumor generation and development may provide a new avenue of tumor immunology, particularly in combination with the harnessing antitumor adaptive immunity.

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Chapter 8
Host Factors Involved in the Propagation and Pathogenesis of Hepatitis C Virus

Toru Okamoto and Yoshiharu Matsuura

Abstract More than 170 million individuals worldwide are infected by hepatitis C virus (HCV) and it is one of the most common etiologic agents of chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma. Current standard therapy combining peg-interferon, ribavirin, and a protease inhibitor has achieved a sustained virologic response in more than 80 % of individuals infected with HCV. In addition, other directly acting antivirals (DAAs) targeting to the viral non-structural proteins, including NS3, NS5A and NS5B, have been gradually applied in clinical settings. Recent reports have shown that combination therapy using different types of DAAs further improves the outcomes. However, previous reports have shown that drug-resistant variants can emerge during treatment with DAAs. In addition, most patients in developing countries are not able to access or afford such advanced therapies. Furthermore, it remains unclear whether elimination of HCV by DAAs can protect against HCV-induced liver diseases. In this review, we summarize our current data regarding molecular mechanisms of HCV-induced liver diseases.

Keywords Hepatitis C virus • Virus-induced liver diseases • PA28γ • Signal peptide peptidase

8.1 Hepatitis C Virus (HCV)

Hepatitis C virus (HCV) belongs to the Flaviviridae family and has a positive- and single-stranded RNA as a genome. The viral RNA is translated into a single large precursor polyprotein that consists of 3,000 amino acids and which is cleaved by viral and host proteases. This results in production of ten viral proteins (Fig. 8.1). Core protein is a component of viral capsids and E1/E2 proteins act as envelope proteins. The p7 protein acts as a proton pump for efficient virus release. Non-structural (NS) 2 and NS3 proteins possess protease activity. NS4 is thought to be a

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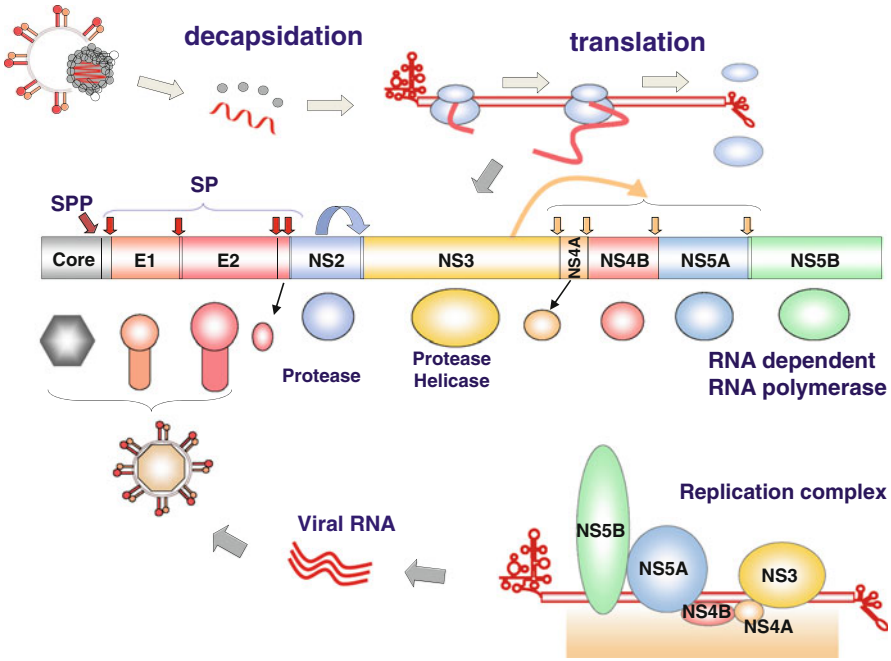

Fig. 8.1 Structure of hepatitis C virus (HCV) genome and viral proteins. Upon infection, uncoated viral RNA is directly translated into a precursor protein through an internal ribosome entry site (IRES)-dependent manner and processed by host and viral proteases into ten viral proteins. The structural proteins forming viral particles are processed by signal peptidase (SP). Core protein is cleaved off from the polyprotein and processed by SP and the signal sequence was further processed by signal peptide peptidase (SPP). The non-structural (NS) proteins are cleaved by NS2 and NS3/4A proteases and recruit various host proteins to make a large replication complex required for viral RNA replication. Replicated viral RNA forms nucleocapsid with core protein and generates HCV particles bearing E1 and E2 envelope glycoproteins

scaffold for viral replication complex. NS5A can interact with several host factors and regulate HCV replication. NS5B has an RNA-dependent RNA polymerase activity (Moriishi and Matsuura 2003; Moradpour et al. 2007).

8.2 Core Protein Plays Crucial Roles in Pathogenesis of HCV

It is well-known that HCV mainly infects hepatocytes and induces steatosis, cirrhosis and hepatocellular carcinoma (Maasoumy and Wedemeyer 2012). Chronic HCV infection is often associated with at least one extrahepatic manifestation (EHM), including mixed cryoglobulinemia, non-Hodgkin lymphoma, lichen planus, thyroiditis, diabetes mellitus, Sjögren syndrome, and arthritis (Galossi et al. 2007). Although precise molecular mechanisms of HCV-induced pathogenesis

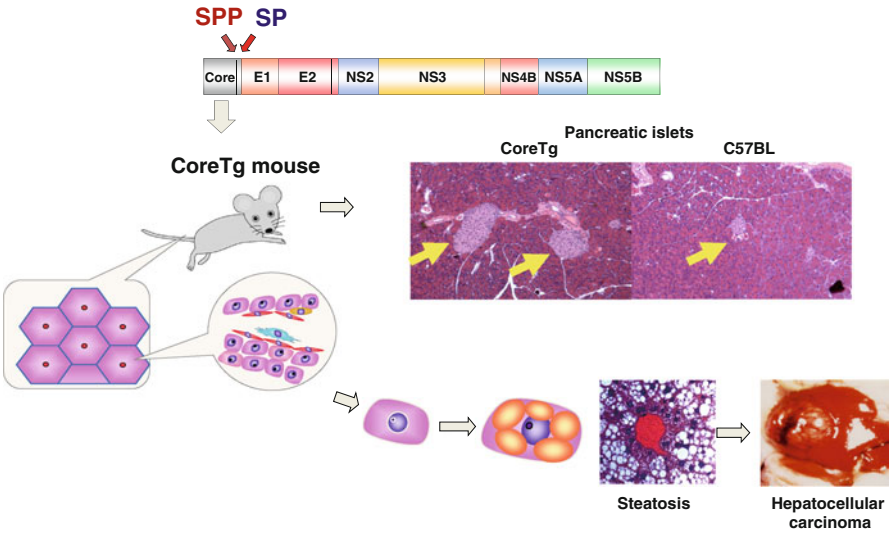

Fig. 8.2 Core protein has major roles in hepatitis C virus (HCV)-induced pathogenesis. HCV core transgenic (CoreTg) mice, which express core protein (genotype 1b) under the hepatitis B virus X promoter, have been developed. The mice show insulin resistance (2 months old~), steatosis (~3 months old), and hepatocellular carcinoma (HCC; 10–16 months old). *Yellow arrows* indicate pancreatic islets in CoreTg and control (C57BL) mice. CoreTg mice exhibit enlarged pancreatic islets, which is the feature of insulin resistance

remain unknown (Li et al. 2011), HCV core transgenic (CoreTg) mice developed insulin resistance, steatosis, and hepatocellular carcinoma (Shintani et al. 2004; Moriya et al. 1998), suggesting that HCV core protein plays a crucial role in liver diseases and EHM (Fig. 8.2).

8.3 HCV Core Protein Interacts with PA28γ in the Nucleus

We have shown that HCV core protein is degraded in the nucleus by the proteasome through the interaction with proteasomal activator 28γ (PA28γ) in ubiquitin/adenosine triphosphate (ATP)-independent manner (Fig. 8.3a, Moriishi et al. 2003; Suzuki et al. 2009). To understand the roles of interaction between HCV core protein and PA28γ on the pathogenesis in mice, we crossed CoreTg mice with PA28γ$^{-/-}$ mice and established CoreTg/PA28γ$^{-/-}$ mice. Although core protein localizes in the cytoplasm in CoreTg mice, the majority of core protein was detected in the nucleus in CoreTg/PA28γ$^{-/-}$ mice (Moriishi et al. 2007). Surprisingly, the insulin resistance, steatosis, and hepatoccellular carcinoma developed in CoreTg mice were not observed in CoreTg/PA28γ$^{-/-}$ mice, despite accumulation of HCV core protein in the nucleus (Moriishi et al. 2007; Miyamoto et al. 2007). Sterol regulatory element binding protein 1c (SREBP-1c), which positively regulates the production of saturated and monounsaturated fatty acids and triglycerides, is enhanced in the liver of

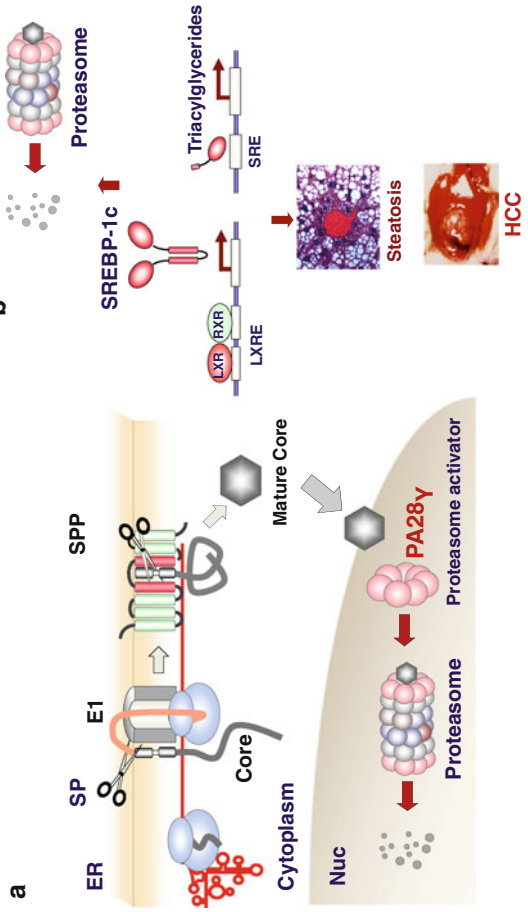

Fig. 8.3 Proteasomal activator 28γ (PA28γ) involved in core-induced pathogenesis. (**a**) PA28γ had been identified as a core-interacting protein by yeast two-hybrid screening. PA28γ degrades hepatitis C virus core protein in the nucleus in a ubiquitin-independent proteasome-dependent manner. (**b**) From our core transgenic (CoreTG)/PA28γ data, we hypothesize the following. Core protein degraded by PA28γ enhanced transcriptional activity of liver X receptor (LXR)/retinoid X receptor (RXR) complex by unknown mechanisms. The activation of LXR/RXR leads to expression of sterol regulatory element binding protein 1c (SREBP-1c), a master regulator of triacylglycerides and fatty acids production. Eventually, CoreTg mice develop steatosis and hepatocellular carcinoma (HCC)

CoreTg mice (Fig. 8.3b), leading to accumulation of lipid droplets in the liver. On the other hand, CoreTg/PA28γ$^{-/-}$ mice exhibit no activation of SREBP-1c. These data suggest that degradation of HCV core protein in the nucleus through the PA28γ-dependent proteasome plays a crucial role in the pathogenesis observed in CoreTg mice (Fig. 8.3a) (Mori et al. 2008).

8.4 Maturation of Core Protein by Signal Peptide Peptidase

The HCV core protein is cleaved from a precursor polyprotein by a signal peptidase (SP) at amino acid position 191 to liberate it from an envelope E1 protein and then is further processed by a signal peptide peptidase (SPP) (Hüssy et al. 1996; McLauchlan et al. 2002). However, the biological significance of the intra-membrane processing of the HCV core protein by SPP remains largely unknown. The C-terminus of the HCV core protein cleaved by SPP was identified to be Phe^{177} by mass spectrometry. SPP cleaves the helix-breaking site of the signal peptide and mutation of Leu^{176} to Ala and Phe^{177} to Leu in HCV core protein (M1) was deduced to acquire the intact α-helix structure in the signal sequence (Fig. 8.4, Okamoto et al. 2004). As we expected, M1 was resistant to SPP cleavage. The hydrophobic

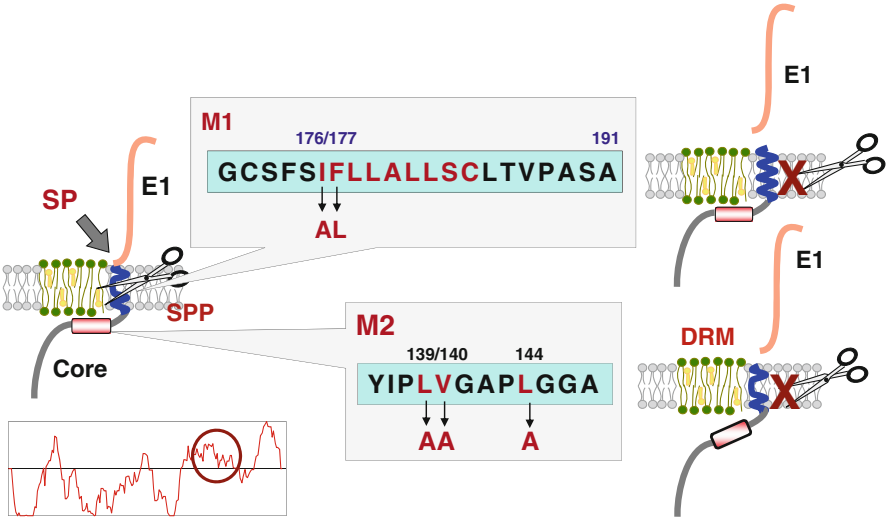

Fig. 8.4 Signal peptide peptidase (SPP) is required for core maturation. To process core protein by SPP, we found two regions of core protein were important for SPP cleavage. The first is the transmembrane region of core; this region is recognized by SPP directly. The transmembrane region of core forms a loose α-helix. Once core protein forms a stable α-helix structure by introducing mutations of I176A and F177L (M1 mutation), SPP cannot cleave core protein. On the other hand, the hydrophobic region in the upstream of the transmembrane region was found to be required for SPP processing. Mutation of the hydrophobic amino acids to alanine (L139A, V140A, and L144A: M2 mutation) protected core protein from SPP cleavage. Mutant hepatitis C virus (HCV) with either an M1 or M2 mutation impaired production of infectious particles in the culture media

amino acid residues located in the upstream of the SPP cleavage site of HCV core protein were also necessary for SPP cleavage and mutation of Ile^{139}, Val^{140}, and Ile^{144} to Ala in HCV core protein (M2) prevented from SPP cleavage. Processing by SPP is required for localization of HCV core protein on the detergent-resistant membrane (DRM) and the recombinant HCV possessing M1 or M2 mutation in core protein abrogated production of infectious particles into the culture supernatants (Okamoto et al. 2008), suggesting that intra-membrane processing of HCV core protein by SPP is required for the localization of the HCV core protein in the DRM and the viral propagation.

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Chapter 9
Hepatitis C Virus (HCV)-Induced Inflammation: The Role of Cross-Talk Between HCV-Infected Hepatocytes and Stellate Cells

Hironori Nishitsuji, Kenji Funami, Yuko Shimizu, Saneyuki Ujino, Tsukasa Seya, and Kunitada Shimotohno

Abstract Hepatitis C virus (HCV) is a major public health problem, as 170 million people worldwide are currently chronically infected with the virus. HCV infection leads to chronic inflammation, which is the initial step toward fibrosis and is a significant risk factor for developing hepatocellular carcinoma. HCV-induced liver inflammation involves several events, such as modification of cytokine and chemokine pathways, oxidative stress, and induction of steatosis. Recent studies have revealed that not only HCV-infected hepatocytes but also neighboring cells, such as lymphocytes, Kupffer cells and hepatic stellate cells (HSCs), play important roles in HCV-induced inflammation. In the current study, we found evidence of cross-talk between HCV-infected hepatocytes and HSCs, revealed by the production of cytokines and chemokines. Upon co-culture of HSCs with HCV-infected hepatocytes in vitro, HSCs stimulated HCV-infected hepatocytes to produce pro-inflammatory cytokines and chemokines, including interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1α and MIP-1β. This cross-talk is likely to be a key feature of inflammatory diseases caused by HCV infection.

Keywords HCV • Hepatic stellate cell • Inflammation

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9.1 Introduction

Many cancers arise from sites of infection and chronic inflammation. In 1863, Rudolf Virchow first suggested that the origin of cancer was at sites of chronic inflammation and tissue injury (Balkwill and Mantovani 2001). A role for inflammation in tumorigenesis is generally accepted, and it is evident that an inflammatory microenvironment is an essential component of all tumors (Mantovani et al. 2008).

Cytokines and chemokines are the most important players in inflammation and cancer, as they can either promote or inhibit tumor development. Cancer cell growth and survival are regulated by tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and interleukin (IL)-6 (Becker et al. 2005; Jing et al. 2011), and tumor progression is promoted by IL-6, IL-17, and IL-23 (Wang et al. 2009b). By contrast, IL-12, IL-21, and interferon (IFN)-γ are anti-tumor cytokines (Weiss et al. 2007).

Hepatitis C virus (HCV) chronically infects approximately 170 million persons worldwide, causing liver fibrosis, which can evolve into cirrhosis and hepatocellular carcinoma (HCC). During development of fibrosis, hepatic inflammation appears to be the major source of pathology. Fibrosis causes an imbalance in extracellular matrix (ECM) production and degradation (Hernandez-Gea and Friedman 2011). Activated hepatic stellate cells (HSCs) are a major source of ECM production. Following liver injury, such as that caused by HCV infection, quiescent HSCs become activated and convert into highly proliferative myofibroblast-like cells, which express the inflammatory and fibrogenic mediators that are responsible for ECM accumulation within the microenvironment (Hernandez-Gea and Friedman 2011).

However, many of the molecular mechanisms underlying the relationship between HCV infection and HSCs remain unclear. Understanding the mechanisms of HCV-related inflammation and development of disease is important for prediction of disease progression and development of new therapeutic approaches.

9.2 Hepatitis C Virus Infection Triggers Liver Inflammation

Chemokines and inflammatory cytokines are key regulators of immunity and inflammation during HCV infection, and HCV infection is frequently associated with chronic liver inflammation. Intrahepatic levels of chemokines and cytokines are elevated in HCV infection (Wald et al. 2007; Zeremski 2007). Increased chemokine and cytokine production reflects the development of HCV-related disease, which may include fibrosis, HCC, and hepatitis C-associated cirrhosis. However, the pathogenesis of HCV-related disease is only partially understood. In particular, the HCV core and some non-structural proteins have been demonstrated to be potent inducers of inflammation in vitro.

The HCV core has been shown to bind to the endoplasmic reticulum and membranes of lipid vesicles (Chang et al. 2007a, 2008; Miyanari et al. 2007). Such

binding may modulate gene transcription, cell proliferation, and cell death and may be involved in the pathogenesis of HCC (Chou et al. 2005; Tanaka et al. 2008).

In in vitro models, ectopic expression of the HCV core in a human hepatoma cell line induces the expression of the transcription factor nuclear factor (NF)-κB, which plays a central role in the inflammatory response to HCV in the liver (Kim et al. 2001; Marusawa et al. 1999; Yoshida et al. 2001). Dendritic cells (DCs) expressing the HCV core protein are induced to produce several inflammatory cytokines, such as IFN-γ, TNF-α, IL-6, IL-2, and IL-12 (Li et al. 2006).

Furthermore, the extracellular HCV core, which is detectable in the blood of HCV-infected patients (Kanto et al. 1995; Sabile et al. 1999), activates p38, extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), activator protein (AP)-1, and NF-κB in monocytes and macrophages via activation of the Toll-like receptor (TLR) 2 pathway (Chang et al. 2007b; Dolganiuc et al. 2003, 2004). Activation of these molecules triggers production of pro- and anti-inflammatory cytokines, such as TNF-α and IL-10, by monocytes and macrophages (Dolganiuc et al. 2004). Increased IL-10 inhibits the production of IL-12 in human macrophages and DCs (Dolganiuc et al. 2003). DCs exposed to the extracellular HCV core prevent the generation of $CD4^+$ T helper (Th) 1 responses (Waggoner et al. 2007). Modulation of immune cell function by the HCV extracellular core involves the activation of signal transducer and activator of transcription 3 (STAT3) in monocytes, macrophages, and DCs (Tacke et al. 2011).

Moriya et al. generated transgenic mice expressing the HCV core protein. These mice developed hepatic steatosis, one of the characteristic histopathological features of chronic hepatitis C, and HCC (Moriya et al. 1998, 2001). Although these transgenic mice did not exhibit inflammation, chronic HCV infection in patients can progress to HCC through inflammation. Thus, the transgenic mouse model may not reflect true clinical progression. To overcome this problem, the HCV core was expressed in mice using the tetracycline system (Chang et al. 2009). These mice expressed intermediate levels (slightly higher than physiological levels) of the HCV core in the liver, but these levels were sufficient to induce not only steatosis but also inflammation and fibrosis.

Clinical and experimental evidence suggests that oxidative stress plays a role in HCV-induced liver disease (Lai 2002; Rockey 2000). Patients with chronic HCV infection produce TNF-α (Larrea et al. 1996; Mühlbauer et al. 2003), a cytokine that can generate oxidative stress by stimulating the release of reactive oxygen species (ROS) (Kizaki et al. 1993). The mitochondrial localization of the HCV core contributes to oxidative stress, and expression of the HCV core stimulates mitochondrial ROS production (Li et al. 2007; Otani et al. 2005). Transgenic mice expressing the HCV core develop oxidative stress and liver steatosis (Korenaga et al. 2005; Okuda et al. 2002). Moreover, products from oxidative stress are found in the serum of patients with HCV-induced chronic liver disease, and antioxidants have some beneficial effects in these patients (Idéo et al. 1999; Vendemiale et al. 2001).

The HCV NS3 protein protease triggers an inflammatory reaction mediated by the TLR pathway. Similar to the effects of the HCV core protein, the recombinant NS3 protein also induces the TLR2-mediated production of inflammatory cytokines,

such as IL-6, IL-8 and TNF-α, in monocytes and macrophages (Chang et al. 2007b; Dolganiuc et al. 2003, 2004). Moreover, the levels of TNF-α and IL-1β are markedly increased in Kupffer cells when the cells are stimulated with recombinant NS3 protein (Hosomura et al. 2011). This increase is inhibited by a TLR4 antibody. In contrast, a recent report has indicated that the NS3 protein directly induces TGF-β1 and collagen expression in HSCs by binding to the TGF-β type I receptor. This activity contributes to the development of liver fibrosis (Sakata et al. 2013).

Accumulating evidence indicates that the HCV NS5A protein may play an important role in the pathological changes that occur in the liver. NS5A induces TLR4 expression in the liver, and TLR4 expression has been observed in livers of NS5A-TG mice and HCV patients (Machida et al. 2009). NS5A-induced TLR4 expression aggravates alcoholic steatohepatitis. ROS levels in NS5A-TG mice are significantly higher than those of littermate controls (Wang et al. 2009a). In addition, NF-κB and STAT3 are highly activated in the livers of NS5A-TG mice. NS5A stimulates cyclo-oxygenase (COX)-2 expression, which is implicated in inflammation and fibrogenesis through production of various prostaglandins (Núñez et al. 2004).

Recently, an alternative mechanism of HCV-induced inflammation was reported; NS5B, the viral RNA-dependent RNA polymerase (RdRp), was shown to catalyze the production of small RNA species, triggering an innate immune response and leading to the production of both IFN and inflammatory cytokines (Yu et al. 2012).

Inflammation is induced not only by HCV proteins but also by HCV RNA. Retinoic acid-inducible gene 1 (RIG-I) and TLR3 are cellular sensors that recognize HCV double-stranded RNA (dsRNA), resulting in production of chemokines, such as IL-8, RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α, and MIP-1β (Li et al. 2012; Wagoner et al. 2007). Recent reports have indicated that HCV RNA triggers IL-1β expression in hepatocytes, macrophages, and Kupffer cells (Burdette et al. 2012; Chen et al. 2014; Negash et al. 2013). In addition, HCV uptake induces a potassium efflux that activates the NLRP3 inflammasome, which results in IL-1β processing and secretion (Negash et al. 2013). Production of IL-1β stimulates production of pro-inflammatory cytokines and chemokines that are associated with liver disease (Negash et al. 2013).

9.3 The Role of Hepatic Stellate Cells (HSCs) in the Progression of Liver Fibrosis

Liver fibrosis is the hallmark of all chronic liver diseases. A number of intrahepatic cell populations are involved in liver fibrogenesis. Immune cells, such as Kupffer cells and recruited lymphocytes, are important for the inflammatory phase of the fibrogenic response. HSCs (previously known as Ito cells, lipocytes, or fat-storing cells) are the predominant cellular source of ECM components and play a key role in development of liver fibrosis. In the normal liver, HSCs are quiescent, long-lived

cells that store vitamin A. Activation or trans-differentiation of HSCs is regulated by growth factors, such as TGF-β, and is associated with pathological conditions, including liver injury, cirrhosis and cancer (Friedman 2008a, b), as a result of the expression of collagen. Activated HSCs may contribute to the growth and progression of HCC by their collagenolytic and angiogenic actions (Corpechot et al. 2002; Olaso et al. 2003; Musso et al. 1997; Torimura et al. 2004). In chronic liver injury, HSCs differentiate into myofibroblast-like cells, which exhibit marked expression of α-smooth muscle actin (SMA). Myofibroblast-like cells have a high fibrogenic capacity in the chronically diseased liver. A previous report has indicated that TLR4 contributes to myofibroblast activation and fibrogenesis in the liver (Seki et al. 2007). TLR4-dependent modulation of TGF-β signaling provides a link between pro-inflammatory and pro-fibrogenic signals. Indeed, HCV-infected patients have higher plasma levels of lipopolysaccharide (LPS) than healthy control patients (Sandler et al. 2011). LPS-stimulated Kupffer cells have a crucial role in hepatic fibrogenesis by stimulating HSCs (Duffield et al. 2005; Rivera et al. 2001). Activated HSCs express TLR4 and are highly responsive to LPS, leading to expression of inflammatory cytokines (Brun et al. 2005; Mühlbauer et al. 2004; Paik et al. 2003). TLR4 is thus a candidate for hepatic fibrosis therapy.

Recently, IL-33, an IL-1-related cytokine, has been found to exhibit pro-fibrotic properties in the liver (McHedlidze et al. 2013). Innate lymphoid cells express the IL-33 receptor (ST2 and IL-1R4) (Chackerian et al. 2007), respond to IL-33 and produce large amounts of the Th2 cytokines IL-5, IL-6, and IL-13 (Moro et al. 2010; Neill et al. 2010). IL-13 triggers the activation and trans-differentiation of HSCs in an IL-4Ra- and STAT6-dependent manner (McHedlidze et al. 2013).

HCV infection is a major cause of chronic liver disease, and a proportion of patients develop progressive fibrosis. HSCs mediate HCV-related liver fibrosis, and HCV infection directly or indirectly induces the activation of HSCs. For example, IL-8 produced by hepatocytes expressing the HCV core induces α-SMA expression in HSCs (Clément et al. 2010). HCV E2-CD81 interaction in HSCs activates ERK/mitogen-activated protein kinase (MAPK) signaling and expression of matrix metalloproteinase (MMP)-2, a major enzyme involved in the degradation of normal hepatic ECM, resulting in the progression of HCV-related hepatic fibrogenesis (Mazzocca et al. 2005; Ming-Ju et al. 2011). In contrast, CX3CR1, which is more highly expressed in patients with HCV and other chronic liver diseases than in healthy control patients (Isse et al. 2005), suppresses tissue inhibitor of metalloproteinase (*TIMP*)-*1* mRNA in HSCs. These processes are risk factors for development of liver fibrosis (Wasmuth et al. 2008).

9.4 Cross-Talk Between Hepatocytes and HSCs

Although a critical role of HSCs in the progression of liver fibrosis is well-understood, the functional impact of the cross-talk between hepatocytes and HSCs remains largely unexplored. A recent study has demonstrated cross-talk between

tumor hepatocytes and activated HSCs in a co-culture model (Coulouarn et al. 2012). When hepatocytes (HepaRG cells) were co-cultured with activated HSCs (LX2 cells), hepatocyte expression of pro-inflammatory cytokines (such as IL-1β and IL-6) and chemokines [such as IL-8 and C-C chemokine ligand 2 (CCL2)] was enhanced. In mouse models of alcoholic liver disease, alcohol feeding significantly upregulated IL-1β mRNA in the livers and enhanced the secretion of IL-1β in the serum. IL-1β-mediated signaling induced the expression of pro-inflammatory cytokines, such as TNF-α and IL-6. IL-1β exerts its pathogenic effects by upregulating lipid synthesis in hepatocytes (Miura et al. 2010), activating HSCs (Zhang and Yao 2012), and maintaining macrophages in an inflammatory state (Hou et al. 2000). By contrast, deficiency of the IL-1β pathway prevents the increase in pro-inflammatory cytokines. These results suggest that the induction of IL-1β plays an important role in the development of alcoholic steatosis, inflammation, injury, and fibrosis (Petrasek et al. 2012).

Moreover, the enhanced expression of chemoattractant chemokines may contribute to the establishment of a permissive microenvironment by recruiting immune cells.

The increase in IL-8 expression affects the intrahepatic immune response due to T cell chemotaxis to the liver. Because IL-8 is elevated in alcoholic hepatitis (McClain et al. 1999), it is tempting to speculate that induction of IL-8 may exacerbate the deleterious effects of ethanol on the liver, contributing to the increased pathological activity in the liver.

The interaction between hepatocytes and HSCs induces the activation of intracellular signaling pathways, such as AKT and ERK, in HSCs (Sancho-Bru et al. 2010). These pathways have been implicated in many biological functions of HSCs, and it also mediates key events in tumor progression, such as angiogenesis. Indeed, the expression of angiogenesis-related genes, such as *MMP2*, *MMP9* and *VEGFA*, is induced in HSCs by hepatocyte–HSC cross-talk (Coulouarn et al. 2012; Théret et al. 1997). These finding suggest that the dynamic interaction between hepatocytes and HSCs may lead to enhancement of ECM remodeling and angiogenesis, resulting in the progression of HCC.

By co-culturing HSCs with HCV-infected hepatocytes, we have recently shown that HSCs can act as inflammatory mediators in HCV-infected cells (Nishitsuji et al. 2013). Intrahepatic chemokines, such as RANTES, MIP-1α, MIP-1β and IP-10, are elevated in HCV patients (Harvey et al. 2003), and these chemokines have been linked to a high degree of liver inflammation (Zeremski 2007). We examined whether the interaction of HSCs with HCV-infected hepatocytes triggered the production of MIP-1β. When HSCs were co-cultured with HCV-infected hepatocytes, but not uninfected hepatocytes, MIP-1β was induced (Fig. 9.1a). Moreover, the conditioned medium of HSCs could stimulate MIP-1β in HCV-infected hepatocytes (Fig. 9.1b). Other pro-inflammatory cytokines and chemokines, such as IL-6, IL-8 and MIP-1α, are also induced by the interaction between HCV-infected hepatocytes and HSCs (Nishitsuji et al. 2013). Of note, treatment with TGF-β, which activates HSCs, augmented inflammation in HCV-infected hepatocytes (Fig. 9.1c). IL-1α in the conditioned medium of HSCs was involved in the HSC-mediated induction of inflammatory cytokines and chemokines, as it was neutralized by an anti-IL-1α

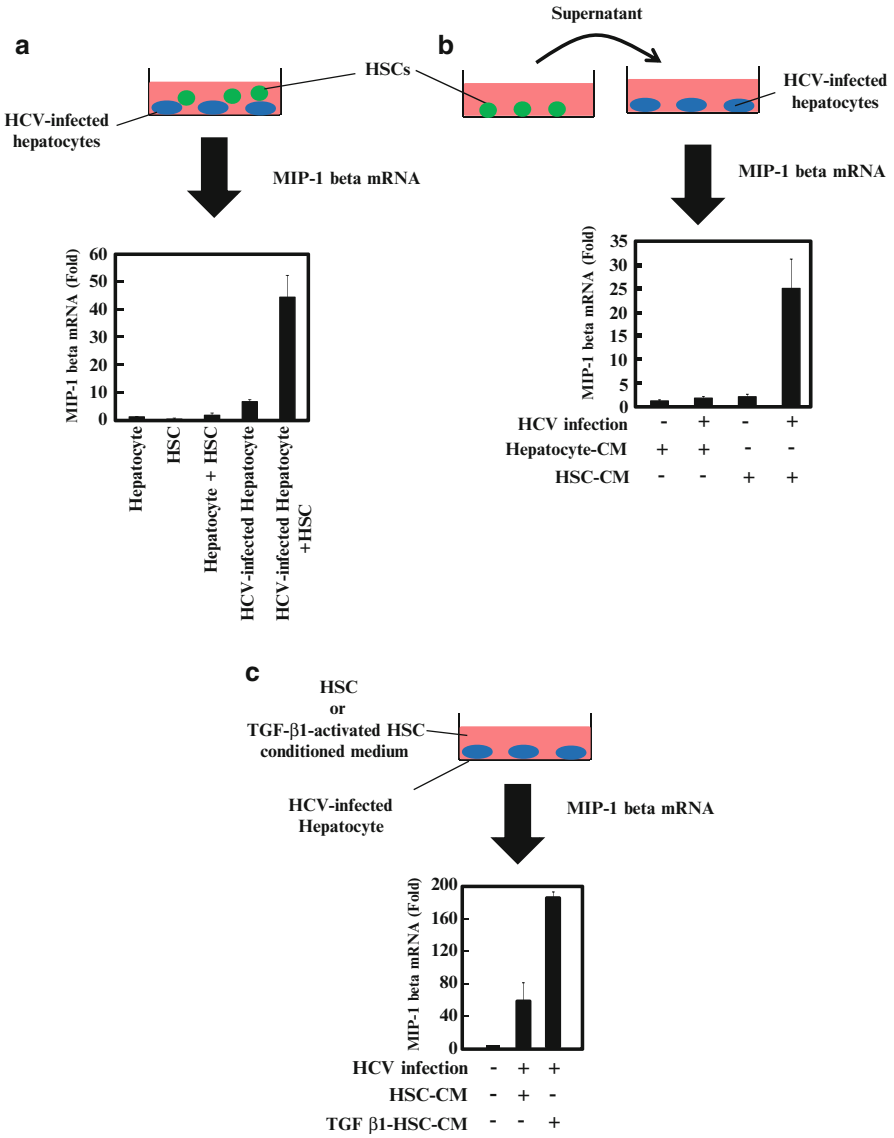

Fig. 9.1 Cross-talk between hepatitis C virus (HCV)-infected hepatocytes and hepatic stellate cells (HSCs) induces macrophage inflammatory protein (MIP)-1β expression. (**a**) Hepatocytes or HCV-infected hepatocytes were cultured alone or in the presence of HSCs for 24 h. The expression of MIP-1β was measured by quantitative real-time polymerase chain reaction (qRT-PCR). (**b**) Hepatocytes or HCV-infected hepatocytes were treated with conditioned medium from hepatocytes (Hepatocyte-CM) or HSCs (HSC-CM) for 24 h. The expression of MIP-1β was measured by qRT-PCR. (**c**) HSCs were treated with transforming growth factor (TGF)-β1 for 24 h. Hepatocytes or HCV-infected hepatocytes were treated with HSC-CM or TGF-β1-stimulated HCS-CM for 24 h. The expression of MIP-1β was measured by qRT-PCR

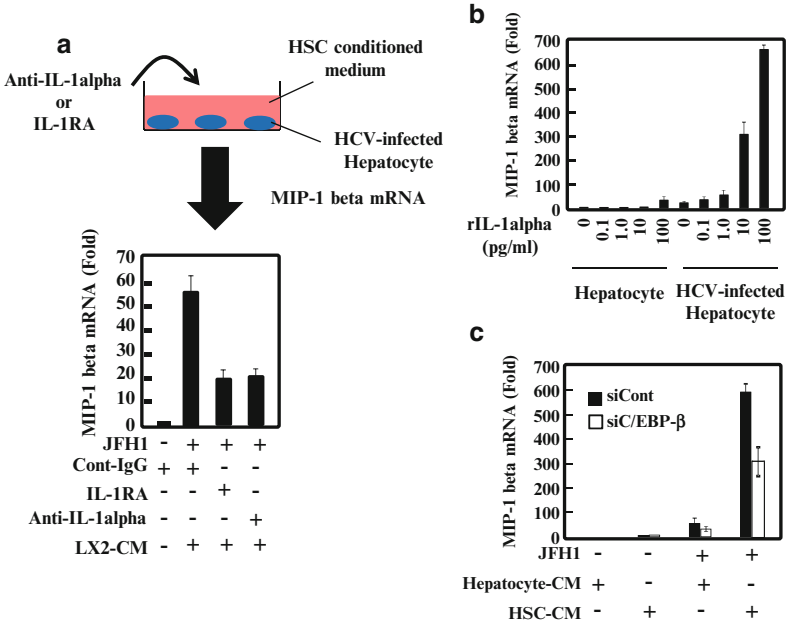

Fig. 9.2 Interleukin (IL)-1α and CCAAT (cytosine–cytosine–adenosine–adenosine–thymidine)-enhancer-binding protein (C/EBP)-β contribute to the hepatitis C virus (HCV)-infected hepatocyte response to hepatic stellate cells (HSCs). (**a**) HCV-infected hepatocytes were cultured with HSCs together with an isotype control, anti-IL-1, or IL-1 receptor antagonist (IL-1RA) for 24 h. Macrophage inflammatory protein (MIP)-1 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). (**b**) Hepatocytes or HCV-infected hepatocytes were treated with various amounts of recombinant IL-1 (0, 0.1, 1.0, 10, or 100 pg/ml) for 24 h. MIP-1 expression was analyzed by qRT-PCR. (**c**) Hepatocytes and HCV-infected hepatocytes were transfected with control small-interfering RNA (siRNA) or small-interfering C/EBP (siC/EBP)-β. After 24 h of transfection, the cells were treated with conditioned medium from hepatocytes (Hepatocyte-CM) or HSCs (HSC-CM) for 24 h. MIP-1 expression was analyzed by qRT-PCR

antibody or IL-1 receptor antagonist (Fig. 9.2a). In addition, recombinant IL-1α was also capable of inducing inflammatory cytokines and chemokines in HCV-infected hepatocytes (Fig. 9.2b). In HCV-infected patients, IL-1α mRNA levels are higher than those in healthy control patients (Kasprzak et al. 2004; Wilkinson et al. 2010). Further, IL-1α induces the acute-phase response and autoactivation of Kupffer cells in the liver (Winwood and Arthur 1993). IL-1α may contribute to HCV-related chronic inflammatory disease. Importantly, uninfected hepatocytes are unaffected by HSC stimulation. It is interesting that HCV-infected hepatocytes, but not uninfected hepatocytes, respond to IL-1α produced by HSCs. To investigate this finding, we searched for transcription factors related to inflammatory cytokines (e.g., IL-6, MIP-1α, and MIP-1β) and identified CCAAT (cytosine–cytosine–adenosine–adenosine–thymidine)-enhancer-binding protein (C/EBP)-β, which stimulates

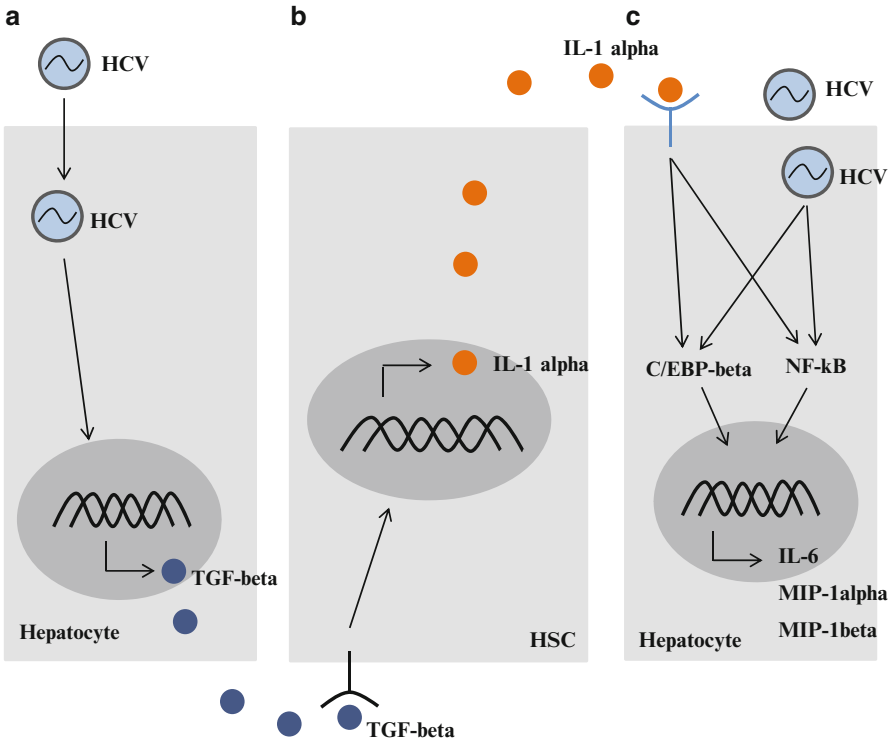

Fig. 9.3 Cross-talk between hepatitis C virus (HCV)-infected hepatocytes and hepatic stellate cells (HSCs). (**a**) HCV infection induces the expression of growth factors, such as transforming growth factor (TGF)-β. (**b**) When quiescent HSCs are activated by growth factors, activated HSCs secrete inflammatory cytokines, such as interleukin (IL)-1α. (**c**) HCV-infected cells are stimulated by IL-1α. An increase in inflammatory cytokine production accelerates inflammation in the liver

production of inflammatory cytokines and chemokines in HCV-infected hepatocytes. Indeed, knockdown of C/EBP-β in HCV-infected hepatocytes reduced the response to HSCs (Fig. 9.2c). A recent study has demonstrated that the HCV NS5A protein induces C/EBP-β expression (Qadri et al. 2012). Another group proposed that ER stress induced by HCV infection leads to the generation of mature sterol regulatory element-binding protein 1 (SREBP-1) (Joyce et al. 2009). The mature SREBP-1c can induce C/EBP-β expression (Le Lay et al. 2002). C/EBP-β is therefore a key molecule in the cross-talk between HCV-infected hepatocytes and HSCs.

