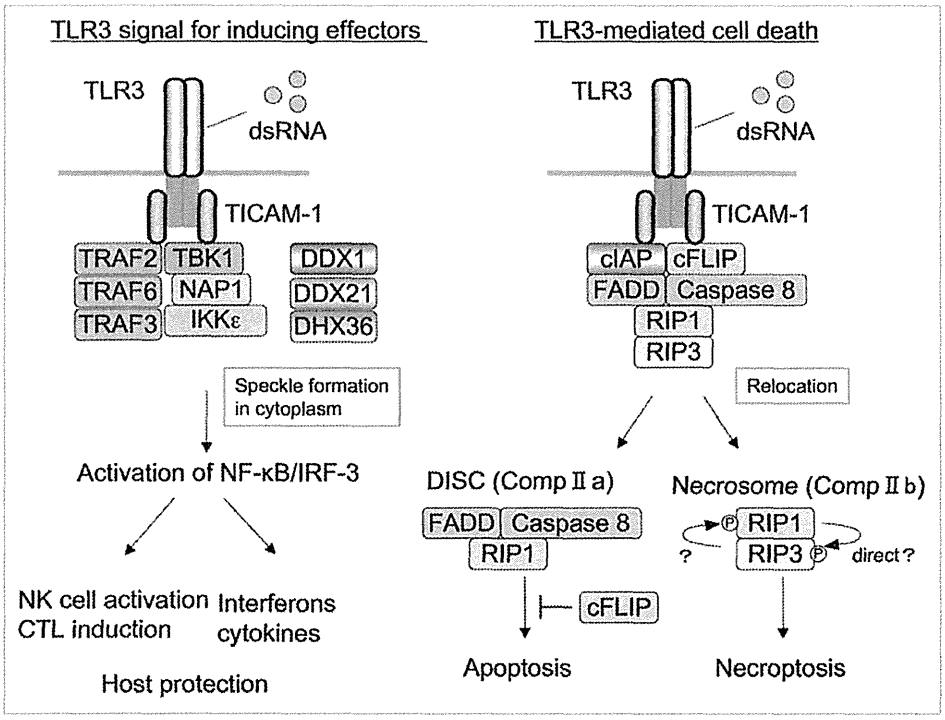

Figure 1. TLR3 signals inducing cell death or effector functions in myeloid cells. Cell survival (left panel) and cell death (right panel) signals are schematically depicted. TICAM-1 assembles in a supramolecular complex around oligomerized Toll-like receptor 3 (TLR3) in the endosome. The complex (named Speckle) then dissociates from TLR3, tranlocating to the cytoplasm. IRF-3 and NFκB are activated by Speckle, leading to their nuclear translocation and induction of Type I interferon (IFN) and inflammatory cytokines, respectively. In dendritic cells (DCs), natural killer (NK) cell-activating ligands and factors for cross-presentation are induced downstream of IRF-3/7 (left panel). In contrast, cell death signaling culminates in apoptosis and/or necrosis depending on downstream signal transducers (right panel). TLR3-dependent apoptosis has been reported in several cancer cell lines,[4] while TLR3-dependent necroptosis has been observed in mouse bone marrow-derived macrophages.[13] These events rely on RIP1/RIP3 activation, similar to those elicited upon ligation of the tumor necrosis factor α receptor 1 (TNFR1). Whether or not the translocation of the TICAM-1 complex is required for the cell death signaling, as well as the mechanisms determining either cytokine secretion or cell death, remain unknown.

dsRNA frequently induces apoptosis in infected cells, a process that in general is known as cytopathic effect.[20] TICAM-1 and RIPs, mainly RIP1, may also be involved in virus-derived necrotic cell death.[5,13] This is relatively rare compared with apoptosis since it occurs only when the viral genome encodes caspase-8 inhibitors.[19] Furthermore, this process requires viral dsRNA to be delivered from the cytosol to the endosomes (where TLR3 is situated) of infected cells. This may happen if the dsRNA is engulfed by autophagosomes, which ensure its transfer to endosomes. The possible involvement of another PRR that sense viral RNA, RIG-I/MDA5, in cell death as induced by viral infection cannot be always ruled out. TNFα can be produced downstream of the TLR3- and RIG-I-mediated RNA-sensing pathways and may induce necrotic cell death. Many RNA viruses trigger cell death,[20] but the factors determining the induction of necroptosis in virus-infected cells remain to be clarified.

DNA viruses can induce necroptosis via another mechanism, which involves the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1/ZBP1).[21] DAI is a DNA sensor[22] and directly activates RIP3 in the absence of Type I IFN induction.[21] This said, the sensing of DNA in the cytoplasm of virus-infected cells is complex, and it may be that DAI is not the only molecule linked to such a necroptotic response. It is unknown whether RIP3-mediated necroptosis can be induced even if caspase-8 is blocked upon the recognition of viral DNA by DAI or via other mechanisms.[20] In fact, this type of virus-derived necrosis has been reported with DNA viruses that encode caspase inhibitors including vaccinia virus (VV), which encodes B13R/Spi2, poxvirus, encoding CrmA, the Kaposi sarcoma-associated herpesvirus (KSHV), encoding K13 and the molluscum contagiosum virus (MCV), which encodes MC159.[20,23] Generally speaking, the mode of cell death secondary to virus infection differ as a function of viral species. The physiological role of TLR3- and DAI-mediated necroptosis should therefore be analyzed in a virus-specific fashion.

Necroptosis in Inflammation

Apoptosis plays a major role in physiological contexts, while necrosis is very common under pathological conditions.[1] Necroptosis differs from accidental necrosis in its programmed nature, and differs from apoptosis in that necroptosis often stimulates inflammation. When virus-infected cells undergo apoptosis, they are removed by phagocytosis. Viral genomes, be they either