These results suggest that TGF-β, which is induced by HCV infection, stimulates quiescent HSCs, and activation or trans-differentiation of HSCs leads to the expression of IL-1α, resulting in increased levels of inflammatory cytokines and chemokines in HCV-infected hepatocytes (Fig. 9.3).

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Chapter 10
Development of Immunotherapy for Hepatocellular Carcinoma

Eishiro Mizukoshi and Shuichi Kaneko

Abstract Hepatocellular carcinoma (HCC) is the sixth most common type of cancer globally. Although many different kinds of treatment are performed for HCC according to the practical guidelines, the prognosis of patients is not still satisfactory because the effects of treatments are limited for advanced tumors and the recurrence rate of HCC, even in early stages, is very high. Therefore, immunotherapy is highly anticipated as a new treatment method for HCC. For the development of a new HCC therapy, we attempted to establish immunotherapy using dendritic cells (DCs) and peptide vaccine. In several clinical trials that we performed, we confirmed that the immunotherapy was safe and well-tolerated by HCC patients. We observed that DC therapy prolonged the recurrence-free survival of patients compared with that of patients without DC infusion, as well as observing the radiological anti-tumor effect in HCC patients with peptide vaccine. In this chapter, we summarize the results of previous studies using DC and peptide vaccine, including our own data, and describe the prospects of immunotherapy for HCC.

Keywords Epitope • Dendritic cell • Cytotoxic T cell • Cancer • Peptide vaccine

10.1 Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer globally. Annually, more than 550,000 people die as a result of HCC (El-Serag and Rudolph 2007; Parkin 2001), about 35,000 of whom are from Japan. The main risk factor for HCC is chronic liver disease, such as chronic hepatitis or liver cirrhosis caused by hepatitis B (HBV) or C virus (HCV). Recently, metabolic abnormalities, such as diabetes, obesity and fatty liver, have been determined to be risk factors of HCC; in association with this, HCC has been increasing in Western and Asian countries and has become a serious health issue.

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For the treatment of HCC, many different kinds of treatment, such as surgical resection, liver transplantation, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), chemotherapy and sorafenib, are performed according to the practical guidelines (El-Serag et al. 2008). However, their effects are limited and the recurrence rate of HCC is very high. As HCC arises from an injured hepatocyte, recurrence occurs at a high rate, even when the existing tumor is removed, unless the background liver disease is completely cured. Moreover, patients who have developed HCC are mostly in an advanced stage at the time of diagnosis, making it difficult for them to receive curative treatment.

Therefore, new treatments are needed to improve the outcome of HCC patients; these include treatment to prevent recurrence after curative treatment and treatment for advanced HCC exhibiting an anti-tumor effect through a mechanism different from that of existing treatments. In this regard, immunotherapy is strongly anticipated as a new treatment method for HCC. For the development of a new HCC therapy, we have tried to establish immunotherapy using dendritic cells (DCs) and peptide vaccine.

10.2 Dendritic Cell Therapy

For the development of immunotherapy for HCC, we first undertook an analysis of the host anti-tumor immunological status in patients with HCC. In this basic study, we identified some tumor-associated antigens (TAAs) and the cytotoxic T lymphocyte (CTL) epitope to which T cells in peripheral blood mononuclear cells (PBMCs) of the HCC patient responded (Mizukoshi et al. 2006a, b, 2008, 2011, 2012). Next, we developed methods to prepare the DCs from the PBMCs of an HCC patient and to deliver the DCs to a local tumor site (Nakamoto et al. 2007). Using these methods and HCC-associated TAA-derived epitope, we investigated the safety and immunological effect of DC immunotherapy in patients with HCC.

In the first clinical trial, DCs were injected into a local tumor site using an arterial catheter in patients with HCC and who had been treated with transarterial embolization (TAE) (Nakamoto et al. 2007). DCs were prepared as follows. PBMCs were plated in six-well tissue culture dishes and allowed to adhere to the plastic for 2 h. Adherent cells were then cultured with 1,000 U/ml recombinant human interleukin (IL)-4 and 100 ng/ml recombinant human granulocyte macrophage–colony-stimulating factor (GM-CSF) for 7 days. On day 7, the cells were harvested. Then, 5×10^6 cells were reconstituted in 5 ml of normal saline containing 1 % autologous plasma, mixed with absorbable gelatin sponge and infused through an arterial catheter following Lipiodol® injection during selective TAE therapy.

In this clinical trial, we identified the following phenomena:

1. DC infusion is associated with no clinical or serological evidence of adverse events, including hepatic failure or autoimmune responses, in addition to those due to TAE alone.

2. Following the infusion of ^{111}indium-labeled DCs, DCs were detectable inside and around the HCC nodules for up to 17 days.
3. T lymphocyte responses were induced against peptides derived from some tumor antigens, human epidermal growth factor receptor 2 (Her-2/neu), multiple drug resistance-associated protein (MRP) 3, human telomerase reverse transcriptase (hTERT), and α-fetoprotein (AFP) in some patients.
4. The cumulative survival rates were not significantly changed by this strategy.

On the basis of these results, we conclude that transcatheter arterial DC infusion into tumor tissues following TAE treatment is feasible and safe for patients with cirrhosis and HCC. Furthermore, the antigen-non-specific, immature DC infusion may induce immune responses to unprimed tumor antigens, providing a plausible strategy to enhance tumor immunity.

In the next step, we designed a clinical trial to examine the protective effect of DC therapy for HCC recurrence after local treatment (Nakamoto et al. 2011). In this study, we used DCs pulsed with OK432, a *Streptococcus*-derived anticancer immunotherapeutic agent, and identified the following phenomena:

1. OK432 stimulation of immature DCs promoted their maturation towards cells with activated phenotypes, induced high expression of a homing receptor, preserved phagocytic capacity fairly well, and greatly enhanced cytokine production and effective tumoricidal activity.
2. Administration of OK432-stimulated DCs to patients was feasible and safe.
3. Administration of OK432-stimulated DCs prolonged recurrence-free survival of patients compared with that of patients without DC infusion.
4. The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15, and tumor necrosis factor-α and the chemokines CCL4 and CCL11.

On the basis of these results, we conclude that a DC-based, active immunotherapeutic strategy in combination with loco-regional treatments exerts beneficial antitumor effects against HCC.

In addition to our studies, several immunotherapies using DCs have been reported in patients with HCC (Table 10.1) (Ladhams et al. 2002; Iwashita et al. 2003; Stift et al. 2003; Lee et al. 2005; Chi et al. 2005; Butterfield et al. 2006; Palmer et al. 2009; Zhou et al. 2011; Qiu et al. 2011; Tada et al. 2012). Most of these studies used DCs generated ex vivo from the peripheral blood of the patient, pulsed with tumor lysate or TAA-derived peptides. These studies have shown that DC therapy is safe and well-tolerated in HCC patients. They have also indicated that tumor-specific immune response is induced by DC infusion, and an anti-tumor effect consisting of prolonged recurrence-free survival after treatment and overall survival is observed in some cases. In several studies, partial radiological response was also observed. However, the strength of the anti-tumor effect and the frequency of patients who have a clinical benefit are still not satisfactory. To establish a treatment using DCs as an immunotherapy for HCC, further clinical trials are necessary to prove the clinical efficacy in more patients with HCC.

Table 10.1 Clinical trials of dendritic cell therapies for hepatocellular carcinoma

Year	Author	No. of patients	Setting for DCs	Responses
2002	Ladhams et al.	2	GM-CSF/IL-4 DC + tumor	1/2 inhibition of tumor growth
2003	Iwashita et al.	10	GM-CSF/IL-4 DC + tumor lysate + TNF-α + KLH	1/10 MR
2003	Stift et al.	2	GM-CSF/IL-4 DC + tumor lysate + TNF-α + IL-2	No PR or CR
2005	Mazzolini et al.	8	GM-CSF/IL-4 DC + tumor + adenovirus IL-12	2/8 SD
2005	Lee et al.	31	DC + tumor lysate	Improved survival
2005	Chi et al.	14	Radiotherapy + DC	Immune response
2006	Butterfield et al.	10	DC with AFP peptides	No PR or CR
2007	Nakamoto et al.	10	TAE + GM-CSF/IL-4 DC	Immune response
2009	Palmer et al.	35	GM-CSF/IL-4 DC + HepG2 cells lysate + TNF-α	25 patients were assessed, 1/25 PR and 6/25 SD
2010	Nakamoto et al.	13	TAE + GM-CSF/IL-4 DC + OK432	Prolonged recurrence-free survival
2011	Zhou et al.	10	PMWA + GM-CSF/IL-4 DC + CIK + CTL	Immune response
2011	Qiu et al.	9	GM-CSF/IL-4 DC + TNF-α + tumor lysate contained α-Gal epitope	3/9 PR and 3/9 SD
2012	Tada et al.	5	GM-CSF/IL-4 DC + recombinant protein (AFP, GPC3, MAGE-1) + cytokine cocktail	1/5 SD
2012	Ansary et al.	15	GM-CSF/IL-4 DC + HepG2 cells lysate	2/15 PR and 9/15 SD

AFP α-fetoprotein, *CIK* cytokine-induced killer cell, *CR* complete response, *CTL* cytotoxic T lymphocyte, *DC* dendritic cell, *GM-CSF* granulocyte-macrophage–colony stimulating factor, *GPC3* glypican-3, *IL* interleukin, *KLH* keyhole limpet hemocyanin, *MAGE* melanoma-associated antigen, *MR* mixed response, *PMWA* percutaneous microwave ablation, *PR* partial response, *SD* stable disease, *TAE* transarterial embolization, *TNF* tumor necrosis factor

10.3 Peptide Vaccine

In addition to DC therapy, we also attempted to develop a peptide vaccine as an immunotherapy for HCC. The first step to establish the treatment is identification of TAA expressed in HCC. Since HCC is complicated by background liver damage in many cases, the establishment of a safe and effective peptide vaccine for HCC depends on whether an immune response killing only tumor cells without injuring normal hepatocytes can be induced, for which it is necessary to discover HCC-specific TAA with high-level immunogenicity. Generally, HCC had not been

considered as an ‘ immunogenic’ tumor, but previous studies reported that the risk of recurrence after treatment was low and the outcome was favorable in HCC patients with many infiltrating lymphocytes in the tumor, suggesting that anti-tumor immunity is also present and tumor progression is inhibited through an immunological mechanism in HCC patients (Wada et al. 1998). Actually, including our data, many TAAs and their CTL epitope expressed in HCC have been discovered over the past 10–15 years (Butterfield et al. 2001; Zerbini et al. 2004; Korangy et al. 2004; Komori et al. 2006; Mizukoshi et al. 2006a, b).

To date, however, there have been no reported studies of T cell responses to previously identified TAAs or their epitopes being measured simultaneously and comparatively in a number of HCC patients. Therefore, to identify suitable epitopes for peptide vaccine, we performed a simultaneous and comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the PBMCs of 31 HCC patients (Mizukoshi et al. 2011). In this study, we made the following findings and selected suitable epitopes: (1) the TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, MRP3, AFP, and hTERT were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic; and (2) HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced de novo T cell responses.

On the basis of the above data, we are now conducting several clinical trials on HCC patients using hTERT-, AFP-, MRP3-, SART2-, and SART3-derived peptides. In the clinical trial of hTERT-derived peptide vaccine, HCC patients treated using RFA were enrolled. The peptide was administered emulsified with incomplete Freund’s adjuvant by subcutaneous immunization three times biweekly. The maximum toxicity observed was grade 2 according to the Common Terminology Criteria and mainly consisted of skin reactions at the vaccination sites. Several immunological assays revealed that the vaccination induced hTERT-specific immunity. Although the HCC recurrence-free survival time after RFA did not differ between patients without vaccination and those with the hTERT-specific immune response after vaccination, the recurrence rate in patients with vaccination was reduced beyond 1 year after RFA.

In the clinical trial of AFP-derived peptide vaccine, we enrolled advanced HCC patients who had been treated by standard therapy including surgical resection, RFA, TACE, chemotherapy, and sorafenib but did not show a clinical benefit. The method of vaccination was almost the same as that for hTERT-derived peptide vaccine but it was continued until the confirmation of tumor progression. The observed toxicity mainly consisted of skin reactions at the vaccination sites, and severe adverse events were not observed. To date, we have observed one patient with complete response (CR) and one patient with long-term stable disease (SD). AFP-specific immune responses were observed in these two patients using several immunological assays. These results are encouraging for the possibility of using peptide vaccines for HCC.

Table 10.2 Clinical trials of peptide vaccines for hepatocellular carcinoma

Year	Author	No. of patients	Setting for peptides	Responses
2003	Butterfield et al.	6	AFP-derived peptides + Montanide adjuvant, HLA-A2	No PR or CR
2010	Greten et al.	40	hTERT-derived peptides + cyclophosphamide + GM-CSF	No PR or CR
2012	Sawada et al.	33	GPC3-derived peptides + Montanide™ adjuvant, HLA-A24 and A2	1/33 PR and 19/33 SD
2012	Mizukoshi et al.	12	SART2-derived peptides + Montanide™ adjuvant, HLA-A24	Immune response
2012	Mizukoshi et al.[a]	14	hTERT-derived peptides + Montanide™ adjuvant, HLA-A24	Prolonged recurrence-free survival and immune response
2012	Mizukoshi et al.[a]	20	AFP-derived peptides + Montanide™ adjuvant, HLA-A24	15 patients were assessed, 1/15 CR and 8/15 SD

AFP α-fetoprotein, *CR* complete response, *GM-CSF* granulocyte-macrophage–colony stimulating factor, *GPC3* glypican-3, *hTERT* human telomerase reverse transcriptase, *PR* partial response, *SART2* squamous cell carcinoma antigen recognized by T cells 2, *SD* stable disease
[a]Present study

Several studies have been reported regarding an immunotherapy using peptide vaccines for HCC (Table 10.2) (Butterfield et al. 2003; Greten et al. 2010a; Sawada et al. 2012). A clinical trial of HCC immunotherapy using AFP-derived peptides has been performed, in which AFP-specific CTL increased after treatment in six of six HCC patients. In addition to AFP, clinical trials of vaccines comprised of the CTL epitopes of hTERT and glypican-3 (GPC3) have been performed in HCC patients (Greten et al. 2010a, b; Sawada et al. 2012). hTERT-derived peptide vaccine was administered in combination with cyclophosphamide to 40 patients with advanced HCC, but no potentiation of immune reactions to the peptide was observed, and, regarding the anti-tumor effect, no patient showed a CR or partial response (PR) (Greten et al. 2010a, b). In the clinical trial of GPC3-derived peptide, vaccine was administered to 33 advanced HCC patients (Sawada et al. 2012). A PR was noted in one patient, and an effect maintaining an SD condition for more than 2 months was noted in 19 patients. In addition, tumor necrosis and size reduction were noted in four of the 19 patients in whom the disease condition was stabilized, supporting the possibility of using TAA-targeting immunotherapy for HCC. No serious adverse events occurred in AFP-, hTERT-, or GPC3-targeted immunotherapy, confirming that immunotherapy by peptide vaccine can be safely applied in advanced HCC patients with reduced hepatic reserve capacity.

10.4 Prospects of Immunotherapy for Hepatocellular Carcinoma

In previous studies, including our own, the anti-tumor effect induced by immunotherapy for HCC was not so strong and frequent. Several mechanisms have been considered as reasons for the insufficient anti-tumor effect of cancer immunotherapy. Similar to other cancer types, HCC has a mechanism to escape from host immune responses. In particular, the presence and mechanism of cells leading to immune responses in the negative direction, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), in HCC have recently been clarified. Tregs are the most strongly characterized suppressor cells shown to inhibit anti-tumor immunity in many studies. Increased Tregs in PBMCs and tumor-infiltrating lymphocytes (TILs) in HCC patients have been reported (Unitt et al. 2005). In a study in which low-dose cyclophosphamide was administered to eliminate Tregs in advanced HCC patients, the frequency and function of peripheral blood Tregs were reduced and AFP-specific T cell reactions resumed (Greten et al. 2010a, b).

MDSCs have been reported to induce Foxp3 and IL-10 through the arginase activity in $CD4^+$ T cells and to inhibit T cell function through the induction of Tregs (Hoechst et al. 2008). A recent study reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were increased in the peripheral blood of HCC patients and negatively correlated with TAA-specific T cell responses (Mizukoshi et al. 2013). Unfortunately, no drug or antibody directly inhibiting this function of MDSCs is available, but elucidation of the detailed T cell-inhibitory mechanism of MDSCs and elimination of this inhibition may lead to the potentiation of anti-tumor immunity.

It is known that inhibition of T cell-induced anti-tumor immunity is also exhibited through inhibitory receptors. The typical molecules are CTL antigen-4 (CTLA-4) and programmed death-1 (PD-1). These molecules transmit inhibitory signals to T cells by binding to specific ligands present on the surface of antigen-presenting or cancer cells and inhibit T cell functions such as proliferation, cytokine secretion, and cytolysis. Many study results support the assertion that potent anti-tumor immunity can be achieved by inhibiting this T cell-inhibitory system using an antibody. Currently, ipilimumab and tremelimumab are available as CTLA-4 antibodies. Ipilimumab has been approved as the first-line treatment for melanoma patients with metastasis (Robert et al. 2011; Ribas 2010). Regarding PD-1, in immunotherapy using an antibody against PD-1 and a ligand of PD-1 (PD-L1), objective responses (CR or PR) were observed in patients with treatment-refractory metastatic solid tumors (melanoma, renal cell cancer, non-small cell lung cancer, and ovarian cancer) (Topalian et al. 2012; Brahmer et al. 2012). At present, no clinical data on these antibodies from a large number of HCC cases have been reported, but they will appear in the future.

As described here, recent studies on anti-tumor immunity have contributed to a marked advancement, and further development is expected in the field of HCC immunotherapy. In particular, there are high expectations for the development of a combination treatment method using DCs, peptide vaccines, and immune-modulating

antibodies to treat advanced HCC. To develop a new effective immunotherapy for HCC, it is necessary to establish the method of DC therapy, identify a highly immunogenic HCC-specific TAA-derived T cell epitope, and elucidate the anti-tumor immunity-inhibitory mechanism of HCC.

Conflict of Interest The authors disclose no conflicts of interest.

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Chapter 11
Development of Glypican-3-Targeted Cancer Immunotherapy

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Abstract Glypican-3 (GPC3) is an onco-fetal antigen expressed in the placenta and embryonic liver and over-expressed in human hepatocellular carcinoma (HCC). We found that GPC3 is useful both as a novel tumor marker and as a target antigen for immunotherapy in several mouse studies. We identified human leukocyte antigen (HLA)-A24-restricted $GPC3_{298–306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144–152}$ (FVGEFFTDV) peptides that can induce GPC3-reactive cytotoxic T lymphocytes (CTLs). Based on results obtained from preclinical mouse studies, we conducted a phase I clinical trial using a GPC3-derived peptide vaccine. Results showed that the GPC3-derived peptide vaccine was well-tolerated. Furthermore, this is the first study to show that the frequency of peptide-specific CTLs correlated with overall survival in patients with HCC receiving peptide vaccines. Next, we conducted a phase II clinical trial of the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC after surgery or radiofrequency ablation. We are now initiating a pilot study of liver biopsies from patients with advanced HCC before and after GPC3 peptide vaccination. In addition, we investigated whether GPC3-based immunotherapy can be applied to other GPC3-expressing cancers, such as clear cell carcinoma of the ovary and pediatric cancers, and are initiating clinical trials to further these studies. We expect that the results of these trials will provide the rationale for a larger clinical trial to determine the efficacy of the GPC3-derived peptide vaccine and to advance future drug development using this approach.

Keywords Glypican-3 • Peptide vaccine • Clinical trial • Cytotoxic T lymphocyte

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11.1 Identification of the Cancer-Specific Antigen Glypican-3

Tumor antigen-specific T cell immunotherapy is a promising new approach to cancer treatment that is more effective and less toxic than previous treatments. Cloning of the human melanoma antigen gene, *MAGE*, affirmed that the human immune system can recognize cancer as a foreign body and exclude it (van der Bruggen et al. 1991). This genetic approach of T-cell epitope cloning led to identification of many genes recognized by tumor-reactive cytotoxic T lymphocytes (CTLs) as tumor antigens and antigenic peptides, which increases the potential for antigen-specific cancer immunotherapy (Rosenberg 2001; Morgan et al. 2003; Maeda et al. 2002; Azuma et al. 2003). Recently, our group (Nakatsura et al. 2001; 2002; Monji et al. 2002) and others (Park et al. 2003; Jager et al. 1998) have together identified more than 1,500 tumor antigen candidates with the SEREX method, which is based on *se*rological analysis of *r*ecombinant complementary DNA (cDNA) *e*xpression libraries.

cDNA microarray technology is progressing rapidly and allows investigators to obtain comprehensive gene expression profile data. This technique has proven useful for the identification of novel cancer-associated genes and for classification of human cancers at the molecular level (Hasegawa et al. 2002; Zembutsu et al. 2002; Kitahara et al. 2001; 2002; Yu et al. 2004). We used cDNA microarrays to identify highly expressed hepatocellular carcinoma (HCC)-specific antigens for tumor immunotherapy that were not expressed in normal adult tissues, except for immune-privileged tissues such as the testis, placenta, or fetal organs. We obtained data comparing expression profiles between 20 HCC and the corresponding non-cancerous liver tissues and various normal human tissues, using cDNA microarrays of 23,040 genes. When we searched for genes over-expressed specifically in HCC, we identified glypican-3 (GPC3).

GPC3 is a member of the glypican family of heparan sulfate proteoglycans attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor (Filmus and Selleck 2001). GPC3 is over-expressed in most HCCs and is not related to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. GPC3 mRNA is highly expressed in the placenta, fetal liver, fetal lung, and fetal kidney and is expressed at low levels in most adult normal tissues. Thus, GPC3 is a novel onco-fetal antigen in HCC. Moreover, we detected soluble GPC3 protein in sera of HCC patients, but not patients with other types of liver diseases (Nakatsura et al. 2003). GPC3 is also a novel tumor marker for melanoma, detectable in the sera of 40 % of melanoma patients, irrespective of clinical stage and detectable even in the sera of patients with stage 0 in situ disease (Nakatsura et al. 2004a). These results indicate that GPC3 is a useful tumor marker for cancer diagnosis in large numbers of patients with HCC and melanoma.

We associated the expression of GPC3 with clinicopathological factors in HCC patients by immunohistochemical analysis and investigated the potential prognostic

value of GPC3 by comparing the survival rates of GPC3-positive and GPC3-negative HCC patients. Multivariate analysis identified GPC3 expression ($P = 0.034$) as an independent prognostic factor for overall survival (OS). Thus, GPC3 expression is correlated with a poor prognosis in HCC patients (Shirakawa et al. 2009).

11.2 Preclinical Studies of Glypican-3 (GPC3)-Targeted Immunotherapy

We found that GPC3 is useful not only as a novel tumor marker, but also as a target antigen for immunotherapy in several mouse studies. Both human and mouse GPC3 are expressed in normal tissues, including placenta and fetal liver, but not in other normal adult tissues. We attempted to identify a GPC3 epitope for CTLs in BALB/c mice by initiating a preclinical study to investigate the usefulness of GPC3 as a target for cancer immunotherapy in vivo. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse K^d are similar (Falk et al. 1991; Maier et al. 1994; Okugawa et al. 2000), and the amino acid sequences of human and mouse GPC3 have 95 % homology. We searched for GPC3-derived peptides with an amino acid sequence that allows binding to HLA-A24 and K^d, selected those carrying binding motifs to both molecules, and used this information to prepare 12 synthetic GPC3 peptides. We observed the effects of adoptive transfer of selected tumor-reactive T cells directed against the $GPC3_{298–306}$ (EYILSLEEL) peptide and peptide-pulsed bone marrow-derived dendritic cells (BM-DCs). We found this onco-fetal protein to be highly immunogenic in mice in that it elicited effective antitumor immunity with no evidence of autoimmunity (Nakatsura et al. 2004b). Similarly, we identified an HLA-A2-restricted $GPC3_{144–152}$ (FVGEFFTDV) peptide using HLA-A2.1 (HHD) transgenic mice. In addition, we investigated whether these GPC3 peptides could induce GPC3-reactive CTLs from peripheral blood mononuclear cells (PBMCs) of HCC patients. In five of eight HLA-$A2^+$ $GPC3^+$ HCC patients, $GPC3_{144–152}$ peptide-reactive CTLs were generated from PBMCs by in vitro stimulation with the peptide. $GPC3_{298–306}$ peptide-reactive CTLs were also generated from PBMCs in four of six HLA-$A24^+$ $GPC3^+$ HCC patients (Komori et al. 2006). We reported a preclinical study using a mouse model with the goal of designing an optimal protocol for clinical trials using a GPC3-derived peptide vaccine. The results suggested that incomplete Freund's adjuvant (IFA) is one of the indispensable adjuvants for peptide-based immunotherapy. Additionally, at least two vaccinations were needed for the induction of GPC3 peptide-specific CTLs, but repeated vaccination with a lower dose of peptide did not induce peptide-specific CTLs. We concluded that the immunologic effect of the peptide vaccines depends on the dose of peptide injected (Motomura et al. 2008).

11.3 Investigator-Initiated Phase I Trial of the GPC3-Derived Peptide Vaccine for Advanced Hepatocellular Carcinoma (HCC)

Based on the results of preclinical studies, we investigated the safety, anti-tumor effects, and immunologic response of a GPC3-derived peptide vaccine in patients with advanced HCC (Sawada et al. 2012). This trial was a non-randomized, open-label, phase I clinical trial with dose escalation (0.3, 1.0, 3.0, 10, and 30 mg/patient) of the GPC3 peptide. The trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number: 000001395). In this trial, 33 patients received peptide vaccines. HLA-A*24:02-restricted $GPC3_{298–306}$ peptide (EYILSLEEL) was used in 17 HLA-A24-positive patients and HLA-A*02:01-restricted $GPC3_{144–152}$ peptide (FVGEFFTDV) was used in 16 HLA-A2-positive patients. Peptides were administered in liquid form, emulsified with IFA (Montanide™ ISA-51VG), by intradermal injection on days 1, 15, and 29. The peptides and IFA were synthesized according to Good Manufacturing Practice guidelines.

The primary endpoint of the trial was the safety of peptide vaccination. The secondary endpoints were immunologic responses, clinical outcomes, and determination of the optimal peptide dose for further clinical trials. Dose-limiting toxicity and dose-specific adverse events were not seen. All patients experienced grade I or II local skin reactions at the injection site. Transient immune-related events, including drug fever, rash, and flushing, were observed in many patients. Grade III hematologic adverse events, such as impaired liver function, were observed in four patients, who had progressively massive liver tumors. The effect and safety evaluation committee, including the external members, judged that these events were not related to the treatment, but rather to disease progression. These results suggest that the GPC3-derived peptide vaccination was well-tolerated.

In this trial, the median time to tumor progression (TTP) was 3.4 months [95 % confidence interval (CI) 2.1–4.6]. The median OS was 9.0 months (95 % CI 8.0–10.0). Among the 33 patients, one patient was judged to have a partial response (PR) and 19 patients stable disease (SD) for 2 months, according to Response Evaluation Criteria In Solid Tumors (RECIST). Four of the 19 patients with SD had tumor necrosis or partial tumor reduction that did not meet the PR criteria. The disease control rate (PR + SD) was 60.6 % after 2 months. The serum levels of α-fetoprotein (AFP) or des-γ-carboxy prothrombin (DCP) decreased temporarily at least once in nine of the 29 patients (31 %) during the 2-month period (Table 11.1).

To determine whether the GPC3 peptide vaccine could induce a specific immune response, we evaluated the GPC3 peptide-specific immune responses by ex vivo interferon (IFN)-γ Enzyme-Linked ImmunoSpot (ELISPOT) assay using PBMCs obtained from all patients before and after each vaccination. We found that the GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 of the 33 patients (91 %). GPC3-specific CTL frequency increased in a peptide dose-dependent manner (Table 11.1). Generally, CTLs for some tumor antigens cannot

Table 11.1 Clinical and immunologic responses in the investigator-initiated phase I clinical trial

Peptide dose	Number of cases	RECIST evaluation[a]	Decreased tumor marker PIVKA-II	CTL frequency[b]
0.3 mg	8	SD 3, PD 5	0/8 (0 %)	7.5
1.0 mg	6	SD 4, PD 2	2/5 (40 %)	31.5
3.0 mg	6	SD 5, PD 1	3/5 (60 %)	70.5
10 mg	7	SD 4, PD 3	1/5 (20 %)	69
30 mg	6	PR 1, SD 3, PD 2	3/6 (50 %)	92.5
Total	33	PR 1, SD 19, PD 13 (PR + SD 60.6 %)	9/29 (31 %)	23

CTL cytotoxic T lymphocytes, *GPC3* glypican-3, *IFN* interferon, *PD* progressive disease, *PR* partial response, *SD* stable disease

[a]The clinical response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines

[b]Peripheral blood was taken from each patient before and after vaccination. GPC3-specific CTLs were measured ex vivo by IFN-γ ELISPOT assay. Median spot number of 5×10^5 peripheral blood mononuclear cells in ex vivo IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) assay for GPC3 for each peptide dosage

be directly detected ex vivo; they can be detected only after expansion by repeated in vitro stimulation with the antigenic peptide on appropriate antigen-presenting cells. This finding can be attributed to the sensitivity of the assay and the low frequency of tumor antigen-specific CTLs. Surprisingly, GPC3-specific CTLs were detected directly ex vivo without in vitro peptide stimulation in almost all patients after GPC3 peptide vaccination.

Vaccination with synthetic peptides occasionally induces ineffective CTL responses due to various underlying mechanisms (Yamshchikov et al. 2001; Matsui et al. 1995; Falo et al. 1992; Amoscato et al. 1998; Chen et al. 1999; Meadows et al. 1997). One possible mechanism is a low affinity of responding T cells such that they recognize only target cells pulsed with high concentrations of the peptide and not tumor cells expressing the relevant epitopes at lower copy numbers. Alternatively, some antigen epitopes were not expressed on the surface of tumor cells (So et al. 2007; Sorensen et al. 2009). When evaluating T cell responses to peptide vaccines, it is important to confirm that responding CTLs lyse human cancer cells. Then, we established several GPC3 peptide-specific CTL clones in PBMCs obtained from vaccinated patients by single cell sorting using a Dextramer® and CD107a antibody. These CTL clones had high avidity with a recognition efficiency of 10^{10} or 10^{11} M resulting in 50 % cytotoxicity, and could recognize HCC cell lines expressing GPC3 in an HLA-class I-restricted manner. We provided substantial evidence that CTLs displayed not only high avidity, but also natural antigen-specific killing activity against HCC cells that could be induced in HCC patients by the peptide vaccine (Yoshikawa et al. 2011). A tumor biopsy was performed with informed consent in seven patients for evaluation of infiltration of CD8-positive T cells by immunohistochemical staining. In five of seven cases, infiltration of CD8-positive T cells into the tumor was increased after vaccination.

We also examined prognostic factors in the trial. We showed that GPC3-specific CTL frequency could be a predictive marker of GPC3 peptide vaccination effects. We compared patients with GPC3-specfic CTL frequencies ≥50 ($n=15$) with those with GPC3-specific CTL frequencies <50 ($n=18$) and found no significant difference in clinical background. We found a significant difference ($P=0.004$) only for vaccine consumption (≥1.0 vs. <1.0 mg). Analysis of all 33 patients showed that the median OS was 12.2 months (95 % CI 6.5–18.0) in patients with GPC3-specific CTL frequencies ≥50, compared with 8.5 months (95 % CI 3.7–13.1) in those with GPC3-specfic CTL frequencies <50 ($P=0.033$). In conclusion, this phase I clinical trial of a GPC3-derived peptide vaccine showed the vaccination to be safe and indicated a plethora of immunologic responses. This study also showed that GPC3-specific CTL frequency was correlated with OS in patients with advanced HCC who received the GPC3 peptide vaccine. These observations suggest that GPC3-derived peptide vaccines represent a novel therapy for patients with HCC, with the potential to improve OS.

11.4 Pilot Study of the GPC3-Derived Peptide Vaccine for Advanced HCC

In the phase I trial, we demonstrated an increase in GPC3 peptide-specific CTLs in peripheral blood, and identified numerous CD8-positive T cell-infiltrated tumors after GPC3 peptide vaccination. However, we could not confirm that the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific CTLs. To determine whether tumor-infiltrating lymphocytes are indeed GPC3 peptide-specific CTLs, we are currently initiating a pilot study of liver biopsies carried out before and after GPC3 peptide vaccination for advanced HCC (UMIN-CTR: 000005093). In this study, we provide evidence of immunologic responses from two cases. In one case, we performed a pathological study, including autopsy, of a patient, which revealed remarkable tumor lysis immediately after the second vaccination (Sawada et al. 2013). In the other case, we provide evidence that GPC3 peptide-specific CTLs have infiltrated the tumor tissue after peptide vaccination (manuscript in preparation). These results serve as a proof-of concept for GPC3 peptide vaccine therapy.

11.5 Investigator-Initiated Phase II Trial of the GPC3-Derived Peptide Vaccine for Treatment of HCC

Immunotherapy is expected to contribute toward cancer therapy, especially during the early stages or in recurrence prevention. Therefore, we have begun an investigator-initiated phase II clinical trial of the GPC3-derived peptide vaccine as

Table 11.2 Ongoing investigator-initiated clinical trials of glypican-3-derived peptide vaccines

Phase	Number of cases	Cancer type	Subject	Primary endpoint	Secondary endpoint
II	40	Hepatocellular carcinoma	After surgery or radiofrequency ablation	1- and 2-year recurrence rates	Immunologic responses
Pilot study	20	Hepatocellular carcinoma	Advanced	Immunologic responses (T cell infiltration into tumor)	Clinical responses
II	130	Ovarian clear cell carcinoma	Advanced, remission, or combined with chemotherapy	Disease control rate, 2-year recurrence rate	Clinical responses, quality of life
I	15 or 30	Pediatric cancer except for leukemia	Advanced or remission	Dose limiting toxicity	Safety and immunologic responses

an adjuvant therapy for patients with HCC (UMIN-CTR: 000002614). Forty patients with initial HCC who had undergone surgery or radiofrequency ablation were enrolled in this phase II, open-label, single-arm trial. Ten 3 mg GPC3-derived peptide vaccinations were performed over 1 year following curative treatment. The primary endpoints were the 1- and 2-year recurrence rates. The secondary endpoints were immunologic responses. Currently, the correlation between the time of recurrence and immunologic responses is being analyzed (Table 11.2).

11.6 The Application of GPC3-Derived Peptide Vaccine for Other GPC3-Expressing Cancers

GPC3 is over-expressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian clear cell carcinoma, and lung squamous cell carcinoma (Nakatsura et al. 2004a; Saikali and Sinnett 2000; Toretsky et al. 2001; Maeda et al. 2009; Aviel-Ronen et al. 2008). In the phase I trial, we established several GPC3 peptide-specific CTL clones from vaccinated patients with HCC. We investigated whether the GPC3-based immunotherapy could be applied to other GPC3-expressing cancers including melanoma, clear cell carcinoma of the ovary, and pediatric cancer using a GPC3 peptide-specific CTL clone. The CTL clone recognized naturally processed GPC3-derived peptide on ovarian clear cell carcinoma cells in a HLA class I-restricted manner. Moreover, we confirmed that the level of GPC3 expression was responsible for CTL recognition and that a subtoxic chemotherapeutic dose caused tumor cells to become more susceptible to the cytotoxic effect of CTL (Suzuki et al. 2011). Based on these results, we are currently conducting an investigator-initiated phase II trial with a GPC3-derived

peptide vaccine in ovarian clear cell carcinoma patients (UMIN-CTR: 000003696). In this study, two cases with chemotherapy-refractory ovarian clear cell carcinoma achieved a significant clinical response (Suzuki et al. 2013). Furthermore, we have begun an investigator-initiated phase I trial with a GPC3-derived peptide vaccine in pediatric cancer patients diagnosed with hepatoblastoma, nephroblastoma, and yolk sac tumor (UMIN-CTR: 000006357) (Table 11.2).

11.7 Development of a Novel Strategy for Peptide-Specific Cancer Immunotherapy

Although the peptide vaccine is a potentially attractive treatment modality, the anti-tumor effects of the peptide vaccine alone are not sufficiently dramatic for advanced HCC. Therefore, the creation of an innovative strategy to link the anti-tumor immune response with the clinical response and to enhance the power of antigen-specific cancer immunotherapy is urgently required. Cellular immunotherapy of solid and hematopoietic malignancies is regarded as a promising approach to deal with common relapse or resistance to conventional treatments. However, isolation and expansion of functionally active T cells is difficult. Developing a new method of CTL expansion may be useful in addressing this problem. We investigated the efficiency of a new method to induce expansion of GPC3 peptide-specific CTLs for adoptive immunotherapy. The expansion of CTLs from PBMCs of vaccinated patients with advanced HCC yields cell numbers sufficient for adoptive transfer (manuscript in preparation).

In the antigen-specific cancer immunotherapy model, antigen-specific CTLs recognize and destroy tumor cells that present antigen-derived peptides using cell surface major histocompatibility complex (MHC) class I molecules. However, the density of endogenously presented antigen-derived peptides on tumor cells is generally sparse, resulting in the inability of antigen-specific CTLs to work effectively. We hypothesize that increasing the density of an antigen-derived peptide would enhance antigen-specific cancer immunotherapy. We have demonstrated that intratumoral peptide injection leads to additional peptide loading onto MHC class I molecules of tumor cells, enhancing tumor cell recognition by antigen-specific CTLs in mouse models. Moreover, we have demonstrated an antigen-spreading effect that occurred after intratumoral peptide injection (Nobuoka et al. 2012). Intratumoral peptide injection enhances tumor cell antigenicity and may be a useful option for improving antigen-specific cancer immunotherapy against solid tumors. An overview of GPC3-targeted immunotherapy is summarized in Fig. 11.1. These translational studies involving innovative immunotherapeutic approaches will lead to the development of novel therapies for HCC.

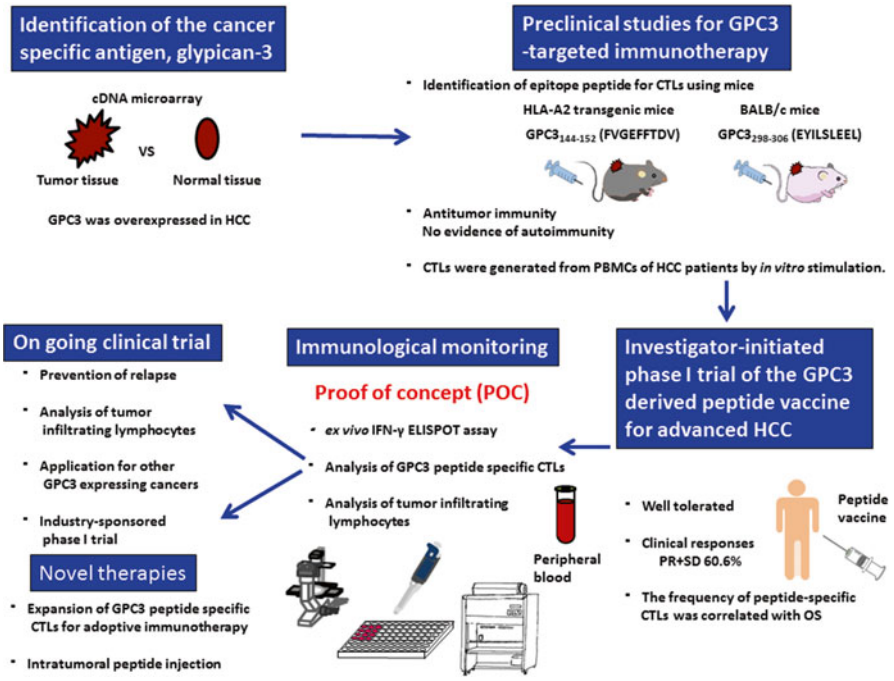

Fig. 11.1 An overview of glypican-3 targeted immunotherapy

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Chapter 12
The Vesicular Traffic System in Plasmacytoid Dendritic Cells as a Target for Immune Regulation

Norimitsu Kadowaki

Abstract Plasmacytoid dendritic cells (pDCs) are a unique immune cell type that produces a vast amount of interferon (IFN)-α in response to virus-derived DNA and RNA through Toll-like receptor (TLR)9 and TLR7, respectively. Nucleic acids derived from self-cells also stimulate pDCs to produce IFN-α in certain conditions, and is implicated in the pathogenesis of inflammatory disorders such as lupus and psoriasis. Recent studies have revealed that elaborate vesicular trafficking machinery in pDCs is the key to the prodigious IFN-α production. Upon stimulation, the nucleic acid-sensing TLRs traffic from the endoplasmic reticulum to the endolysosomal compartments together with a multiple membrane-spanning protein UNC93B1 (Unc-93 homolog B1). Aggregated DNA endocytosed by pDCs is retained in early endosomes for an extended period of time or moves to lysosome-related organelles well-equipped with the machinery for IFN-α production. The nucleic acid then meets the TLR in endolysosomes in a way that produces a large amount of IFN-α. Such exquisite 'receptor transport' and 'ligand transport' systems are vulnerable to pharmacological interventions, the proteasome inhibitor bortezomib, and the tyrosine kinase inhibitor dasatinib, respectively. Elucidating precise molecular mechanisms by which to control vesicular trafficking of TLRs and their ligands in pDCs will reveal new aspects of physiology of vesicular traffic and novel targets to treat pDC-mediated inflammatory disorders.

Keywords Plasmacytoid dendritic cells • Type I interferon • Toll-like receptors • Vesicular traffic

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12.1 Introduction

Plasmacytoid dendritic cells (pDCs) are a distinctive immune cell type that produces a vast amount of interferon (IFN)-α in response to virus-derived CpG DNA (DNA containing unmethylated CpG motifs) or single-stranded RNA through Toll-like receptor (TLR) 9 and TLR7, respectively (Liu 2005), thus playing an important role in antiviral immunity. pDCs also produce IFN-α in response to nucleic acids derived from damaged self-tissues, and are thereby implicated in provoking inflammatory disorders such as lupus and psoriasis (Gilliet et al. 2008; Ganguly et al. 2009). Thus, on the one hand, immunostimulatory CpG DNA has been developed as a promising adjuvant for prevention and treatment of infection and cancer (Kanzler et al. 2007) and on the other hand, pharmaceutical agents that suppress IFN-α production by pDCs may become novel therapies for inflammatory disorders that involve pDCs.

The critical question is how do pDCs produce a vast amount of IFN-α? Recent studies have shown that a unique vesicular traffic system in pDCs appears to be the key to the prominent IFN-α production. Here I discuss (1) recent progress of the studies into the mechanisms by which pDCs produce a large amount of IFN-α; and (2) pharmacological intervention to suppress the activity of pDCs by targeting the vesicular traffic system.

12.2 Distribution of Nucleic Acid-Sensing Toll-Like Receptors (TLRs) Among Antigen-Presenting Cells

The innate immune system is endowed with pattern recognition receptors that recognize molecular signatures common to a broad array of microbial pathogens (Takeuchi and Akira 2010). The TLRs are a family of transmembrane pattern recognition receptors (10 in humans, 12 in mice) that recognize various molecular patterns contained in microbes (Beutler et al. 2006). Some of the TLRs are expressed on cell surfaces and recognize molecules on bacterial cell walls foreign to the host. In contrast, TLR3, 7, 8, and 9 are expressed in intracellular vesicles and recognize pathogen-derived DNA or RNA in endolysosomal compartments (Blasius and Beutler 2010).

The nucleic acid-sensing TLRs are mainly expressed in antigen-presenting cells, such as conventional dendritic cells (cDCs) [also called myeloid dendritic cells (mDCs) in humans], pDCs, monocytes/macrophages, and B cells. Importantly, these cells have different expression profiles of nucleic acid-sensing TLRs. In humans, pDCs selectively express TLR7 and 9, whereas mDCs preferentially express TLR3 and 8 (Kadowaki et al. 2001). Notably, pDCs express only low levels, if any, of non-nucleic acid-sensing TLRs, whereas myeloid antigen-presenting cells express several (Kadowaki et al. 2001; Jongbloed et al. 2010; Hémont et al. 2013).

Such different TLR profiles indicate that these cells respond to different types of pathogens and induce appropriate types of immune responses to eliminate given pathogens. In particular, the restricted TLR profile of pDCs suggests that these cells largely specialize in antiviral immunity.

Mice have small but important differences in expression profiles of nucleic acid-sensing TLRs compared to humans. The most notable difference is the distribution of TLR9 among antigen-presenting cells; myeloid antigen-presenting cells express TLR9 in mice but not in humans. Thus, it should be kept in mind that the effects of TLR9 ligands in mice do not necessarily reflect those in humans.

12.3 Intracellular Localization and Trafficking of Nucleic Acid-Sensing TLRs

While nucleic acids are reliable targets to detect viruses, they are common to pathogens and hosts. Thus, nucleic acid-sensing TLRs have the potential to respond to self-ligands. To avoid this risk of recognizing self-nucleic acids, these TLRs exhibit distinctive intracellular compartmentalization and trafficking (Barton et al. 2006; Ewald et al. 2008). Namely, after trafficking through the endoplasmic reticulum (ER) and the Golgi apparatus, nucleic acid-sensing TLRs localize to the endosomal compartments (Fig. 12.1), in contrast to the other TLRs that localize to the plasma membrane. This results in two safeguard mechanisms against recognition of self-nucleic acids. One is intracellular sequestration of nucleic acid-sensing TLRs, thus preventing them from encountering self-nucleic acids present in an extracellular milieu (Barton et al. 2006). The other is ectodomain proteolysis of TLRs by endosomal proteases active in an acidic condition, thus generating a functional receptor that initiates downstream signals (Ewald et al. 2011).

Trafficking of nucleic acid-sensing TLRs from the ER to the endosomal compartments is controlled by distinctive mechanisms (Fig. 12.1). The multiple membrane-spanning protein UNC93B1 (Unc-93 homolog B1) is a crucial regulator of TLR trafficking from the ER (Kim et al. 2008). UNC93B1 physically associates with TLRs in the ER and facilitates delivery of TLR9 to COPII (coat protein complex II) vesicles, which transport cargo from the ER to Golgi (Lee et al. 2013). Notably, UNC93B1 remains associated with TLRs through post-Golgi sorting steps. Furthermore, the sorting routes are different among TLRs (Lee et al. 2013). TLR9 traffics via the cell surface en route to endosomes, accompanying UNC93B1 and adaptor protein (AP)-2, a complex that directly interacts with UNC93B1 and facilitates endocytosis from the cell surface. TLR7 does not go through this route but traffics from Golgi directly to endosomes, accompanying UNC93B1 and AP-4, which has been implicated in vesicular trafficking between the trans-Golgi network and endosomes. Such a difference in trafficking may result in access of TLR9 and TLR7 to compartments with different functional properties.

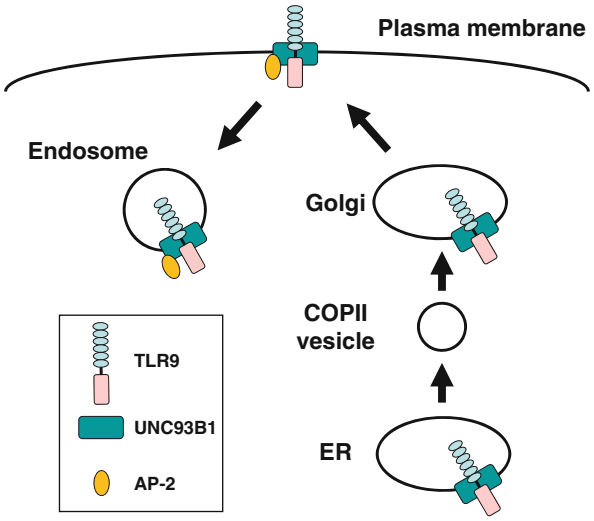

Fig. 12.1 Trafficking of nucleic acid-sensing Toll-like receptors (TLRs). To avoid risks of recognizing self-nucleic acids in an extracellular milieu, nucleic acid-sensing TLRs exhibit distinctive intracellular compartmentalization and trafficking. Unc-93 homolog B1 (UNC93B1) physically associates with nucleic acid-sensing TLRs in the endoplasmic reticulum (ER) and facilitates delivery of TLR9 to COPII (coat protein complex II) vesicles, which transport cargo from the ER to Golgi. TLR9 traffics via the cell surface en route to endosomes, accompanying UNC93B1 and adaptor protein (AP)-2, a complex that directly interacts with UNC93B1 and facilitates endocytosis from the cell surface. TLR7 does not go through this route but traffics from Golgi directly to endosomes, accompanying UNC93B1 and AP-4 (not depicted)

12.4 Endosomal Transport of CpG DNA

After arriving at the endosomal compartments, nucleic acid-sensing TLRs recognize endocytosed DNA or RNA. When it occurs in dendritic cells (DCs) and macrophages, they produce two classes of cytokines: type I IFN (e.g., IFN-α, IFN-β) and pro-inflammatory cytokines [e.g., tumor necrosis factor (TNF), interleukin (IL)-6, IL-12]. Importantly, pDCs produce a vast amount of type I IFN, particularly IFN-α, together with pro-inflammatory cytokines in response to TLR7 and TLR9 engagement (Kadowaki et al. 2000; Ito et al. 2002), whereas myeloid antigen-presenting cells, such as cDCs and macrophages, produce a comparatively small amount of type I IFN (Ito et al. 2002). Here, important questions are as follows: (1) what is the mechanism by which the same cell differentially produces type I IFN and pro-inflammatory cytokines?; and (2) what is the mechanism by which pDCs produce a vast amount of type I IFN? A seminal work by Honda et al. presented an initial conceptual breakthrough for these questions (Honda et al. 2005). It had been shown that different classes of oligonucleotides (ODNs) elicit different cytokine production by pDCs (Krieg 2002). CpG-A ODNs form large multimeric aggregates and strongly induce type I IFN, whereas CpG-B ODNs remain monomeric and

preferentially induce pro-inflammatory cytokines (Kerkmann et al. 2005). Remarkably, Honda et al. has shown using mouse pDCs that this phenomenon is due to differential trafficking of the two classes of ODNs to endosomal compartments where TLR9 signaling leads to activation of distinct transcription factors (Fig. 12.2a). CpG-A preferentially traffics to and is retained for a long period in early endosomes where TLR9 signaling recruits IFN regulatory factor (IRF) 7, leading to the production of type I IFN. In contrast, CpG-B preferentially traffics to late endosomes where TLR9 signaling recruits nuclear factor (NF)-κB, leading to the production of pro-inflammatory cytokines. Guiducci et al. have shown similar results using human pDCs (Guiducci et al. 2006). These studies indicate that the structural conformations of ODNs determine their distinct localization, which leads

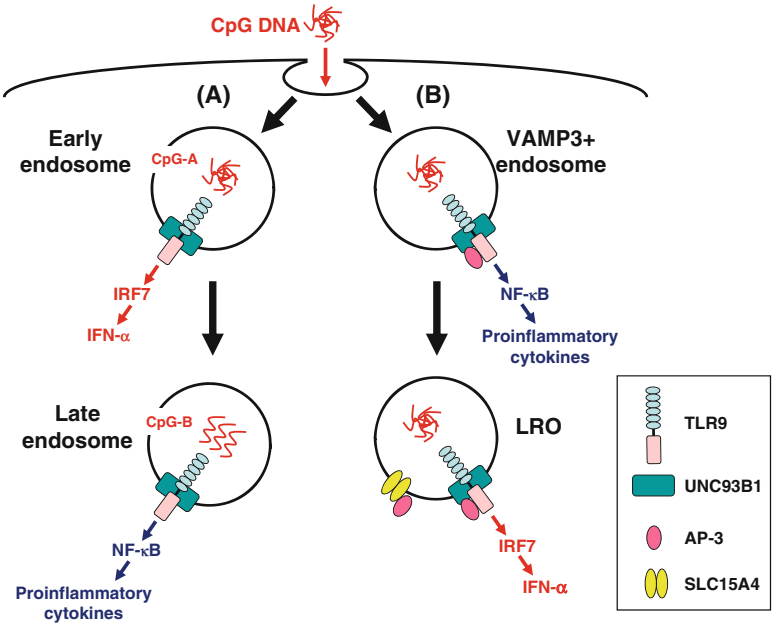

Fig. 12.2 Endosomal transport of CpG DNA. Two models have been proposed regarding the mechanisms by which plasmacytoid dendritic cells (pDCs) produce a large amount of interferon (IFN)-α and differentially produce IFN-α and pro-inflammatory cytokines. (**a**) CpG-A [multimeric aggregated oligonucleotides (ODNs)] preferentially traffics to and is retained for a long period in early endosomes where Toll-like receptor (TLR) 9 signaling recruits interferon regulatory factor (IRF) 7, leading to the production of IFN-α. In contrast, CpG-B (monomeric ODNs) preferentially traffics to late endosomes where TLR9 signaling recruits nuclear factor (NF)-κB, leading to the production of pro-inflammatory cytokines. (**b**) Adaptor protein (AP)-3 interacts directly with TLR9 and facilitates trafficking from vesicle-associated membrane protein (VAMP) 3^+ endosomes to specialized lysosome-related organelle (LRO) compartments. $VAMP3^+$ endosomes recruit NF-κB signaling and initiate pro-inflammatory cytokine production, whereas LROs recruit IRF7 signaling and initiate IFN-α production. AP-3 also interacts with SLC15A4 and may recruit it to LROs, where SLC15A4 plays an essential role in IFN-α production

to different cytokine production. Consistent with this, complexing CpG-B with a cationic lipid alters the localization of CpG-B to early endosomes in pDCs, resulting in type I IFN production (Honda et al. 2005). Furthermore, whereas CpG-A induces cDCs and macrophages to produce pro-inflammatory cytokines alone, complexing with a cationic lipid induces these cells to produce type I IFN. Thus, pDCs and the myeloid cells share functionally distinctive signaling components for type I IFN production. However, some unique biological characters of pDCs are likely to provide these cells with the capacity to preferentially produce a large amount of type I IFN.

More recently, two groups have shown that AP-3 is crucial for TLR7-/9-dependent type I IFN production (Sasai et al. 2010; Blasius et al. 2010). AP-3 is a heterotetrameric adaptor complex that associates with clathrin coats on the trans-Golgi network and endosomes and directs cargo to late endosomes/lysosomes or, in certain cell types, to lysosome-related organelles (LROs) (Bonifacino and Traub 2003). LROs comprise a heterogeneous set of organelles that share features with late endosomes/lysosomes but have specialized functions (Raposo et al. 2007). Examples of LROs are lytic granules in cytotoxic lymphocytes, MHC class II compartments in antigen-presenting cells, and melanosomes in melanocytes. Sasai et al. have shown that AP-3 interacts directly with TLR9 and facilitates trafficking from endosomes marked by an early endosome marker vesicle-associated membrane protein (VAMP) 3 to specialized LRO compartments marked by lysosome-associated membrane protein (LAMP) 2 (Sasai et al. 2010) (Fig. 12.2b). pDCs from Ap3b1 (a subunit of the AP-3 complex)-deficient mice fail to produce type I IFN in response to TLR7/9 ligands, whereas these cells exhibit normal or moderately enhanced production of IL-12p40. Sasai et al. concluded that TLR9 in $VAMP3^{+}$ NF-κB endosomes transmits a pro-inflammatory cytokine-inducing signal, whereas TLR9 conveyed by AP-3 to $LAMP2^{+}$ LROs (IRF7 endosomes) transmits a type I IFN-inducing signal. Blasius et al. reported cytokine profiles of pDCs that disagree with this; mice deficient in any of three protein complexes involved in Hermansky-Pudlak syndrome [AP-3, biogenesis of LROs complex (BLOC)-1, BLOC-2] fail to produce both type I IFN and a pro-inflammatory cytokine TNF (Blasius et al. 2010). Furthermore, mice deficient in solute carrier family 15 (SLC15) A4, a proton-coupled histidine and oligopeptide co-transporter, also fail to produce both type I IFN and pro-inflammatory cytokines TNF, IL-6, and IL-12p40 (Blasius et al. 2010). Importantly, neither AP-3 nor SLC15A4 is necessary for TLR7/9 signaling in cDCs. As SLC15A4 has an AP-3-binding motif in its cytoplasmic domain, Blasius et al. proposed that LRO trafficking capacitates the endosome by directing the incorporation of proteins, including SLC15A4, that allow TLR signaling within the specialized environment of pDCs (Fig. 12.2b). Of note, Sasawatari et al. have shown that exogenously added histidine suppresses TLR9-triggered cytokine production by wild-type but not SLC15A4-deficient DCs (Sasawatari et al. 2011). As SLC15A4 transports histidine out of lysosomes, Sasawatari et al. proposed that SLC15A4 deficiency may result in accumulation of histidine in lysosomes, which may abrogate acidification of lysosomes and type I IFN production by pDCs.

The involvement of LROs in TLR9-mediated type I IFN signaling is difficult to reconcile with the previous studies identifying early endosomes as the key compartment (Honda et al. 2005; Guiducci et al. 2006) because these two organelles are distinct. Nonetheless, a series of studies have revealed that pDCs possess specialized endosomal compartments, apparently LROs, that are well-equipped with machinery for a vast amount of type I IFN production upon TLR7/9 triggering.

12.5 A Proteasome Inhibitor Bortezomib Suppresses the Activity of Plasmacytoid Dendritic Cells (pDCs) by Targeting Intracellular Trafficking of TLR9

The elaborate vesicular traffic system in pDCs is likely to be maintained by multiple layers of signaling pathways, and may thus be susceptible to pharmacological intervention.

Proper functioning of highly secretory cells such as pDCs depends on the unfolded protein response (UPR), i.e., coordinated handling of ER stress caused by a burden of unfolded proteins in the lumen of the ER (Todd et al. 2008). After sensing unfolded proteins, an ER-resident transmembrane endoribonuclease IRE1 splices the mRNA of a transcription factor XBP1, which results in the conversion of an inactive unspliced XBP1 (XBP1u) to an active spliced XBP1 (XBP1s) protein (Fig. 12.3a). XBP1s induces transcription of a broad array of UPR genes that assist in protein synthesis and secretion (Lee et al. 2003b). It has been shown that development of XBP1-deficient pDCs is reduced, likely due to their increased sensitivity to apoptosis induced by ER stress (Iwakoshi et al. 2007).

A selective inhibitor of the 26S proteasome bortezomib has been established as an effective drug for plasma cell myeloma (Richardson et al. 2006). The selectivity of bortezomib for myeloma appears to be due to increased susceptibility of myeloma cells to ER stress-induced apoptosis (Lee et al. 2003a; Obeng et al. 2006). Proteasome inhibitors suppress the activity of IRE1 and stabilize the dominant negative XBP1u protein, resulting in a decrease in the activity of XBP1s in myeloma cells (Lee et al. 2003a) (Fig. 12.3a). Proteasome inhibitors also prevent retrograde translocation of misfolded proteins in the ER to the cytosol, resulting in the accumulation of a large amount of misfolded immunoglobulin in the ER in myeloma cells (Mancini et al. 2000). Such overloading of the ER might compromise physiological functions of the ER. Importantly, pDCs resemble plasma cells, in that both have the developed ER (Grouard et al. 1997), are highly secretory, and depend on ER homeostasis, particularly on XBP1, for their development and survival (Reimold et al. 2001; Iwakoshi et al. 2003, 2007). Furthermore, coordinated trafficking of the two ER-resident proteins, TLR and UNC93B1, is necessary for pDCs to respond to the TLR ligands (Kim et al. 2008). Therefore, it is possible that bortezomib suppresses the activity of pDCs by targeting the two critical events in the ER: the UPR and the coordinated function of the ER-resident TLRs and UNC93B1. Indeed, we

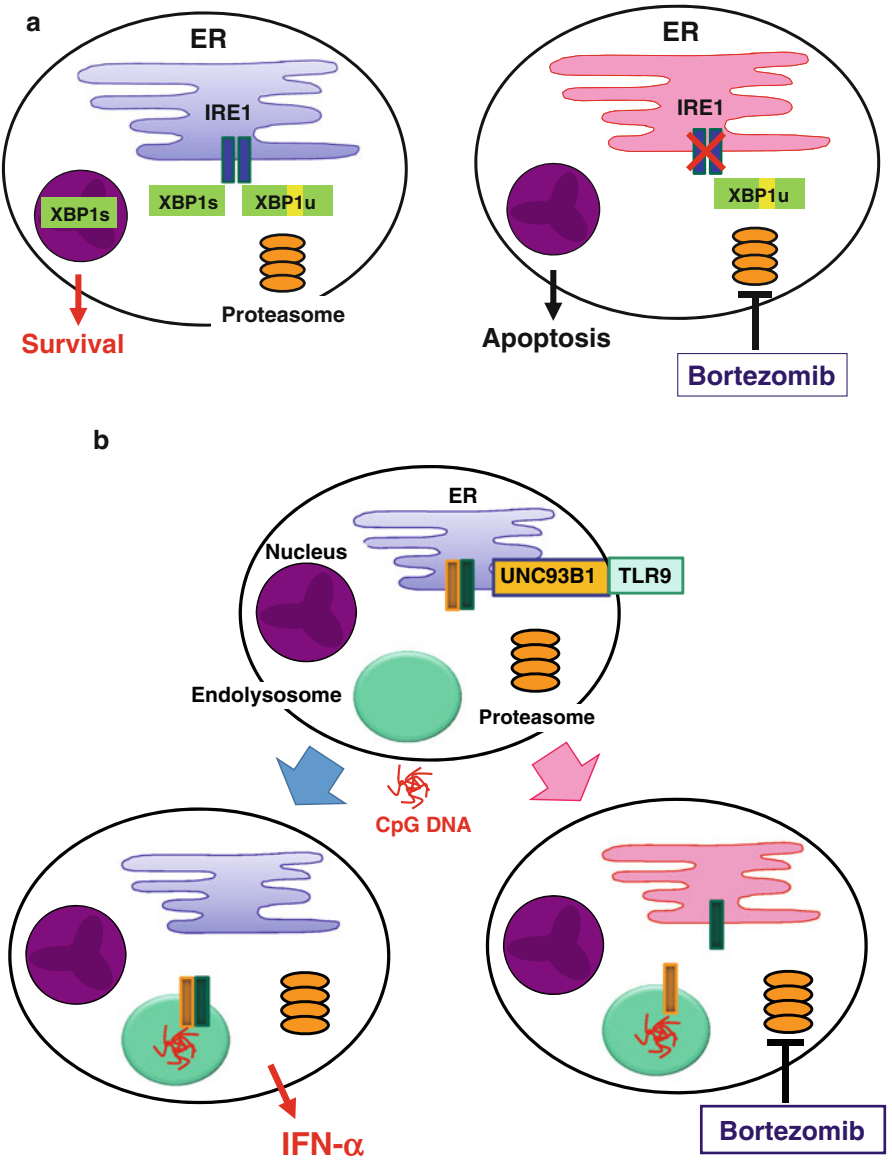

Fig. 12.3 Suppression of plasmacytoid dendritic cells (pDCs) by a proteasome inhibitor, bortezomib. (**a**) In highly secretory cells such as pDCs and plasma cells, coordinated handling of endoplasmic reticulum (ER) stress is necessary for maintaining survival and function. For example, an ER-resident endonuclease IRE1 transforms unspliced XBP1 (XBP1u) into spliced XBP1 (XBP1s), which induces transcription of a broad array of unfolded protein response (UPR) genes. Bortezomib increases ER stress and suppresses the activity of IRE1, leading to apoptosis. (**b**) Stimulation with CpG DNA induces coordinated trafficking of Toll-like receptor (TLR)9 and Unc-93 homolog B1 (UNC93B1) from the ER to endolysosomes, which may depend on proper function of the ER. Bortezomib disturbs the ER function and dissociates TLR9 from UNC93B1, leading to suppression of IFN-α production

found that pDCs were most susceptible to the killing effect of bortezomib among immune cells in human blood (Hirai et al. 2011). This correlates with a decrease in the generation of XBP1s (Fig. 12.3a). Bortezomib suppressed the production of IFN-α and IL-6 by pDCs activated with a TLR9 ligand, which appears to be partially independent of apoptosis. Importantly, bortezomib inhibited translocation of TLR9, but not of UNC93B1, from the ER to endolysosomes (Fig. 12.3b). Thus, bortezomib suppresses the activity of pDCs by disturbing ER homeostasis, which results in disruption of the coordinated trafficking of TLR9 and UNC93B1 from the ER to endolysosomes and eventually in apoptosis of pDCs. This study illustrates that proper functioning of pDCs depends on undisturbed ER, which may represent a target to treat pDC-mediated inflammatory disorders.

12.6 A Tyrosine Kinase Inhibitor Dasatinib Suppresses the Activity of pDCs by Targeting Endosome Transport of CpG DNA

In addition to trafficking of TLRs from the ER to endolysosomes, trafficking of TLR ligands through the endosomal compartments may be susceptible to pharmacological intervention. Protein phosphorylation is at the heart of controlling endosomal trafficking and of integrating them with signal transduction networks of the cell (Liberali et al. 2008). Thus, protein kinases may represent another target to suppress the activity of pDCs.

Dasatinib, a tyrosine kinase inhibitor for chronic myeloid leukemia, is capable of inhibiting a broad array of tyrosine kinases, among which SRC family kinases (SFKs) are prominent targets (Lombardo et al. 2004). We found that dasatinib and SFK inhibitors strongly suppressed the production of IFN-α and pro-inflammatory cytokines (TNF, IL-6) by pDCs stimulated with CpG-A without reducing viability, whereas these inhibitors did not suppress the cytokine production induced by CpG-B as strongly in comparison (Fujita et al. 2013). Neither dasatinib nor SFK inhibitors inhibited endocytosis of CpG ODNs or the trafficking of TLR9 from the ER to endosomes induced by CpG ODNs. Whereas chloroquine, which inhibits proteolysis of TLRs in endolysosomes, did not suppress global tyrosine phosphorylation induced by CpG ODNs, dasatinib and SFK inhibitors suppressed it, indicating that these inhibitors suppress intracellular events upstream of TLR9. Importantly, dasatinib, but not SFK inhibitors, abrogated prolonged localization of CpG-A in early endosomes (Fig. 12.4). These data suggest that dasatinib inhibits SFK-independent endosomal retention of CpG-A as well as certain SFK-dependent steps upstream of TLR9, resulting in suppressing the production of IFN-α and pro-inflammatory cytokines. This study provides a clue to being able to dissect molecular mechanisms for the distinctive behavior of endosomes in pDCs as well as possibilities to develop novel therapies for inflammatory disorders by targeting the endosomal trafficking in pDCs.

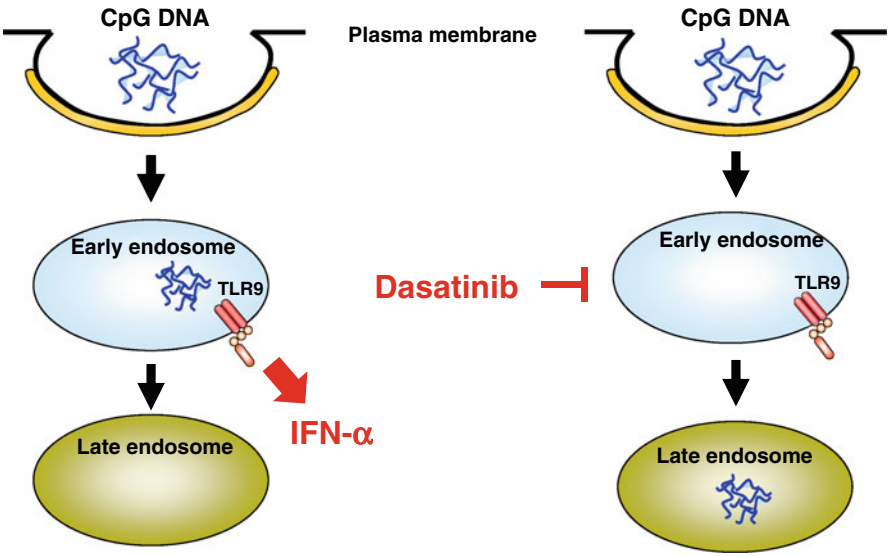

Fig. 12.4 Suppression of plasmacytoid dendritic cells (pDCs) by the tyrosine kinase inhibitor dasatinib. It has been shown that the prodigious production of interferon (IFN)-α depends on prolonged localization of CpG-A in early endosomes. Dasatinib abrogates prolonged localization of CpG-A, which appears to contribute to the suppression of IFN-α production by pDCs

12.7 Conclusions and Perspectives

Recent studies have clarified that the highly elaborate machinery of vesicular trafficking of TLRs and their ligands enables pDCs to produce a vast amount of type I IFN. Such distinctive machinery may represent a possible target to treat pDC-mediated inflammatory disorders. If fact, we have shown that the proteasome inhibitor bortezomib and tyrosine kinase inhibitor dasatinib suppress the activity of pDCs by targeting ER homeostasis ('receptor transport' from the ER to endosomes) and endosomal trafficking of a TLR ligand ('ligand transport'), respectively (Fig. 12.5). However, many important questions remain to be answered regarding molecular mechanisms by which TLRs and their ligands are transported between vesicles. For example, what is the exact molecular mechanism of the dissociation between TLR9 and UNC93B1 upon treatment with bortezomib? To understand it, we need to clarify the mechanisms by which external stimuli trigger the departure of TLR9 and UNC93B1 from the ER and unidentified molecular components of the TLR–UNC93B1 complex. The next question is: what are the kinases inhibited by dasatinib when it suppresses cytokine production by pDCs? In particular, the kinases responsible for the retention of CpG DNA in early endosomes may be the key to elucidating the mechanism by which pDCs produce a large amount of type I IFN. In

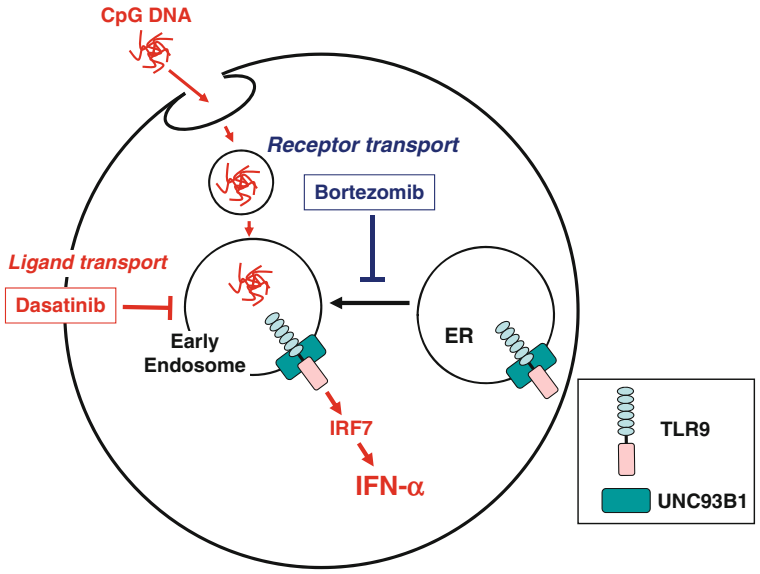

Fig. 12.5 Transport of nucleic acid-sensing Toll-like receptors (TLRs) and their ligands as targets to suppress plasmacytoid dendritic cells (pDCs). The highly elaborate machinery of vesicular trafficking of TLRs (receptor transport) and their ligands (ligand transport) is vulnerable to pharmacological interventions by bortezomib and dasatinib, respectively. Such interventions may lead to development of novel therapies for pDC-mediated inflammatory disorders

line with this, recent studies have shown that several molecules that are involved in actin remodeling, and may thus play a role in endosomal trafficking, are critical for regulating type I IFN production by pDCs: a Rac activator, DOCK (dedicator of cytokinesis) 2 (Gotoh et al. 2010); a cytoplasmic phosphoprotein, PACSIN (protein kinase C and casein kinase substrate in neurons protein) 1 (Esashi et al. 2012), which interacts with several proteins implicated in vesicular trafficking; and a scaffold protein that regulates actin dynamics, WASP (Wiskott-Aldrich syndrome protein) (Prete et al. 2013). pDCs from WASP patients or WASP-deficient mice are hyperresponsive to CpG-A and produce a higher level of IFN-α. Notably, accumulation of CpG-A in early endosomes and LAMP-1$^+$ endosomes, possibly LROs, is enhanced in these pDCs. Thus, WASP-mediated actin polymerization appears to promote endosomal trafficking of TLR9 ligands in pDCs. Dasatinib may suppress cytokine production by pDCs by inhibiting protein kinases that regulate such actin dynamics. Elucidating precise molecular mechanisms by which to control vesicular trafficking of nucleic acid-sensing TLRs and their ligands in pDCs will reveal new aspects of physiology of vesicular traffic and novel targets to treat pDC-mediated inflammatory disorders.

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Chapter 13
In Vivo Targeting of Dendritic Cells with Artificial Adjuvant Vector Cells (aAVC) as a Novel Cancer Immunotherapy

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Abstract An ideal cancer vaccine would inhibit tumor cells by inducing both innate and adaptive immunity, allowing for specific and long-term cancer immunosurveillance.

Dendritic cells (DCs) and invariant natural killer T (iNKT) cells are excellent targets for this type of tumor immunotherapy development. DCs are innate immune cells that initiate and shape adaptive immunity through naïve T cell priming and chemokine/cytokine secretion, while iNKT cells induce the maturation of endogenous DCs in situ, thereby linking innate and adaptive immunity.

Our laboratory has established a unique method of targeting in vivo DCs using a cell vector system comprised of glycolipid-loaded, mRNA-transfected allogeneic cells. These cells provide iNKT cell activation, DC maturation, and tumor-specific T cell immunity. This unique tool could prove clinically beneficial in the development of immunotherapies against malignant and infectious diseases.

Keywords iNKT cells • Adjuvant effects • Dendritic cells • α-Galactosylceramide • Cross-presentation

13.1 Introduction

Invariant natural killer T (iNKT) cells have several unique features that differentiate them from T cells and natural killer (NK) cells. iNKT cells express a nearly invariant T cell receptor (TCR) encoded by Vα14Jα18 in mice and Vα24Jα18 in humans and can rapidly produce both T helper (Th) 1- and Th2-type cytokines after ligand stimulation (Fujii et al. 2007; Terabe and Berzofsky 2008). Mice lacking iNKT cells are susceptible to many types of infectious diseases, such as herpes simplex virus

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(HSV)-1 infection (Tupin et al. 2007; Van Kaer 2007). Humans with X-linked inhibitor of apoptosis (XIAP) deficiency lack iNKT cells and develop X-linked lymphoproliferative disorders once infected with Epstein-Barr virus (EBV) (Rigaud et al. 2006). These examples underline the important role iNKT cells play in the first host defense. An exogenous glycolipid, α-galactosylceramide (α-GalCer), is widely used as a synthetic ligand for activating iNKT cells and is presented to them by the monomorphic, human leukocyte antigen (HLA)-class I-like molecule CD1d. iNKT cells act not only by mediating their own distinctive protective functions, but also by interacting with the different types of immune cells, such as macrophages and dendritic cells (DCs) to optimize the subsequent response against infectious pathogens and tumors.

It has been previously shown that DCs loaded ex vivo with α-GalCer (DC/Gal) provoke a better anti-tumor immune response than α-GalCer alone (Fujii et al. 2002). iNKT cells from mice treated with DC/Gal have an altered cytokine profile, secreting more interferon (IFN)-γ than interleukin (IL)-4. Injection of DC/Gal activates both iNKT cells and NK cells in mice and humans, with a positive correlation between anti-tumor effects and the number of IFN-γ producing innate lymphocytes and anti-tumor effects (Nieda et al. 2004; Chang et al. 2005; Motohashi and Nakayama 2009). However, not only mature DCs but also macrophages and B cells express CD1d and therefore can act as potent antigen-presenting cells (APCs) for iNKT cells (Shimizu et al. 2007a; Fujii et al. 2007). In this chapter, we discuss strategies using 'iNKT cell-triggered mature DCs in situ' as a novel mode of cancer immunotherapy by linking innate and adaptive immunity.

13.2 Harnessing the Adjuvant Effects of Invariant Natural Killer T (iNKT) Cell-Activated Dendritic Cells (DCs) in Cancer Immunotherapy

13.2.1 Co-administration of Tumor Antigen with iNKT Cell Ligand

During steady-state conditions and in the absence of maturation stimuli, a subset of DCs induce T cell tolerance to protein from dying cells (Liu et al. 2002). Delivery of foreign protein in conjunction with α-GalCer leads to antigen-specific T cell immunity in vivo through the maturation of in situ DCs by activated iNKT cells (Fujii et al. 2003). This maturation process of DCs involves not only phenotypic changes, but functional changes as well (Fujii et al. 2007; Fujii 2008). In addition to increases in co-stimulatory (CD40, CD70, CD80, and CD86) and major histocompatibility complex (MHC) class II molecules, DCs acquire the potential to produce inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-12, as well as chemokines, such as CCL17 and CCL21, which induce migration of both T cells and iNKT cells (Semmling et al. 2010). Furthermore, iNKT cell-matured DCs

stimulate robust T cell proliferation in the mixed leukocyte reaction (MLR) (Fujii et al. 2003, 2004). Using this method of DC maturation through iNKT cell activation (Fujii et al. 2007; Fujii 2008), several immunotherapeutic strategies have been evaluated for their effectiveness in eliciting in vivo anti-tumor responses.

An initial approach combined tumor antigen with α-GalCer. Mice given irradiated tumor cells (J558 plasmacytoma cells) and α-GalCer were protected from subsequent tumor cell re-challenge (Liu et al. 2005). Both anti-tumor $CD4^+$ and $CD8^+$ T cell responses were induced using this method. Thus, co-administration of dying cell-associated antigen together with α-GalCer is effective in eliciting protective antigen-specific T cell immunity (Fujii et al. 2003, 2007; Fujii 2008).

This strategy can also be used for the induction of immunity to infectious agents. Mice immunized with α-GalCer and inactivated influenza A virus or HSV-2 glycoprotein D (gD) demonstrated antigen-specific $CD8^+$ T cell immunity as well as systemic virus-specific IgG (Youn et al. 2007; Kamijuku et al. 2008; Guillonneau et al. 2009; Lindqvist et al. 2009). Furthermore, vaccination with antigen-specific DNA plus α-GalCer led to protection against *Leishmania* and HIV-1 infection (Dondji et al. 2008; Huang et al. 2008).

13.2.2 Use of Adjuvant Vector Cells in Therapies Targeting In Situ DCs

Despite its early promise, there are limitations to using α-GalCer and irradiated tumor cells or protein antigen alone for eliciting T cell immunity in vivo. The problem with this approach is that in situ targeting of DCs requires a relatively large dose of irradiated tumor cells or protein in mice. Therefore, we tested a method that would use α-GalCer-loaded tumor cells (tumor/Gal) to stimulate a tumor antigen-specific response.

To test this approach, we used several tumor models, including B16 melanoma, J558 plasmacytoma, WEHI3B myelomonocytic leukemia, and EL4 thymoma. All tumor models successfully induced iNKT and NK cell activation (Shimizu et al. 2007a). iNKT- and NK-mediated lysis of tumor cells led to cross-presentation of tumor antigens by host DCs matured by iNKT cells. Interestingly, intravenous injection with tumor/Gal induced long-term protection against subcutaneous challenge with the same tumor in mice (Fujii et al. 2007; Shimizu et al. 2007b).

13.2.3 Development of Artificial Adjuvant Vector Cells Expressing Tumor Antigen and iNKT Ligand

In the above studies, tumor/Gal acted as powerful adjuvants for host immunity in tumor-bearing mice. However, adoptive transfer of tumor/Gal may not always be an available option for immunotherapy in cancer patients because it depends on the

tumor cell number in patients. We reasoned that any cell expressing both glycolipid and tumor antigens could serve as an adjuvant vector cell and therefore attempted to develop an approach that could be more readily applicable for use in patients. First, we developed a murine 'artificial adjuvant vector cells' (aAVC) model using allogeneic fibroblasts loaded with α-GalCer and transfected with mRNA encoding tumor antigen (Fujii et al. 2009; Shimizu et al. 2013), thus combining the adjuvant effects of iNKT cell activation with delivery of antigen to DCs in situ (Fig. 13.1) (Shimizu et al. 2007a; Fujii et al. 2009). We found a direct correlation between the level of CD1d expression on adjuvant vector cells (AVCs) and the level of T cell response (Fujii et al. 2007, 2009; Fujii 2008).

We then demonstrated that human cells transfected with ovalbumin (OVA) antigen and loaded with α-GalCer could also generate both innate and adaptive immunity in wild-type mice. We also assessed the safety of this approach in a canine large animal

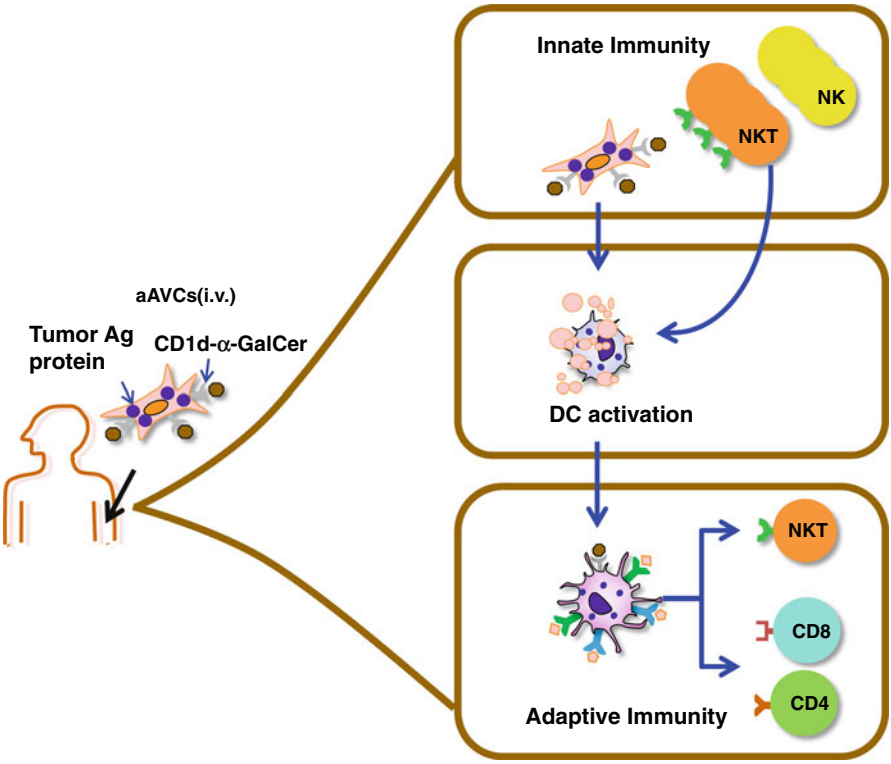

Fig. 13.1 Invariant natural killer T (iNKT) cell-mediated adjuvant effects on T cells are dependent on the innate immunity. Intravenously administered artificial adjuvant vector cells (aAVCs) are killed by iNKT and natural killer (NK) cells (*upper*, *right*), and cellular debris is captured by endogenous dendritic cells (DCs) (*right*, *middle*). α-Galactosylceramide (α-GalCer) on the aAVCs activates iNKT cells, which secrete cytokines that lead to DC maturation (*right*, *middle*). Mature DCs are then able to induce an antigen-specific T cell response (*right*, *lower*). They also cross-present glycolipid from phagocytosed aAVCs to iNKT cells in a CD1d-dependent manner

model. Physical examination, blood chemistry analysis, and autoantibody tests, as well as tissue biopsies from liver, lung, and other organs from dogs given high-dose aAVCs, confirmed the safety of this method in addition to its efficacy in generating immunologic responses (Shimizu et al. 2013).

A final preclinical study was performed to determine whether tumor antigen-specific T cell responses seen in allogeneic studies could also be generated in an autologous setting. For this purpose, we developed a novel humanized mouse model using *NOD/Shi*-scid*/IL-2Rγc*^{null} (NOG) mice. T cells from healthy HLA-A2+ donors were transduced with the MART (melanoma antigen recognized by T cells)-1 tumor antigen *TCR* gene and adoptively transferred into NOG mice. This was followed by intravenous transfer of syngeneic immature DCs and iNKT cells. Finally, aAVCs transduced with MART-1 mRNA and loaded with α-GalCer were transferred into one group of mice but not controls. As expected, the transferred *TCR* gene-transduced T cells expanded robustly in mice receiving MART-1 expressing aAVC but not controls (Shimizu et al. 2013).

Thus far, our current pre-clinical studies demonstrate the safety and efficacy of aAVCs in harnessing the innate and adaptive immune systems through targeting of in situ DCs. This model system, which uses different arms of the immune system to generate a systemic and specific anti-tumor response, could prove clinically beneficial as immunotherapies against a number of malignant diseases.