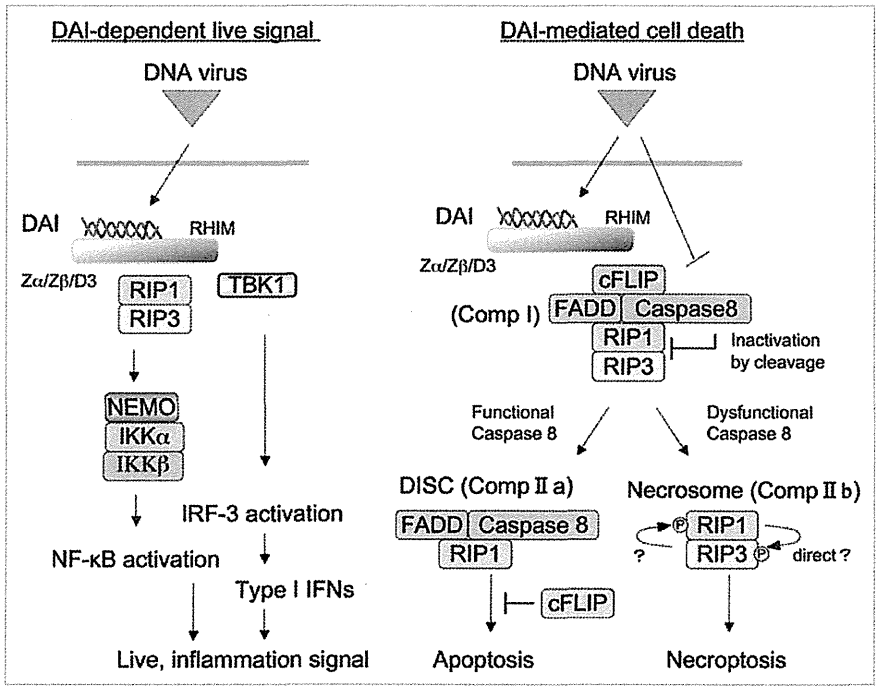

Figure 2. Necroptosis induced by the DAI pathway. Cell survival (left panel) and cell death (right panel) signals transmitted by the DNA-dependent activator of IFN-regulatory factors (DAI) are schematically depicted. Pro-survival signaling involves the activation of IRF-3 and NFκB to support antiviral responses (left panel). Type I IFNs and inflammatory cytokines are the main effectors induced by IRF-3/NFκB activation. In contrast, DAI activates RIP3 to induce necroptosis during viral infection, provided that caspases are inhibited. When viruses express caspase inhibitors, the RIP1/RIP3 necrosome plays a dominant role in the activation of cell death via necroptosis (right panel). If caspase-8 is active, RIP3 should get inactivated and apoptosis should be the dominant phenotype, though this scheme has not yet been experimentally confirmed. The mechanisms determining the choice between these two signaling pathways are unknown.

DNA- or RNA-based, are degraded in infected cells, thus being able neither to stimulate phagocytes including macrophages and DCs, nor to favor the liberation of DAMPs. In contrast, non-apoptotic cell death is accompanied by the release of DAMPs and viral products, resulting in the activation of macrophages,[13] as it occurs during chronic infection, in which viruses produce caspase inhibitors or render infected cells resistant to apoptosis.[24] A typical model of necroptosis evokes two effectors, namely, viral nucleic acids and DAMPs, to modulate immune and bystander cells of the host. In the context of necroptosis, these effectors allow for the amplification of inflammatory responses by myeloid phagocytes (mDCs and macrophages). These cells accumulate in inflammation as induced by persistent viral infection, and mediate the secondary release of cytokines and other biologically active molecules. In addition, viral factors can result in incipient inflammation, as observed in chronic infections by the hepatitis B or C virus.[24] This, in conjunction with viral nucleic acids and DAMPs, may modify the features of the infectious milieu. Further studies are needed to clarify the importance of viral nucleic acids and DAMPs in the context of virus-dependent chronic inflammation, as it may facilitate tumor progression.

Necroptosis and Oncogenesis

Accumulating evidence indicates that pro-inflammatory signals, including those following the activation of NFκB, are crucial for oncogenesis. Moreover, DAMPs have been associated with tumorigenesis as well as with antitumor immune responses.[25,26] Tumor progression is not always accompanied by viral infections, and it remains unclear whether DAMPs released from non-infected tumor cells are sufficient to support tumor growth. It has been reported that self mRNA acts as a TLR3 ligand[14] and that self DNA can stimulate host cell sensors.[22,27] Due to the uncomplete identification and functional characterization of DNA sensors and their signaling pathways, however, it is unknown whether host nucleic acids are potent inducers of inflammation as compared with viral RNA or unmethylated CpG DNA of bacterial origin. Moreover, the role of RNA sensors in the tumor microenvironment has not yet been clarified (**Table 2**).

DAMPs have recently been characterized at the molecular level[11] and representative DAMPs (**Table 1**) include HMGB1,[28] uric acid crystal,[10] S100 proteins,[29] naked actin[30,31] and heat-shock proteins (HSPs).[32] The functional features of DAMPs and the mechanisms whereby they provoke inflammation have been delineated,[11,28,29] and these studies have introduced the concept of "inflammasome" in the field of innate immunity.[33] Caspase-1 is activated upon the administration of NOD-like receptor (NLR) ligands, which include some DAMPs as well as inorganic PAMPs. Active caspase-1, together with the upregulation of the immature variants of IL-1 family proteins that ensues TLR stimulation, accelerates the robust release of IL-1β, IL-18 and IL-33.[34] There are many kinds of NLRs as well as TLRs, and the common pathways (including those centered around the adaptor ASC) can be activated by a variety of cytoplasmic DAMPs and PAMPs.[33,34] The cytoplasmic immature forms of the abovementioned cytokines are activated by limited caspase-1-mediated proteolysis, and then are secreted into the extracellular microenvironment.[34] Hence, IL-1 family proteins require two DAMPs/PAMP signals for their upregulation and activation.[35] Of note, the tumorigenic properties of asbestos and silica are in part attributable to the activation of the inflammasome, leading to the secreton of IL-1 family proteins. However, not all DAMPs operate as inflammasome activators, even in the broad sense of this term.