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Chapter 14
Enhancement of Efficacy of Wilms' Tumor Gene *WT1* Product-derived Peptide Cancer Vaccine by Co-administration with Immunopotentiating Agents: Lessons from Mouse Models

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Abstract To induce and activate tumor-associated antigen-specific cytotoxic T lymphocytes (CTLs) for cancer immunity, it is important not only to select potent CTL epitopes but also to combine them with appropriate immunopotentiating agents. Wilms' tumor gene *WT1* is expressed at high levels in many kinds of hematological and solid malignancies. *WT1* gene products have high immunogenicity and have been reported to serve as a promising cancer antigen for tumor-specific immunotherapy. We have started WT1 peptide vaccine clinical trials since 2001, and demonstrated that WT1 peptide can induce WT1-specific immunologic

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responses and the associated clinical responses. To enhance the WT1 peptide vaccine's therapeutic efficacy, we investigated various immunopotentiating agents that co-administer with WT1 peptide vaccine, using mice models for WT1 peptide cancer immunotherapy. *Mycobacterium bovis* bacillus Calmette-Guérin cell wall skeleton (BCG-CWS), which is well-known to activate dendritic cells (DCs), i.e., activate innate immunity, could induce and/or activate WT1-specific CTLs in combination with WT1 peptide vaccination. Interferon (IFN)-β is a type I IFN, and is known for its various anticancer properties. Co-administration of WT1 peptide and IFN-β enhanced tumor immunity mainly through the induction of WT1-specific CTLs, enhancement of natural killer (NK) activity, and promotion of major histocompatibility complex (MHC) class I expression on the tumor cells. WT1 peptide vaccination combined with BCG-CWS or IFN-β can thus be expected to enhance the clinical efficacy of WT1 immunotherapy.

Keywords WT-1 • BCG-CWS • Dendritic cells • Type I interferon • Toll-like receptors • Peptide vaccine therapy • Cytotoxic T lymphocytes • NK cells

14.1 Wilms' Tumor Gene *WT1* and the Background for *WT1* Gene Products Targeting Cancer Immunotherapy

Wilms' tumor gene *WT1* was originally isolated as a gene responsible for a pediatric renal cancer, Wilms' tumor (Call et al. 1990; Gessler et al. 1990). This gene encodes a zinc finger transcription factor involved in organ development, cell proliferation and differentiation, as well as apoptosis. The *WT1* gene products regulate the expression of various genes either positively or negatively, depending on how it combines with other regulatory proteins in different types of cells. Although the *WT1* gene was categorized at first as a tumor-suppressor gene (Menke et al. 1998), we have proposed that the wild-type *WT1* gene plays an oncogenic rather than a tumor-suppressor gene function in many kinds of malignancies on the basis of the following findings: (1) the wild-type *WT1* gene was highly expressed in hematological and solid malignancies (Sugiyama 2001, 2002, 2005, 2010; Oka et al. 2006, 2007, 2008a, b, 2009; Oka and Sugiyama 2010; Miwa et al. 1992; Briegar et al. 1994; Inoue et al. 1994a, b; Menssen et al. 1995; Bergmann et al. 1997; Oji et al. 1999, 2002, 2003, 2004; Loeb et al. 2001; Ogawa et al. 2003); (2) high expression levels of *WT1* mRNA correlated with poor prognosis in leukemia and several kinds of solid cancer (Inoue et al. 1994b; Bergmann et al. 1997); (3) growth of WT1-expressing leukemia and solid cancer cells was inhibited by treatment with *WT1* antisense oligomers in vitro (Yamagami et al. 1996); and (4) in wild-type *WT1* gene-transfected myeloid progenitor cells, differentiation was blocked but proliferation was induced in response to granulocyte colony-stimulating factor (Inoue et al. 1998; Tsuboi et al. 1999).

These findings indicate that *WT1* over-expression and leukemogenesis or tumorigenesis may be closely related, which suggests that the wild-type *WT1* gene

products could be a promising tumor rejection antigen for cancer immunotherapy. Tumor escape from immune surveillance as a result of downregulation of WT1 expression is unlikely to occur, because expression of WT1 seems to have an essential role in leukemogenesis or tumorigenesis, and seems to be required to maintain the transformed phenotype and function. This is a theoretical advantage for using WT1 protein as a target antigen for cancer immunotherapy. In fact, we and others have generated human WT1-specific cytotoxic T lymphocytes (CTLs) in vitro (Sugiyama 2001, 2002, 2005, 2010; Oka et al. 2000a, 2006, 2007, 2008a, b, 2009; Oka and Sugiyama 2010; Gao et al. 2000; Ohminami et al. 2000), and we were able to show that mice immunized with major histocompatibility complex (MHC) class I-restricted WT1 peptide or with WT1 plasmid DNA elicited WT1-specific CTLs and rejected the challenge of WT1-expressing cancer cells in vivo (Sugiyama 2001, 2002, 2005, 2010; Oka et al. 2000b, 2006, 2007, 2008a, b, 2009; Oka and Sugiyama 2010; Tsuboi et al. 2000). In addition, leukemia stem cells have been shown to express WT1, which supports the superiority of WT1 as a cancer antigen, because it indicates the possibility that WT1-directed immunotherapy can target cancer stem cells (Saito et al. 2010). Furthermore, a series of investigations using clinical samples such as patients' peripheral blood demonstrated that WT1-directed cellular and humoral immune responses were generated in patients with malignancies (Sugiyama 2001, 2002, 2005, 2010; Oka et al. 2006, 2007, 2008a, b, 2009; Oka and Sugiyama 2010; Elisseeva et al. 2002; Wu et al. 2005; Oji et al. 2009). These preclinical results urged us to perform a WT1-targeting cancer immunotherapy trial, and we started a WT1 peptide vaccine clinical trial in 2001 and demonstrated that WT1 peptide can induce WT1-specific immunologic responses and the associated clinical responses (Sugiyama 2001, 2002, 2005, 2010; Oka et al. 2003, 2004, 2006, 2007, 2008a, b, 2009; Oka and Sugiyama 2010; Tsuboi et al. 2004, 2007, 2012; Morita et al. 2004; Iiyama et al. 2007; Kawakami et al. 2007; Izumoto et al. 2008; Ohta et al. 2009; Hashii et al. 2010, 2012; Chiba et al. 2010). On the basis of these features, WT1 has been ranked as the most promising cancer antigen in a recent prestigious paper (Cheever et al. 2009).

14.2 Bacillus Calmette-Guérin Cell Wall Skeleton (BCG-CWS)

14.2.1 Strategy of WT1 Peptide Vaccination Combined with BCG-CWS

Mycobacterium bovis bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) has been used for cancer immunotherapy in clinical settings for a long period (Ohno et al. 1978; Azuma and Yamamura 1979; Yamamura et al. 1979; Yasumoto et al. 1979; Veronesi et al. 1982; Lipton et al. 1983, 1991; Ochiai et al. 1983; Lamm et al. 1991; Hayashi 1994; Hayashi et al. 1998; Azuma and Seya 2000; Seya et al. 2000), and the mechanisms of the enhancement of immunity against tumor were

accumulated (Hayashi et al. 1998; Azuma and Seya 2000; Seya et al. 2000, 2001; Thurnher et al. 1997; Underhill et al. 1998; Begum et al. 1999; Muzio et al. 2000; Tsuji et al. 2000; Matsumoto et al. 2001; Hirahashi et al. 2002; Okamoto and Sato 2003; Uehori et al. 2003; Akazawa et al. 2004; Ishii et al. 2005). Toll-like receptor (TLR) 2 and 4, which are expressed on dendritic cells (DCs), are the main receptors for BCG-CWS. BCG-CWS binds to TLR2 and 4 and follows signaling of gene regulation such as MHC class I, co-stimulatory molecules, and cytokines.

The ability of BCG-CWS to activate DCs led us to a strategy in which we could use BCG-CWS as adjuvant to enhance tumor antigen-specific immune responses. In this method, BCG-CWS and WT1 peptide are intradermally (i.d.) injected. Injected BCG-CWS activates immature DCs into a mature form that upregulates MHC class I and co-stimulatory molecules such as CD80 and CD86 (Thurnher et al. 1997; Tsuji et al. 2000). Under these conditions, injected WT1 peptide binds to upregulated MHC class I molecules on matured DCs, and efficiently induces WT1-specific CTLs (Fig. 14.1).

In the WT1 peptide vaccination combined with BCG-CWS study (Nakajima et al. 2004), WT1 peptide was injected 1 day after injection of BCG-CWS for the following reasons: (1) it takes some time for BCG-CWS to activate DCs from immature to mature forms that have higher potency to induce antigen-specific immune responses; and (2) the activated DCs are required to stay at the site of BCG-CWS injection until the injection of WT1 peptide. In fact, immunization with a mixture of BCG-CWS and WT1 peptide did not provide better effects on tumor rejection and growth inhibition than immunization with WT1 peptide alone in our preliminary studies (data not shown).

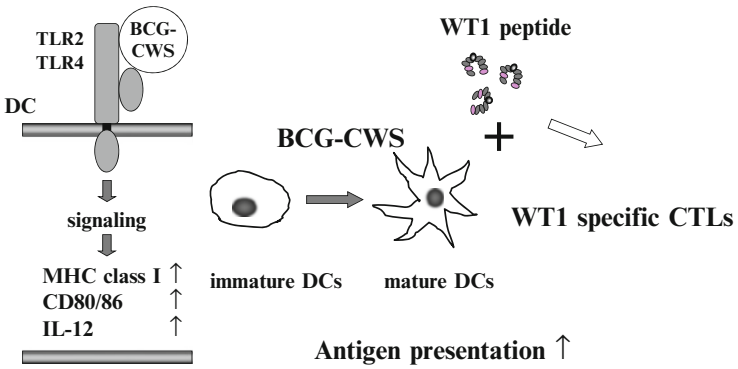

Fig. 14.1 Strategy of Wilms' tumor gene (*WT1*) product-derived peptide vaccination combined with bacillus Calmette-Guérin cell wall skeleton (BCG-CWS). Intradermally (i.d.) injected BCG-CWS binds to Toll-like receptor (TLR) 2 and 4 on dendritic cells (DCs), and activates immature DCs into mature form. Major histocompatibility complex (MHC) class I, CD80, and CD86, on matured DCs are upregulated. And matured DCs produce interleukin (IL)-12. Under this condition, i.d. injected WT1 peptide binds to upregulated MHC class I molecules on matured DCs, and efficiently induces WT1-specific cytotoxic T lymphocytes (CTLs)

14.2.2 BCG-CWS Enhances Potency of WT1 Peptide Vaccination

14.2.2.1 Effect of WT1 Peptide Vaccination Combined with BCG-CWS on Rejection of Implanted Tumors

To investigate the in vivo effect of vaccination with WT1 peptide combined with BCG-CWS, mice were implanted with WT1-expressing leukemia cells (mWT1-C1498) and treated with the WT1 peptide vaccination combined with BCG-CWS. The tumor implantation and vaccination schedules are shown in Fig. 14.2. On day 0, mice were intraperitoneally (i.p.) implanted with 5×10^5 mWT1-C1498 tumor cells in 100 µl of phosphate buffered saline (PBS). On day 1, 100 µg of BCG-CWS was i.d. injected in the abdomen, followed by i.d. injection of 100 µg of WT1 peptide [MHC class I (H-2D^b)-binding peptide, Db126, a.a. 126–134 RMFPNAPYL] at the same site as that of BCG-CWS injection on day 2. This treatment was repeated four times at weekly intervals. In control groups, either WT1 peptide, BCG-CWS, or PBS was injected on days 1, 8, 15, and 22. Tumor growth was monitored by measuring the longest diameter of the palpable mass.

Four of the 13 BCG-CWS- and WT1 peptide-treated mice rejected implanted tumor and survived until day 65. On the other hand, none of the BCG-CWS-treated, WT1 peptide-treated, and non-treated mice did not reject implanted tumors (Fig. 14.3a). Disease-free survival of BCG-CWS- and WT1 peptide-treated mice

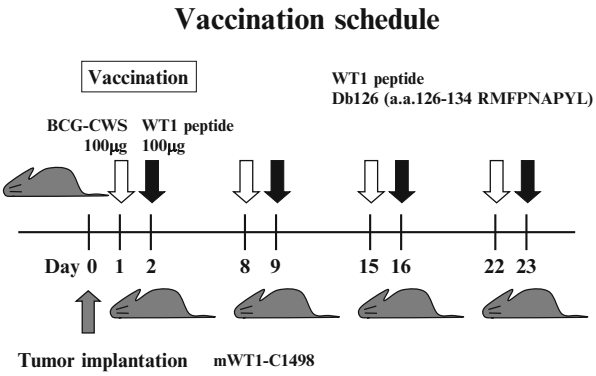

Fig. 14.2 In vivo tumor challenges and vaccination schedule. Mice were intraperitoneally (i.p.) implanted with 5×10^5 mWT1-C1498 cells on day 0. In the bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) and Wilms' tumor gene (*WT1*) product-derived peptide group, 100 µg of BCG-CWS was intradermally (i.d.) injected in the abdomen on days 1, 8, 15, and 22, followed by i.d. injection of 100 µg of WT1 peptide at the same site as that of BCG-CWS injection on days 2, 9, 16, and 23. In control groups, either 100 µg of BCG-CWS, 100 µg of WT1 peptide, or phosphate buffered saline (PBS) was i.d. injected in the abdomen on days 1, 8, 15, and 22

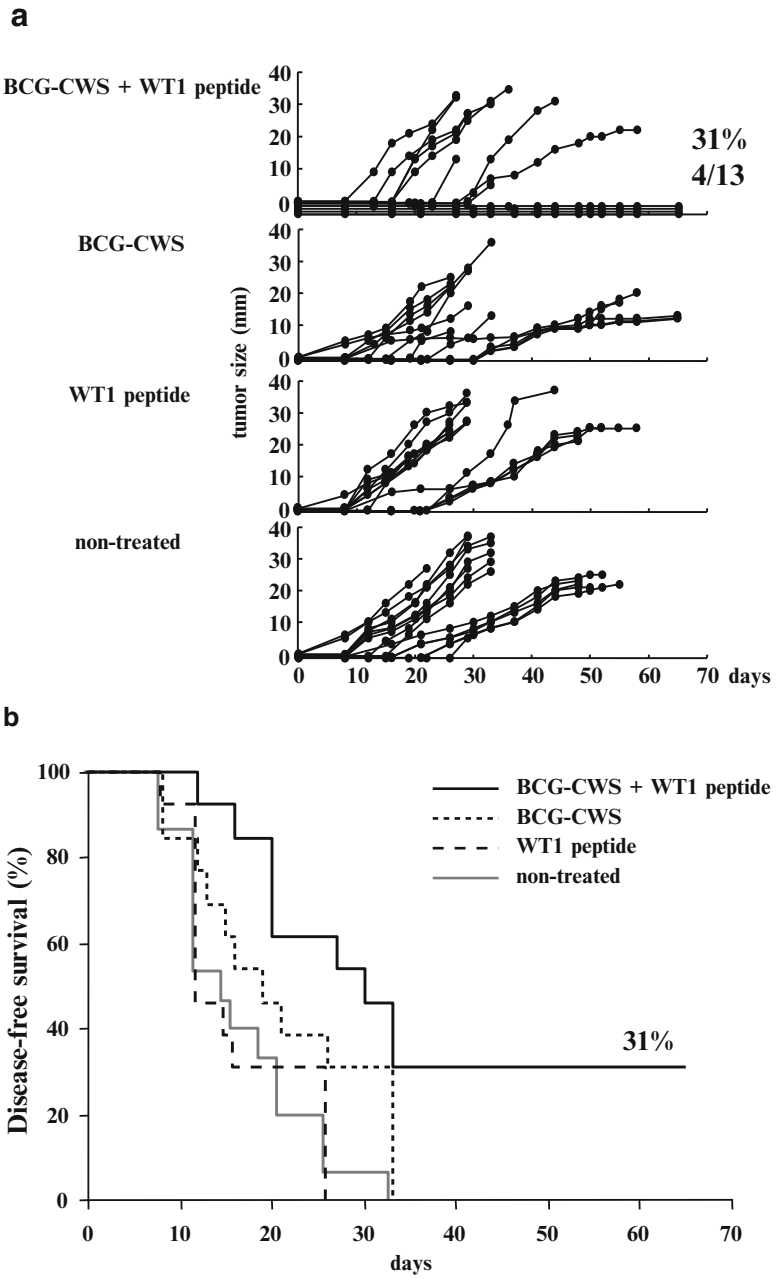

Fig. 14.3 Effect of Wilms' tumor gene (*WT1*) product-derived peptide vaccination combined with bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) on rejection of implanted WT1-expressing tumor cells. (**a**) The tumor growth curves of the four groups. Each *line* represents the tumor size of individual mice. Tumor sizes represent the longest diameters. (**b**) Disease-free survival curves of the four groups. *Solid black*, *dotted*, *broken*, and *solid gray lines* represent disease-free survival curves of mice treated with BCG-CWS and WT1 peptide, BCG-CWS alone, WT1 peptide alone, and non-treated mice, respectively

was 31 % and was significantly higher than that of the other three groups ($p < 0.05$) (Fig. 14.3b). WT1 peptide vaccination combined with BCG-CWS effectively rejected implanted tumors.

14.2.2.2 Induction of WT1-Specific Cytotoxic T Lymphocytes (CTLs) by Immunization with BCG-CWS and WT1 Peptide

Splenocytes from the mice treated four times with BCG-CWS and WT1 peptide or from non-treated mice were in vitro re-stimulated with WT1 peptide and assayed for cytotoxic activity against WT1-expressing mWT1-C1498 and WT1-nonexpressing C1498 cells (Fig. 14.4). The splenocytes from the immunized mice showed a significant WT1-specific lysis against WT1-expressing mWT1-C1498 compared with that against WT1-non-expressing C1498. In contrast, the splenocytes from the non-treated mice did not show a significant WT1-specific lysis against the target cells. These results demonstrated that WT1 peptide and BCG-CWS treatment could induce WT1-specific CTL responses, resulting in suppression of the growth of the implanted tumor cells.

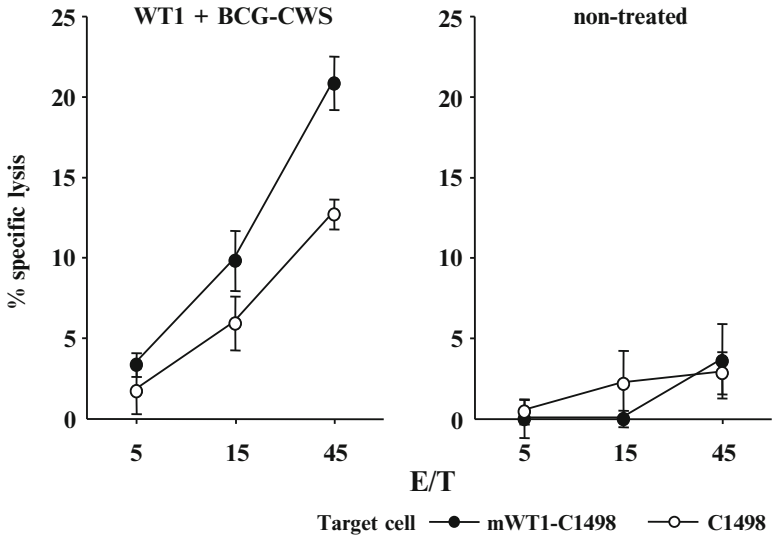

Fig. 14.4 Induction of Wilms' tumor gene (*WT1*) product-derived peptide-specific cytotoxic T lymphocytes (CTLs) by immunization with bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) and WT1 peptide. Splenocytes from the mice immunized four times with BCG-CWS and WT1 peptide or from non-treated mice were in vitro re-stimulated with WT1 peptide-pulsed synergistic splenocytes. Their cytotoxic activities against WT1-expressing mWT1-C1498 or WT1-non-expressing C1498 were tested by ^{51}Cr-release cytotoxicity assay at the indicated effector cell to target cell (E/T) ratios in triplicate. *Closed* and *open circles* represent cytotoxic activities against mWT1-C1498 and C1498 cells, respectively. *Bars* indicate standard errors

14.3 Type I Interferon (IFN): IFN-β

14.3.1 Strategy of Co-administration of WT1 Peptide Vaccine and IFN-β

Interferon (IFN)-β is a type I interferon, and is known for its various anticancer properties: (1) enhancement of the expression of many surface molecules that are essential for binding and/or activation of CTLs, in particular the MHC class I as well as the receptors B7-1 (CD80) and intercellular adhesion molecule-1 (ICAM-1) (Dhib-Jalbut and Cowan 1993; Dezfouli et al. 2003), on antigen-presenting cells (APCs) or cancer cells; (2) activation of natural killer (NK), B, and T cells (Kayagaki et al. 1999; Sato et al. 2001; Kirkwood et al. 2002); (3) a direct anti-proliferation effect on cancer cells by promoting cell cycle arrest at the G1 phase (Tanabe et al. 2000); (4) induction of apoptosis of cancer cells (Chen et al. 2001); and (5) inhibition of angiogenesis (Streck et al. 2004). In fact, it was reported in mouse models that type I IFN was essential in the induction and augmentation of CTLs (Wakita et al. 2006; Gehring et al. 2005). Furthermore, type I IFN, IFN-β, and/or IFN-α has already been in use for cancer immunotherapy in clinical settings (Kirkwood et al. 2002; Mani et al. 1996; Fine et al. 1997; Beppu et al. 2003; Watanabe et al. 2005; Gresser 2007; Anguille et al. 2011), and the mechanism for the enhancement of immunity against cancer has been thoroughly investigated (Dhib-Jalbut and Cowan 1993; Dezfouli et al. 2003; Kayagaki et al. 1999; Sato et al. 2001; Kirkwood et al. 2002; Tanabe et al. 2000; Chen et al. 2001; Streck et al. 2004). Considering these reports, IFN-β should be considered as one of the most promising immunopotentiating agents for use with TAA-directed cancer vaccines.

We focused on the immunopotentiating ability of IFN-β, and planed the co-administration of WT1 peptide vaccine and IFN-β (Nakajima et al. 2012). IFN-β increases MHC class I molecules on tumor cells, and activates T cells and NK cells (Kayagaki et al. 1999; Sato et al. 2001; Kirkwood et al. 2002). Activated NK cells produce IFN-γ, which in turn activates DCs, T cells, and NK cells (Degli-Esposti and Smyth 2005; Fedele et al. 2004; He et al. 2007). Under this condition, injected WT1 peptide vaccine induces WT1-specific CTLs efficiently. Furthermore, these reactions gave CTLs an advantage in killing MHC high-expressing tumor cells (Dhib-Jalbut and Cowan 1993; Dezfouli et al. 2003). On the other hand, IFN-β-activated NK cells kill the remaining MHC non- or low-expressing tumor cells (Fig. 14.5).

14.3.2 IFN-β Promotes Efficacy of WT1 Peptide Vaccination

14.3.2.1 Effect of WT1 Peptide Vaccination in Co-administration with IFN-β on Rejection of Implanted Tumors

To investigate whether IFN-β promotes tumor rejection by WT1 peptide vaccination, mice were twice treated with incomplete Freund's adjuvant (IFA, Montanide™ ISA51)-emulsified WT1 peptide with or without IFN-β administration before

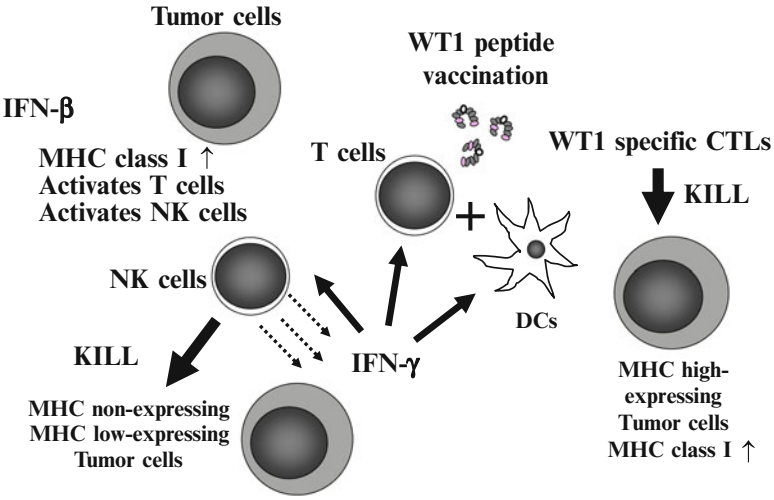

Fig. 14.5 Strategy of co-administration of Wilms' tumor gene (*WT1*) product-derived peptide vaccine and interferon (IFN)-β. Injected IFN-β increases major histocompatibility complex (MHC) class I molecules on tumor cells, and activates T cells and natural killer (NK) cells. Activated NK cells produced IFN-γ, which in turn activates dendritic cells (DCs), T cells, and NK cells. Under this condition, injected WT1 peptide vaccine induces WT1-specific cytotoxic T lymphocytes (CTLs) effectively. Induced WT1-specific CTLs efficiently recognize and kill MHC high-expressing tumor cells. On the other hand, IFN-β-activated NK cells kill the remaining MHC non- or low-expressing tumor cells

implantation of WT1-expressing tumor cells (mWT1-C1498) and then WT1 peptide vaccination was repeated four times. The tumor implantation and vaccination schedules are shown in Fig. 14.6. Mice were pre-treated with i.d. injection of 100 μg WT1 peptide emulsified with IFA on days -14 and -7. On day 0, mice were subcutaneously (s.c.) implanted with 3×10^5 mWT1-C1498 cells in 100 μl of PBS at the abdomen, followed by i.d. injection of 100 μg WT1 peptide emulsified with IFA on days 1, 8, 15, and 22. During the treatment, 50,000 units of murine IFN-β was i.p. injected three times per week. Mice in control groups were treated with WT1 peptide/IFA and PBS (WT1 peptide alone group); PBS/IFA and IFN-β (IFN-β alone group); and PBS/IFA and PBS (non-treated group). Tumor growth was monitored by measuring the longest diameter of the palpable mass.

Six of the 15 mice treated with WT1 peptide vaccine and IFN-β rejected implanted tumors and survived on day 75 (Fig. 14.7a). In contrast, 14 of the 15 mice treated with WT1 peptide vaccine alone, 14 of the 15 mice treated with IFN-β alone, and all of the 15 non-treated mice had died of tumor growth by day 75. Overall survival rates on day 75 were 40 % for mice treated with WT1 peptide vaccine and IFN-β, but 7, 7, and 0 % for mice treated with WT1 peptide alone, IFN-β alone, or for non-treated mice, respectively. The overall survival rates of mice treated with WT1 peptide vaccine and IFN-β were significantly higher than those of the other three groups (WT1 peptide vaccine and IFN-β vs. WT1 peptide vaccine alone, IFN-β alone, or non-treated: $p < 0.05$, $p < 0.05$, and $p < 0.0005$, respectively). The

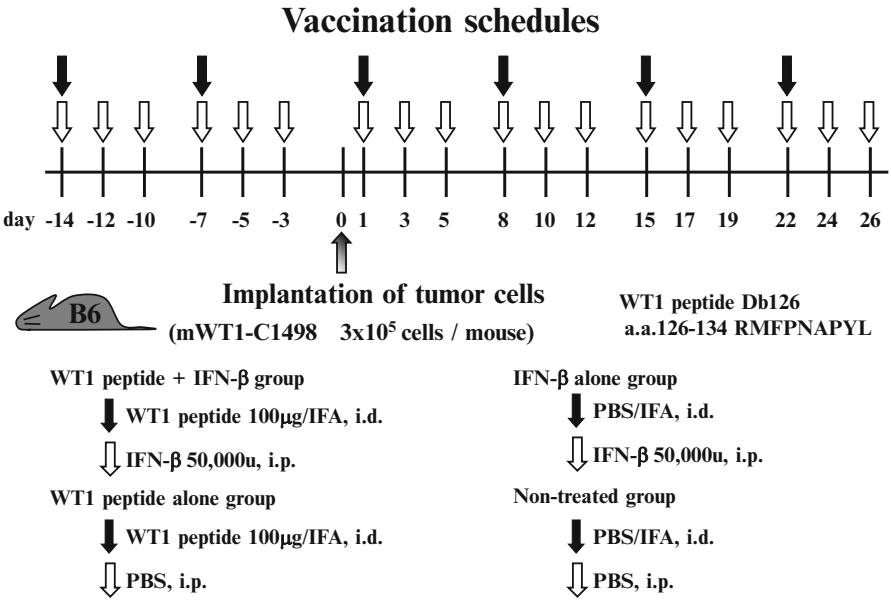

Fig. 14.6 In vivo tumor challenges and vaccination schedule. Mice were pre-treated with intradermal (i.d.) injection of 100 μg Wilms' tumor gene (*WT1*) product-derived peptide emulsified with incomplete Freund's adjuvant (IFA) on days -14 and -7. On day 0, mice were subcutaneously (s.c.) implanted with 3×10^5 mWT1-C1498 cells in 100 μl of phosphate buffered saline (PBS) in the abdomen, followed by i.d. injection of 100 μg WT1 peptide emulsified with IFA on days 1, 8, 15, and 22. During the treatment, 50,000 units of murine interferon (IFN)-β was intraperitoneally (i.p.) injected three times per week until day 26. Mice in the control groups were injected with WT1 peptide emulsified in IFA and PBS (WT1 peptide vaccine alone group), PBS emulsified in IFA and IFN-β (IFN-β alone group), or PBS emulsified in IFA and PBS (non-treated group)

overall survival rates of mice treated with WT1 peptide vaccine alone or IFN-β alone were significantly higher than those of non-treated (WT1 peptide vaccine alone vs. non-treated, IFN-β alone vs. non-treated: $p < 0.05$ and $p < 0.005$, respectively). There was no significant difference in survival rate between WT1 peptide vaccine alone and IFN-β alone (Fig. 14.7b).

14.3.2.2 WT1 Peptide Vaccine and IFN-β Enhances Induction of WT1-Specific CTLs and Activation of Natural Killer (NK) Cells

In order to analyze immune responses, we performed in vivo experiments independently from those for the assessment of survival. Tumor-bearing mice treated with WT1 peptide vaccine and IFN-β, as shown in Fig. 14.6, were sacrificed on day 30 (8 days after the last vaccination). The splenocytes of each mouse were stimulated in vitro with WT1 peptide and assayed for WT1 peptide-specific CTL activity

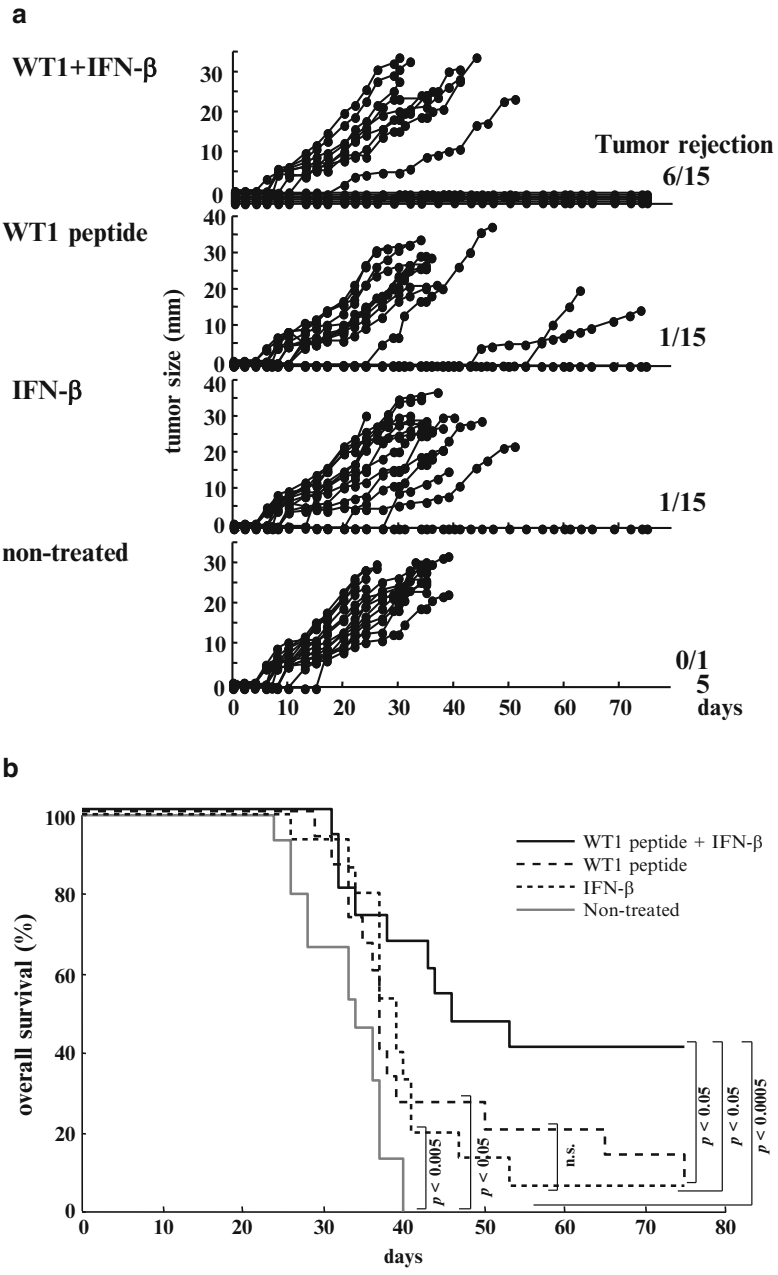

Fig. 14.7 Effect of Wilms' tumor gene (*WT1*) product-derived peptide vaccination in co-administration with interferon (IFN)-β on rejection of implanted WT1-expressing tumor cells. (**a**) The tumor growth curves of the four groups. Each *line* represents tumor size of individual mice. Tumor sizes represent the longest diameters. (**b**) Overall survival curves of the four groups. *Solid black*, *broken*, *dotted*, and *solid gray lines* represent overall survival curves of mice treated with WT1 peptide vaccine and IFN-β, WT1 peptide vaccine alone, IFN-β alone, and non-treated mice, respectively

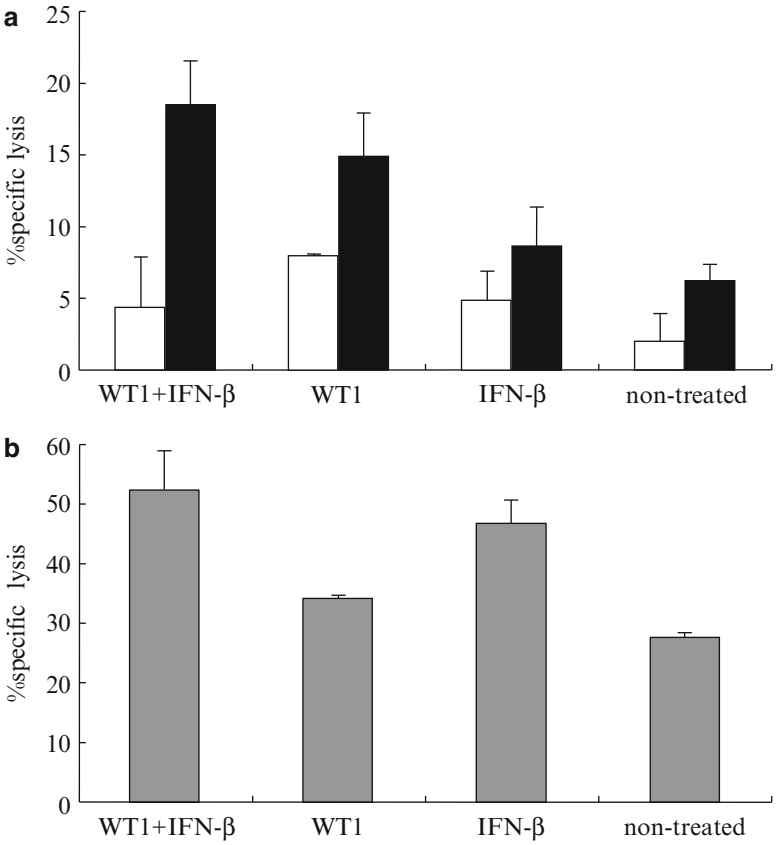

Fig. 14.8 Induction of Wilms' tumor gene (*WT1*) product-derived peptide-specific cytotoxic T lymphocytes (CTLs) and enhancement of natural killer (NK) activity by co-administration of WT1 peptide vaccine and interferon (IFN)-β. Eight days after the last vaccination, splenocytes from the mice in each group were stimulated in vitro with WT1 peptide. WT1-specific CTL and NK cell activities were assayed in triplicate as cytotoxic activities against WT1 peptide-pulsed, -unpulsed RMAS, or YAC-1 cells, respectively, at the effector cell to target cell (E/T) ratio = 15. (**a**) WT1-specific CTL activity. *Closed* and *open column* represent cytotoxic activities against WT1 peptide-pulsed or -unpulsed RMAS, respectively. (**b**) NK activity is shown as cytotoxic activities against YAC-1 cells. *Bars* indicate standard errors

against WT1 peptide-pulsed and -unpulsed RMAS cells and for NK activity against YAC-1 cells. Splenocytes from mice treated with WT1 peptide vaccine and IFN-β showed the strongest WT1 peptide-specific cytotoxic activity compared with three control groups. It appeared that the WT1-specific CTL activities in splenocytes from IFN-β-treated or non-treated mice were endogenously induced as a result of immunologic stimulation by WT1-expressing tumor cells implanted (Fig. 14.8a). Furthermore, NK cell activity was higher in both the WT1 peptide vaccine and IFN-β, and IFN-β alone groups. These results suggested that NK activity was

endogenously induced in WT1-expressing tumor-bearing mice and that this activity was enhanced by administration of IFN-β, which is a potent enhancer of NK activity (Fig. 14.8b). Taken together, these results indicated that the strongest rejection of implanted tumor cells in the mice treated with WT1 peptide vaccine and IFN-β resulted from the generation of the highest levels of both WT1-specific CTLs and NK cells.

14.3.2.3 WT1-Specific CTLs and NK Cells Play Crucial Roles in the Treatment by WT1 Peptide Vaccine and IFN-β

To confirm that WT1-specific CTLs and NK cells played crucial roles in the tumor rejection, in vivo depletion of $CD8^+$ T and/or NK cells was performed. Mice that were implanted with mWT1-C1498 cells and vaccinated with WT1 peptide and IFN-β as shown in Fig. 14.6 were treated with both or either of anti-CD8 and anti-NK monoclonal antibodies (mAbs).

Three of five mAb-non-treated mice rejected implanted tumors and survived. In contrast, all of the mice that were treated with both or either of anti-CD8 and anti-NK mAbs and vaccination-non-treated mice died of tumor development. The efficacy of WT1 peptide vaccine and IFN-β was completely cancelled by the administration of anti-CD8 and/or anti-NK mAbs. It should be noted that appearance of tumors in mice treated with both or either anti-CD8 and anti-NK mAbs was earlier than that in mAb-non-treated mice (Fig. 14.9).

These results strongly indicated that both WT1-specific $CD8^+$ CTLs and NK cells played crucial roles in the rejection of tumor cells.

14.3.2.4 Enhancement of Major Histocompatibility Complex Class I ($H\text{-}2D^b$) Expression on Implanted Tumor Cells by the Administration of IFN-β

Since WT1 (Db126) peptide is produced from WT1 protein through processing in tumor cells and presents on the cell surface in association with MHC class I ($H\text{-}2D^b$) (Oka et al. 2000b), $H\text{-}2D^b$ expression levels of target cells are thought to exert a major influence on the susceptibility of the cells to attack by vaccination-induced WT1 (Db126)-specific CTLs. For this reason, the $H\text{-}2D^b$ expression levels on the implanted tumor cells (WT1-expressing C1498 cells) were examined. Tumor-bearing mice were sacrificed 30 days after tumor cell implantation, the tumors were resected, and the tumor cells were stained with anti-$H\text{-}2D^b$ antibody (Fig. 14.10). The expression levels of $H\text{-}2D^b$ on tumor cells was significantly higher in mice treated with WT1 peptide vaccine and IFN-β or IFN-β alone than in those treated with WT1 peptide vaccine alone or non-treated mice ($p < 0.05$) (Fig. 14.10b). These results indicated that IFN-β administration enhanced the expression of $H\text{-}2D^b$ on tumor cells, which should make tumor cells more susceptible to attack by WT1-specific CTLs.

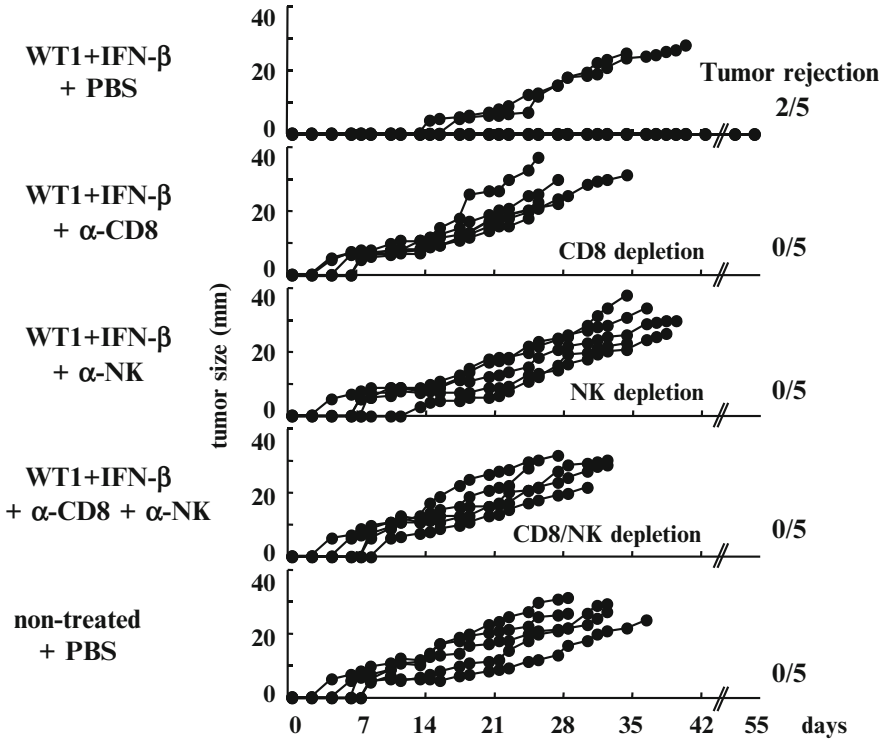

Fig. 14.9 Cancellation of the efficacy of Wilms' tumor gene (*WT1*) product-derived peptide vaccine and interferon (IFN)-β by the administration of anti-CD8 and/or anti-natural killer (NK) monoclonal antibodies (mAbs). Mice were implanted with 3×10^5 mWT1-C1498 cells and treated with WT1 peptide vaccine and IFN-β as shown Fig. 14.6. The WT1- and IFN-β-treated mice were injected with phosphate buffered saline (PBS) or 200 μg of anti-CD8 and/or 200 μg of anti-NK mAbs on days -15, -8, -1, 4, 7, 11, 14, 18, 21, and 25. Each *line* on the tumor growth curves of the five groups represents tumor size of individual mice. Tumor sizes represent the longest diameters

14.4 Conclusion and Future Works

Co-administration of appropriate immunopotentiating agents, including adjuvants or cytokines, together with WT1 peptide vaccine could enhance its therapeutic efficacy. WT1 peptide vaccine combined with BCG-CWS enhanced both innate and WT1-specific immune responses (acquired immunity). Co-administration of WT1 peptide vaccine and IFN-β enhanced the induction of WT1-specific CTLs, NK activity, and MHC class I expression on the tumor cells. These synergistic effects increased the survival rate of WT1 peptide vaccine and IFN-β treated mice.