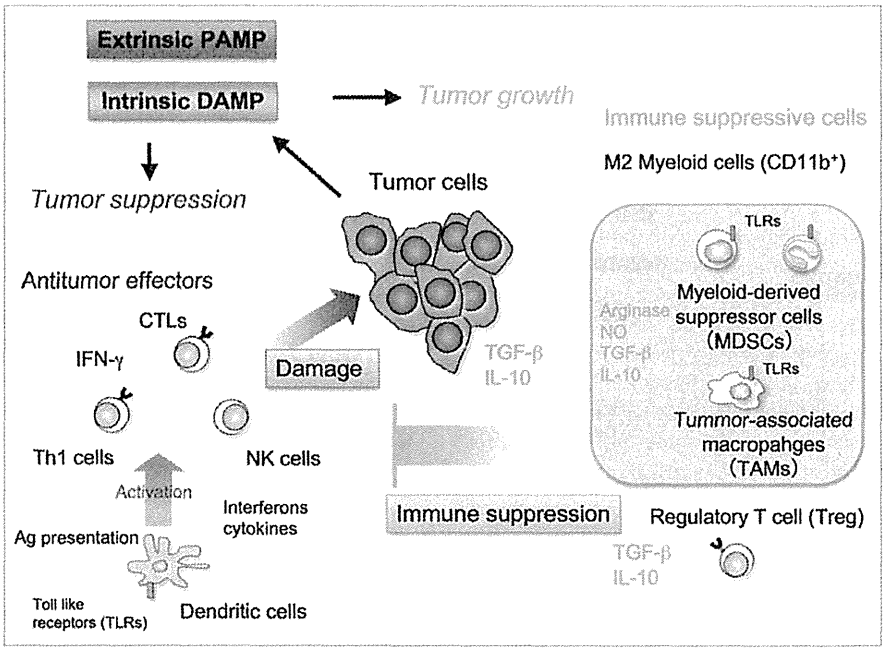

Figure 3. Inflammation provides the microenvironment for infection-related cancer. Immune cells infiltrating the tumor mass may modulate the local microenvironment upon the recognition of pathogen- or damage-associated molecular patterns (PAMP/DAMPs). Cancer cells undergoing necrosis liberate DAMPs and debris containing nucleic acids, which recruit immune cells stimulating an inflammatory response. In some cases, tumors benefit from the inflammatory response, while in other cases they regress following inflammation. The mechanisms determining this switch remain to be clarified.

Immune Response Elicited by the Phagocytosis of Dead Cells

Phagocytosis of dead cells involves not only cell clearance but also the initiation of an immune response. Dead cell antigens are rapidly presented on MHC Class II molecules after internalization by DCs, driving the recruitment and activation of various $CD4^+$ T cell subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs) (**Fig. 1**). In the presence of a second co-stimulatory signal provided by TLRs, working as an adjuvant, DCs cross-present antigens on MHC Class I molecules to induce the proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs).[36] The presentation of exogenous antigens by DCs is therefore dependent on the presence of PAMPs/DAMPs.[36] Accordingly, necrotic debris appears to result in CTL cross-priming more efficiently than apoptotic bodies. Cross-presentation is enhanced by molecules such as Type I IFN and CD40, and by immune cells including $CD4^+$ T, NK and NKT cells. Hence, the use of adjuvants to affect many cell types of the immune system other than antigen-presenting cells, and a precise evaluation of the total cross-priming activity appear to be indispensable for the development of efficient adjuvant therapies.

The TLR3/TICAM-1 axis is best known as an inducer of cross-presentation in vivo.[37] The cross-presentation activity of the TLR3 ligands polyI:C and viral dsRNA was first described by Schulz et al. in 2005.[38] While the potency of polyI:C as an adjuvant has been reported by Steinman and colleagues,[37,39] the precise identity of the DAMPs participating in cross-presentation and possessing latent cross-priming (CTL-inducing) capacities has not yet been determined.

It is known that phagocytosis induces functional changes in mDCs and macrophages (**Fig. 2**): phagocytes are skewed toward a regulatory phenotype accompanied by the production of IL-10 and TGFβ during the phagocytosis of apoptotic cell debris, even in the presence of PAMP.[40,41] This suggests that material that cannot be taken up exerts different effects on mDCs than internalizable material during their phagocytic interactions. Phagocytes undergo cytoskeletal rearrangement when they take up cell debris, involving cell adhesion molecules that accelerate the interaction between the phagocyte membrane and cell debris. The opsonization of dead cells further enhances phagocytosis as well as the induction of an immune outcome.[42] Complement-mediated opsonization of dead cells strongly alters the functional properties of mDCs and macrophages.[43] Yet, it has been impossible to discriminate apoptotic and necroptotic cells based on this.[44] Thus, the mechanisms whereby necroptotic cells initiate an immune response upon phagocytosis by mDCs and macrophages, compared with apoptotic cells, remain largely uncharacterized. Elucidating the role of necroptotic cells and DAMPs as adjuvants for NK-cell activation and antigen presentation is highly relevant for antitumor therapy. Since the phagocytosis of dead cells by mDCs usually leads to the generation of tolerogenic mDCs, additional adjuvants appear to be required for mDCs to present tumor antigens in an immunogenic fashion, leading to the induction of an effective immune response against cancer.

Termination of Inflammation

Inflammation often drives tissue repair and regeneration, and the microenvironment formed during inflammation serves a basis for assembling cells that initiate tissue development and reorganization (**Fig. 3**). The pro-inflammatory microenvironment facilitates cell growth as well as genome instability, thus being prone to the accumulation of cells with multiple mutations. Furthermore, incipient inflammation compromises the immune system so that the abnormal proliferation of transformed cells is tolerated. Thus, malignant cells build up a tissue that involves tumor-associated macrophages serving a scaffold for invasion and metastasis.[45] In this context, a region harboring DAMP-mediated persistent inflammation provides a perfect nest for tumor progression (**Fig. 3**). Therapeutics for suppressing inflammation, such as aspirin, may constitute an immune therapy irrespective of the presence of infection.[46] We surmise that two types of inflammation exist, namely tumor-supporting and tumor-suppressing, implying that inflammation is a complex phenomenon consisting of multiple distinct aspects. We have shown that some adjuvants can induce tumor-suppressing inflammation, thereby limiting tumor proliferation by DAMPs.[47] The adjuvant-induced switch of cell death/inflammation signals to an antitumor outcome is an intriguing approach for cancer therapy, particularly in view of the fact that the mechanisms of adjuvant signaling are being increasingly characterized at the molecular level.[48,49] The clarification of the role of adjuvant signaling in compromising tumor progression will lead to the discovery of non-toxic synthetic tumor-regressing molecules with potential as novel anticancer therapeutics.[50]

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Immunotherapeutic benefit of α-interferon (IFNα) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients

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Survivin, a member of the inhibitor of apoptosis protein (IAP) family containing a single baculovirus IAP repeat domain, is highly expressed in cancerous tissues but not in normal counterparts. Our group identified an HLA-A24-restricted antigenic peptide, survivin-2B80–88 (AYACNTSTL), that is recognized by CD8 + CTLs and functions as an immunogenic molecule in patients with cancers of various histological origins such as colon, breast, lung, oral, and urogenital malignancies. Subsequent clinical trials with this epitope peptide alone resulted in clinical and immunological responses. However, these were not strong enough for routine clinical use as a therapeutic cancer vaccine, and our previous study of colon cancer patients indicated that treatment with a vaccination protocol of survivin-2B80–88 plus incomplete Freund's adjuvant (IFA) and α-interferon (IFNα) conferred overt clinical improvement and enhanced the immunological responses of patients. In the current study, we further investigated whether this vaccination protocol could efficiently provide not only improved immune responses but also better clinical outcomes for advanced pancreatic cancers. Tetramer and enzyme-linked immunosorbent spot analysis data indicated that more than 50% of the patients had positive clinical and immunological responses. In contrast, assessment of treatment with IFNα only to another group of cancer patients resulted in no obvious increase in the frequency of survivin-2B80-88 peptide-specific CTLs. Taken together, our data clearly indicate that a vaccination protocol of survivin-2B80-88 plus IFA and IFNα is very effective and useful in immunotherapy for this type of poor-prognosis neoplasm. This trial was registered with the UMIN Clinical Trials Registry, no. UMIN000000905. (*Cancer Sci* 2013; 104: 124–129)

Recent progress in human tumor immunology research has presented us with the possibility that immunotherapy could be established as an effective cancer therapy in the very near future.[1–6] Indeed, since the first discovery of a human tumor antigen in 1992,[7] many clinical trials for cancer vaccines have been carried out, and these studies have suggested that active immunization using HLA class I restricted tumor antigenic peptides and the whole or part of the tumor antigenic protein could work as activators of antigen-specific CTLs, at least in some cancer patients.[8–16] However, even in effective cases, vaccination with these molecules alone is not sufficient to evoke a potent and stable immune response and subsequent strong clinical effect. Thus, it is crucial to develop various methods for enhancing the immunological efficacy of tumor antigens.

We have studied how tumor antigenicity can be efficiently enhanced in cancer patients since 2003. In our studies, the HLA-A24-restricted peptide survivin-2B80-88 was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, and urinary bladder cancers, and lymphomas. Clinically, certain patients with colon, lung, and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed by computed tomography (CT).[8–12] These effects, however, were not strong enough for the clinical requirements as decided by the criteria for cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors, which requires more than 30% regression of tumors on CT, only one patient each of 15 with colon cancers and three with urinary bladder cancers had a positive clinical response, indicating that the therapeutic potential was obviously not strong enough for routine clinical use as a cancer treatment.

In a previous study,[8] to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we carried out and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear clinical improvement and enhanced the immunological responses of patients. We also analyzed CTLs of these patients by single-cell sorting, and found that each CTL clone from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Pancreatic cancer is still one of most difficult malignant neoplasms to treat, so in the current study we investigated whether the most effective protocol for colon cancer patients, namely survivin-2B80-88 plus IFA and IFNα, could work similarly in pancreatic cancers as in colon cancers. Furthermore, we carried out frequency monitoring of survivin-2B80-88 peptide-specific CTL in cases of cancer patients treated with IFNα alone, and found no overt increase of these CTLs. Once the survivin-2B80-88 peptide was administered with IFNα, patients showed strong clinical and immunological responses as assessed by tetramer and enzyme-linked immunosorbent spot (ELISPOT) analyses. Taken together, our current data strongly suggest that vaccination using survivin-2B80-88 plus IFA and IFNα is actually very effective in patients with advanced pancreatic cancers from both the clinical and immunological points of view.

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Materials and Methods

Patients. Patient selection was done as reported in our previously published work. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University (Sapporo, Japan).[8–12] All patients gave informed consent before being enrolled. Patients who participated in this study were required to: (i) have histologically confirmed pancreatic cancer; (ii) be HLA-A*2402 positive; (iii) have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) be between 20 and 85 years old; (v) have unresectable advanced cancer or recurrent cancer; and (vi) have Eastern Cooperative Oncology Group performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection, or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, in the Sapporo Medical University Primary Hospital from December 2005 through to November 2010.

Peptide, IFA, and IFNα preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8–10,12] The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by HPLC analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) and stored at −80°C until just before use. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan).

Patient treatment. In this clinical study, we used the protocol illustrated in Fig. 1, with the survivin-2B80-88 peptide plus IFA and IFNα. In this trial, the primary endpoint was safety. The second endpoint was investigation of the antitumor effects and clinical and immunological monitoring.

In this protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, IFNα at a dose of 3 000 000 IU was given s.c. twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination (Fig. 1).

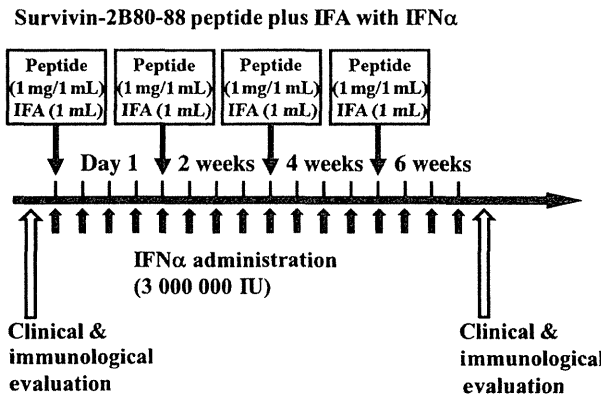

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination.

Toxicity evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria.[8–10]

Clinical response evaluation. Physical examinations and hematological examinations were carried out before and after each vaccination.[8–10] A tumor marker (Ca19-9) was examined. Changes in the tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary pancreatic cancer tissues was done with anti-HLA class I heavy chain mAb EMR-8-5[13] (Funakoshi, Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response was defined as a $\geq$30% decrease from the baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for complete response, partial response, or PD.[8–10] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC, tetramer staining, and ELISPOT assay.** The samples for tetramer analysis and ELISPOT analysis were simultaneously obtained at the time of the hematological examination before and after each vaccination. These experiments were carried out as in our previous report. The PBMCs were isolated from blood samples by Ficoll–Conray density gradient centrifugation. Then they were frozen and stored at −80°C. As needed, frozen PBMCs were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM V (Life Technologies Corp, Grand Island, NY, USA) medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days, and 6 days after the addition of the peptide. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

The FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) and phycoerythrin (PE)-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from Medical and Biological Laboratories (MBL) Co., Ltd (Nagoya, Japan). For flow cytometric analysis, PBMCs, stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a PE-Cy5-conjugated anti-CD8 mAb (BD Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was carried out using FACSCalibur and CellQuest software (BD Biosciences). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,10,12]

The ELISPOT plates were coated overnight in a sterile environment with an IFNγ capture antibody (BD Biosciences) at 4°C. The plates were then washed once and blocked with AIM V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well) that were stimulated *in vitro* as above were then added to each well along with HLA-A24-transfected T2 cells (T2-A24) (5×10^4 cells/well) that had been preincubated with or without survivin-2B80-88 (10 mg/mL) or

with an HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFNγ antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Oberkochen, Germany). In this study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-postive spots as compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTLs. Survivin-2B80-88 peptide tetramer-positive CTLs were sorted and subsequently cloned to single cells using FACS (Aria II Special Order; BD Biosciences). The peptide-specific cytotoxicity of each of these CTLs was determined by pulsing T2A24 cells[8,17] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides, as previously described.