Mice models for WT1 peptide cancer immunotherapy are very useful to investigate immunopotentiating agents and analyze their mechanisms. There are a lot of promising agents, including another TLR agonist, cytokines, co-stimulatory molecule-targeting antibodies, and helper peptide. In the future, we will be seeking ways to enhance the efficacy of WT1 peptide vaccine using these mice models.

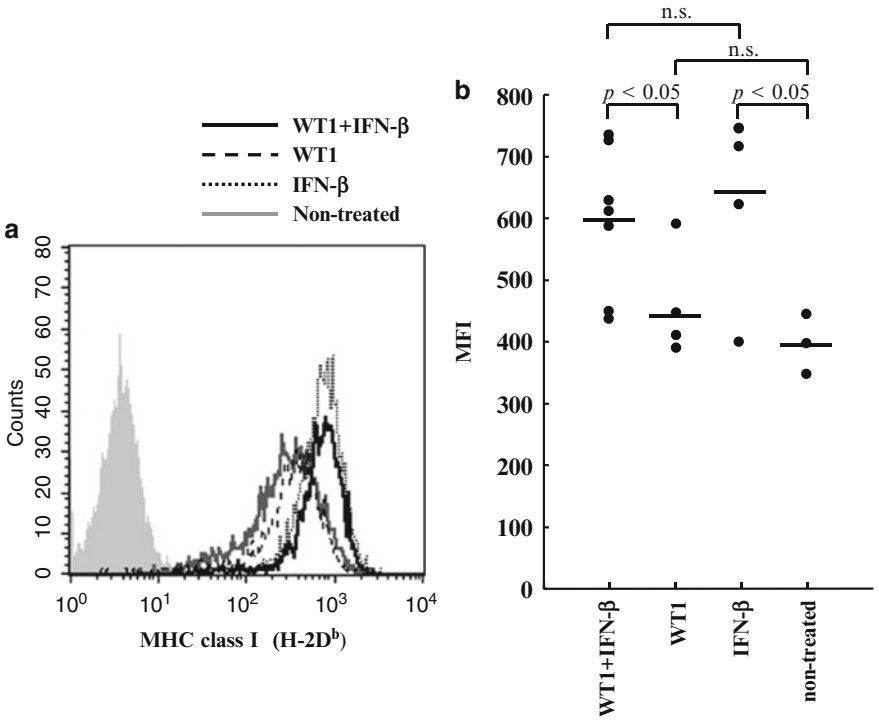

Fig. 14.10 Interferon (IFN)-β enhanced major histocompatibility complex (MHC) class I (H-2D^b) expression of tumor cells in vivo. (**a**) H-2D^b expression levels of tumor cells recovered from mice. *Solid black*, *broken*, *dotted*, and *solid gray lines* represent the expression levels of tumor cells from mice treated with Wilms' tumor gene (*WT1*) product-derived peptide vaccine and IFN-β, WT1 peptide vaccine alone, or IFN-β alone, and non-treated mice, respectively. (**b**) The mean fluorescence intensity (MFI) of H-2D^b expression of tumor cells from mice

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Chapter 15
Regulation of Antigen Presentation by Dendritic Cells and Its Application to Cancer Immunotherapy

Hidemitsu Kitamura, Junya Ohtake, Shun Kaneumi, Yosuke Ohno, Takuto Kishikawa, Satoshi Terada, and Kentaro Sumida

Abstract Dendritic cells (DCs) are one of the most powerful antigen-presenting cells and play a crucial role in bridging between innate and acquired immunity. Cancer antigens or the long peptides are engulfed by DCs, digested into helper and killer epitope peptides, transported to the cell surface, and presented to $CD4^+$ and $CD8^+$ T cells through major histocompatibility complex (MHC) class II and MHC class I to induce effector T helper cells and cytotoxic killer T cells, respectively. In addition, DCs produce type 1 cytokines such as interleukin (IL)-12 and interferon (IFN)-α/-β to facilitate differentiation of naïve T cells into effector T cells and activation of memory T cells. Therefore, proper regulation of DC function is essential for induction and augmentation of anti-tumor immunity in cancer patients. Generally, DCs are immediately activated by various maturation signals including Toll-like-receptor (TLR) ligands, type 1 cytokines, and CD40/40L interaction. On the other hand, IL-6 produced in tumor microenvironments caused dysfunction of DCs through reduction of MHC class II expression and IL-12 production. It has recently been reported that zinc transporter-mediated intracellular zinc levels and neuropeptide signaling through the receptors are involved in the regulation of the antigen-presenting function of DCs in type 1 immune responses, including TLR-mediated inflammatory response. In this chapter, we report on regulation of the antigen-presenting function and the potential benefit of DC-mediated cancer immunotherapy.

Keywords Dendritic cells • MHC class II • IL-6 • Type-1 cytokine

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15.1 Dendritic Cells (DCs) as Professional Antigen-Presenting Cells

In 1973, dendritic cells (DCs) were first identified as a novel cell type, different to macrophages, in adherent cell populations by Steinman's group (Steinman and Chon 1973). It has been demonstrated that DCs are critical not only for innate immunity but also for antigen-specific immune responses through activation of $CD4^+$ and $CD8^+$ T cells as professional antigen-presenting cells (APCs) (Steinman and Hemmi 2006). Generally, it is characteristic of DCs to initiate strong T cell immune responses because of signals from co-stimulatory molecules and cytokines in addition to antigen-loaded major histocompatibility complex (MHC) cells rather than other APCs. DCs in steady state, so-called 'immature DCs', are activated by various stimuli, including Toll-like receptor (TLR) ligands such as CpG-oligodeoxynucleotide (ODN), poly I:C, and lipopolysaccharide (LPS), cytokines such as tumor necrosis factor (TNF)-α, type 1 interferons (IFNs), and IFN-γ, cell-to-cell interactions through CD40/40L or adhesion molecules, and so on. The activated DCs, so-called 'mature DCs', highly express surface MHC class II and co-stimulatory molecules and induce cytokines such as IFN-α/-β and interleukin (IL)-12. In addition, endocytosis of antigens is reduced, and transport of peptide-bound MHC class I and II molecules to cell surface is promoted during the maturation of DCs, facilitating T cell activation through antigen presentation (Fig. 15.1).

Generally, cancer antigens or the long peptides are engulfed by DCs, digested into helper and killer epitope peptides by the cellular enzymes such as cathepsins in

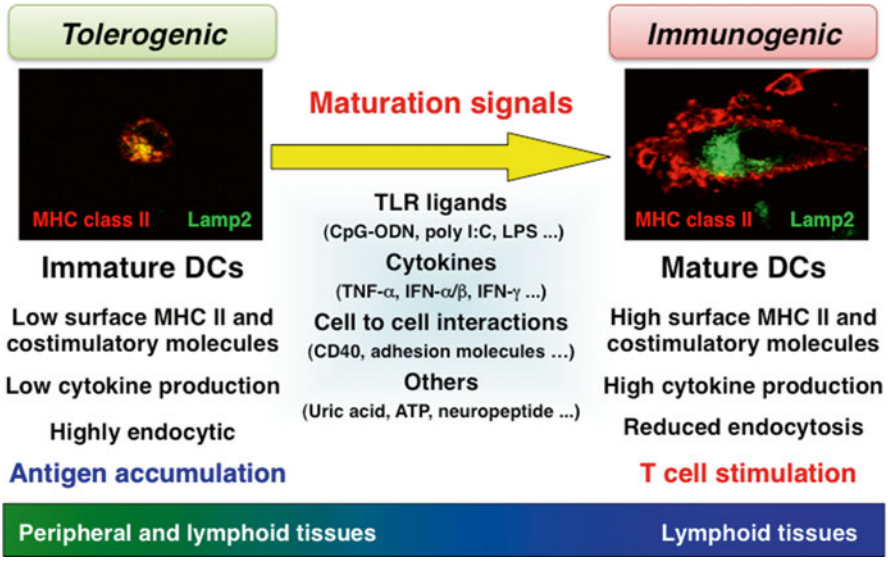

Fig. 15.1 Characteristic features of immature and mature dendritic cells (DCs)

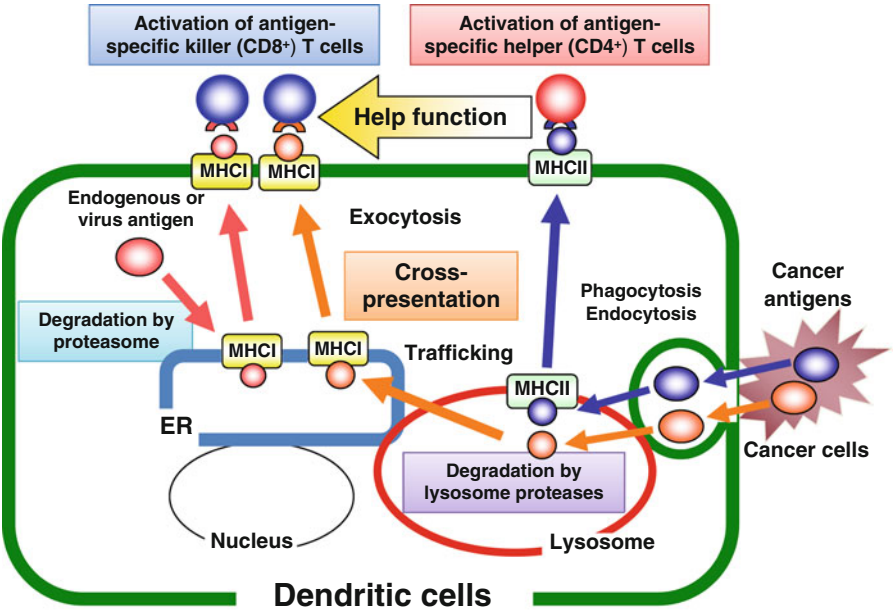

Fig. 15.2 Antigen presentation pathways in dendritic cells (DCs)

lysosome, transported to the cell surface by exocytosis, and presented to T cell receptors (TCRs) on $CD4^+$ T cells through MHC class II to induce effector T helper (Th) cells. On the other hand, endogenous antigens are degraded by proteasome and transported to the cell surface to present to $CD8^+$ T cells through MHC class I to induce cytotoxic killer T (Tc) cells. Some antigens engulfed by DCs are digested in lysosome to give killer epitopes. The killer epitope peptides are then transported to endoplasmic reticulum (ER) and bind to MHC class I presented to $CD8^+$ T cells, so-called 'cross presentation'. Antigen-specific Th cells producing cytokines such as IL-2 and IFN-γ encourages the induction of Tc cells (Fig. 15.2).

15.2 Regulation of Antigen-Presenting Function of DCs

DCs, representative APCs, effectively induce anti-tumor immune responses through activation of antigen-specific $CD4^+$ and $CD8^+$ T cells. In tumor microenvironments, DCs engulf cancer-derived antigens and generate Th and Tc cells in the draining lymph node. The induced effector Tc cells recognize and kill the target cancer cells in response to the antigens. In vaccination, long peptide-pulsed DCs strongly prime both $CD4^+$ and $CD8^+$ T cells capable of recognizing and killing tumor cells in response to the antigens on the target cells. Therefore, controlling the DC activation and the subsequent effective antigen presentation is required for application of

DC-mediated cancer immunotherapy. In this chapter, we report on the regulating mechanisms of the antigen-presenting function of DCs mediated by TLR ligands, type 1 cytokines, and CD40/40L interaction and the possibility of their application in cancer immunotherapy.

15.2.1 TICAM-1 (TRIF) Signaling in DCs Regulates Major Histocompatibility Complex (MHC) Class II Expression and CD4⁺ T Cell Activation

Stimulation with TLR ligands such as LPS strongly upregulates surface MHC class II expression levels on DCs, which play a central role in immune responses by presenting antigenic peptides to $CD4^+$ T cells. It has been reported that surface expression of MHC class II is mainly regulated by the post-transcriptional mechanism in DCs. TICAM-1 [TRIF (TIR-domain-containing adapter-inducing IFN-β)]-, but not the MyD88-, dependent pathway of LPS signaling in DCs is crucial for the surface expression of MHC class II, followed by $CD4^+$ T cell activation. LPS enhanced RhoB activity, but not RhoA, Cdc42, or Rac1/2 in a dependent manner. Transduction of dominant-negative (DN) form of the *RhoB* gene or small-interfering RNA (siRNA) against RhoB significantly blocked the LPS-induced surface expression of MHC class II on DCs. In addition, GEFH1 associated with RhoB and DN-GEFH1 or siRNA of GEFH1 suppressed the LPS-mediated RhoB activation and surface expression of MHC class II. Furthermore, DN-RhoB attenuated the antigen-presenting function of DCs against $CD4^+$ T cells. Therefore, these results not only provide a molecular mechanism relating to how the surface expression of MHC class II is regulated during the maturation of DCs, but also suggest that the activation of the TICAM-1 –GEFH1–RhoB pathway in DCs might be a promising target for controlling the activation of antigen-specific $CD4^+$ T cells in cancer immunotherapy (Kamon et al. 2006).

15.2.2 Zinc Homeostasis Is Involved in DC Maturation

Zinc, a trace element, is required for the function of many enzymes and transcription factors, controlling cell growth, development, and differentiation. Generally, lack of zinc causes defects in innate and acquired immune responses. In DCs, stimulation with LPS, the TLR4 agonist, induced the expression of zinc exporters (Znt family) and reduced zinc importers (Zip family), and thereby decreased intracellular free zinc. The LPS-induced alterations in zinc transporter expression were dependent on TICAM-1 (TRIF) but not MyD88. TPEN, a zinc chelator, mimicked the effects of LPS, whereas zinc supplementation with pyrithione in DCs or transduction of the *Zip6* gene, a zinc transporter whose expression was reduced by LPS,

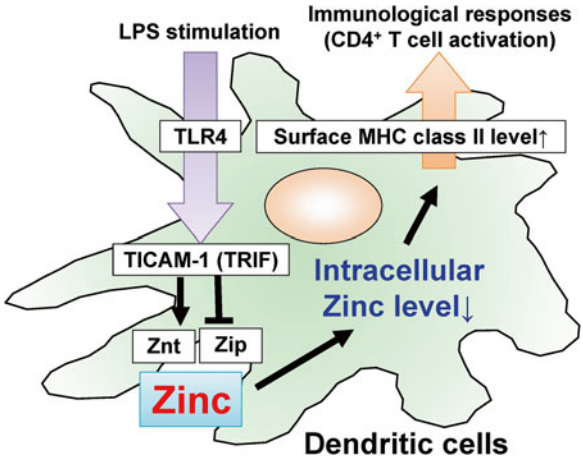

Fig. 15.3 TICAM-1 [TRIF (TIR-domain-containing adapter-inducing interferon-β)]-dependent alteration of zinc transporters in dendritic cells (DCs) controls antigen presentation to $CD4^+$ T cells

inhibited LPS-induced surface expression of MHC class II and co-stimulatory molecules. In addition, TPEN-treated DCs were capable of activating antigen-specific $CD4^+$ T cells. In the presence of antigen peptide, TPEN- or LPS-treated DCs induced augmented IL-2 production by $CD4^+$ T cells compared with untreated DCs. Thus, LPS-induced reduction of intracellular free zinc levels is a critical step in the antigen-presenting function of DCs (Fig. 15.3). These results suggest a correlation between TLR–TICAM-1 (TRIF) signaling cascade and transporter-mediated intracellular zinc homeostasis in DC maturation (Kitamura et al. 2006).

15.2.3 Neuropeptide Signaling in DCs Activates Type-1 Immune Responses

Substance P (SP) and neurokinin (NK) A, both neurotransmitters, are widely distributed in both the central and peripheral nervous system. Recently, it was demonstrated that IFN-γ remarkably induced NK-2 receptor (NK2R) expression of DCs. This finding strongly suggests that neuropeptide signaling may be closely related to regulation of DC-mediated immune responses. The effect of neuropeptide signaling on function of DCs was then investigated. DCs treated with IFN-γ or LPS remarkably induced the *NK2R* gene in a STAT (signal transducer and activator of transcription) 1-dependent manner. LPS-induced *NK2R* gene expression was dependent on IFNAR1, suggesting that type 1 IFNs induced by LPS were critical in the induction of the *NK2R* gene. Moreover, it was confirmed that surface NK2R expression and NKA production levels were significantly elevated after IFN-γ or LPS stimulation. Transduction of the *NK2R* gene into DCs augmented the expression level of surface MHC class II and promoted antigen-specific IL-2 production by $CD4^+$ T cells. Furthermore, blockade of NK2R by an antagonist significantly suppressed IL-2 and

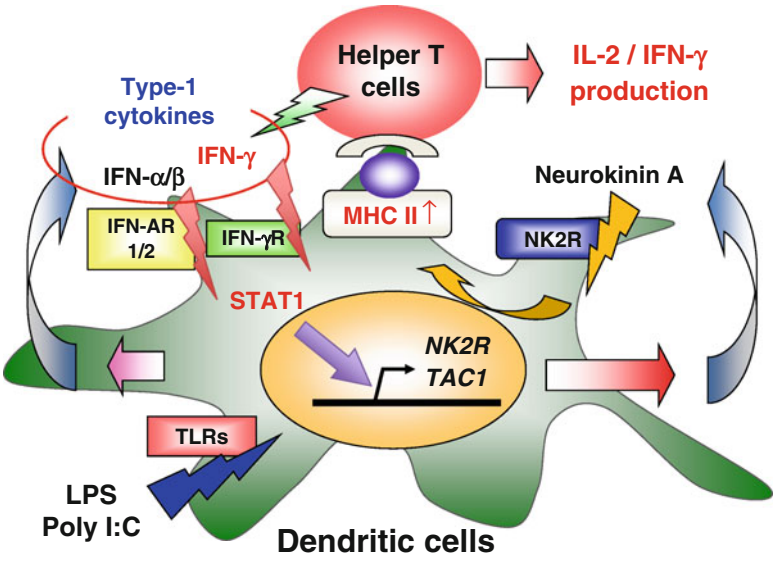

Fig. 15.4 Neurokinin-2 receptor (NK2R)-dependent neuropeptide signaling activates dendritic cell (DC) function in type 1 immune responses

IFN-γ production by $CD4^+$ or $CD8^+$ T cells after stimulation with the antigen-loaded DCs. Finally, it was confirmed that human DCs also enhanced expression levels of the *NK2R* and *TAC-1* genes, encoding both SP and NKA, after the IFN-γ or poly I:C stimulation (Fig. 15.4). Thus, these findings indicate that NK2R-dependent neuropeptide signaling regulates type 1 immunity through the activation of DC function including antigen presentation to effector T cells (Kitamura et al. 2012).

15.2.4 CD40/40L-Mediated Cell-to-Cell Interaction Augments DC Function

In vivo injection with α-galactosylceramide (α-GalCer), the ligand for natural killer T (NKT) cells shows strong anti-tumor activities. Because these activitieswere similar to those of IL-12, it was thought that the involvement of IL-12 in the activation of NKT cells was caused by α-GalCer. In fact, production of IFN-γ by NKT cells in response to α-GalCer was required for IL-12 produced by DCs and direct interaction between NKT cells and DCs through CD40/CD40L. The combination therapy using suboptimal doses of α-GalCer together with suboptimal doses of IL-12 synergistically enhanced natural killing activity and IFN-γ production. These findings indicate an important role of CD40/40L interaction for inducing IL-12 production by DCs in the activation of NKT cells by α-GalCer. Therefore, the antigen-loaded NKT cells may be able to activate DCs for subsequent immune responses, suggesting a promising strategy for immunotherapy of cancers (Kitamura et al. 1999).

15.3 Dysfunction of DCs in Tumor Microenvironments

Immunosuppression in tumor microenvironments is one of the critical issues for cancer immunotherapy. To develop more effective treatment, it is essential to overcome the dysfunction of immunity in cancer patients. It has been demonstrated that myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) have crucial roles for such immunosuppression. Recent reports have indicated that IL-6 production and STAT3 activation was caused in tumor microenvironments. In this section, we focused on the effect of IL-6/STAT3-signaling pathway on the antigen-presenting ability of DCs.

15.3.1 Interleukin (IL)-6 Blocks DC Maturation Through STAT3 Activation In Vivo

After stimulation by pathogens, DCs immediately differentiate into mature cells that initiate immune responses, while in the absence of such maturation signals, most DCs remain in an immature form that induces tolerance to self-antigens. Elucidation of the regulating mechanism of mature and immature status is essential for effective vaccine development and prevention of undesirable immune responses by DCs. It was found that IL-6 knockout mice had increased numbers of mature DCs, indicating that IL-6 played a major role in blocking DC maturation in vivo. STAT3 activation by IL-6 was involved in the suppression of LPS-induced DC maturation and activation. Furthermore, it was confirmed that DC-mediated T cell responses were enhanced in IL-6 knockout mice. These findings suggest that IL-6 is a potent negative regulator of DC maturation in vivo, and IL-6/STAT3 signaling cascade in DCs may represent a critical target for controlling T cell-mediated immune responses in tumor-bearing state (Park et al. 2004).

15.3.2 IL-6/STAT3 Controls Intracellular MHC Class II Through Activation of Lysosome Protease in DCs

Previous reports have indicated that the IL-6/STAT3 signaling pathway suppresses MHC class II expression on DCs and attenuates T cell activation. In this study, it was shown that IL-6/STAT3 signaling reduced intracellular MHC class II αβ-dimer levels in DCs. IL-6-mediated STAT3 activation decreased cystatin C level, an endogenous inhibitor of cathepsins, and enhanced cathepsin activities. Cathepsin S inhibitors blocked the reduction of MHC class II αβ-dimer in the IL-6-treated DCs. Transduction of cystatin C gene suppressed IL-6-/STAT3-mediated increase of cathepsin S activity and reduction of MHC class II αβ-dimer levels in DCs. In addition, over-expression of cathepsin S gene in DCs decreased intracellular MHC class II αβ-dimer levels and LPS-mediated surface expression of MHC class II and

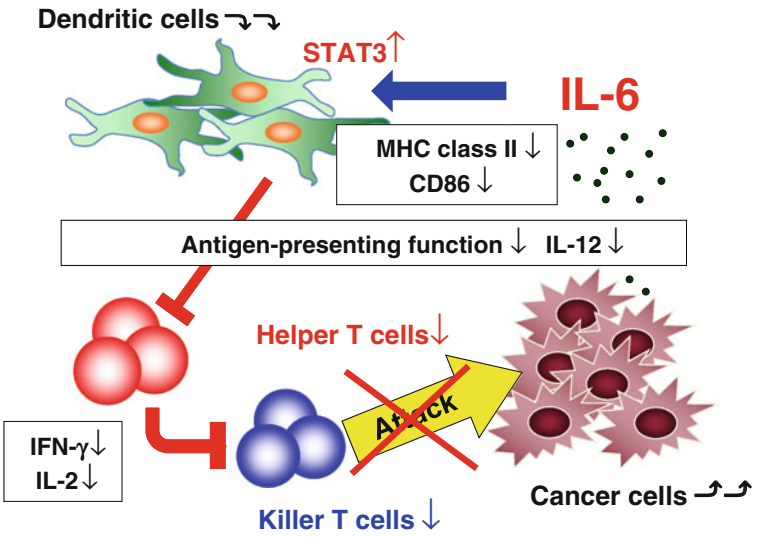

Fig. 15.5 Dysfunction of dendritic cell (DC)-mediated anti-tumor immunity induced by interleukin (IL)-6/signal transducer and activator of transcription (STAT) 3 activation in tumor microenvironments

suppressed $CD4^+$ T cell activation. Thus, IL-6-/STAT3-mediated increase of cathepsin S activity reduces the MHC class II αβ-dimer levels in DCs, and suppresses $CD4^+$ T cell-mediated immune responses (Fig. 15.5). These data indicate that IL-6-/STAT3-induced activation of lysosomal proteases regulates immunosuppressive function of DCs, which will be a promising target for improving the effects of cancer immunotherapy (Kitamura et al. 2005).

15.3.3 IL-6-Dependent Arginase Activation Causes Dysfunction of DCs in Tumor-Bearing Mice

Dysfunction of the immune system in the tumor-bearing state is a critical issue in development of effective cancer immunotherapy. IL-6, produced in tumor microenvironments, suppressed $CD4^+$ T cell-mediated immunity through downregulation of MHC class II by enhanced arginase activity of DCs in tumor-bearing mice. Then, the administration of monoclonal antibody against IL-6 receptor (anti-IL-6R mAb)-enhanced T cell responses and inhibited tumor growth in vivo. Tumor-infiltrating $CD11c^+$ DCs enhanced the arginase-1 mRNA expression level and reduced surface expression of MHC class II in parallel with the increase in serum IL-6 levels at the late stage in tumor-bearing mice. It was found that the administration of anti-IL-6R

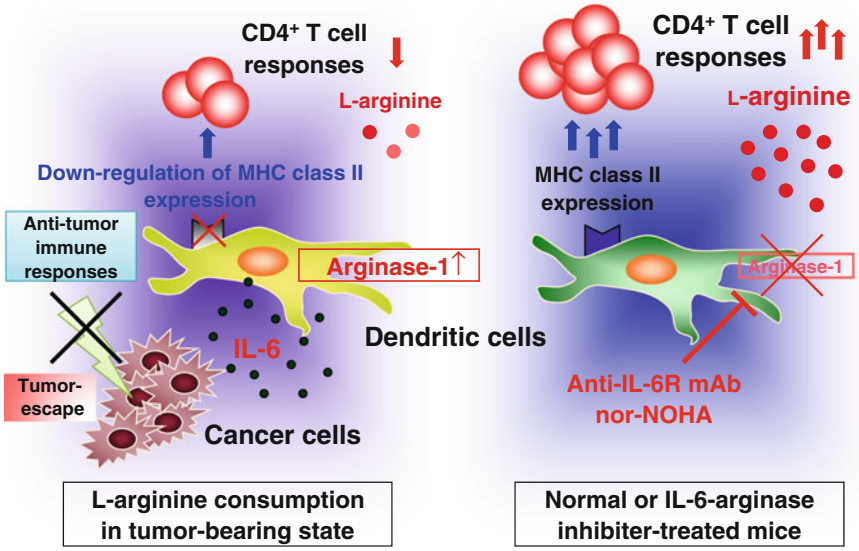

Fig. 15.6 Interleukin (IL)-6-induced arginase activation downregulates major histocompatibility complex (MHC) class II expression on dendritic cells (DCs) in the tumor-bearing state

mAb into tumor-bearing mice inhibited the reduced expression of MHC class II and the enhanced arginase-1 mRNA levels in DCs. Furthermore, N(ω)-hydroxy-L-arginine, an arginase-1 inhibitor, blocked the reduction in MHC class II levels on $CD11c^+$ DCs during the tumor-bearing state. In vivo injection of arginase inhibitor at the peritumor site significantly enhanced $CD4^+$ T cell responses and inhibited tumor growth (Fig. 15.6). Therefore, IL-6-mediated arginase activation and the subsequent reduction in MHC class II expression may be involved with dysfunction of the DC-mediated immune system in the tumor microenvironments, suggesting that anti-IL-6R blockade of the IL-6–arginase cascade is a promising tool to overcome the dysfunction of anti-tumor immunity in tumor-bearing hosts (Narita et al. 2013).

15.4 Development of DC-Based Immunotherapy for Cancer Patients

DCs engulf dying cancer cells, cancer antigens, and antigen-derived peptides in tumor microenvironments. The DCs digest the antigens into helper and killer epitope peptides to present $CD4^+$ and $CD8^+$ T cells through MHC class II and I, respectively. However, the tumor microenvironments cause failure of antigen-presenting function of DCs by IL-6/STAT3 signaling. Therefore, blockade of the IL-6 signaling pathway in DCs will be a promising strategy for improving immunotherapy for cancer patients. On the other hand, it is well-known that cancer antigen-pulsed DCs

also strongly generate Th cells and Tc cells, which recognize and kill the target tumor cells in response to the antigens. Thus, proper design of antigen peptides and selection of immunologic adjuvants are also required for the application of DC-mediated cancer immunotherapy.

Many investigators have performed clinical trials of cancer vaccine immunotherapy. Numerous vaccinations with cancer antigen-derived peptides have been able to induce tumor-specific immune responses to eradicate cancer with superior specificity and without severe adverse effects. However, the therapeutic efficacy of cancer vaccine therapy using MHC class I-binding short peptides for Tc cells have had limited results in inducing complete regression in cancer patients. To overcome this limitation of anti-tumor effects, strong and persistent activation of tumor-specific Tc cells is required for eradication of tumor tissues to induce a complete cure in cancer patients.

It has recently been demonstrated that a long peptide composed of helper and killer epitopes in human cancer antigen, survivin-2B, effectively induced the antigen-specific Th cells and killer T cells via sustained antigen presentation by DCs compared to short peptides (Ohtake et al. 2014). In this study, treatment of DCs with immunologic adjuvant OK-432, which contains TLR4 ligands, effectively induced the antigen-specific $CD4^+$ T cells compared to IFN-γ-treated DCs.

In a phase I clinical study, vaccination using a long peptide of survivin-2B or MAGE-A4 combined with OK-432 induced antigen-specific immune responses including T cell activation and antibody formation in cancer patients (Ohtake et al. 2014; Takahashi et al. 2012). These results indicate that long peptides containing helper and killer epitopes may be critical for inducing effective anti-tumor immunity in cancer patients, suggesting that long peptide-pulsed DCs will become a promising strategy for cancer immunotherapy (Fig. 15.7).

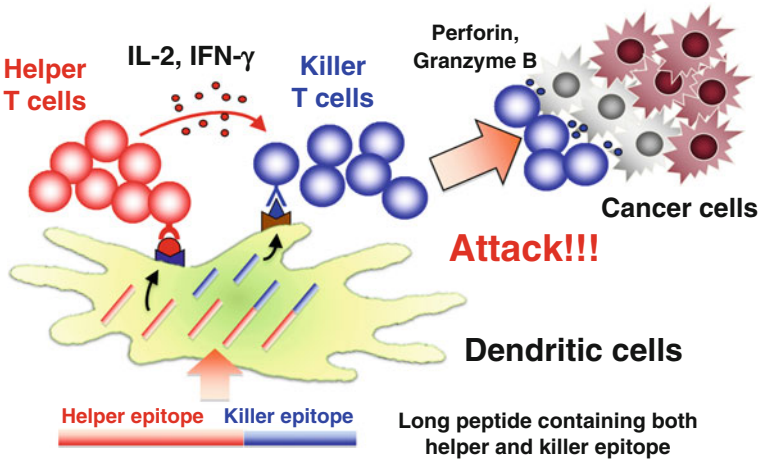

Fig. 15.7 Vaccine using dendritic cells (DCs) with long peptides containing helper and killer epitopes may become a promising immunotherapy for cancer patients

In addition to vaccination using cancer antigen peptides with or without adjuvants, ex vivo-generated cancer antigen-loaded DCs are designed as a powerful tool to induce effector Th and Tc cells in cancer immunotherapy (Palucka et al. 2011; Palucka and Banchereau 2012). DCs generated in the presence of granulocyte-macrophage–colony-stimulating factor (GM-CSF) and IL-4 or FLT3 ligand are pulsed with antigen peptides, α-GalCer, or tumor lysates, and vaccinated into patients with metastatic prostate cancer, melanoma, renal cell carcinoma, or glioma. Efficacy and immunogenicity of the vaccination with ex vivo DCs have been extensively evaluated and reported in numerous clinical trials. Several studies have suggested a correlation between DC-based vaccination and the induction of antigen-specific effector T cells. Although we have to evaluate the precise clinical efficacy in DC-based therapy, there is no doubt that DCs play a crucial role in providing a bridge between innate and acquired immunity against cancer. Thus, DCs are not only a critical target to induce anti-tumor immunity but also a powerful tool of cancer immunotherapy.

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Chapter 16
A New Mode of Cytotoxic T Lymphocyte Induction Secondary to Alteration of the Peptide/Major Histocompatibility Complex Class I Repertoire by Antigen Processing Defects

Takayuki Kanaseki, Yosuke Shionoya, and Noriyuki Sato

Abstract The cell surface repertoire of peptide/major histocompatibility complex (MHC) class I (pMHCI) provides potential ligands for circulating $CD8^+$ cytotoxic T lymphocytes (CTLs). Because the action of antigen-processing machineries inside cells are necessary for optimizing pMHCI formation, it has been believed that antigen-processing defect (APD) attenuates any CTL responses. However, recent evidences demonstrate that the cells with APD often present a unique pMHCI repertoire harboring immunogenic peptides that are never displayed on normal cells. Here we focus on the absence of endoplasmic reticulum (ER)-resident editors ERAAP or tapasin and discuss a new mode of CTL induction secondary to it.

Keywords MHC class I • Antigen processing • ERAAP • Tapasin

16.1 Major Histocompatibility Complex (MHC) Class I Antigen Processing

When the cells are infected by microbes or develop into tumors, peptides derived from pathogenic or mutant proteins are processed and loaded onto the major histocompatibility complex class I (MHC I) molecules, and are consequently eliminated by the $CD8^+$ cytotoxic T lymphocytes (CTLs). Therefore, the peptide/MHCI (pMHCI) repertoire determines the consequence of adaptive immune surveillance in vivo (Shastri et al. 2002). MHC class I processing pathway comprises multiple

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proteolytic events. The processing begins in the cytoplasm; proteasomes/immunoproteasomes, or the other proteases such as tripeptidyl peptidase II or thimet oligopeptidase, digest endogenous proteins and produce peptide fragments. Somehow, a small population of the fragments are associated with cytoplasmic chaperones such as TRiC to avoid complete degradation, and are eventually transported into the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP), where the second proteolytic and selection event for appropriate MHC I binding takes place (Pamer and Cresswell 1998; Rock and Goldberg 1999; Kunisawa and Shastri 2003; Reits et al. 2004; York et al. 2006; Kessler et al. 2011).

In the ER, an ER aminopeptidase associated with antigen processing (ERAAP, ERAP1) plays a key role in optimizing the length of MHC I peptides (Serwold et al. 2002; Saric et al. 2002; York et al. 2002). The role of ERAAP in the ER is unique and cannot be compensated by the other proteases because antigenic short peptides are no longer produced from their N-terminally extended precursors in ERAAP-deficient cells. Moreover, ERAAP's enzymatic activity has been shown to even destroy antigenic peptides in the absence of appropriate MHC I for binding (Kanaseki et al. 2006). Hence, an aminopeptidase ERAAP is capable to both generate and destroy antigenic peptides. On the other hand, nascent and empty MHC I molecules are properly folded in the ER, following which it forms the peptide-loading complex (PLC) together with ER-resident TAP, calreticulin, ERp57, and tapasin (Cresswell et al. 1999). Tapasin, which is a core molecule for forming PLC, is covalently linked to ERp57 and plays a role in tethering MHC I molecule to TAP (Ortmann et al. 1997; Tan et al. 2002). A proposed role of PLC is to retain empty MHC I inside the ER or to edit peptides, until the MHC I molecule meets the peptide that perfectly fits in its peptide-binding groove and makes the pMHCI complex stable (Elliott and Williams 2005; Garbi et al. 2006; Wearsch and Cresswell 2007). Thus, the antigen processing in the ER profoundly influences the quality of peptides on the cell surface in a synergistic and sophisticated way, and contributes to form a normal peptide repertoire with appropriate length (typically 8–10 amino acids) and appropriate anchors for binding to MHC I molecules (Hammer et al. 2007b).

16.2 Antigen-Processing Defect (APD) and a New Mode of Cytotoxic T Lymphocyte Induction

The antigen-processing defect (APD) is the condition in which the function of molecules related to MHC antigen processing has been lost due to several reasons, and often observed in pathological conditions such as viral infection and tumor transformation. For instance, the infected cell protein (ICP) 47 protein from herpes simplex virus (HSV) 1 prevents peptide binding to TAP, HCMV micro RNA miR-US4-1 reduces ERAAP expression, while E19 protein from adenovirus and US3 protein from human cytomegalovirus (HCMV) inhibit tapasin's function (Park et al. 2004; Lilley and Ploegh 2005; Lybarger et al. 2005; Kim et al. 2011). $CD8^+$ T cell responses to the cells with APD are generally attenuated because the cells present a

quantitatively limited number of pMHCI due to compromised antigen processing. The experiments using knockout mice demonstrated that MHC I expression on the surface was significantly (to less than 10 % of wild type) reduced in both TAP- and tapasin-deficient cells, while it was reduced to around 70–80 % of wild type in ERAAP-deficient cells. In any cases, APD didn't interfere MHC class II expression (Van Kaer et al. 1992; Garbi et al. 2000; Grandea et al. 2000; Hammer et al. 2006).

On the other hand, there is a continuous flow of peptide into the ER even in the absence of infections, and cells are always ready to present, or do present, pMHCI complexes originated from self proteins. Intriguingly, reciprocal immunizations using knock-out mice and biochemical analysis using a mass spectrometry have demonstrated that the pMHCI repertoire of the cells with APD is not a quantitatively-reduced copy of normal cells, but is a qualitatively distinct one. Although many conventional peptide presentations are reduced and compromised on ERAAP-deficient and tapasin-deficient cells, they, instead, present a unique set of MHC I peptides which are never presented on normal cells without APD (Hammer et al. 2007a; Kanaseki et al. 2013). Naturally, wild-type mice are no longer tolerant to MHC-matched ERAAP-deficient or tapasin-deficient cells, and elicit vigorous $CD8^+$ T cell responses. Induced specific CD8+ T cells are able to lyse ERAAP-deficient or tapasin-deficient cells in vivo, thus alteration of pMHCI repertoire caused by the change in the process through which proteins are converted to MHC I-bound peptides results in a novel mode of CTL induction that is independent of antigen expressions. The cells with APD decrease conventional antigenic peptide presentation to escape from immune surveillance, meanwhile, they present unique antigenic peptides which can be recognized by the host's immune cells. Such unique $CD8^+$ T cell ligands, which are most likely derived from self proteins and produced through a post-translational modification due to altered MHC I antigen processing, should be distinguished from conventional peptides whose presentations are reduced by APD and we refer to the unique ligands as APD peptides (Fig. 16.1).

The generation of APD peptides has been known in the absence of TAP, ERAAP, or tapasin function. Van Hall and colleagues demonstrated that TAP-deficient cells are immunogenic to wild-type mice, and identified the H-$2D^b$-restricted antigenic peptide derived from the *Lass5* gene, which encodes ER-membrane spanning protein (van Hall et al. 2006). Shastri and colleagues identified the another APD peptide encoded in Fam49b protein that is presented in the absence of ERAAP (Nagarajan et al. 2012). This particular peptide is of interesting because of its Qa-1^b-restricted presentation. Qa-1^b in mouse (HLA-E in humans), which belongs to non-classical MHCI family, is known to present non-polymorphic peptides derived from the signal sequence of classical MHCI and interacts with NKG2A/CD94 heterodimer on natural killer (NK) cells or a small subset of $CD8^+$ T cells (Braud et al. 1998; Vance et al. 1998). This finding clearly indicates that the immune system takes advantage of non-classical MHCI for surveillance of APD peptide, along with the fact that Qa-1^b is capable of presenting a diverse pMHCI repertoire (Oliveira et al. 2010). Recently, we have found that cells lacking tapasin also display the qualitatively altered pMHCI and are able to elicit new $CD8^+$ T cell responses in immunized wild-type mice (Kanaseki et al. 2013). Tapasin is covalently bound to

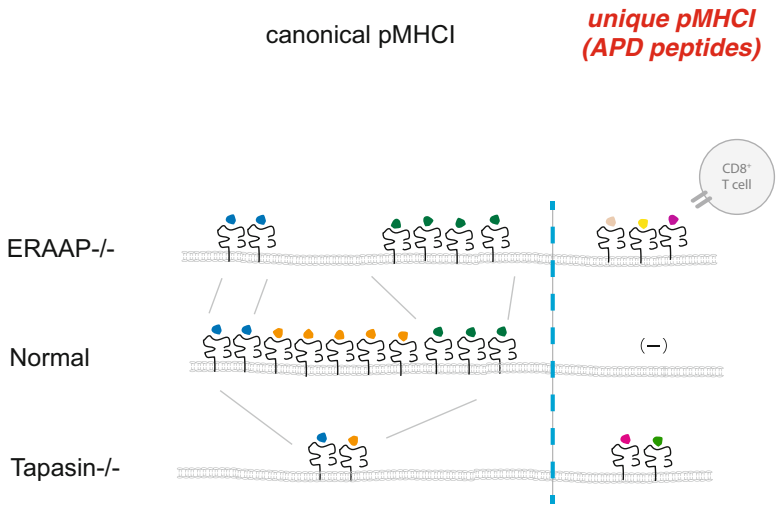

Fig. 16.1 The altered peptide/major histocompatibility complex class I (pMHCI) repertoire caused by antigen-processing defect (APD). In ERAAP-deficient or tapasin-deficient cells, the total amount of pMHCI on the cell surface is reduced to 70–80 % and 10 % of wild type, respectively. Both of two APD conditions present quantitatively reduced pMHCI repertoire, however, the quality of presented peptides is largely altered from that of wild-type peptides. While canonical peptides are reduced, APD peptides which are never seen on wild type are preferentially presented in the absence of ERAAP or tapasin. APD peptides are immunogenic and able to elicit $CD8^+$ T cell responses when immunized into wild-type animals.

Erp57 and recruits an empty MHCI next to TAP, thus the absence of tapasin could represent the compromised condition of PLC formation in the cells. Angiotensin converting enzyme (ACE) is a carboxypeptidase that is a potential candidate of an another pMHCI editor, and, in fact, new $CD8^+$ T cell responses specific to ACE-deficient cells are also generated (Shen et al. 2011). Rock and colleagues reported the altered pMHCI repertoire in the cells lacking three immunoproteasome catalytic subunits; however, new $CD8^+$ T cell responses specific to it are not induced, indicating the distinct nature of APD in the cytoplasm (Kincaid et al. 2011).