Results

Patient profiles, safety, and clinical responses. In the present protocol with the survivin-2B80-88 peptide plus IFA and IFNα, six patients were enrolled in the study (Table 1). None dropped out because of adverse events due to the vaccination. They consisted of three men and three women, whose age range was 50–80 years.

With respect to the safety, vaccination was well tolerated in all patients. Four patients had fever reaching nearly 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination except for induration at the injection site, which was conduced by IFA.

The clinical outcomes for the six patients treated with survivin-2B80-88 plus IFA and IFNα are summarized in Table 1. In some patients, particularly No. 1, the postvaccination Ca19-9 value was clearly decreased as compared with prevaccination, and was within the normal limit. Other patients (Nos. 2, 4, and 6) also had decreased or stable postvaccination levels of Ca19-9, although not as large. As for tumor size evaluated by CT, four patients (Nos. 1, 2, 4, and 6) were considered to have SD, but the other two patients (Nos. 3 and 5) had PD. Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced or stable Ca19-9 level.

Immune responses, single-cell cloning, and subsequent functional assessment of tetramer-positive CTLs. As in our previous study with colon cancer patients, we determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequencies before the first vaccination (prevaccination) and after the last vaccination (postvaccination) were assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with an HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

As summarized in Table 1, four of the six patients (Nos. 1, 2, 4, and 6) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and all four had SD by CT evaluation, suggesting that immune responses might appropriately reflect clinical responses with the current vaccination protocol.

As in our previous work, we also analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Fig. 2, patient No. 1 (62 years old, female) had a reduced serum Ca19-9 level, and obvious immune responses as assessed by the survivin-2B80-88 ELISPOT and tetramer analyses (Fig. 3) after vaccination.

Subsequently, CD8 T cells of the tetramer-positive fraction were sorted by FACS, then cultured with 1, 3, and 10 cells/well for 7–10 days. Almost all growing T cells were survivin-2B peptide-specific T cells (data not shown), and we next assessed peptide-specific cytotoxicity by using these T cells. As Fig. 4 clearly shows, all T cells had very high peptide-specific cytotoxic potential. Taken together, these data clearly indicated that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα was capable of inducing a strong CTL response and for some pancreatic cancer patients might result in clinical effectiveness.

Assessment of treatment effect with IFNα alone. The above data strongly suggested that the current vaccination protocol

Table 1. Profiles of patients with advanced pancreatic cancer enrolled in the study and their clinical and immunological responses to vaccination with survivin-2B80-88 peptide, incomplete Freund's adjuvant and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre/post (CA19-9 U/mL)	CT eval.	Tetramer staining†		ELISPOT‡	
					Pre/post	% Increase	Pre/post	% Increase
1	62/F	Induration	136.5/31.4	SD	23/246	1069.6	27/294	1088.9
2	61/F	Induration Fever	63.6/60.6	SD	1/157	15700.0	25/71	284.0
3	56/M	Induration Fever Thrombopenia	171.4/978.8	PD	22/19	86.3	19/525	2763.2
4	80/F	Induration Fever	30.0/22.7	SD	9/1030	11444.4	1/101	10100.0
5	58/M	Induration Fever	436.0/2885.0	PD	3/0	0.0	34/20	58.8
6	50/M	Induration	4389.0/4295.0	SD	2/7	350.0	27/85	314.8

†Cytotoxic T-lymphocyte frequency of prevaccinated (pre) and postvaccinated (post) patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The numbers of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 × CD8 T cells are shown. ‡γ-Interferon (IFNγ) secretion of pre- and postvaccinated patients' CD8 T cells was assesed with enzyme-linked immunosorbent spot (ELISPOT) assay using T2-A24 cells pulsed with survivin-2B80-88 peptide. The numbers of spots in 5 × 10^3 CD8 T cells are shown. CT eval., evaluation by computed tomography; IFNα, α-interferon; PD, progressive disease; SD, stable disease.

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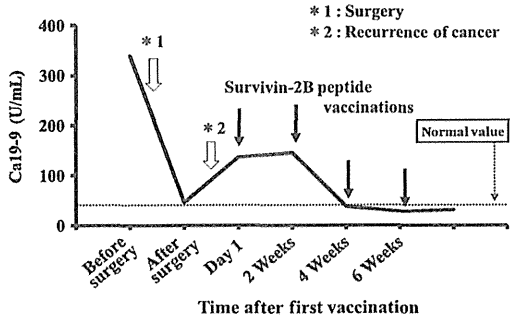

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

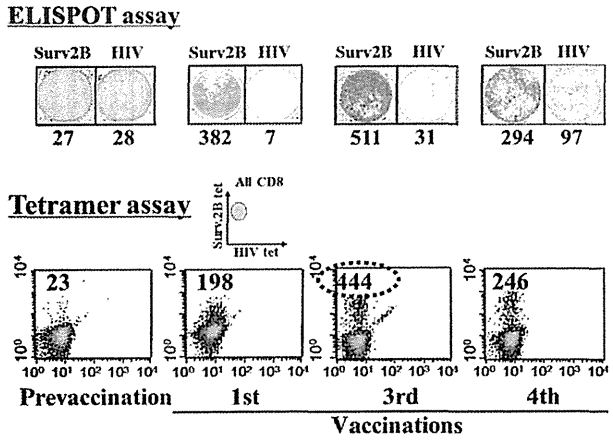

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against survivin2B80-88 or HIV peptide pulsed T2-A24 cells in $10^4\times$ $CD8^+$ T cells. Numbers in tetramer analysis indicate survivin-2B80-88 peptide-specific $CD8^+$ T cells among $10^4\times$ $CD8^+$ T cells.

with the survivin-2B80-88 peptide plus IFA and IFNα could work as a potential therapeutic regimen in pancreatic cancers. However, it remained to be clarified if IFNα alone without the peptide could function in a similar manner, at least to some extent, as this cytokine is considered to be the most potent for the activation and maturation of dendritic cells (DCs) as well as upregulation of HLA class I in tumor cells. To this end, we studied this profile in three patients with colon cancer, not pancreatic cancer, whose condition was similar to those in this study, that is, patients with unresectable advanced or recurrent cancer. This was done because patients with the latter cancer had highly advanced clinical cases, making this type of study impossible. As shown Table 2, all three patients showed no obvious increases, but rather reductions, in the frequency of survivin-2B peptide-specific T cells as assessed by tetramer analysis before and after two to four treatments with IFNα alone. Furthermore, this was also true for ELISPOT analysis. These data supported the idea that IFNα alone did not actively participate in the activation of survivin-2B peptide-specific T cells.

Discussion

Our group previously showed that the vaccination protocol of survivin-2B80-88 plus IFA and IFNα could work as a potent

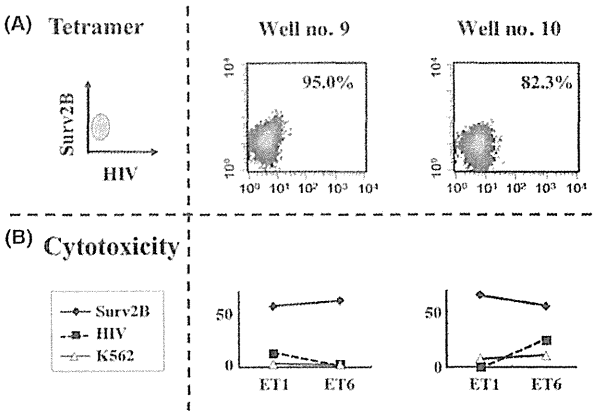

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer (Surv2B) (A) and against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide and against control K562 cells (B). ET, effector/target ratio.

immunotherapeutic regimen in colon cancers.[8] In addition to colon cancer, survivin2B protein is expressed in most tumor cells of various tissue origins, such as those in the gastrointestinal and biliary tracts and pancreas, therefore, there is a possibility that the survivin2B peptide could work as a potential therapeutic tumor vaccine in cancer patients with these neoplasms.

In this present study, we assessed whether the vaccination protocol using survivin-2B80-88 plus IFA and IFNα could be effective in pancreatic cancer patients from immunological and clinical points of views. Consequently, our data strongly suggested that this protocol was very effective and useful in immunotherapy for advanced pancreatic cancers as in colon cancers. Actually it was shown that more than 50% of patients with pancreatic cancers showed positive clinical and immunological responses in tetramer and ELISPOT analyses. In some cases, the immunological response of survivin-2B80-88 peptide-specific CTLs was elucidated at the single-cell level. Taken together, the current data implied that our vaccination protocol was very useful in immunotherapy for pancreatic cancers.

As shown in Fig. 3, the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced from the third to the fourth vaccination. We speculate that there could be various reasons for this reduction. One might be immune escape by the downregulation of HLA expression, cytokines, or regulatory T cells. Another might be an activity of the stored samples, or differences between the environment of the peripheral circulation and the tumor. In other words, the peptide-specific CTL responses were reduced in immunological monitoring in the peripheral circulation, but maintained in the local cancer environment. In this case, the clinical responses, such as tumor marker (CA19-9) level and tumor size evaluated by CT, had been maintained also after that, even though the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced between the third and fourth vaccinations. Therefore, CA19-9 levels had been kept within normal limits and new cancer lesions had not appeared.

We evaluated immunological monitoring of this clinical protocol by tetramer staining and IFNγ ELISPOT assay. Tetramer staining recognizes the structure of the T cell receptor, and

Table 2. Frequency monitoring of the number of survivin-2B80-88 peptide tetramer-positive CTLs in cancer patients treated with IFNα alone

Patient no.	Tumor	Age/sex	Number of treatment	Tetramer staining†		ELISPOT‡	
				Pre/post	% Increase	Pre/post	% Increase
1	Colon	60/M	3	1/0	0.0	111/75	67.6
2	Colon	63/M	4	11/9	81.8	44/20	45.5
3	Colon	77/F	2	13/3	23.1	26/40	153.8

†CTL frequency before and after treatment with IFNα alone in patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. An HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 CD8 T cells is shown. ‡γ-Interferon (IFNγ) secretion of pre and post IFNα treatmnet were assesed with ELISPOT assay using T2-A24 cells pulsed with survivin2B80-88 peptide. The number of spots in 5×10^3 CD8 T cells are shown. IFNα, α-interferon.

detects naive T cells, memory T cells, and activated CTLs. The ELISPOT assay detects more the functional aspects of T cells by IFNγ release, therefore, ELISPOT detects memory T cells and CTLs. In this study, the tetramer-positive cases are also positive in the ELISPOT study. Therefore, these results indicate that memory T cells and CTLs can be effectively induced by this peptide vaccination protocol.

In this present study, we also assessed evidence concerning the extent to which peptide-specific CTL responses in pancreatic cancer patients treated with peptide vaccines could occur at the single-cell level. To assess this, CTLs of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed peptide-specific in the context of the expression of HLA-A24.

Type-I interferons such as IFNα are known to work in various immunological manners to activate T cell responses.[18–25] The maturation of DCs and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Although we could not actually compare these features of patients' DCs and primary pancreatic tumor tissues before and after treatment with IFNα, the obvious enhancement of CTL responses and improvement of clinical responses in our previous and current studies favors the two main actions described above. These observations strongly suggest that the action of IFNα is remarkable from the aspect of being an immunogenic enhancer for human cancer peptide vaccines.

It is widely known that IFNα is involved in DC maturation and activation.[18,21] This particular cytokine is also potent for increasing the expression of MHC class I molecules.[26–29] Indeed, our previous study of the expression of HLA class I molecules in pancreatic cancer indicated that many tumor tissues heterogeneously expressed such molecules, with some tumor cells showing high expression, whereas others had only weak expression. Interferon-α is presumed to actually enhance their expression even in those tumor tissues with weak expression. Moreover, because tumor patients generally show overt expression of survivin protein in their tumor tissues and, although in small numbers, survivin-2B peptide-specific T cells in peripheral blood, it is considered that IFNα alone may increase the frequency of these T cells in peripheral blood as well. These features of this particular cytokine lead to the possibility that treatment with IFNα alone could result in, at least to some extent, certain immunological and clinical effects of survivin-2B peptide-specific T cells in tumor-bearing patients. However, we analyzed three colon cancer patients, and our data strongly suggested that there was no increase of these T cells as assessed by tetramer and ELISPOT analyses.

Taken together, our results highly suggest that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα is very effective for pancreatic and colon cancers, and that this protocol might be useful as a standard, general immunotherapy modality for human cancers. However, further clinical studies involving many patients are necessary in order to consolidate the immunotherapeutic benefit of this vaccination protocol.

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Disclosure Statement

The authors have no conflict of interest.

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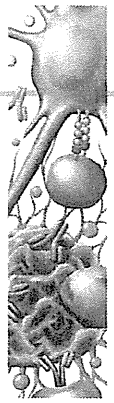

Cytotoxic T lymphocytes: the future of cancer stem cell eradication?