16.3 Unique Peptide/MHC Class I (pMHCI) Repertoire Produced by APD

We next consider how the pMHCI repertoire is altered from the original repertoire in the cells with APD in the ER, particularly in the absence of ERAAP, or tapasin. The source peptides coming from the cytoplasm could be comparable among these two APD conditions and wild type without APD, contrary to the absence of TAP, in which the peptide supply is extremely limited and membrane proteins are preferentially processed. In addition, wild-type $CD8^+$ T cells induced by immunizations

against tapasin-deficient cells do not cross-respond to ERAAP-deficient cells, and vice versa, which suggests that the immunogenic repertoires of APD peptides produced in ERAAP- or tapasin-deficient cells are exclusive to each other. Therefore, the nature of the altered pMHCI repertoire reflects the roles of the defected molecule in MHCI antigen processing, and the immune system potentially distinguishes the cells with different APD conditions.

Both of the immunogenic pMHCIs presented on ERAAP- and tapasin-deficient cells are unstable, and the specific $CD8^+$ T cell responses to them are readily diminished by Brefeldin A treatment. In addition, an immunization of ERAAP-deficient cells to wild-type mice elicits B cell responses which produce specific antibodies. Considering that, the unique pMHCIs formed with APD peptides are structurally distinguished from conventional ones (Hammer et al. 2007a). Furthermore, the large-scale peptide sequencing by the use of mass spectrometry has demonstrated that the H-$2D^b$ and H-$2K^b$ peptides produced in the absence of ERAAP are unusually long on average, most likely due to the loss of their aminopeptidase activity to trim the N-terminal end of precursors (Blanchard et al. 2010). It is well characterized that unusually long peptides bound to MHCI molecule make a bulge at the center and often elicit strong $CD8^+$ T cell responses (Tynan et al. 2005). Meanwhile, the APD peptides produced in the absence of tapasin frequently lack consensus anchor residues for binding to MHCI (Kanaseki et al. 2013). Their MHCI binding scores are low according to the prediction and indeed consistent with the proposed role of tapasin in selecting high-affinity peptides. The crystal structure of tapasin–Erp57 complex in the PLC estimates that tapasin interacts with the mobile α2-1 segment of MHCI, that is, interaction with tapasin potentially influences the shape of peptide-binding groove (Lewis and Elliott 1998; Dong et al. 2009). Thus, the unusual anchorless pMHCI which were supposed to be retained inside the ER are unexpectedly sorted and displayed on the surface in the absence of tapasin. The molecular mechanisms by which APD peptide are preferentially processed in the absence of tapasin should be further investigated.

16.4 APD and Immunotherapy Against Tumors

ERAAP and tapasin are usually expressed in eukaryotic cells expressing MHC I on the cell surface, therefore most of normal cells do not present APD peptides and they are not targeted by specific CTLs induced by APD. However, the loss of ERAAP, and in particular, tapasin expression has been reported in many malignant tumors including lung cancers, colorectal cancers, and head and neck cancers (Ogino et al. 2006; Jiang et al. 2010; Lou et al. 2005; Atkins et al. 2004). These findings suggest that not a few tumors are considered to be the heterogeneous mixture consisting of tapasin-deficient variants. Precise molecular mechanisms by which tapasin expression is down-regulated in tumors are under debate for now, but a fact is that tapasin-deficient variants of tumors grow up in vivo despite of the very low level of MHCI expression which makes cells susceptible to NK cell lysis.

Moreover, a positive correlation between the loss of tapasin expression and poorer prognosis has been reported. As is expected, CTLs targeting a conventional peptide are able to lyse human tumor cells that express tapasin, however, CTLs no longer lyse the tapasin-deficient variant of that tumor cells, even when the antigen is expressed within (Kanaseki et al. 2013). Such tumor cells lacking tapasin expression is an immune escape variant of that tumor and could become a serious issue in CTL immunotherapy. Because CTLs induced by APD distinguish tapasin-deficient tumor cells from tapasin-proficient normal cells and are able to lyse them in vivo, we propose to apply the CTL responses induced by APD peptides for targeting tumor cells. In mouse case, it has been shown that CTL responses to the peptide derived from lass5 gene mentioned above successfully lysed TAP-deficient variant of tumor cells. Exploiting APD peptides and a new mode of CTL induction is definitely a candidate solution against the immune-escape variants of tumors.

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Chapter 17
Progress in Clinical Use of CC Chemokine Receptor 4 Antibody for Regulatory T Cell Suppression

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Abstract Recent clinical research advances in tumor immunity provide hope for the development of a novel tumor immunotherapy. Many clinical studies of cancer vaccines over the past 10 years have shown the augmentation of tumor immunity in some patients, although the clinical effects have been low. In addition, it was reported that blocking regulatory molecules involved in the immune checkpoint is important for immune activation in both in vitro and in vivo studies. In particular, studies using blocking antibodies to cytotoxic T lymphocyte-associated antigen (CTLA)-4, programmed cell death protein (PD)-1, or PD-1 ligand 1 (PD-L1) observed significant clinical effects including complete remission, suggesting that regulation of regulatory signals in the immune checkpoint is a new direction for the development of new drugs based on tumor immunity. As regulatory T cells (Tregs) also plays a key role in immune regulation, just as the regulatory molecules in the immune checkpoint become targets for the development of novel tumor immunotherapy, they may also become a target for development. Several reports have suggested that Tregs that infiltrate tumor sites as well as regulatory signals for immune checkpoints can inhibit tumor immunity, which is one explanation why treatment by cancer vaccine is limited. CC chemokine receptor 4 (CCR4) is selectively expressed on effector Tregs, and humanized monoclonal antibodies to CCR4 have been developed as a drug, 'mogamulizumab', to treat adult T cell leukemia/lymphoma (ATLL). Mogamulizumab depletes both ATLL cells and normal Tregs, resulting in the recovery of immune function in ATLL patients. Mogamulizumab is now expected to be accepted for use as a novel immuno-activator for tumor therapy by depletion of

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Tregs and is undergoing phase Ia/Ib studies for treatment of solid tumors in the lung, esophagus, intestine, ovary, and melanoma.

Keywords Regulatory T cell • CCR4 • Mogamulizumab • Immune checkpoint • Regulatory molecules • Immunotherapy • Adult T cell leukemia/lymphoma • ADCC

17.1 Introduction

Despite the remarkable progress in cancer research in the past decade, both the incidence (618.5 per 10,000 in 2007) and mortality (283.2 per 100,000 in 2011) of cancer are still high in Japan, and are increasing yearly (Matsuda et al. 2013). Accordingly, the development of radical cancer therapy is an urgent issue. Recent advances in tumor immunology have led to the development of cancer immunotherapy and some form of immunotherapy is expected to be the next promising therapeutic for cancer treatment with high target specificity and killing activity. For example, antibody medicines have contributed to improved cure and survival in several types of cancers in recent years, e.g., rituximab (anti-CD20) for B cell lymphoma (Coiffier et al. 2002; Pfreundschuh et al. 2006), trastuzumab [anti-human epidermal growth factor receptor 2 (HER2)] for breast cancer (Slamon et al. 2001; Robert et al. 2006), cetuximab [anti-epidermal growth factor receptor (EGFR)] for multiple cancers (Van Cutsem et al. 2009; Cunningham et al. 2004), mogamulizumab, defucosylated humanized anti-CC chemokine receptor 4 (anti-CCR4) monoclonal antibody (mAb), for adult T cell leukemia/lymphoma (ATLL) (Ishii et al. 2010; Ishida et al. 2012; Yamamoto et al. 2010), amongst others (Table 17.1). Most of these antibodies recognize target antigens expressed on the tumor cells and kill them by antibody-dependent cell cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), anticancer drug or radio isotope conjugated with antibody, or inhibition of cell growth signaling. ADCC is mediated by natural killer (NK) cells via binding of IgG with FcγIIIa expressed on NK cells. It is reported that defucosylated human IgG1 exhibits to enhance ADCC. Although detailed knowledge of the mechanism to enhance ADCC by defucosylation is not known, it is considered that absence of Fuc provided a more suitable conformation for the binding of IgG1 to FcγIIIa. Mogamulizumab is the first therapeutic antibody produced by defucosylation technology called POTELLIGENT® (Shinkawa et al. 2003; Niwa et al. 2004).

In contrast, target antigens recognized by antibodies listed in Table 17.2 are immunomodulatory receptors or ligands (Pardoll 2012; Weber 2010; Ngiow et al. 2011a). These receptors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; also known as CD152), programmed cell death protein 1 (PD-1; also known as CD274), and lymphocyte activation gene 3 (LAG3), are expressed in activated T cells (Schwartz 1992; Lenschow et al. 1996; Rudd et al. 2009; Ishida et al. 1992), and the ligands, including CD80 (also known as B7-1), CD86 (also known as B7-2), and PD-1 ligand 1 (PD-L1, also known as B7-H1 and CD273), are commonly expressed on antigen-presenting cells (APC), tumors, or non-transformed

Table 17.1 Therapeutic monoclonal antibodies for tumors

Target antigens	Antibody medicines	Tumor types	Mechanisms of tumor killing
Hematopoietic cell differentiation antigens			
CD20	Rituximab	Non-Hodgkin's lymphoma	ADCC
	Tositumomab	Non-Hodgkin's lymphoma	^{131}I
	Ibritumomab		^{90}Y
CD22	Inotuzumab	Non-Hodgkin's lymphoma	Ozogamicin
CD30	Brentuximab	Hodgkin's lymphoma	Monomethyl auristatin E
CD33	Gemtuzumab	Acute myelogenous leukemia	Ozogamicin
CD52	Alemtuzumab	Chronic lymphocytic leukemia	CDC
Chemokine receptor			
CCR4	Mogamulizumab	Adult T cell leukemia/lymphoma	ADCC
Tyrosine kinase receptors			
EGFR	Cetuximab, panitumumab, nimotuzumab, and 806	Glioma, lung, breast, colon, and head and neck tumors	Inhibition of growth signaling, ADCC and CDC
ERBB2 (HER2)	Trastuzumab and pertuzumab	Breast, colon, lung, ovarian, and prostate cancers	ADCC
ERBB3	MM-121	Breast, colon, lung, ovarian, and prostate cancers	Inhibition of growth signaling
VEGFR	IM-2C6 and CDP791	Epithelium-derived solid tumors	Inhibition of growth signaling
MET	AMG 102 and METMAB	Breast, ovary and lung cancers	Inhibition of growth signaling
IGF1R	AVE1642, IMC-A12, MK-0646, and R1507	Glioma, lung, breast, head and neck, prostate, and thyroid cancers	Inhibition of growth signaling
EPHA3	IIIA4	Lung, kidney and colon cancers, melanoma and glioma	Disrupting the tumor stroma and microvasculature
Death receptors			
TRAILR1 (DR3)	Mapatumumab	Colon, lung and pancreas tumors and hematological malignancies	Death signal
TRAILR2 (DR4)	HGS-ETR2 and CS-1008		
RANKL	Denosumab	Prostate cancer and bone metastases	Blockade of RANKL/RANK interaction

(continued)

Table 17.1 (continued)

Target antigens	Antibody medicines	Tumor types	Mechanisms of tumor killing
Glycoproteins			
CEA	Labetuzumab	Breast, colon, and lung cancers	^{131}I
EpCAM	Adecatumumab	Breast and prostate cancers	ADCC and CDC
CA-125	Oregovomab	Ovarian cancer	Enhancement of tumor-specific humoral and cellular immune responses
CAIX	cG250	Renal cell carcinoma	^{131}I, ^{90}Y
Others			
VEGF	Bevacizumab	Tumor vasculature	Disrupting the tumor microvasculature
Integrin aVb3	Etaracizumab	Tumor vasculature	
Integrin a5b1	Volociximab	Tumor vasculature	
FAP	Sibrotuzumab	Colon, breast, lung, pancreas, and head and neck cancers	Disrupting the tumor stromal fibroblasts

ADCC antibody-dependent cellular cytotoxicity, *CAIX* carbonic anhydrase IX, *CCR4* CC chemokine receptor 4, *CDC* complement-dependent cytotoxicity, *CEA* carcinoembryonic antigen, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *EPHA3* ephrin receptor A3, *FAP* fibroblast activation protein, *HER2* human epidermal growth factor receptor 2, *IGF1R* insulin-like growth factor 1 receptor, *RANK* receptor activator of nuclear factor-κB, *RANKL* RANK ligand, *TRAILR* tumor necrosis factor-related apoptosis-inducing ligand receptor, *VEGF* vascular endothelial growth factor, *VEGFR* VEGF receptor

Table 17.2 Therapeutic antibodies targeting immune checkpoints

	Targets		Counterparts	
Antibodies	Antigens	Cells	Antigens	Cells
Ipilimumab	CTLA-4	T cell	CD80 or CD86 (B7-1) (B7-2)	APC
Tremelimumab				
Nivolumab (BMS-936558)	PD-1	T-cell	PD-L1 or PD-L2 (B7-H1) (B7-DC)	APC
Pembrolizumab (MK-3475)				
CT-011				
AMP-244				
BMS-936559	PD-L1 (B7-H1)	Tumor	PD-1, CD80	T cell
MEDI4736		T cell		
BMS-986016	LAG3	T cell	MHCII	APC
MGA271	B7-H3	APC	?	T cell ?
Under development	B7-H4	APC	?	T cell ?
Under development	TIM3	T cell	Galectin 9	Tumor
BMS-986015	KIR	T cell	MHCI	Tumor

APC antigen-presenting cell, *CTLA-4* cytotoxic T lymphocyte-associated antigen, *LAG3* lymphocyte activation gene 3, *MHCI* major histocompatibility complex class I, MHCII major histocompatibility complex class II, *PD-1* programmed cell death protein 1, *PD-L* PD-1 ligand, *TIM3* T cell membrane protein 3, ? unknown

cells in the tumor microenvironment (Zou and Chen 2008). Over-expression of these molecules and their receptor/ligand pathways are a major cause of exhaustion in CD8 T cells. Exhausted CD8 T cells are functionally deficient and have decreased proliferative capacity, cytokine production, and cytotoxic activity and are metabolically deficient (Barber et al. 2006). The antibodies can block the receptor/ligand pathway between infiltrating T cells in tumors and the tumor cells to restore T cell cytotoxic activity against the tumors. Indeed, significant clinical effects by single drug therapy has been reported for ipilimumab (anti-CTLA-4), nivolumab/BMS-936558 (anti-PD-1), lambrolizumab/MK-3475 (anti-PD-1), and BMS-936559 (anti-PD-L1) (Hodi et al. 2010; Topalian et al. 2012; Hamid et al. 2013; Brahmer et al. 2012). Thus, turning the immune balance to an active state by blocking immune inhibitory signals with antagonistic antibodies is a new direction for the development of antibody drug therapy against tumors.

Regulation of regulatory T cells (Tregs) is also considered important for the development of new therapeutics against tumors because many reports indicate infiltrating Tregs in tumors suppress tumor immune responses and Treg depletion by administration of CD25 mAb causes tumor regression in a mouse tumor model (Onizuka et al. 1999; Ohmura et al. 2008). CCR4 is commonly expressed on Tregs; thus, mogamulizumab can diminish not only ATLL cells but also normal Tregs in vivo (Yano et al. 2007; Ishida and Ueda 2006, 2011; Ishida et al. 2003). Therefore, mogamulizumab might be a unique regulator of immune balance to resolve tumor immune suppression, resulting in new therapeutics for tumors. This review focuses on the regulation of Tregs by mogamulizumab and discusses the theory and possibility of a new tumor therapy using mogamulizumab compared with antibodies to immune checkpoint-related molecules such as ipilimumab, nivolumab, and BMS-936559.

17.2 Regulation of Immune Balance in Immune Checkpoints

Immune checkpoints during the sequential steps of immune responses are crucial events to maintain self-tolerance and to protect tissues from damage caused by hyperimmune reactions during pathogenic inflammation (Pardoll 2012; Zou and Chen 2008). CTLA-4 is transiently expressed on effector T cells after activation and functions as an inhibitory receptor in the first immune checkpoint, which regulates antigen presentation from dendritic cells (DC) in primary or secondary lymph nodes (Zou and Chen 2008). When antigen is presented by DCs to naïve or memory T cells, T cells recognize antigen by binding to the T cell receptor (TCR) and epitope peptide complexed with major histocompatibility complex (MHC) expressed on the DC. Simultaneously, co-stimulatory receptor CD28, exclusively expressed on T cells, binds to CD80/CD86 on DCs and stimulates immune responses (Zou and Chen 2008). CTLA-4 shares identical ligands, CD80/CD86, with CD28, and counteracts the activity of CD28 (Schwartz 1992; Rudd et al. 2009). Although the mechanisms of T cell suppression by CTLA-4 are not clear, two different mechanisms have been demonstrated. CTLA-4 is thought to compete with CD28 in binding CD80 and/or CD86 because the binding affinity of CTLA-4 for both ligands is higher than that of CD28 (Linsley et al. 1994; Egen and Allison 2002). Another explanation is the activation of protein phosphatases, Src homolog domain-containing phosphatase 2 (SHP2, also known as PTPN11) and protein phosphatase 2 (PP2, also known as PP2A), which inhibit kinase signaling downstream of TCR and CD28 (Schwartz 1992). CTLA-4 is constitutively expressed on Tregs as well as CD8+ cytotoxic T cells and CD4+ T helper (Th) cells, and is a target gene of the forkhead transcription factor FOXP3 (Hori et al. 2003; Fontenot et al. 2003), the master gene of Treg. FOXP3 regulates the differentiation and functions of Treg (Hill et al. 2007; Gavin et al. 2007) and is one of the mechanisms of effector T cell suppression that downregulates CD80 and CD86 on APCs. This is caused by the binding of CTLA-4 on Treg counteracting CD28 binding CD80/CD86, resulting in the inhibition of antigen presentation to T cells (Vignali et al. 2008) (Fig. 17.1).

PD-1 is another important co-inhibitory receptor stably expressed on effector-phase T cells, which regulates T cell activation by interactions with PD-L1 and/or PD-L2 expressed on tumor cells, APC, and non-transformed cells in the tumor microenvironment (Dong et al. 2002; Konishi et al. 2004; Curiel et al. 2003; Kuang et al. 2009). PD-1 engagement inhibits kinase signaling downstream of TCR through phosphatase SHP2 (Dong et al. 2002). PD-1 is also highly expressed on Tregs and enhances the proliferation of Tregs in the presence of ligand (Freeman et al. 2000; Francisco et al. 2009).

As well as the molecules described above, various inhibitory receptors and ligands are involved in immune checkpoints. T cell membrane protein 3 (TIM3), LAG3 (also known as CD233), B and T Lymphocyte attenuator (BTLA; also known as CD272), adenosine A2a receptor (A2aR), and the family of killer inhibitory receptors are inhibitory receptors expressed on T cells. Galectin 9, MHC class II, herpesvirus entry mediator (HVEM), adenosine, and subsets of human leukocyte antigens (HLA) have been respectively identified as their ligands (Pardoll 2012).

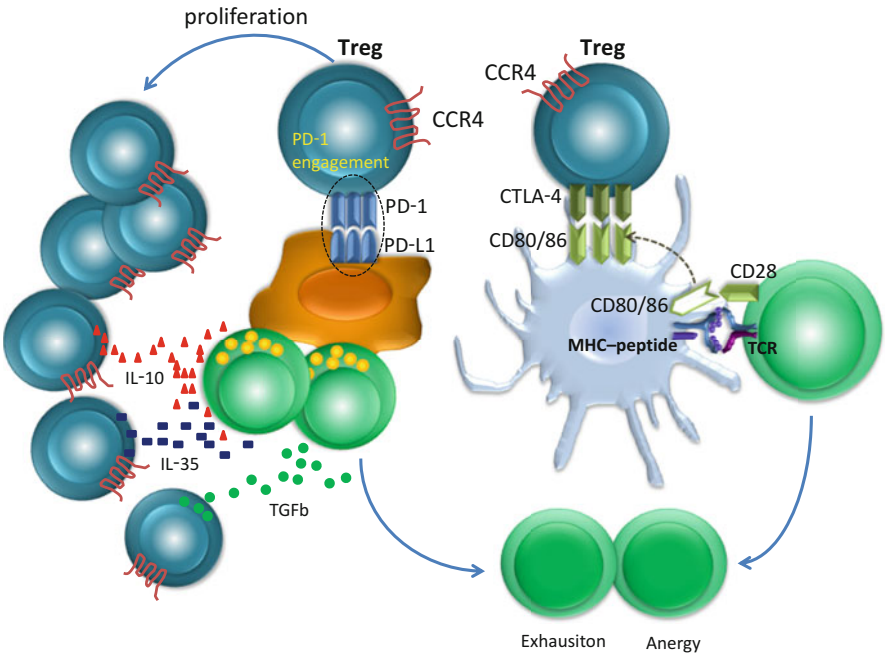

Fig. 17.1 T cell exhaustion mechanisms induced by regulatory T cells (Tregs). Tregs constitutively express cytotoxic T lymphocyte-associated antigen (CTLA)4. The affinity of CTLA-4 to CD80 or CD86 is higher than CD28, thus CTLA-4 outcompetes CD28, and co-stimulatory signals via CD28 are blocked in antigen-stimulated T cells, resulting in anergy. Tregs highly express programmed cell death protein 1 (PD-1), the proliferation of Tregs is enhanced in the presence of ligand, and the effector T cells are exhausted by the release of interleukin (IL)-10, IL-35, and transforming growth factor (TGF)-β from Tregs

These receptors are also expressed on activated T cells, and the ligands are expressed on many types of tumor cells (Ngiow et al. 2011a; Grosso et al. 2007; Cedeno-Laurent and Dimitroff 2012; Fourcade et al. 2012; Baixeras et al. 1992). Furthermore, antibodies to these enhance anti-tumor immunity in mouse models and human in vitro experiments (Baghdadi et al. 2013; Ngiow et al. 2011b). LAG3 is expressed on Tregs as well as effector T cells and enhances Treg functions. A2aR functions in the development of Tregs by inducing FOXP3 expression in CD4+ T cells (Huang et al. 2004; Zarek et al. 2008).

17.3 Clinical Application of the Blockade of Immune Checkpoints

Thus, co-inhibitory receptors/ligands regulate immune responses of effector T cells at sites of inflammation or tumors (Fig. 17.2), and drugs, such as antibodies and small molecules, which block receptor/ligand interactions are expected to be novel

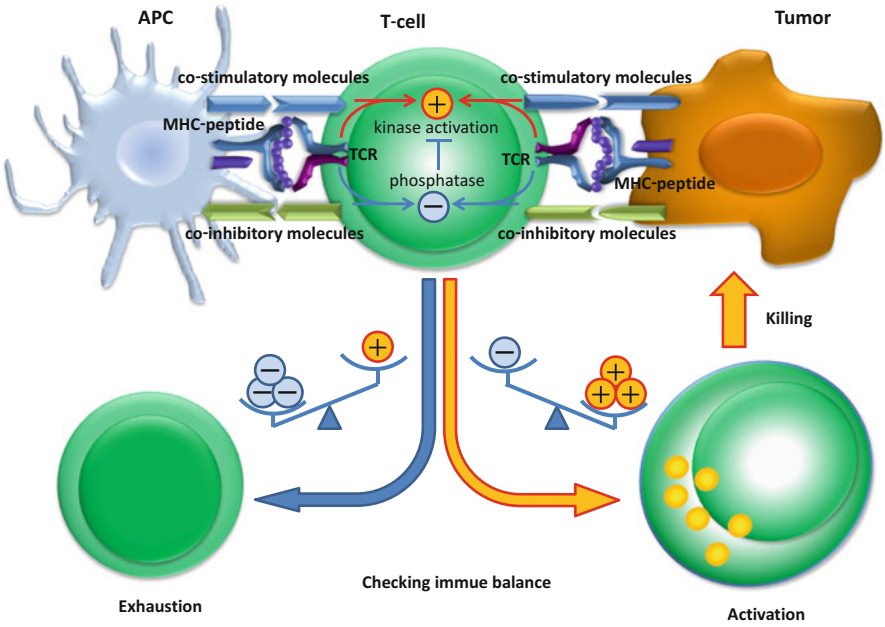

Fig. 17.2 Immune checkpoint determines the balance of stimulatory and inhibitory signals and the future state of T cells. After T cells recognize antigen epitope peptides presented on human leukocyte antigen (HLA) class I molecules, the signal is transduced in addition to co-stimulatory and co-inhibitory molecule signaling. Co-stimulatory receptors (CD28, CD137, ICOS, OX40, CD27) and co-inhibitory receptors [cytotoxic T lymphocyte-associated antigen (CTLA)-4, programmed cell death protein 1 (PD-1), T cell membrane protein 3 (TIM3), killer cell immunoglobulin-like receptor (KIR), lymphocyte activation gene 3 (LAG3), adenosine A2a receptor (A2aR)] are expressed on T cells, and their co-stimulatory ligands (CD80, CD86, CD137L, B7PR1, OX40L, CD70) and co-inhibitory ligands [CD80, CD86, PD-1 ligand (PD-L) 1, PD-L2, galectin 9, major histocompatibility complex (MHC) class II, adenosine) are expressed on antigen-presenting cells or tumor cells. The balance of stimulatory and inhibitory signals determines whether T cells are activated or exhausted. Once tumor-specific T cells are activated, they elicit killing activity to the tumor cells

beneficial drugs for tumor therapy (Fig. 17.3). Clinical trials of anti-CTLA-4 humanized mAb, ipilimumab, and tremelimumab were conducted from 2000. Although objective clinical responses were observed in ~10 % of patients with melanoma using both antibodies, no survival benefit was observed in comparison with standard melanoma chemotherapy treatment (dacarbazine) in a randomized phase III trial for tremelimumab (Ribas 2010). However, in a randomized, three-arm phase III trial of patients with advanced melanoma using (1) gp100 peptide vaccine alone, (2) gp100 plus ipilimumab, or (3) ipilimumab alone, 3.5-month survival benefit was observed in the ipilimumab alone group compared with the gp100 peptide vaccine alone or gp100 plus ipilimumab groups (Hodi et al. 2010). Furthermore, Grade 3 or 4 immune-related adverse events (AEs) occurred in 10–15 % of patients treated

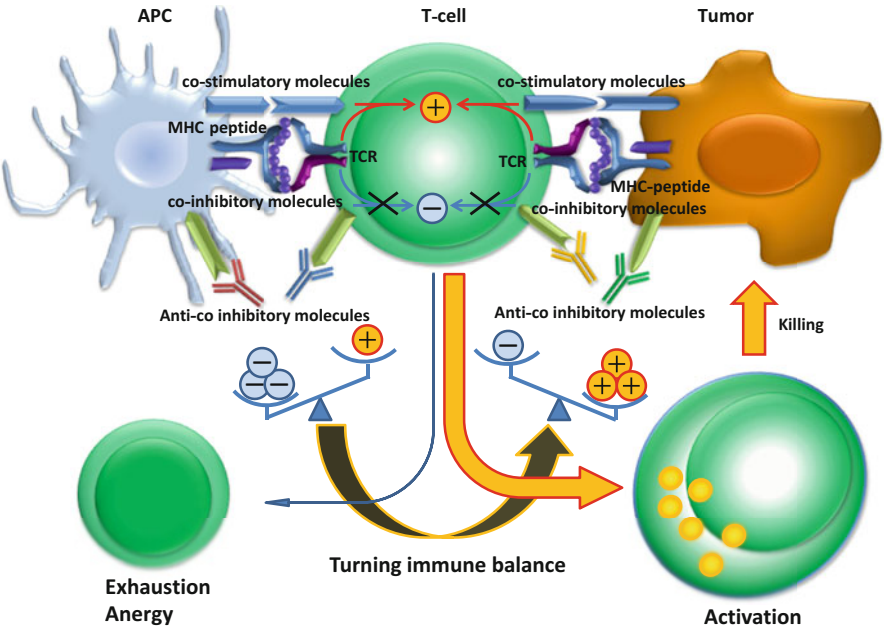

Fig. 17.3 Antibodies to immune checkpoint molecules block co-inhibitory signals turning the immune balance to an active state. In the tumor site, the immune balance is considered inhibitory, caused by inhibitory signals that exceed stimulatory signals. Antagonistic antibodies such as ipilimumab [anti-cytotoxic T lymphocyte-associated antigen (CTLA)-4], nivolumab [anti-programmed cell death protein 1 (PD-1)], lambrolizumab (anti-PD-1), and BMS936559 [anti-PD-1 ligand 1 (PD-L1)] block co-inhibitory signals of T cells, turning the immune balance towards an active state. Exhausted T cells in the tumor site show restored tumor killing functions and kill tumor cells

with ipilimumab, including deaths associated with immune-related AEs. Ipilimumab was the first drug to demonstrate a survival benefit for metastatic melanoma, and was approved by the US Food and Drug Administration (FDA) for the treatment of advanced melanoma in 2010. Both humanized mAb to PD-1 (nivolumab/BS936558) and PD-L1 (BS936559) demonstrated very promising results in a phase I clinical trial for multiple tumors (Topalian et al. 2012; Brahmer et al. 2012). Objective response rates to BMS936558 were observed in 18 % of patients with non-small cell lung cancer (14 of 76 patients), 28 % of patients with melanoma (26 of 94 patients), and 27 % of patients with renal cell cancer (9 of 33 patients). Interestingly, no objective response was observed in 17 patients with PD-L1-negative tumors, although 9 of 25 patients (36 %) with PD-L1-positive tumors had an objective response. In a phase I trial using anti-PD-L1 (BMS936559), an objective response (a complete or partial response) was observed in 17 % of patients with melanoma (9 of 52), 12 % of patients with renal cell cancer (2 of 17), 10 % of patients with non-small cell lung cancer (5 of 49), and 6 % of patients with ovarian cancer (1 of 17). Furthermore, responses were durable for both antibodies. Responses lasting 1 year

or more were shown in more than half of the patients with 1 year or more of follow-up. Immune-related AEs occurred in 14 % of patients treated with anti-PD-1 and 9 % of patients treated with anti-PD-L1. Interestingly, immune-related AEs frequently occurred in the skin and intestine in patients receiving any of ipilimumab, nivolumab, or BMS936559.

17.4 Immune Tolerance in Tumors Caused by Regulatory T Cells

It is clarified that FOXP3 elicits Treg functions (Hori et al. 2003; Fontenot et al. 2003). Because FOXP3 knockout mice develop autoimmune diseases involving multiple organs, and patients with FOXP3 homozygous mutations develop immunodysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome (Fontenot et al. 2003; Bennett et al. 2001), Tregs are considered to be crucial for the maintenance of self-tolerance and for maintaining immune balance to prevent damage of healthy cells from excessive immune reactions in cooperation with the immune checkpoint system described above. It was reported that Tregs are recruited and accumulate in tumor tissues by chemokine-induced chemotaxis via interaction between CCR4 and its ligands produced by cells of the tumor microenvironment (Curiel et al. 2004; Zou 2006). Hodgkin's lymphoma (HL) is a representative example of Treg recruitment to the tumor site by chemokine-induced chemotaxis resulting in immunosuppression. HL is characterized by the presence of a small number of tumor cells in a rich background of T and B cells, macrophages, and other inflammatory cells (Küppers 2009). The question of why a very small number of HL cells can survive in the presence of a large excess of host immune cells was unclear. HL cells express high levels of the CCR4 ligand and thymus and activation-regulated chemokine (TARC)/CCL17 (van den Berg et al. 1999), and elevated serum levels of TARC/CCL17 were reported as an unfavorable prognostic factor in patients with HL (Weihrauch et al. 2005). Considering these findings, we have shown that HL tumor cells attracted CD4+CCR4+ T cells by interactions with TARC/CCL17 and macrophage-derived chemokine (MDC)/CCL22. Migratory CD4+ cells attracted by HL tumor cells were hyporesponsive to TCR stimulation and suppressed the activation and proliferation of effector CD4+ T cells in an autologous setting in vitro. Furthermore, double staining showed that HL cells in the affected lymph nodes were surrounded by a large number of lymphocytes expressing both CCR4 and FOXP3 (Ishida et al. 2006). Collectively, these findings imply that the migratory cells induced by HL function as Tregs to create a favorable environment for the tumor cells to escape from host immunity. It was proposed that Treg-mediated immunosuppression is a crucial tumor immune-evasion mechanism in ovarian, gastric, breast, and pancreatic cancers, and may be one of the main obstacles to successful tumor immunotherapy (Curiel et al. 2004; Zou 2006). Therefore, Tregs are a potential target for novel tumor therapy by inhibition of immunosuppression in the tumor site.

17.5 CC Chemokine Receptor 4 Is a Promising Target to Regulate Regulatory T Cells (Tregs)

CD4+/FOXP3 cells can be functionally divided into three sub-fractions by double staining of CD45RA and FOXP3. Naïve Tregs and effector Tregs can be separated into CD45RA+/FOXP3+ and CD45RA–/FOXP3++ fractions, where the CD45RA–/FOXP3+ fraction (fr.) represents conventional effector T cells (Tconv) (Miyara et al. 2009). CCR4 is expressed on effector Tregs but not naïve Tregs. We also analyzed the CCR4 expression of CD4+ T cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy subjects by flow cytometry using 4-color staining for CD4, CD45RA, CCR4, and FOXP3 (Fig. 17.4). CD4+ T cells were separated into five fractions: fr. I, CD45RA + FOXP3+; fr. II, CD45RA–/FOXP3++; fr. III, CD45RA–/FOXP3+; fr. IV, CD45RA+/FOXP3–; and fr. V, CD45RA–/FOXP3–. CCR4 expression on CD4+ T cells was restricted to the CD45RA– fractions (fr. II, fr. III, and fr. V), and the expression levels correlated with FOXP3. CCR4 expression

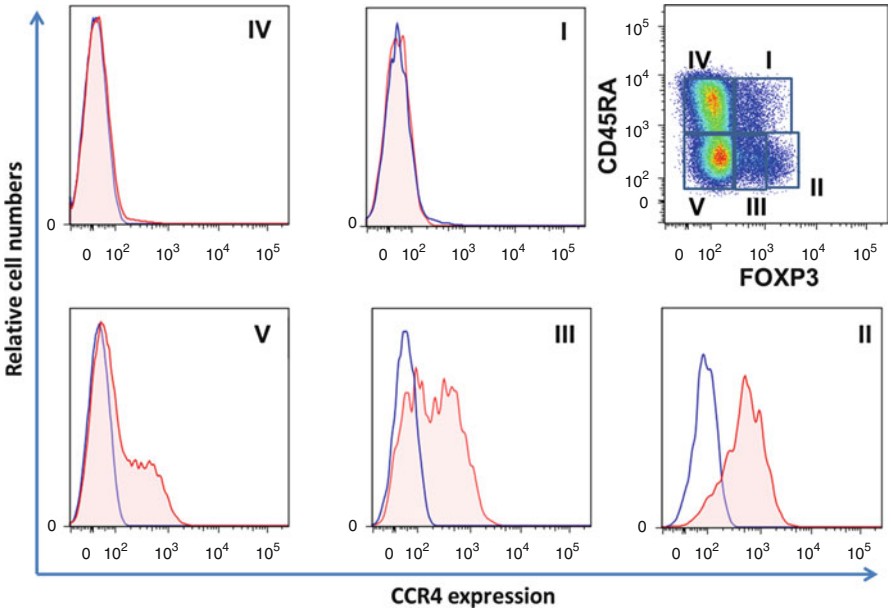

Fig. 17.4 CC chemokine receptor 4 (CCR4) expression on CD4+ T cell subsets delineated by CD45RA and FOXP3. Peripheral blood mononuclear cells isolated from healthy donors were stained by fluorochrome-conjugated monoclonal antibodies to CD4, CD45RA, CCR4, and FOXP3, acquired by flow cytometry (FACScantoII™), and analyzed with Flowjo software. CD4+ T cells were delineated by the expression CD45RA and FOXP3 into five subsets: fraction (fr.) I, CD45RA+/FOXP3+; fr.II, CD45RA–/FOXP3++; fr.III, CD45RA–/FOXP3+; fr.IV, CD45RA+/FOXP3–; and fr.V, CD45RA–/FOXP3–. The histograms show CCR4 expression in the cells of each fraction. *Red* and *blue lines* indicate CCR4 expression and isotype controls, respectively

Table 17.3 Global development plan of mogamulizumab for malignant lymphoma

Indication	Region	Development phase		Status
		Pilot (phase I/IIa)	Pivotal (phase IIb/III)	
ATL (relapsed/refractory)	Japan	NCT00355472	NCT00920790	Complete/complete
ATL (1st-line)	Japan		NCT01173887	Complete
ATL (relapsed/refractory)	US/EU		NCT01626664	Recruiting
PTCL/CTCL (relapsed)	Japan		NCT01192984	Complete
CTCL (relapsed/refractory)	North America/EU	NCT01226472	NCT01728805	Complete/recruiting
PTCL/CTCL (relapsed/refractory)	EU	NCT01611142		Recruiting

ATL adult T cell leukemia/lymphoma, *CTCL* cutaneous T cell lymphoma, *PTCL* peripheral T cell lymphoma

was highest in fr. II, which corresponded to effector Tregs, but was negative in fr. I, which corresponded to naïve Tregs. Therefore, anti-CCR4 mAb mogamulizumab might target effector Tregs that dominantly infiltrate the tumor site. The clinical trials of mogamulizumab for patients with relapsed, aggressive CCR4-positive malignant lymphoma such as ATLL, cutaneous T cell lymphoma (CTCL), and peripheral T cell lymphoma (PTCL) are being conducted in Japan, EU, and the USA. Table 17.3 lists clinical trials of mogamulizumab for malignant lymphoma in the pipeline. The excellent treatment results have been reported in all lines. It is reported that objective responses were noted in 13 of 26 evaluable patients, including eight complete responses, with an overall response rate of 50 % (95 % confidence interval 30–70) in a multicenter phase II study for patients with relapsed, aggressive CCR4-positive ATLL (Ishida et al. 2012). It was also reported that the overall response rate was 39 % for 38 patients (23 with mycosis fungoides; 15 with Sézary syndrome) and 12 of 15 SS patients had a response in the blood, including seven complete responses in a phase I/II trial (NCT01226472) for patients with previously treated PTCL and CTCL (Duvic et al. 2010). In addition, the normal Treg elimination effect of mogamulizumab in peripheral blood of the patients is reported in these clinical trials. In a phase II clinical trial of mogamulizumab for recurrent ATLL patients (NCT00920790), a significant reduction of CD4+/CD25+/FOXP3+ cells in peripheral blood was observed, and they were not recovered at 4 months even after the completion of protocol treatment (Fig. 17.5) (Ishida et al. 2012). Additionally, the induction of human T-lymphotropic virus type I (HTLV-1) Tax-specific cytotoxic T lymphocytes were observed in some patients receiving mogamulizumab (Suzuki et al. 2012; Masaki et al. 2013). Also, in NCT00920790, a reduction of Tregs in

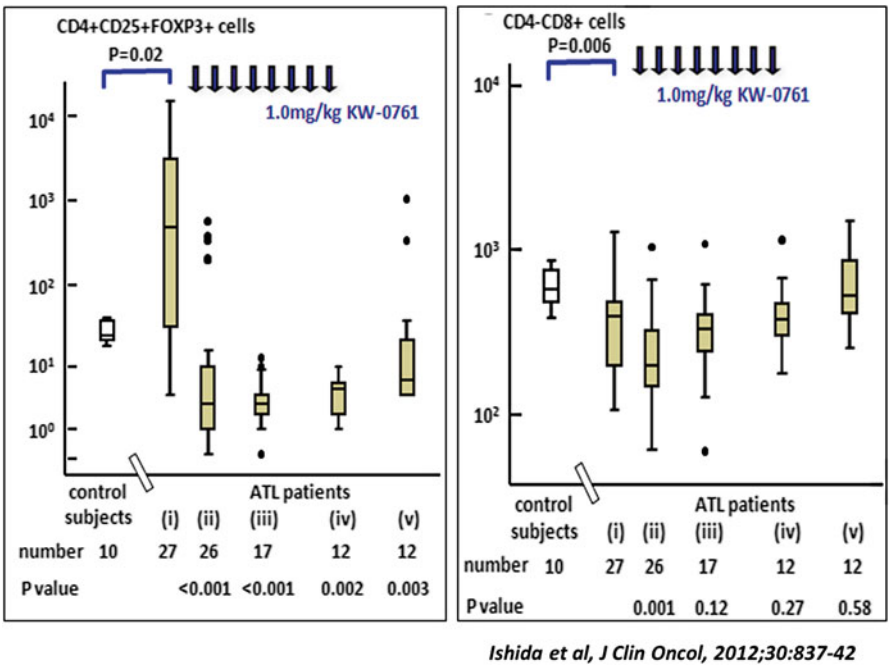

Fig. 17.5 Delay of regulatory T cell (Treg) (CD4+/CD25+/FOXP3+) recovery in phase II study of mogamulizumab for adult T cell leukemia/lymphoma (ATLL) patients. Differences between the patients' values before KW-0761 treatment and those of the controls are indicated as a *P* value (Mann-Whitney *U*-test) in the graph. The number of samples used for analysis at each point is indicated below the graph. The differences between before and each point after KW-0761 treatment [(i) just before the first KW-0761 infusion, (ii) just before the second infusion, (iii) just before the fifth infusion, (iv) 1 week after the eighth infusion, and (v) 4 months after the eighth infusion] are indicated as a *P* value (Wilcoxon signed-rank test) below the graph. Recovery of the number of Treg(CD4+/CD25+/FOXP3+) was clearly delayed rather than that of CD4–/CD8+ cells. Treg(CD4+/CD25+/FOXP3+) number is not recovered to normal range at even 4 months after the eighth infusion of 1 mg/kg once a week

most CTCL patients and a subsequent increase in NK numbers and function in some patients are reported (Xiao et al. 2011). These observations suggested that Treg reduction led to restoration of the tumor-specific immunity, resulting in long-term remissions. Thus, the humanized mAb to CCR4, mogamulizumab, is expected as novel agent not only for ATLL but also for various types of cancer patients (Fig. 17.6).