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"Cancer stem-like cells/cancer-initiating cells are immunogenic to cytotoxic T lymphocytes and express several tumor-associated antigens that can be recognized by cytotoxic T lymphocytes."

KEYWORDS: cancer stem cell ▪ cytotoxic T lymphocyte ▪ immunotherapy ▪ tumor antigen

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are a hot topic in cancer research since they are highly tumorigenic and are resistant to standard cancer therapies. Recent studies have revealed that cancer immunotherapy is a possible and promising candidate to target CSCs/CICs. Among the various immunological effector cells, cytotoxic T lymphocytes (CTLs) are a good candidate for CSC/CIC-targeted immunotherapy as CTLs are antigen-specific effector cells. In this article, we summarize advances in studies on CTLs and discuss the future of CSC/CIC-targeted therapy.

Since the first identification of leukemia stem cells from an acute myeloid leukemia sample, studies of CSCs/CICs have made great advances [1]. CSCs/CICs are a small population of cancer cells that have tumor-initiating, self-renewal and differentiation abilities. Recent studies have revealed that CSCs/CICs are resistant to cancer therapies because they are in a quiescent cell-cycle state, they express high levels of transporters and apoptosis inhibitors, and they express low levels of reactive oxygen species. CSCs are therefore regarded as major causes of cancer recurrence, distant metastasis and treatment resistance; studies of CSCs/CICs have been focusing on how CSCs/CICs can be targeted efficiently.

Cancer immunotherapy: a possible option for CSC/CIC-targeted therapy

Cancer immunotherapy is expected to become the fourth main cancer therapy following surgery, chemotherapy and radiotherapy. Several cancer immunotherapy protocols have been tested in clinical trials, and the first cancer immunotherapy drug Provenge® (Dendreon, WA, USA) was approved by the US FDA in 2010 for treatment of advanced prostate cancer. It had not been clear whether the immune system can recognize therapy-resistant CSCs/CICs, but recent studies have revealed that both the innate and acquired immune system can recognize CSCs/CICs [2]. CTLs, NK cells, γδT cells and antibodies have been shown to be able to target CSCs/CICs. CTLs, NK cells and γδT cells kill target cells by cytotoxic granules including perforin and granzymes, therefore treatment-resistant CSCs/CICs would be susceptible to death. CTLs are a key player in the aquired immune system and recognize target cells in an antigen-specific manner. On the other hand, NK cells and γδT cells are players in the innate immune system and are not specific for antigens. Therefore, CTLs might be useful for CSC/CIC-targeted immune therapy.

CTLs: key player for eradicating CSCs/CICs

Since CTLs recognize antigenic peptides presented by MHCs, it is essential that CSCs/CICs express both MHCs and tumor-associated antigens (TAAs). A previous study revealed that colon cancer stem cells isolated as side population cells express MHC molecules at levels similar to those in non-CSCs/CICs [3]. In a recent study, Carbone's group found that colon CSCs/CICs derived as spheroid cultures express lower levels of MHC molecules than parental colon tumor cells, which enables NK cells to recognize CSCs/CICs [4]. They reported that NK cells could recognize colon CSCs/CICs because they express higher levels of ligands of NK cell-activating receptors, including NKp30 and NKp44. However, colon CSCs/CICs used in the study expressed MHC molecules to some extent, which may be sufficient to be recognized by CTLs. In fact, we and other groups have reported that CSCs/CICs can be recognized by CTLs [3,5,6] and CSCs/CICs might

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therefore express sufficient levels of MHCs to be recognized by CTLs.

CSCs/CICs have been shown to express several TAAs. We previously classified TAAs into three groups according to expression profiles in CSCs/CICs and non-CSCs/CICs [7]. These three groups are: CSC/CIC antigens, which are expressed preferentially in CSCs/CICs; shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs; and non-CSC/CIC antigens, which are expressed preferentially in non-CSCs/CICs. Thus, CSC/CIC antigens and shared antigens are expressed in CSCs/CICs. The CSC/CIC antigens so far reported include MAGEA3, MAGEA4, DNAJB8, SOX2, OCT3/4, BMI1 and ALDH1A1 [6,8–11]. SOX2, OCT3/4 and ALDH1A1 are also expressed in the normal stem cell fraction, whereas MAGEA3, MAGEA4 and DNAJB8 are cancer testis (CT) antigens that are expressed in CSCs/CICs and normal testis cells, but not in normal stem cells. SOX2, OCT3/4 and ALDH1A1 can be candidates for CSC/CIC-targeted immunotherapy; however, there is a risk of also targeting normal stem cells, which may make the patient's condition severe. Therefore, CT antigens may be good candidates for CSC/CIC-targeted immunotherapy. Interestingly, large numbers of CT antigens are preferentially expressed in CSCs/CICs [12]. The testis is an 'immunologically privileged organ', and CT antigens are regarded as immunogenic TAAs [13]. The biological significance of testis gene products in CSCs/CICs is still elusive; however, they should be better candidates for CSC/CIC-targeted immunotherapy as they are not expressed in normal stem cells.

> *"...both the innate and acquired immune system can recognize cancer stem-like cells/cancer-initiating cells."*

Since both CSC/CIC antigens and shared antigens are expressed in CSCs/CICs, it raises the question, which one is better for targeting CSCs/CICs? We have identified a novel CSC/CIC antigen, DNAJB8, which is expressed in kidney CSCs/CICs [11]. To answer the above question, we compared the potency of DNAJB8 with that of survivin, which is a well-established TAA and is a shared antigen [14]. Interestingly, DNAJB8 was more potent than survivin in a tumor prophylactic DNA vaccination model. However, it may be too early to reach a conclusion; these results suggest that CSC/CIC antigens are better at targeting cancers than shared antigens. Several other studies have demonstrated the potency of CSC/CIC-targeted immunotherapy by *in vivo* animal models [5,6,15]. Therefore, CSC/CIC-targeted immunotherapy using CSC/CIC antigens is a feasible and promising approach.

> *"...cancer stem-like cell/cancer-initiating cell antigens are better at targeting cancers than shared antigens."*

There are several molecular mechanisms by which CSCs/CICs may escape from CTLs. Heinberger's group reported that glioma stem cells express high levels of immunosuppressive molecules, including B7-H1 and soluble galectin-3 [16]. Kim's group reported that CSCs/CICs express high levels of NANOG, which induces CTL resistance by activation of Nanog/Tcl1a/Akt signaling [17]. The results of these two studies demonstrate the molecular mechanisms of suppression of CTL induction phase and suppression of CTL effector phase, respectively. Since CSCs/CICs express high levels of CT antigens, which are regarded as highly immunogenic TAAs, CSCs/CICs must be a relatively immunogenic cancer cell population. However, CSCs/CICs in clinical cancer specimens survive immune pressure, indicating that there are mechanisms for CSCs/CICs to escape the immune system. These two mechanisms may be aspects of CSC/CIC immune escape. Therefore, to overcome the problem of immune escape of CSCs/CICs and target CSCs/CICs in cancer immunotherapy, an immune potentiator (adjuvant) may be necessary to achieve significant antitumor effects.