We must simultaneously be alert to the dangers of depleting CCR4 positive Tregs, which possibly lead to the immune-related AEs. In fact, skin rashes, which could be immune-related AEs, were observed frequently (63 %) in our phase II study (Ishida et al. 2012). Furthermore, during only the first 4 months after mogamulizumab went on sale, nine skin-related severe AEs, including four cases of

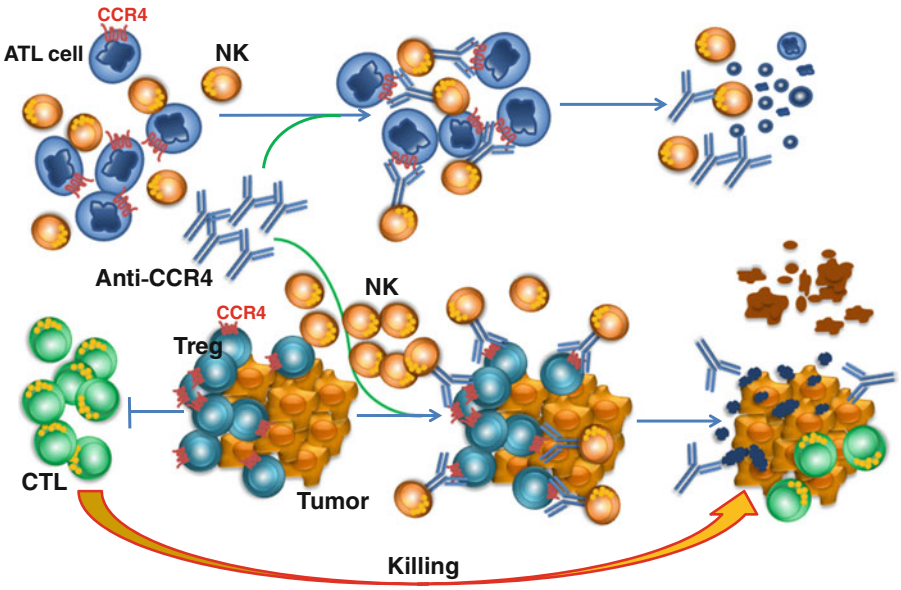

Fig. 17.6 Mogamulizumab induces the killing of adult T cell leukemia/lymphoma (ATLL) cells and normal regulatory T cells (Tregs) and might enhance tumor immunity. CC chemokine receptor 4 (CCR4) is expressed by ATLL cells and normal Tregs so mogamulizumab can bind to the surface of both cell types. Natural killer (NK) cells recognize mogamulizumab by interactions between the Fc portion of mogamulizumab and FcγIII on NK cells, and kills them by antibody-dependent cell cytotoxicity (ADCC). In ATLL treatment, mogamulizumab can kill ATLL cells directly. In contrast, in solid tumors that do not express CCR4, tumor immunity is enhanced by the elimination of Tregs infiltrating into the tumor site by mogamulizumab. Tumor-specific CTLs that are exhausted in tumor sites, caused by Tregs, show restored functions by the elimination of Tregs, and can kill tumor cells

Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) were reported, with one SJS/TEN fatality. Therefore, it is an urgent issue to identify which factors determine the severity of immune-related skin disorders associated with mogamulizumab treatment (Ishida et al. 2013). In the patient with SJS, skin biopsy revealed marked liquefaction, degeneration, and perivascular inflammation with dominant CD8-positive cells but almost a complete lack of FOXP3-positive cells, and at SJS onset the proportion of cells in the Treg subset was further reduced. Although these observations suggest Treg reduction is a cause of severe immune reactions in the skin, the reduction of the Treg subset was not specific to the SJS case, but is commonly observed in ATLL patients. This may explain why Th2 reduction by mogamulizumab results in a Th1/Th2 balance alteration as well as Treg reduction. Further investigation is necessary to establish the safest and most effective

treatment strategies for mogamulizumab in ATLL patients and other types of cancer, to maximize benefit and minimize risk.

As the skin and intestine are the main locations in which the immune system is in direct contact with the outside environment, it would be reasonable that immunotherapy targeting immune checkpoints or Tregs causes adverse reactions at these locations. In ATLL patients, ATLL cells are more likely to migrate to the skin because they express CCR4 that is necessary for homing to the skin, and therefore it is presumed that specific immune reactions most likely occur in this location. Thus, when mogamulizumab is infused to ATLL patients, NK cells are activated by ADCC in the skin, release inflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and kill normal Treg and Th2 cells causing a shift in immune balance towards an active state. Therefore, immune AEs in the skin are often observed in ATLL patients receiving mogamulizumab. Why mogamulizumab does not cause AEs in the intestine in ATLL patients despite many CCR4-expressing Tregs and Th2 cells homing to the intestine is unknown. An explanation might be that effector Tregs reappear soon after depletion by mogamulizumab because naïve Tregs not expressing CCR4 are not affected by mogamulizumab.

17.6 Mogamulizumab Combination Therapy with Other Drugs

Combination therapy of ipilimumab with dacarbazine prolonged the overall survival of patients with previously untreated metastatic melanoma compared with those receiving dacarbazine plus placebo, with higher survival rates at 1 year (47.3 vs. 36.3 %), 2 years (28.5 vs. 17.9 %), and 3 years (20.8 vs. 12.2 %) (Robert et al. 2011). This combination synergy indicates that reactivation of tumor immunity enhances the drug therapy effect, indicating the potential for immunotherapy as a base for tumor therapy. Recently, a phase I clinical trial of nivolumab combined with ipilimumab was conducted, and 53 % of patients had an objective response, all with a tumor reduction of 80 % or more at the maximum doses (Wolchok et al. 2013). Since February 2013, we have been conducting a phase Ia/Ib clinical trial of mogamulizumab by monotherapy for standard therapy-resistant multiple malignant tumors [Health and Labor Sciences Research Grants, Research on Applying Health Technology (NCT01929486)] (Fig. 17.7). Because mogamulizumab treatment is considered to reactivate tumor immunity at the tumor site by eliminating Tregs as discussed, the clinical trial of combination therapy with mogamulizumab and standard therapy such as surgery, irradiation, and drug agents, as well as tumor antigen vaccines and monoclonal antibodies to immune checkpoint molecules will be necessary for the development of novel therapies against malignant tumors.

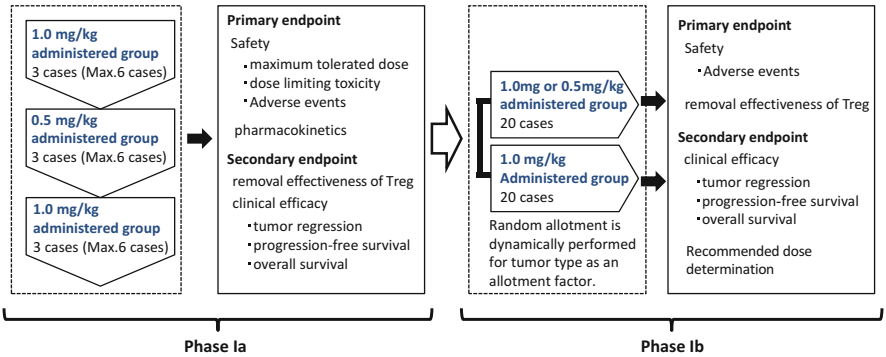

Fig. 17.7 Protocol of phase Ia/Ib clinical trial of mogamulizumab for solid tumors. *Phase Ia*: dose-rising study of mogamulizumab is performed among advanced/recurrence solid tumors (lung cancer, an esophagus cancer, stomach cancer, an ovarian cancer, and a malignant melanoma) with standard treatment resistance. Mogamulizumab is prescribed once for the patient a total of eight times every week, and the dosage to which tolerability is accepted is determined from examination of safety and pharmacokinetics. It starts from three 0.1 mg/kg medication groups (a maximum of six cases), and shifts to three 0.5 mg/kg medication groups (a maximum of six cases) and three 1.0 mg/kg medication groups (a maximum of six examples). Safety is evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0, Japanese translation Japan Clinical Oncology Group (JCOG)/Japanese Society of Clinical Oncology JSCO version. Moreover, regulatory T cell (Treg) removal effect is also examined. *Phase Ib*: mogamulizumab is prescribed once for the patient a total of eight times every week among the medication groups in which tolerability was checked for in a high dose (20 cases) and 0.1 mg/kg (20 cases) group [just one group of 0.1 mg/kg when maximum tolerated dose (MTD) was 0.5 mg/kg in phase Ia]. Treg removal effect in peripheral blood and safety are made into a primary endpoint, and clinical efficacy is estimated in a secondary endpoint. Clinical efficacy is evaluated by RECIST (Response Evaluation Criteria In Solid Tumors), and progression-free survival and overall survival are also observed in either phase Ia or Ib

17.7 Conclusions

To date, immunotherapy has not been successful in the treatment of solid tumors. However, successful clinical results using ipilimumab, BMS-936558, and BMS-936559 indicate immunotherapy may be useful for tumor therapy following surgery, radiotherapy, and chemotherapy. Mogamulizumab will also develop immunotherapy like these monoclonal antibodies to checkpoint molecules. Although the regulation of immune checkpoint molecules and Tregs are recognized as key points for immunotherapy, their integration with former research progress outcomes, including the enhancement of specific tumor immunity by tumor antigen vaccine and the enhancement of antigen presentation by stimulation of innate immunity, are required for further progression and establishment of tumor immunotherapy.

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Chapter 18
Adjuvant Immunotherapy for Cancer: From Basic Research to Clinical Bench

Misako Matsumoto, Masahiro Azuma, and Tsukasa Seya

Abstract Microbial infection is usually accompanied by inflammation and tissue wounding. These local symptoms are dependent on a host's immune response rather than the direct cell damage by invading microbes. A microbe usually contains pattern molecules that enhance the host immune response. Clinical tests medicating cancer patients with this microbial component (namely adjuvant) were tried prior to the twentieth century, when the outline of the immune system had not yet been unveiled molecularly. These tests showed effect in some patients, though accompanied by side effects. The response to adjuvants was also useful for understanding the host–defense mechanism, later elucidated as innate immunity. Adjuvant is a general term for molecules that imitate a host immune-stimulator of microbes that activates pattern recognition receptors in the innate system to enhance interferons/cytokines and activation of the cellular immune system. This chapter outlines the history of adjuvant and the updated understanding by which an adjuvant is rationally introduced into anti-tumor immunity.

Keywords Pattern recognition • Innate immunity • Dendritic cells • Macrophages • Interferons • Pam2 lipopeptides • polyI:C • Immunotherapy

Abbreviations

AP1	Activator protein-1
BCG	Bacillus Calmette-Guérin (a live attenuated strain of *Mycobacterium bovis*)
BCG-CWS	BCG–cell wall skeleton
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dsRNA	Double-stranded RNA
GMP	Good Manufacturing Practice

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IFN	Interferon
IFNAR1	Interferon (α and β) receptor 1
IL	Interleukin
INAM	IRF3-dependent natural killer-activating molecule
IRF	Interferon regulatory factor
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation-associated protein-5
MDP	Muramyl dipeptide
NK	Natural killer
Pam2CS	*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-*S*-serine
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglycan, polyI:C, polyinosinic–polycytidylic acid
RIG-I	Retinoic acid-inducible gene-I
TAA	Tumor-associated antigen
TDM	Trahalose dimycolate
TICAM-1	Toll–IL-1 receptor domain-containing adaptor molecule 1
TLR	Toll-like receptor

18.1 Introduction

Immunity represents the response to a non-self antigen in a host (Medzhitov and Janeway 1997). An antigen may come from an altered form of endogenous proteins as well as most substances in foreign cells other than self (Kono and Rock 2008). Antigenicity originates in proteins or cells being recognized as 'not self' by the host irrespective of the origin. The mammalian immune system is highly sophisticated, consisting of innate and acquired arms (Koyasu et al. 2010), the latter of which acquired the huge diversity of non-self recognition by gene recombination. The recognition repertoire differs among species. An antigen epitope can be formed, if the immunological system responds to it, even if it is unlikely to be an antigen only with a delicate one amino acid variation. In contrast, whatever strange or unusual molecule there is, it would not be an antigen if an immunological system does not detect it with significant avidity. Additionally, due to nucleotide polymorphisms, each human individual possesses their own immunological system peculiar to them. In earlier times when ethics regulation was unrestricted, microbial rough products were used for patients as adjuvant for a long time with insufficient theoretical back-up or toxicity testing (Coley 1891). Those data became a precious key to unveiling the response to microbes, i.e., the human innate immune system, which has been established recently (Honda et al. 2006; Desmet and Ishii 2012). In this chapter we summarize the history of research regarding adjuvants, show the general function of the adjuvant in responding to the antigen to the human immune system, and discover the principal by which anti-tumor immunity is evoked in response to a cancer antigen.

18.2 Discovery of Innate Immunity as an Essential for Anti-tumor Immunity

The circumstances preceding lymphocyte research are related to immunology. Experience of the "nothing two-times phenomenon" (i.e. a child once infected with the measles is safe from having it a second time) of infection reflects the essence of immunological memory. The acquired immune system of the lymphocytes, terminally differentiated cells, was identified first, and the regulation system of soluble mediators, such as IFNs, cytokines and chemokines, has been continuously discovered at the molecular level. Almost 15 years have passed since it was shown that they interlock under the control of innate immunity (Medzhitov and Janeway 1997). In this sense, the history of immunology research accomplished development by 'putting the cart before the horse'. The field in which immunology research is fused to the larger view of life science has been developed because it was urgently required.

Phenomenal understanding of innate immunity goes back to an early stage. Janeway and coworkers showed that signal transfer of innate immunity started according to activation of a specific molecular receptor, and advocated the concept of cooperation of the innate and acquired immune system (Medzhitov and Janeway 1997). In a broad sense of the immune system, self–non-self recognition is a critical issue and there is both the non-self of innate immunity (patterns) and non-self of acquired immunity (antigen). Given that the construction of the immune system is decoded from phylogeny, it is presumed that the system has progressed by PAMP sensing (by myeloid cells), natural lymphocyte response, and thymus-dependent lymphocyte response (Koyasu et al. 2010). In mammals, the latter is fundamentally influenced by the system made previously (Spits et al. 2013), which is followed by activation of the next system. As a result of the struggle between a parasite and an immunological system, infection is realized if the parasite is predominant. Therefore, the immune response battles with the extreme. However, since cancer occurs in somatic cell mutations and epigenetic events, it usually acquires immune resistance through immune surveillance and becomes established (Pardoll 2002). Administration of tumor antigen peptides to patients by themselves for lymphocyte activation does not cure cancer in principle (Rosenberg et al. 2004). It should be noted that a non-self response is completed only after the help of inflammation secondary to pattern recognition (Seya et al. 2003). The method by which infection controls cancer progression is in fact the reflection of this principle. However, infection is also a double-edged sword promoting tumor formation, and its role in innate pattern sensing, i.e., innate immunity, and its relationship to the cancer microenvironment has not been resolved.

Since this chapter deals with the particulars of an anticancer adjuvant, it discusses two TLRs, TLR2 and TLR3, in relation to cancer immunotherapy (Fig. 18.1). Although the monumental work by Japan's Dr. Tokunaga (Tokunaga et al. 1999) first described CpG DNA as a TLR9 adjuvant, research into DNA sensors appears too premature to discuss regarding immunotherapy (Desmet and Ishii 2012). The reader is referred to previous publications for more information on that topic (Desmet and Ishii 2012).

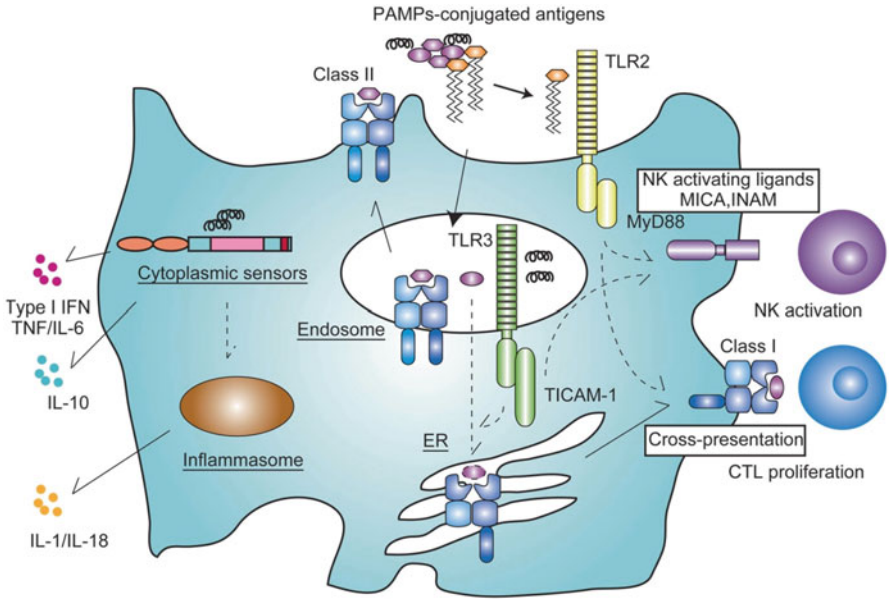

Fig. 18.1 Maturation of the antigen-presenting DCs by an adjuvant. Myeloid cells appear to represent the response of innate immunity. Pattern molecules of microbes and denatured self cells behaving like non-self material, activate DCs, and tissue-resident macrophages. IFNs and cytokines are liberated from these cells in response to adjuvant. In addition, activation of DCs result in enhancement of the expression of the co-stimulators and major histocompatibility complex class I, leading to activation of a cellular effectors (NK, CD4 T cells, CTL, etc.). Here we especially outline the roles of the TLR2–MyD88 and TLR3–TICAM-1 pathways in activation of the cellular immunity followed by activation of innate immunity by the adjuvant for cancer immunotherapy

18.3 Bacterial Adjuvants for Therapeutics Revisited

The phenomenon of infection-inducing tumor regression was known experientially in the eighteenth century. William Coley performed a trial in which bacteria was injected into humans (cancer patients) with the goal of treatment in the 1880s, when the mechanism of understanding lymphocyte antigen recognition had not yet been defined. In 1891, the therapeutic results of the bacterial therapy (mixed bacillus of *Streptococcus pyogenes* and *Serratia marcescens*) for cancer patients was published (Coley 1891). In today's terms, it is so-called 'adjuvant immunotherapy', although problems, such as TAAs being dependent on the patient (van der Bruggen et al. 1991), were undetermined. This method prescribed simultaneous administration of bacterial foreign antigens and PAMP, in contemporary terms, to the cancer patients, and was activating acquired immunity from innate immunity. Although anti-tumor CTL induction was not proven, it was effective in some cases. However, there were severe side effects and cases of death. Since the Federal Institute for Drugs and

Medical Devices of Germany was not able to reproduce this vaccine's validity, it was withdrawn in 1990. In the future, attenuating bacteria incorporating the genes of TAAs will be a device that is sufficient as a therapeutic.

Since the 1960s in Japan, cancer immunotherapy has been developed using BCG of the tubercular vaccine (Yasumoto et al. 1979; Kodama et al. 2009). These examples are BCG-CWS by Yamamura and Azuma (Azuma et al. 1974) and Maruyama vaccine (Maruyama 1971), both of which were crude extracts of BCG bacillus. There are case reports of effective monotherapy use of BCG-CWS administration by Kodama and coworkers at the Osaka Medical Center for Cancer (Kodama et al. 2009). The BCG treatment raised the rate of complete remission to 70 % or more in a bladder cancer (transitional–epithelial cancer) as a standard treatment (Lamm et al. 1991). BCG-CWS was reported as an agonist of TLR2/4 by Tsuji et al. in 1999 (Tsuji et al. 2000). BCG-CWS consists of mycolic acid moieties with TDM, arabinogalactan, and peptidoglycan (containing MDP) (Seya et al. 2002). Ishikawa et al. and Schoenen et al. have reported that TDM is a ligand for a C-type lectin receptor, Mincle (Ishikawa et al. 2009; Schoenen et al. 2010). The peptidoglycan portion contains MDP, which is reported to be the active center for TLR2 agonistic activity, but, unexpectedly, synthesized MDP failed to contribute to TLR2 agonistic activity (Uehori et al. 2005). The MDP portion therefore will not act as a center of the activity of BCG-CWS. MDP will hardly confer effective anticancer activity to the patients unless the other region of PGN is attached. To date, the whole PGN has been impossible to synthesize chemically. Sumitomo Pharmaceuticals recently provided highly refined BCG-CWS under GMP regulation and called SMP105, which showed anticancer activity in mouse tumor-implant models (Murata 2008). There was, however, inconsistency, and tumor regression depended upon MyD88 but was independent of TLR2. These results suggest that multifarious or complicated immune responses induced by the mixture of BCG origin were more advantageous to the anticancer immune effect than the single isolated or synthesized compound. The reader is referred to the literature for further details of the mechanism of activation of TLR2 in the immune system by PAMP for immunotherapy (Vacchelli et al. 2013).

18.4 Viral Adjuvants for Therapeutics Revisited

It has been known for a long time that viral infection affects the prognosis of patients with cancer. Since discovery of IFN (later defined as type I IFN) in 1957, study of this mechanism has been underway (Taniguchi et al. 1980). From the 1960s, Levy and others started anti-tumor immunotherapy that prescribes a virus product (dsRNA analog polyI:C) as an inducing factor of IFN for treatment with tumor-implant mice (Levy et al. 1969). PolyI:C has been used since the 1970s for patients with cancer. In 1979, IFN-β complementary DNA was cloned and applied to cancer patients for the purpose of direct immuno-cytokine therapy (Levine et al. 1979). Although these reagents contributed to tumor regression in a number of cancers in

humans, severe side effects were exhibited (fever, arthralgia, myalgia, neurological manifestation, endotoxin-like shock, etc.). Therefore, it is not yet clinically applied, except for in some restricted types of cancers (metastatic renal cancer, hairy cell leukemia). Another problem was its expense.

By that time, it had become clear that IFN is expressed by stimulation with dsRNA of the virus. Nevertheless, polyI:C as a dsRNA analog was used because the sequence-specific synthesis of long RNA was impossible throughout the studies of dsRNA medication. Indeed, polyI:C was greeted at the beginning in the 1970s with great expectation as a promising medicine including cancer immunotherapy. However, it being stated as "untolerable" (proof of being impossible to administer) increased as a clinical trial progressed. In order to reduce the side effects of polyI:C, Levy and coworkers mixed the RNase inhibitor LC (polyL-Lysine and methylcellulose) into polyI:C, and used it in a clinical trial. However, the maximal therapeutic dose was still very low, and patients died before an effective amount was determined. An example of the severe side effects was renal failure causing death at a dose of 27 mg/m^2, which represented a sign of cytokine storm. PolyI:CLC (Hiltonol®) and polyI:C_{12}U (Ampligen®) are still used for immunotherapy. The cytoplasmic RIG-I/MDA5 pathway may be activated by these reagents, although both are provisionally called TLR3 agonists.

Although IFN-inducing activity was proved in human volunteers using 1.6 mg subcutaneous administration, in this low dose cellular effectors appear to be hardly guided (Caskey et al. 2011). The phase II examination was still ongoing in 2012.

18.5 Innate Immune Adjuvants Revisited

Although Coley and Levy started their anticancer treatments wholly independently, both reached the TLR adjuvant therapy by PAMP. Moreover, both obtained suggestions from the observation of infection-mediated tumor regression and developed adjuvant therapies for cancer by artificial administration of PAMP to patients. Because adjuvants were regarded as evil principles in the cancer therapy field as compared with surgery, radiation, and chemotherapy, common agreement to this therapy could not be reached easily. Yet, scientific understanding and reconsideration of cancer immunity are called for nowadays based on the fact that eligible patients are extremely restricted by the sole peptide vaccine therapy. As the 2000s have progressed, an understanding of the importance of an adjuvant has gradually permeated in association with the knowledge of DC maturation in the field of cancer immunotherapy (Fig. 18.1). If we made an effort to faithfully reproduce the results of the clinical study of Coley and Levy as science, the adjuvant must have been effective against cancer. In accordance with this view, the practical research regarding TLR2 and TLR3 as adjuvants (for human use) is discussed below.

18.6 TLR2 as an Adjuvant Receptor

The development of crystal structure analysis made it clear that TLR generally formed a dimer to recognize specific PAMPs. TLR2 discriminates and recognizes a detailed difference of PAMP structure by heterodimerization (Kang et al. 2009). TLR2/TLR6 recognize Pam2 lipopeptides and TLR2/TLR1 recognize Pam3 lipopeptides (Fig. 18.2). Seemingly, PGN will be recognized by the TLR2 dimer, although it remains unproven since chemical synthesis of PGN is unfeasible.

Pam2 and Pam3 differentially activate TLR2 signaling, which should reflect the difference in the TLR2 signal caused by PGN according to the distinct TLR complex. Pam2 lipopeptides have various peptide sequences depending upon bacterial species, and bacteria express various Pam2 lipopeptides on the cell membrane. This will be one of the reasons why activation of TLR2 changes with bacterial species. Although the diversity of the lipopeptide sequences in which amino acids from the second amino acid (the first is always Cys) differ among bacterial species is natural

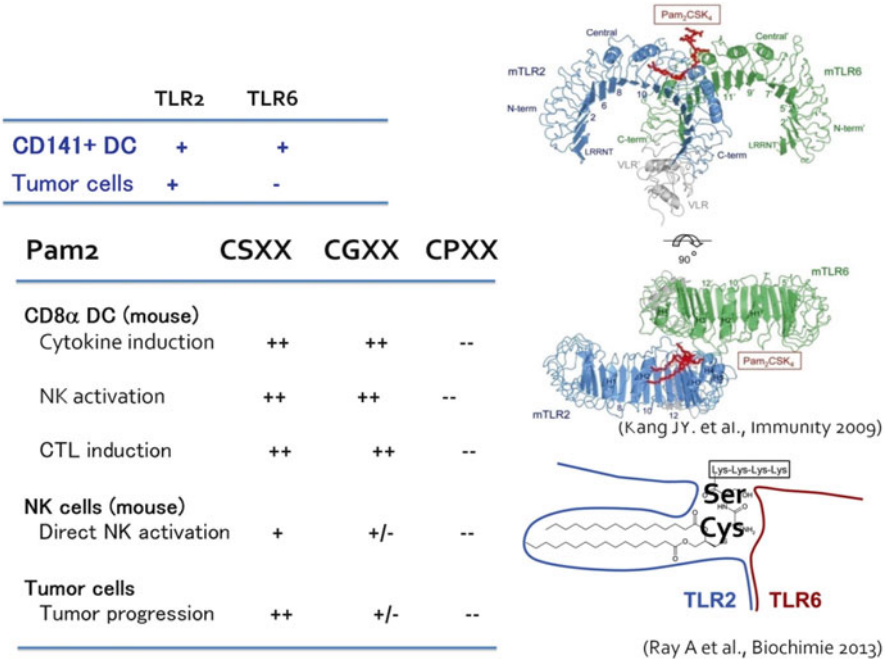

Fig. 18.2 The crystal structure of TLR2 and its ligand recognition. Kang et al. (2009) first submitted the crystal structures of TLR2/TLR6 and TLR2/TLR1 in the presence of binding ligands. Taken together with the result of cristalized protein analysis (Ray et al. 2013), the amino acid sequences of the peptide portion of conventional TLR2 lipopeptide will regulate the response of the macrophage in a tumor (TLR2-positive), and CD141+ DCs (TLR2-/TLR6-positive). Development of TLR2 adjuvant which does not have tumor proliferation activity may be feasible based on this concept

providence, it will be a problem of the Pam2/3 adjuvants if those differences have any physiological implication (Azuma et al. 2010).

Unexpected findings were obtained when we compared the functions of DC TLR2 with tumor-infiltrating macrophage TLR2 (Maruyama et al. 2015). NK activation and IL-10 secretion are dependent by DC TLR2 on a peptide sequence of the Pam2 lipopeptide (Azuma et al. 2010; Yamazaki et al. 2011). TLR2 in tumor-resident macrophages is engaged in tumor progression and metastasis (Kim et al. 2009). Macrophages in tumors are a unique target for therapy. That is, unlike TLR2 of other healthy cells, the TLR2/TLR6 pocket for antigen-presenting DCs recognizes Pam2-Cys-Ser-X-X and Pam2-Cys-Gly-X-X, and induces cytokine production and NK activation (Fig. 18.2), but the activity disappeared in Cys-Pro and Cys-Leu substitution (Azuma et al. 2010). On the other hand, the peptide sequence recognized by TLR2 of tumor cells and NK cells is only Pam2-Cys-Ser-X-X. In the case of Pam2 lipopeptides, NK activation usually initiates cell damage other than IFN-γ and CD69 upregulation by NK cells (Azuma et al. 2010). Type I IFN is never induced by TLR2/MyD88 signal.

If Pam2 lipopeptides with a peptide sequence of the DC type (Cys-Gly) are used for medication, based on the above knowledge, this TLR2 agonist can selectively activate DCs and then cellular immunity without involving tumor implication (Takeda et al. 2015). In the case of tumor-associated macrophage-dependent ligands, such as versican, TLR2 exerts protumor activity (Kim et al. 2009). DC-type Pam2-Cys-Gly-X-X simultaneously cross-primes CTL in a MyD88-dependent manner (Takeda et al. 2015). Although Pam2/3 lipopeptides allow myeloid cells to liberate a lot of cytokines, NK cell activation ability is not as strong in these TLR2 agonists as in TLR3 agonists, which should be attributable to the functional properties of Pam2 that block NK cell activation (Yamazaki et al. 2012). DCs mature via the p38 MAPK pathway in this context through activation of AP1 transcription family proteins (Takeda et al. 2015). Future research is needed to determine what kind of molecular mechanism lies in the MyD88 downstream that participates in DC maturation and CTL cross-priming.

18.7 TLR3 as an Adjuvant Receptor

It has become clear that TLR3 forms dimers from crystal structure analysis, and recognizes dsRNA in a dimeric form (Bell et al. 2006). The extracellular domain (consisting of LRRs (Leucine rich repeat)) of TLR3 recognizes a dsRNA structure at two places, and even if the other spacer RNA part is an imperfect stem (with many mismatches), TLR3 recognizes dsRNA without any functional problem (Tatematsu et al. 2013). The structure of the 5′/3′ end of RNA does not participate in TLR3's RNA recognition either. That is, TLR3 recognizes the dsRNA structural motif (Fig. 18.3), which is not recognized by RIG-I/MDA5. Based on these background data (Matsumoto and Seya 2008), it is expected that a TLR3-specific ligand will not have cytokine toxicity, which is totally due to RIG-I/MDA5 signaling, in

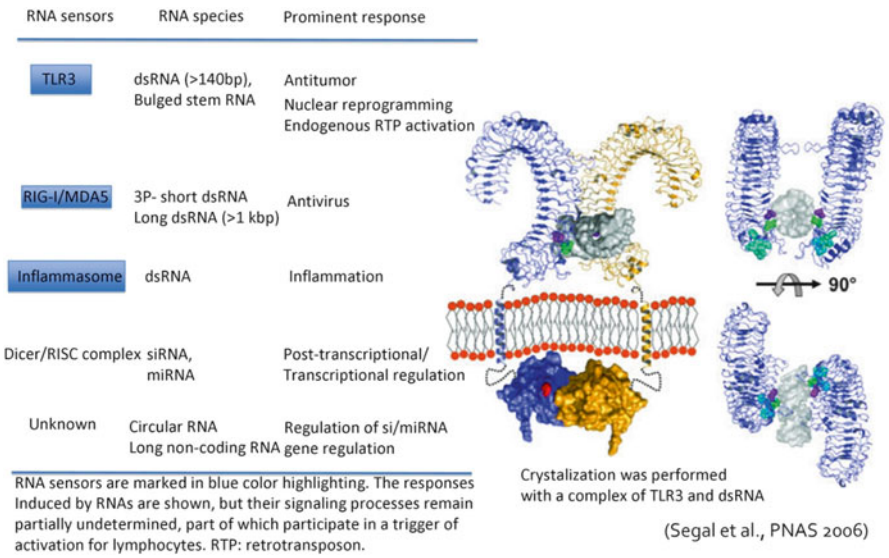

Fig. 18.3 The crystal structure of TLR3 and its ligand recognition. The crystal structure of the complex of TLR3 and dsRNA was presented by Liu et al. 2008. The figure shows the dsRNA-binding site on the C terminus region of the extracellular TLR3 protein. Another dsRNA-binding site is situated in the N terminus of TLR3. Neither dimer formation nor TICAM-1 signal transmission is spoiled, although DC TLR3 receives enzymatic processing in the endosome. The physiological significance of the limited proteolysis of TLR3 by endosomal cathepsins remains unknown. Although the TLR3 pathway in DCs performs strong driving of cellular effectors, other unresolved activities remain to be defined, such as promotion of nuclear reprogramming and activation of endogenous retrotransposon in epithelial cells and fibroblasts, which was described recently (*left figure*). Here we compare these reported RNA responses of TLR3 with those of other RNA sensors

systemic administration (Seya et al. 2013). A design completed from in vitro synthetic compounds of RNA and the TLR3-specific agonist will be perfect for chemical synthesis (Matsumoto et al. 2015).

Systemic administration of polyI:C resulted in cytokinemia in mice bearing implant tumors. In those models, however, TLR3 plays a pivotal role in tumor regression. PolyI:C activates NK cells (Akazawa et al. 2007), the mechanism of which is thought to be by induction of INAM via the IRF-3 and IFNAR1 pathways (Ebihara et al. 2010; Kasamatsu et al. 2014). Importantly, early induction of IRF-3 activation by polyI:C is indispensable for introduction of CD8α DC (mouse) and CD141–DC-mediated cross-presentation (Azuma et al. 2012, 2015; Kasamatsu et al. 2015). Strikingly, polyI:C converts tumor-supporting macrophages to tumor-suppressing ones (Shime et al. 2012, 2014). In addition, polyI:C expresses direct anti-tumor activity by inducing apoptosis/necroptosis to some types of tumors (Paone et al. 2008; Conforti et al. 2010; Seya et al. 2012; Takemura et al. 2015).

If systemic administration of this TLR3-agonistic RNA is carried out and only TLR3 response is reproduced in humans, the importance of TLR3 in the evoking of cellular immune response by RNA can be proved (Galluzzi et al. 2012). Although we have reproduced this theory in mice, a future study is needed in humans (since GMP material is underdeveloped). The reason that old clinical trials of polyI:C were intolerable to patients is thought to be the toxicity by high cytokinemia. Since TLR3 is expressed only in myeloid and epithelial cells (Matsumoto et al. 2003), TLR3 barely causes systemic IFN induction like RIG-I/MDA5. The lessons from the nature of such TLR3 agonists may enable us to develop a new therapeutics involving NK/CTL inducers with few side effects for humans (Fig. 18.3). That is, the cellular immune effecters can be induced by a synthetic RNA in vivo, independent of cytokinemia (Matsumoto et al. 2015). Anti-tumor immunotherapy will be attained by extensive administration of this TLR3 agonist.

18.8 Concluding Remarks

For many years, cancer immunotherapies involved the injection of bacteria, viruses, or their components into the body, as it was revealed that infections resulted in a large increase in immunocompetence. Adjuvants, which are substances that enhance the systemic immune response, have been widely used for immunotherapies; however, the molecular mechanism causing immune activation remained unknown until recently. Findings on TLRs and DC maturation in innate immunity and mechanisms whereby inflammation are associated with tumor progression allow us to hypothesize that the receptors that recognize adjuvants are pattern recognition receptors of the innate immune system, including the TLRs. In this chapter we have summarized recent advances on synthetic adjuvants for TLR2 and TLR3. We anticipate that the use of immunotherapy together with surgery, radiation, and chemotherapy will result in great improvements in 5-year survival in patients with cancer.

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Chapter 19
Immunological Regulation of Human Cancer Stem Cells/Cancer-Initiating Cells

Noriyuki Sato, Yoshihiko Hirohashi, Tomohide Tsukahara, Takayuki Kanaseki, Vitaly Kochin, Yasuaki Tamura, and Toshihiko Torigoe

Abstract Recent cancer biology research has suggested that cancer stem cells/cancer-initiating cells (CSCs/CICs) exist in most tumor tissues. They show self-renewal, differentiation, and, most importantly, tumorigenic potential. Thus, the possibility of regulating CSCs/CICs immunologically is highly intriguing. Our recent studies suggest that CSC/CIC-specific antigens, designated 'cancer testis stem cell antigens', are generally highly immunogenic, and should be useful for immunotherapeutic as well as prophylactic treatment of cancers. Since the clonal expansion of peripheral T lymphocytes is limited *in vivo*, immunological targeting of CSCs/CICs by cytotoxic T lymphocyte is thought to be the most rational and efficient approach for the treatment of and prophylaxis against cancers. In particular, cancer testis stem cell antigens might act as primordial tumor antigens in the basic immune surveillance mechanisms against tumors.

Keywords Cancer stem cells/cancer-initiating cells (CSCs/CICs) • Cancer stem cell antigen • Testis antigen • Immune response • Immune surveillance

19.1 Introduction

Recent human tumor immunology studies suggest that human tumor vaccines can evoke clinical and immunological anti-tumor responses in patients (Hirohashi et al. 2002, 2009a; Idenoue et al. 2005; Kitamura et al. 2013a; Kameshima et al. 2011, 2013; Kawaguchi et al. 2012; Miyazaki et al. 2011; Tsukahara et al. 2009;

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Sato et al. 2009; Honma et al. 2009a; Kobayashi et al. 2009; Tsuruma et al. 2008). However, the tumor regression effect is generally low, and it is obvious that the current vaccination protocols are insufficient to provide substantial clinical outcomes. This means that other more potent immunotherapeutic protocols, including novel tumor antigens, are required in cancer immunotherapy. To develop such efficient and sophisticated protocols we need to conduct basic immunological and biological studies of cancers.

Recent cancer biology research has clarified that cancer stem cells/cancer-initiating cells (CSCs/CICs) indeed exist in all tumor tissues (Sato et al. 2009; Hirohashi et al. 2009a; Yasuda et al. 2013). They show self-renewal, differentiation, and, most importantly, tumorigenic potential. Thus, the possibility of regulating CSCs/CICs immunologically is highly intriguing.

In this chapter, we introduce our recent analysis of human CSC/CIC-associated antigens that are expressed specifically in these particular cells. Some of these antigens are commonly expressed in tumors derived from developmental germ layers, namely those with endodermal, mesodermal, and ectodermal origins. In terms of the expression in normal tissues, most are expressed only in testis, and hence categorized as cancer testis antigens. We propose that these potential antigens be newly categorized as novel 'cancer testis stem cell antigens', and also suggest that these exclusive antigens might be able to act as 'general, fundamental, and primordial' tumor antigens in the immune surveillance of tumors by the host.

19.2 Human Cancer Stem Cells (CSCs), Cancer-Initiating Cells (CICs), and Expression of Specific Tumor Antigens

Tumors are generally considered to be composed of heterogeneous cells (Yasuda et al. 2013; Kuroda et al. 2013; Michifuri et al. 2012, 2013; Nishida et al. 2012, 2013, Kitamura et al. 2013b; Hirohashi et al. 2009b, 2010, 2012; Nishizawa et al. 2012; Mori et al. 2012; Nakatsugawa et al. 2011; Kano et al. 2011; Inoda et al. 2009, 2011a, b; Murase et al. 2009; Honma et al. 2009b). Actually, this is true for many tumors from the patho-histological point of view. Cellular and structural observations are usually not uniform but rather diverse and heterogeneous. For some tumors, epithelial (cancer-like) as well as sarcoma-like components exist within the same specimens, and cells isolated from each part can produce tumors in immune-deficient animals such as the non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mouse. This means that common progenitor, cancer stem-like, or at least CICs must exist.

Indeed, the search for and identification of these CSCs/CICs has been ongoing for a long time, since these cells, if present, could be considered to be ideal molecular and immunological target cells. To this end, as illustrated in Fig. 19.1, more than 20 years ago we investigated cell transformation-associated antigens that were induced and newly expressed by activated ras oncogene introduction into murine fetal fibroblasts (Yagihashi et al. 1988; Konno et al. 1989; Cho et al. 1991; Kanki et al. 2000). One of these was CD44, and many recent studies support our findings that this molecule can act as a cell surface marker of CSCs/CICs (Cho et al. 1991;

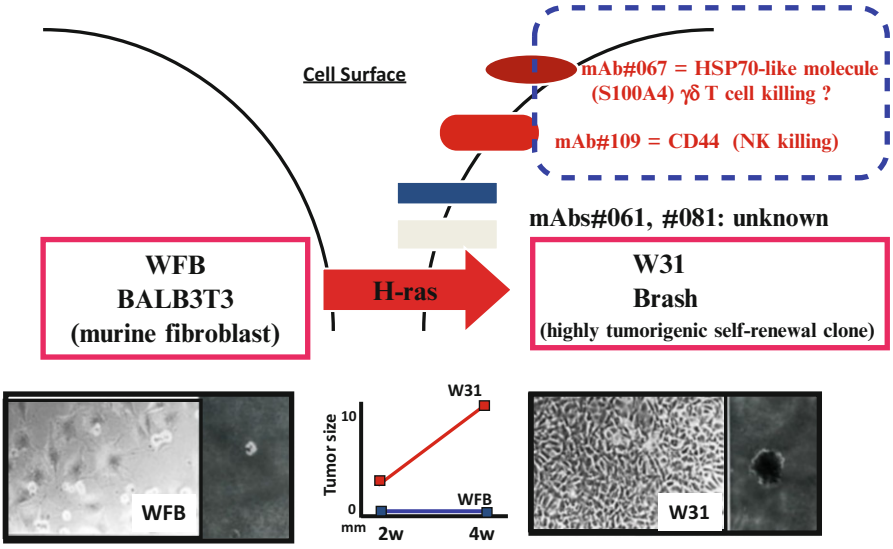

Fig. 19.1 Search for transformation-associated cell surface molecules induced by activated oncogene introduction into murine fetal fibroblasts, rat WFB cells and mouse BALB3T3 cells

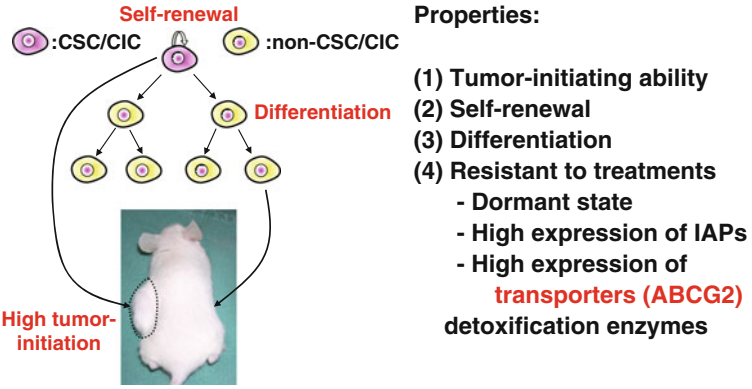

Fig. 19.2 Properties of cancer stem cells/cancer-initiating cells (CSCs/CICs)

Kanki et al. 2000). However, CD44 is obviously not adequate for molecular and immunological targeting of CSCs/CICs since it is widely expressed in vascular endothelial cells as well as different subsets of many inflammatory cells.

Meanwhile, as shown in Fig. 19.2, recent progress in regenerative stem cell research has also clarified substantial aspects of the biological features and similarities of CSCs/CICs. It is now known that regenerative stem cells and CSCs/CICs may commonly express various sets of stress-related molecular chaperones, transporters, and detoxification enzymes.

The above characteristics can be utilized for isolating CSCs/CICs. For example, the adenosine triphosphate (ATP)-binding transporter, ABCG2, is highly expressed

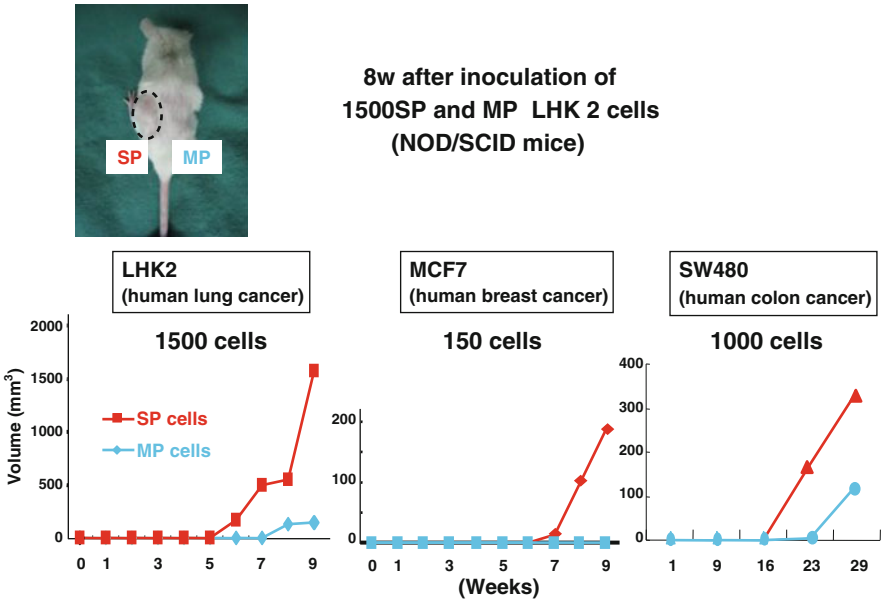

Fig. 19.3 In vivo tumorigenicity assays of non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mouse cells fractionated from the side population (SP) and main population (MP) in cell sorter analysis

in CSCs/CICs. Therefore, a dye such as Hoechst 33342 cannot stain CSCs/CICs because of the high expression of the transporter, and consequently these unstained cells can be efficiently isolated using a cell sorter (Yasuda et al. 2013; Murase et al. 2009). As shown in Fig. 19.3, cells in the unstained side population (SP) fractions of human cancer cell lines such as LHK2 lung cancer, MCF7 breast cancer, and SW480 colon cancer, obviously have higher capabilities in in vivo tumorigenicity assays using NOD-SCID mice than those in the stained main population (MP) fractions. This is also true in isolation of SP fraction cells from primary colon, lung, and ovarian cancer tissues. Taken together, our experiments indicate that CSCs/CICs are enriched or purified at least to a certain degree in the SP fraction. This fact allows subsequent studies of gene profiles that are expressed specifically in cells of the SP fraction which contain purified CSCs/CICs.

19.3 Classification of Molecules Expressed in Human CSCs/ CICs

By DNA microarray, we have analyzed genes of cells from the SP and MP fractions in a total of 60 human cancer lines, including tumors of endodermal (colon, lung, oral cancers), mesodermal (osteosarcomas, myxofibrosarcoma, bladder, prostate, ovarian cancers) and ectodermal (breast cancer and melanomas) origin, as well as

primary cancers such as colon, lung, and gynecological malignancies. Consequently, we can summarize and classify genes that are preferentially or specifically expressed in human CSCs/CICs as shown in Box 19.1.

Box 19.1: Classification of Molecules Expressed in Human CSCs/CICs

1. Cancer Testis Stem Antigens
   - Or7c1
   - DNAJB8
   - Others [SMCP, UBQLN3 (ubiquilin 3), etc.]
2. Stem Cell Related (iPS-Related)
   - Sox-2
   - Oct3/4
   - Klf4
3. Stress Response-Related
   - ABC transporters (ABCB1, ABCG2, etc.)
   - Detoxification enzymes (ALDH1, aldoketo reductase)
   - Heat shock proteins (DNAJB8, HSP70A1B, HSP70B9)
4. Epithelial–Mesenchymal-related
   - Snail
   - Twist
   - CDH2
5. Others

As suggested in previous works, CSCs/CICs express stem cell- and iPS (induced pluripotent stem) cell-related molecules such as SOX2, Oct3/4, and KIF4. It is interesting that CCSs/CICs also express diverse sets of cellular stress response-related genes. These include ATP-binding cassette (ABC) transporters (typically ABCB1 and ABCG2), detoxification enzymes [aldehyde dehydrogenase (ALDH) 1 and aldoketo reductase], and heat shock proteins (DNAJB8, HSP70A1B, and HSP70B9) (Nishizawa et al. 2012; Torigoe et al. 2009, 2013; Takahashi et al. 2012). We also found that molecules related to epithelial–mesenchymal transition (Snail, Twist, and CDH2) were highly expressed in the SP fraction containing purified CSCs/CICs, when compared with the MP fraction, which contains non-CSCs/CICs.

These gene profiles are indeed very interesting for the biological nature and characteristics of CSCs/CICs. These genes are also expressed in many non-cancerous, regenerative tissue-derived stem cells, suggesting that stem cells, irrespective of their non-cancerous and cancerous aspects, maintain their intrinsic biological actions using these molecules.

Meanwhile, in terms of gene expression that occurs specifically in CSCs/CICs, but not in normal and regenerative stem cells such as mesenchymal stem cells, we found that a substantial number of testis-specific genes were expressed in CSCs/CICs. These include Or7c1 (olfactory receptor family 7 subfamily C member 1), DNAJB8 (DnaJ Hsp40 homolog, subfamily B, member 8), and SMCP (sperm mitochondrial cysteine rich protein), which are highly expressed only in CSCs/CICs, and their expression in normal tissues is limited to testicular cells when analyzed by quantitative polymerase chain reaction (PCR) and immunohistochemistry, indicating that these molecules are typical "cancer testis antigens" (Yamada et al. 2013; Morita et al. 2013).

Interestingly, our subsequent studies also tentatively identified other novel cancer testis antigens that were expressed selectively in CSCs/CICs. The expression of these molecules seems to be regulated via epigenetic mechanisms, particularly DNA methylation and demethylation.

We have also recently clarified that HLA-A24-restricted natural antigenic peptides (NAPs) are specifically expressed in colonic CSCs/CICs. This was determined by comparing cell lines established from SP and MP single clones, by analyzing NAP with mass spectrometry. Interestingly, some of the parental proteins of NAP are cancer testis antigens. Such NAPs are highly immunogenic, as assessed using patients' peripheral T cells, suggesting the possibility that they could work as potent immunotherapeutic and prophylactic cancer vaccines targeting CSCs/CIs.

19.4 Immunogenic Profiles of Human CSC/CIC-Specific Molecules

At first there was a question whether CSCs/CICs were susceptible to the action of cytotoxic T lymphocytes (CTLs), although CSCs/CICs are generally resistant to chemotherapeutic treatment. To solve this issue, we successfully established an autologous pair of CSC/CIC and CTL clones from soft-part sarcomas. Our data clearly indicated that autologous CTL could lyse CSCs/CICs in the context of HLA class I molecules (Kano et al. 2011). This was the first-ever demonstration that human autologous CTLs were cytotoxic to CSCs/CICs. In other words, the T cell immune system can fulfill the immunological surveillance against CSCs/CICs.

We also investigated whether CSC/CIC antigens such as Or7c1 and DNAJB8 were immunologically potent as well. Their relative capabilities to induce CTL response were compared with non-CSCs/CICs or overlapping shared (common) tumor antigens, i.e., those expressed both in CSCs/CICs and non-CSCs/CICs. To this end, in subsequent experiments we used survivin and Cep55 molecules as shared (common) tumor antigens.

In Fig. 19.4, the data comparing the relative immunoprophylactic potencies to inhibit the in vivo tumorigenic incidence of challenged murine renal tumor cells are shown. In this syngeneic murine immunoprophylactic tumor model, we designed

DNA vaccines encoding both DNAJB8 CSC/CIC-specific antigen and shared (common) survivin molecule. When DNA vaccinations were administered twice before tumor implantation, the results clearly indicated that the relative potency to inhibit tumor development was much stronger with CSC/CIC-specific DNAJB8 than with shared survivin (Nishizawa et al. 2012).

This was true when we studied the relative capability to inhibit already established human tumor growth in a CTL adoptive transfer model using NOD-SCID mice. In this experiment we established CTL clones that reacted with the HLA-A24-restricted Or7c1 CSC/CIC-specific peptide antigen. To compare the action of CTL we also established CTL clones specifically reacting with the Cep55 shared tumor antigen. Our immunotherapeutic results also clearly demonstrated that adoptive transfer of an Or7c1-specific CTL clone into mice was more potent in inhibiting human SW480 colon tumor growth than Cep55-specific CTL.

As such, CSC/CIC antigens have an obvious advantage in comparison with shared (common) or non-CSC/CIC-specific tumor antigens. Results of both immunoprophylactic and immunotherapeutic experiments strongly suggest that CSC/CIC-specific tumor antigens have theoretical and practical predominance in immunological cancer treatment.

19.5 Cancer Testis Stem Cell Antigens as the New Immunological Milestone

Our recent studies also confirmed that another diverse set of testis-specific antigens, with at least ten or more different molecules, is preferentially expressed in CSCs/CICs (Yasuda et al. 2013; Kuroda et al. 2013; Michifuri et al. 2013; Nishida et al. 2012, 2013; Kitamura et al. 2013b; Michifuri et al. 2012; Hirohashi et al. 2009b, 2010, 2012; Nishizawa et al. 2012; Mori et al. 2012; Nakatsugawa et al. 2011; Kano et al. 2011; Inoda et al. 2009, 2011a, b; Murase et al. 2009; Honma et al. 2009b; Yamada et al. 2013; Morita et al. 2013). Functionally they are suggested to play roles in sperm meiosis, maturation, mobility, and fertilization. Some molecules may also be directly involved in the epigenetic mechanism of spermatogenesis. Thus, it is becoming clear that genes pivotal to maintaining the nature of CSCs/CICs may substantially overlap genes that are indispensable for spermatogenesis and fertilization. Biologically, this is highly intriguing, and here we can propose a novel paradigm for the biological linkage between maintenance of the CSC/CIC phenotype and the molecular mechanisms in spermatogenesis and fertilization.

Meanwhile, from an immunological point of view, most of these molecules are immunogenic. They can induce CTL responses in cancer patients in vitro and in vivo. The Or7c1 molecule is now being used in clinical trials as a novel CSC/CIC cancer vaccine. The DNJB8 vaccine works as a potent immunoprophylactic cancer vaccine in animal tumor models. As shown in Fig. 19.4, its immunogenic potency is actually higher than those of conventional and common tumor (shared) antigens

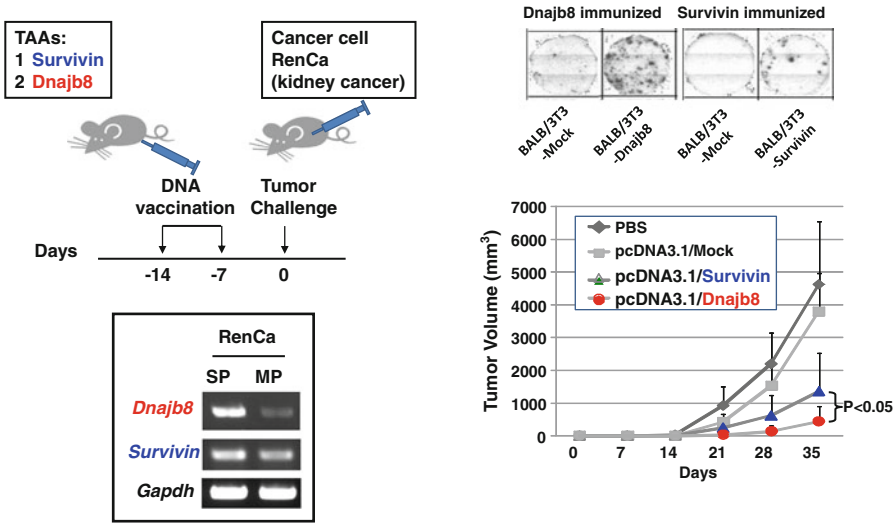

Fig. 19.4 Comparison of relative immunoprophylactic potentials to inhibit the in vivo tumor incidence of challenged murine renal cells in mice prevaccinated twice with DNA vaccines containing cancer stem cell/cancer-initiating cell (CSC/CIC)-specific DNAJB8 and survivin shared between CSCs/CICs and non-CSCs/CICs

that are equally expressed both in CSCs/CICs and non-CSCs/CICs. Thus, we propose a new category of tumor antigens, called 'cancer testis stem cell antigens' as distinct immunobiologic entities.

It is also intriguing that epidemiological data in many countries have shown relatively high mortality in male cancer patients but low mortality in female cancer patients. However, it is not known if the actual CTL response against 'cancer testis stem cell antigens' is higher in female patients than in males, so we cannot draw any definitive conclusion as to whether the male immune system is in fact more tolerant of these antigens. Nevertheless, it is possible that 'cancer testis stem cell antigens' could play a more pivotal role in female cancer patients than in male cancer patients.

19.6 Summary

Taken together, as illustrated in Fig. 19.5, our studies suggest that CSC/CIC-specific antigens, designated 'cancer testis stem cell antigens', are generally highly immunogenic, and should be useful for immunotherapeutic as well as prophylactic treatment of cancers. Since the clonal expansion of peripheral T lymphocytes is limited in vivo, immunological targeting of CSCs/CICs by CTL is thought to be the most rational and efficient approach for the treatment of and prophylaxis against cancers. More importantly, cancer testis stem cell antigens might act as "general,

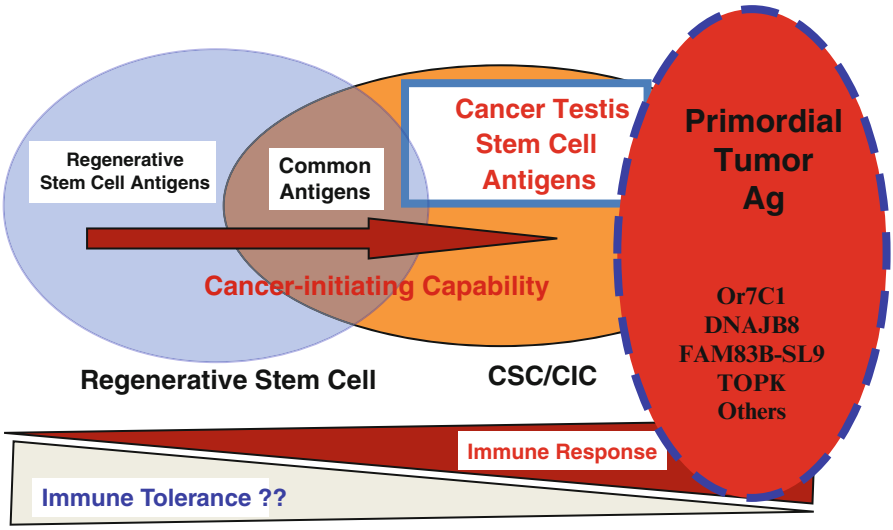

Fig. 19.5 Cancer testis stem cell antigens may create a new immunological category in the immune surveillance mechanism against human cancer stem cells/cancer-initiating cells (CSCs/CICs)

fundamental and primordial" tumor antigens in the basic immune surveillance mechanisms against tumors, particularly CSCs/CICs, by the host immune system (Sato et al. 2009; Hirohashi et al. 2009a; Nakatsugawa et al. 2011).

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Chapter 20
Development of Personalized Combination Cancer Immunotherapy Based on the Patients' Immune Status

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Abstract Cancer immunotherapies, particularly immune-checkpoint blockade and T cell-based adoptive cell therapy, have recently been recognized as cancer treatments that show strong and durable responses even for advanced cancer patients with multiple metastases. The major issues in the development of cancer immunotherapy are the identification of biomarkers to distinguish responders and non-responders, and the improvement of efficacy of immunotherapy possibly by combination with appropriate immune interventions targeting different key regulating points in the anti-tumor immune responses. Interestingly, pretreatment T cell immune status varies among cancer patients, and appears to correlate with responses to various cancer treatments including surgery, chemotherapy, radiation therapy, and immunotherapy. Balance of anti-tumor T cell induction pathway and immunosuppressive pathway, which are regulated by characteristics of both cancer cells and patients' immune reactivity, may define the differential immune status among cancer patients along with environmental factors such as intestinal microbiota. The analysis of such mechanisms may lead to the identification of immune biomarkers and immune-modulating strategies for combination immunotherapies. Further research on human cancer immunopathology will lead to the development of effective personalized combination immunotherapies based on the evaluation of cancer patients' immune status.

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20.1 Introduction

During the long process of cancer development, various interactions occur between cancer cells and stromal cells, including various immune cells and other stromal cells (e.g., fibroblasts, mesenchymal stem cells, endothelial cells). T cells and natural killer (NK) cells have the ability to eliminate cancer cells (immune surveillance), while innate immune cells such as macrophages and mast cells and other stromal cells often promote proliferation and invasion of cancer cells. However, cancer cells that have lost highly immunogenic tumor antigens and acquired immune-suppressive and resistant ability through their genetic instability, selectively grow (immune editing). Cancer diagnosed in the clinic has evaded the immune system (tumor escape) (Schreiber et al. 2011). In tumor-associated microenvironments including tumors and sentinel lymph nodes, tumor-promoting and immunosuppressive conditions are established. There are various immunosuppressive mechanisms in which various cell components, including cancer cells and their subpopulations [e.g., cancer-initiating cells, epithelial–mesenchymal transition (EMT) cells], immune cells [e.g., cytotoxic T lymphocytes (CTL), T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), regulatory dendritic cells (DCregs)], and other stromal cells (e.g., cancer-associated fibroblasts, mesenchymal stem cells, endothelial cells) (Yaguchi et al. 2011; Kawakami et al. 2013a; Kudo-Saito et al. 2009) are involved, and they are different among cancer patients. Therefore, personalized, strong immune interventions, which may accompany immunological adverse effects, may be required to eliminate such cancer cells.

20.2 Recent Progress of Cancer Immunotherapy

Recently, two immunological strategies, immune-checkpoint blockade [e.g., programmed death protein (PD)-1/PD ligand (PD-L)1, CTL-associated protein (CTLA)-4] and T cell-based adoptive cell therapy (ACT) [e.g., tumor-infiltrating T-cells (TILs), T-cell receptor (TCR)/chimeric antigen receptor (CAR)-transduced T cells], have shown strong and durable anti-tumor clinical effects even for patients with advanced cancer with multiple metastases in various cancer types [e.g., melanoma, kidney cancer, lung cancer, bladder cancer, head and neck cancer, synovial sarcoma, B cell malignancies such as lymphoma, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL)] (Topalian et al. 2013; Brahmer et al. 2012; Hodi et al. 2010; Rosenberg et al. 2011; Robbins et al. 2011; Kalos et al. 2011). In melanoma, durable responses by these immunotherapies are impressive, in contrast to relatively short response by molecular target therapy (e.g., BRAF inhibitors) (Wagle et al. 2011) despite their early good tumor reduction ability.

However, these immunotherapies do not work for all cancer types, nor all patients. Therefore, two major issues remain to be solved for cancer immunotherapy: (1) identification of immune biomarkers to select the patients who are likely to respond to immunotherapy; and (2) improvement of efficacy of immunotherapies possibly by combination of interventions targeting different key regulating points in the anti-tumor immune responses.

20.3 Differential Immune Status Among Cancer Patients

Interestingly, pretreatment immune status varies among cancer patients and appears to correlate with patients' prognosis after various cancer treatments For example, high infiltrations of $CD3^+$ and $CD8^+$ T cells in tumors are correlated with favorable prognosis after cancer therapies in patients with various cancers (e.g., colon cancer, lung cancer, ovarian cancer, head and neck cancer, melanoma) (Fridman et al. 2012). In Japanese patients with colon cancer, we found high infiltrations of $CD3^+$ T cells, $CD8^+$ T cells, $FOXP3^+$ (forkhead box $P3^+$) T cells, and $CD20^+$ B cells in tumor tissues are correlated with favorable prognosis after curative surgery. Currently, by international collaboration (Immunoscore Validation Task Force) initiated by Jerome Galon (Research Director, INSERM) and organized by the SITC (Society for Immunotherapy of Cancer), the significance of TILs (Immunoscore: $CD3^+$ and $CD8^+$ T cells) in colon cancer is being evaluated in large numbers of patients using customized analysis software. The immunoscore may change future clinical management of patients by improving the prognostic diagnostic method, which includes evaluation of immune status (Galon et al. 2014). In addition to immune status in tumor microenvironments, immune status of peripheral blood also varies among cancer patients. For example, cytokine levels are different among patients, and appear to correlate with responses to various cancer treatments. We have shown that patients with high pretreatment plasma interleukin (IL)-6 and IL-8 have a poor prognosis after various cancer vaccines [e.g., peptide vaccine, dendritic cell (DC) vaccine].

20.4 Mechanisms for the Differential T Cell Immune Status Among Cancer Patients

One of the major questions in human cancer immunology is the mechanism for the differences in T cell immune status among cancer patients. Our previous studies suggested that the immune status may be defined by the balance of positive and negative immune pathways in the anti-tumor immune responses (Fig. 20.1). Recent studies have indicated that environmental factors, including intestinal microbiota which regulate not only intestinal mucosal immunity but also systemic immune responses (e.g., anti-tumor immunity), diet/obesity which promotes chronic

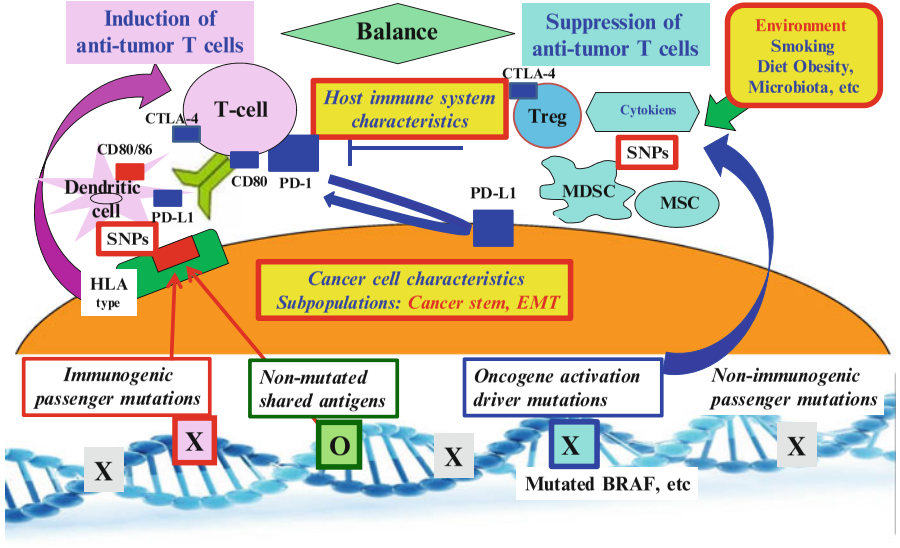

Fig. 20.1 Mechanisms for the differential immune status among cancer patients. Balance of the anti-tumor T cell induction pathway and immunosuppressive pathway along with environmental factors may define the differences in T cell immune status among cancer patients. Passenger mutations may induce anti-tumor T cells, while driver mutations promote immunosuppressive cascades

inflammation, and smoking which introduces various chemicals, affect the immune system.

In the positive anti-tumor immune pathway, various factors are involved in its strength. We have previously shown that TILs recognize mutated peptides derived from genome DNA mutations (Robbins et al. 1996; Kawakami et al. 2001) of cancer cells. It was recently shown that melanoma TILs frequently recognize such mutated peptides derived from passenger mutations by systemic exome DNA sequencing, and that these immunogenic mutations are different among patients (Robbins et al. 2013), indicating a possible necessity for personalized therapies. We have reported that immune responses occur to the frameshift mutations caused by DNA slippage through dysfunction of DNA mismatch repair enzymes such as MLH1 (mutL homolog 1) in MSI (microsatellite instability)$^+$ colon cancer (Ishikawa et al. 2003). Interestingly, the patients with MSI$^+$ colon cancer have high CD8$^+$ T cell infiltration in tumors, and relatively good prognosis after surgery even though they show a histologically malignant appearance. Therefore, differences in DNA mutation status in cancer cells may result in the different T cell responses. It was also shown that loss of production of a cytokine (IL-15) and chemokine [chemokine (C-X-C motif) ligand (CXCL) 13] through DNA deletions in cancer cells cause fewer T cell infiltrations in colon cancer (Bindea et al. 2013; Mlecnik et al. 2014). In addition to these cancer cell characteristics, difference in patients' immune reactivity partly defined by polymorphism of immune systems including HLA type, may also influence the anti-tumor T cell induction pathway.

In the negative immunosuppressive pathway, we have previously shown that simple overproduction of immunosuppressive molecules such as transforming growth factor (TGF)-β in tumor affects the immune status of tumor microenvironments (e.g., DC impairment, increase of Tregs and MDSCs in tumors and draining lymph nodes) and subsequent decrease of anti-tumor T cell induction and accumulation in tumors (Nakamura et al. 2014). In addition, we have shown that oncogene activation (e.g., driver mutations, over-expression) rather promotes multiple immunosuppressive cascades and generates immunosuppressive tumor microenvironments. Oncogene activation status is different in cancer patients, and the immunosuppressive pathway is also possibly influenced by polymorphisms in the immunosuppressive pathway. These factors may define the strength of the immunosuppressive pathway for anti-tumor T cell responses. Altogether, cancer cell characteristics (e.g., gene alterations), patients' immune reactivity (e.g., immune polymorphism), and environmental factors (e.g., microbiota, diet, smoking) may generate the differential T cell immune status among cancer patients.

20.5 Immune Biomarkers for Cancer Therapies

There are differences in immune status, including T cells, B cells, and various myeloid cells, in cancer patients, and it may be correlated with patients' prognosis after cancer therapies, indicating that these are immune biomarkers for various cancer therapies, including immunotherapies.

Recently PD-1/PD-L1 blockade therapy [e.g., anti-PD-1 antibody (Ab) anti-PD-L1 Ab] showed durable clinical responses in patients with advanced cancers, including melanoma, kidney cancer, lung cancer, bladder cancer, ovarian cancer, head and neck cancer, and Hodgkin disease. In the search for immune biomarkers for the PD-1/PD-L1 blockade therapy, PD-L1 expression on cancer cells as well as $CD8^+$ T cell infiltrations in tumors were found to correlate with good response to anti-PD-1 Ab treatment (Topalian et al. 2013). Interestingly, PD-L1 on cancer cells appears to be induced by interferon (IFN)-γ produced by tumor-infiltrating $CD8^+$ T cells in melanoma (Taube et al. 2014). Combination of both PD-L1 expression and $CD8^+$ T cell tumor infiltration appears to be a better immune biomarker to predict response to the PD-1 Ab therapy in melanoma. T cells induced against mutated peptides may reject melanoma cells after PD-1/PD-L1 blockade. Numbers of DNA mutations are much higher in UV-related melanoma and smoking-related lung cancer, which are relatively responsive cancers to the PD-1/PD-L1 blockade, suggesting again that mutated peptide-specific T cells may be involved in the PD-1/PD-L1 therapies. In this regard, MSI^+ colon cancer, which also has frequent DNA mutations, may be a good candidate for the PD-1/PD-L1 therapies. These observations indicate that pre-existing anti-tumor T cell immunity may be essential for the PD-1/PD-L1 blockade therapy. $CD8^+$ T cell infiltrations were also reported to correlate with responses to cancer vaccines and IL-2 therapy in melanoma.

T cell infiltration in tumors is also a possible immune biomarker for other cancer therapies in various cancers. It is correlated with favorable prognosis of colon cancer patients after curative surgery and is possibly a better prognostic marker than TNM (tumor, nodes, metastasis) staging criteria (Pagès et al. 2009). There is also a report showing that T cell infiltrations in liver metastases of colon cancer is correlated with chemotherapy response. Similarly, we and others have shown that T cell infiltration correlates with favorable prognosis after various cancer therapies including surgery, radiation, and chemotherapy in various cancers (e.g., lung, ovarian, and cervical cancer). In addition to TILs, plasma cytokines such as IL-6 and IL-8 are reported to be associated with responses to various cancer therapies, including chemotherapy and molecular target therapy. Therefore, immune status is a possible biomarker for various cancer therapies. Further confirmation in a large numbers of patients and consideration of inclusion of these immune biomarkers into current prognostic diagnosis such as TNM staging criteria are warranted (Fridman et al. 2012).

20.6 Combination Immunotherapy

The immune-checkpoint blockade and T cell-based ACT are not effective in all patients. It is important to increase the anti-tumor effects of these immunotherapies. In PD-1/PD-L1 therapy, both PD-L1 expression on cancer cells and $CD8^+$ T cell infiltration in tumors are correlated with good response. Two strategies should be considered: one is to increase the efficacy in the patients with both T cell infiltration

and PD-L1 expression, the other is to change the immune status of the non-responders (without T cell infiltration and PD-L1 expression) to responsive immune status (e.g., induction of anti-tumor T cells and accumulation into tumors). We have previously identified key regulation points in the anti-tumor T cell responses through the identification of human tumor antigens recognized by TILs (Kawakami et al. 1994a, b, c), and analyses of anti-tumor antigen-specific T cell responses in patients in immunotherapy clinical trials (Salgaller et al. 1995; Rosenberg et al. 1998). We proposed the development of following methods for modulation of the different key regulation points, and their appropriate combinations for effective cancer immunotherapy (Kawakami et al. 2013b) (Fig. 20.2): (1) identification of better tumor antigens such as the antigens involved in cancer cell proliferation and survival, and expressed in cancer-initiating cells [e.g., Wilm's tumor gene 1 (WT-1), SOX2, SOX6); (2) in situ tumor destruction methods to induce immune response to endogenous tumor antigens including unique mutated peptides (immunogenic cancer cell death) (e.g., chemotherapy, molecular targeted drugs, anti-tumor Ab, irradiation, cryoablation, radiofrequency ablation, oncolytic viruses); (3) enhancing methods for antigen-presenting function of DCs [e.g., adjuvants (Toll-like receptor (TLR)/stimulator of IFN genes (STING)], cytokines [IL-12, tumor necrosis factor (TNF)-α], agonistic antibodies (anti-CD40 Ab); (4) in vivo activation and expansion methods for anti-tumor T cells [e.g., cytokines (IL-2, IL-7, IL-15, IL-21), agonistic antibodies against costimulatory molecules on T cells (anti-CD134, CD137 Ab), T cell-based ACT]; (5) reversal methods for cancer-induced immunosuppression [neutralizing and depleting antibodies (e.g., TGF-β, Treg), immune-checkpoint blockers (e.g., anti-CTLA-4, anti-PD-1/PD-L1, anti-LAG3, anti-TIM3), chemotherapy, molecular-targeted drugs (e.g., tyrosine kinase inhibitors, transcription factor inhibitors)].

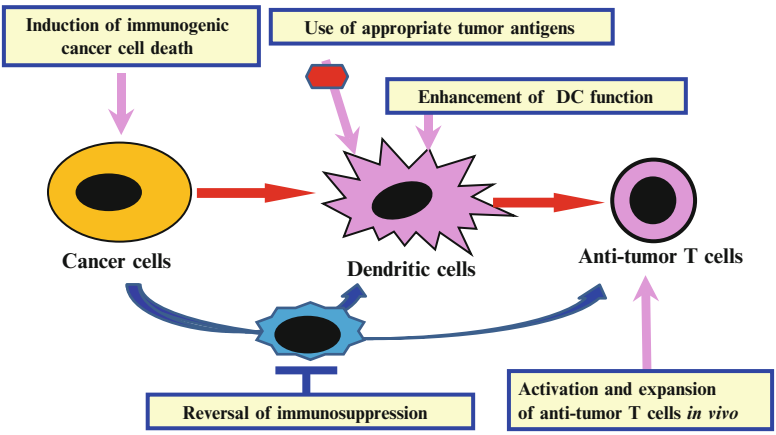

Fig. 20.2 Combinations of immune interventions targeting key regulation points in the anti-tumor T cell responses. Development of immune interventions targeting different key regulation points in the anti-tumor immune responses and their appropriate combination is important to develop effective cancer immunotherapy

We have been working on these immune-modulating methods, including the identification of better tumor antigens (e.g., SOX6, mutant BRAF) (Ueda et al. 2004, 2010), DC-stimulating new adjuvants [e.g., *Mycobacterium bovis* Bacillus Calmette-Guérin–cell wall skeleton (BCG-CWS)] (Udagawa et al. 2006), oncolytic herpes simplex virus (HSV) (Toda et al. 2002; Ohkusu-Tsukada et al. 2011), new TLR-stimulating compounds, cultured anti-tumor T cells (e.g., anti-tumor TILs, new TCR/CAR transduced T cells), various molecular-targeted drugs which act on both cancer cells, and activating and inhibiting immune cells (Iwata-Kajihara et al. 2011; Yaguchi et al. 2012; Kudo-Saito et al. 2012; Nishio et al. 2014; Sumimoto et al. 2006). We have previously reported that mutant BRAF depletion not only inhibits cell proliferation and invasion of human melanoma cells, but also inhibits production of multiple immunosuppressive cytokines such as IL-10, IL-6, and vascular endothelial growth factor (VEGF). Inhibitory effects of human melanoma cells on DC functions such as IL-12 production and T cell-stimulatory ability was reduced by mutant BRAF depletion in melanoma cells by specific short hairpin RNAs (shRNAs) or mitogen-activated protein kinase (MAPK) signaling inhibitors such as MAPK kinase (MEK) inhibitors. Interestingly, it has recently been reported that administration of selective mutant BRAF inhibitors alone induces abundant $CD8^+$ T cell accumulation in regressing tumors, but not in progressing tumors (Wilmott et al. 2012). Administration of BRAF inhibitors appears to augment anti-tumor $CD8^+$ T cells through multiple mechanisms, including immunogenic cancer cell death to release immunogenic antigens, enhanced expression of melanoma antigens [melanocyte differentiation antigens MART-1 (melanoma antigen recognized by T cells 1), gp100], and inhibition of production of multiple immunosuppressive cytokines, while the mutant BRAF selective inhibitor had fewer T cell inhibitory effects. We have shown that STAT3 (signal transducer and activator of transcription 3) has similar properties. In addition, immunosuppressive cytokines produced by melanoma cells impair immune cells by activating STAT3 and make them immunosuppressive cells such as tolerogenic DCs and immunosuppressive macrophages. Therefore, administration of STAT3 inhibitors may also be useful for augmentation of anti-tumor T cell immunity (Iwata-Kajihara et al. 2011). We have shown that some combinations increase the efficacy of immunotherapies in mouse tumor models, including cryoablation plus intratumoral injection of TLR stimulating BCG-CWS pretreated cultured DCs (Udagawa et al. 2006) and molecular-targeted drugs plus PD-1/PD-L1 blockade.

20.7 Personalized Immunotherapies Based on the Patients' Immune Status

Immune status varies among cancer patients, and it correlates with responses to cancer therapies, including immunotherapies, indicating that personalized therapy should be exploited for cancer immunotherapy based on the analysis of individual patients' immune status (Fig. 20.3). For example, in patients with pre-existing

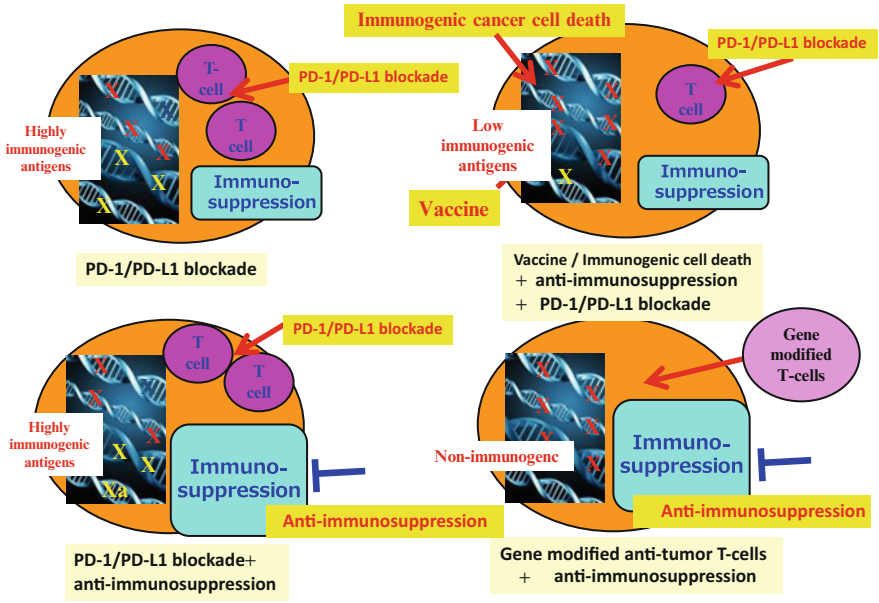

Fig. 20.3 Personalized combination immunotherapy for cancer based on the patients' immune status, which should be considered in order to develop effective cancer immunotherapy

anti-tumor T cell immunity and PD-1-/PD-L1-related immune suppression, PD-1/PD-L1 blockade is a reasonable strategy. If immune suppression mechanisms other than PD-1/PD-L1 are dominant, other methods to overcome the immunosuppression are needed even for patients with T cell immunity. For colon cancer patients, the PD-1/PD-L1 therapies have not shown good responses. This may be explained by either the fact that TILs are not tumor-specific T cells or that immunosuppression mechanisms other than PD-1/PD-L1 are dominant. For the patients with less T cell response but who still have immunogenic tumor antigens, immune-stimulating interventions [e.g., immunogenic cancer cell death to release immunogenic antigens, cancer vaccine with strong adjuvants, T cell-stimulating reagents (e.g., agonistic Ab, anti-CTLA Ab)] may promote T cell immunity and make it more responsive to the PD-1/PD-L1 blockade. For patients with almost no immunogenic antigens, ACT with artificially generated anti-tumor T cells such as TCR /CAR-transduced T cells may be applied. Altogether, personalized combination immunotherapy should be considered for future improvement in cancer immunotherapy.

20.8 Concluding Remarks

The recent progress of cancer immunotherapy, particularly immune-checkpoint blockade and T cell-based ACT, has demonstrated clear directions for the future improvement of cancer immunotherapy. To solve the major issues in cancer

immunotherapy, fine analysis of human immunology using clinical samples, particularly those obtained from patients in the immunotherapy clinical trials, is essential. In this human immunology research, various new technologies, including systems biological approaches (e.g., genome, transcriptome, proteome, metabolome), new flow cytometric technology for systematic analysis of human immune cell subsets, various immunologically humanized mice for in vivo analysis, and iPS cell technology, will be utilized. We believe these efforts will lead to development of more effective cancer immunotherapy for broader populations of patients with various cancers.

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Epilogue

The idea that utilization of host immunity for prevention and/or treatment of cancer patients has been very attractive, thus massive efforts were put into this field for more than half a century without much success. However, in 1991, the identification of the first human tumor antigens was reported by Boon's group. Thereafter, many tumor antigens were reported and the nature of these antigens have been elucidated. In the 1990s and 2000s, some of those antigens were utilized for immunization as antigenic proteins and/or peptides mostly with incomplete Freund adjuvant in clinical trials, but in general the response rate was found to be very low. These results are certainly disappointing but were not unexpected, since it is conceivable that the immunogenicity of most tumor antigens is much weaker than that of non-self microorganisms, as will be described below. More importantly, by vaccination alone it is more difficult to induce growth suppression of tumors, which had once escaped from the host immune surveillance, than to prevent infection in healthy donors. During the course of these clinical studies, fortunately rapid progress has been made in the field in basic immunology, which now can be employed for improvement of cancer immunotherapy.

By the technique of X-ray crystallization, the study of "antigen presentation" entered the age of structure analysis of MHC molecules and antigenic peptides. Since then the biology of dendritic cells has been extensively studied, together with research advances in clonal selection, development of methods for identification of antigen peptides, epitope/agretope analysis, and the genetic mechanism of the diversity of T cell receptors.

It was also argued in connection with autoimmunity that MHC class I presents an auto-antigen, and that peptides of self-origin do not always induce an intense immune response like the non-self peptide shown in the case of infection. Concepts such as cryptic antigen (non-recognizing antigen below a threshold value) and tolerance (like immune tolerance) were introduced, in part explaining immunologic unresponsiveness to self-origin antigens including tumor antigens. It needs to be studied, however, whether cancer antigens can trigger initiation of specific immunity against cancer without causing autoimmune diseases.

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It also became clear from recent studies on microbial infection that immune activation was not started even if a "non-self" antigen is present apart from other microbial factors, namely, pattern molecules. Systematic understanding of the innate immune system (TLR, NLR, RLR, CLR, and others) and immuno-regulatory systems (regulatory T cells, Treg, and checkpoint inhibitors PD-1/PDL-1) suggests that antigen uptake and the immune-activating (or -suppressing) mechanism are caused by different machinery. Surprisingly, the recent success of anti-PD-1/PD-L1 antibody therapy for patients with progressive cancer unequivocally revealed that the immune system has a potential to look out for growing tumors when it is awakened. There would be differential ways for immunological waking, which we should select depending on the nature of the tumors to be targeted for immunotherapy. I believe that vaccine immunotherapy will contribute to the future eradication of cancer.

As described above, to date a number of tumor antigens have been defined and a part of them have been or are ready for clinical use. In contrast, as for immune adjuvants, a relatively small number of them have been used, thus only a small amount of information is available. Based on new findings and concepts described above, now is the time to study the next generation of immune adjuvants and to find a suitable way to select proper adjuvants to potentiate immunogenicity of tumor antigens in the interest of inducing antitumor immunity in cancer patients.

This book overviewed cancer immunity from broad scientific fields, based on the concept that cancer is a sort of by-product of infection, inflammation, and host–immune response. In this book, authors actively involved in the field of antitumor immunity study in Japan and Taiwan were invited to contribute. We do hope that the knowledge summarized in the book will encourage readers to understand and satisfy patient's urgent wishes, that is, we believe in the establishment of immunotherapy that brings high QOL to patients with cancer.

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