Conclusion

CSCs/CICs are immunogenic to CTLs and express several TAAs that can be recognized by CTLs. CSC/CIC-targeted immunotherapy using these TAAs might be feasible. Antigenic peptide vaccination and CTL adoptive transfer are possible approaches to target CSCs/CICs. In the near future, chemotherapy- and radiotherapy-resistant CSCs/CICs may be able to be targeted by CTLs in clinical settings.

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Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA

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Endosomal Toll-like receptor 3 (TLR3) serves as a sensor of viral infection and sterile tissue necrosis. Although TLR3 recognizes double-stranded RNA, little is known about structural features of virus- or host-derived RNAs that activate TLR3 in infection/inflammatory states. Here we demonstrate that poliovirus-derived single-stranded RNA segments harbouring stem structures with bulge/internal loops are potent TLR3 agonists. Functional poliovirus-RNAs are resistant to degradation and efficiently induce interferon-α/β and proinflammatory cytokines in human and mouse cells in a TLR3-dependent manner. The N- and C-terminal double-stranded RNA-binding sites of TLR3 are required for poliovirus-RNA-mediated TLR3 activation. Like polyriboinosinic:polyribocytidylic acid, a synthetic double-stranded RNA, these RNAs are internalized into cells via raftlin-mediated endocytosis and colocalized with TLR3. Raftlin-associated RNA uptake machinery and the TLR3 RNA-sensing system appear to recognize an appropriate topology of multiple RNA duplexes in poliovirus-RNAs. Hence, TLR3 is a sensor of extracellular viral/host RNA with stable stem structures derived from infection or inflammation-damaged cells.

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Type I interferon (IFN) production is crucial for controlling virus infections[1,2]. Toll-like receptors (TLRs) 3, 7, 8 and 9 and RIG-I-like receptors detect viral nucleic acids in the endosomes and cytoplasm, respectively, and induce cytokine production, including type I IFNs (IFN-α and IFN-β), via distinct adaptor proteins[3]. TLR3 recognizes double-stranded RNA (dsRNA), a viral replication intermediate that is produced by positive-strand RNA viruses and DNA viruses, and signals to produce IFN-β and proinflammatory cytokines through the Toll-interleukin-1 (IL-1) receptor domain-containing adaptor molecule-1 (TICAM-1, also known as TRIF)[4–8]. TLR3 is expressed at high levels in myeloid dendritic cells (DCs), especially in professional antigen-presenting DCs, such as mouse splenic $CD8\alpha^+$ DCs and human $CD141^+$ DCs, and localizes to the early endosome[9–11]. Although macrophages, fibroblasts and epithelial cells also express TLR3 on the cell surface as well as the endosomal membrane, TLR3-mediated signalling initiates from the endosomal compartment[12]. Thus, TLR3 activation depends on the uptake of extracellular virus-derived RNA molecules.

Studies using TLR3-deficient mice demonstrated that TLR3 mediates a protective response against positive-strand RNA virus infection, including coxsackie virus group B serotype 3, encephalomyocarditis virus and poliovirus (PV), all of which belong to the *Picornaviridae* family[13–16]. TLR3- or TICAM-1-deficient human PV receptor (PVR)-transgenic mice were more susceptible than normal PVR-transgenic mice to intraperitoneal or intravenous inoculation with a low titre of PV[15,16]. TLR3-dependent type I IFN production by splenic $CD11c^+$ DCs and macrophages was essential for the protection of PVR-transgenic mice against PV infection. In addition to mouse studies, genetic studies in the patients with herpes simplex encephalitis demonstrated that the TLR3-TICAM-1 pathway is involved in the protection against herpes simplex virus-1 encephalitis in children[17–19]. Collectively, these findings indicate that TLR3 has an important role in antiviral responses in both humans and mice. However, several studies have also demonstrated that TLR3-mediated signalling exacerbates RNA virus infection including West Nile virus (positive-strand RNA virus), influenza virus and phelebovirus (negative-strand RNA viruses)[20–22]. TLR3-dependent inflammatory cytokine and chemokine productions have an impact on virus-induced pathology and host survival, and, remarkably, RNA from necrotic cells or host mRNA are also recognized by TLR3[23,24]. However, RNA species detected by TLR3 have not been analysed and little is known about the essential structural elements of virus- or host-derived RNAs capable of activating TLR3 in an infection/inflammatory state.

In the current study, we analysed the RNA structure recognized by TLR3 using *in vitro*-transcribed RNAs derived from PV. We found that, in addition to dsRNA, PV-derived structured RNAs harbouring ds regions with bulges and internal loops are potent TLR3 ligands. The PV-RNAs activated endosomal TLR3 in mouse and human cells, leading to the production of IFN-α/β and proinflammatory cytokines.

Results

Identification of RNA structures recognized by TLR3. To examine whether viral RNA activates TLR3 extracellularly, mouse splenic DCs from wild-type (WT) or TLR3-deficient mice were stimulated with total RNA isolated from PV-infected or uninfected Vero cells, or a synthetic viral dsRNA analogue, polyriboinosinic:polyribocytidylic acid (poly(I:C)), in FCS-free medium. Poly(I:C) induced type I IFN production by mouse splenic DCs in a TLR3-independent manner (Fig. 1a). The results reflect a current notion that cytosolic RNA helicase, melanoma differentiation-associated gene 5 (MDA5), in addition to endosomal TLR3, senses poly(I:C) to induce type I IFNs in mouse DCs[25]. However, this is not the case in RNA from PV-infected cells, which induces type I IFNs and proinflammatory cytokines in splenic DCs largely through their TLR3 (Fig. 1a and Supplementary Fig. S1). Notably, RNA from PV-uninfected cells had no potential for IFN/cytokine induction (Fig. 1a and Supplementary Fig. S1). Given that PV infection leads to a rapid inhibition of host-cell RNA synthesis[26], the main RNA species in PV-infected Vero cells were approximately 8,000-nucleotide (nt) RNA corresponding to the PV genome and its replicative form, which were segmented into approximately 1,000–2,000-nt RNAs in the FCS-free culture medium (Fig. 1b). The ~8,000-nt PV-RNAs were degraded by treatment with single-stranded RNA (ssRNA)- and dsRNA-specific RNase, suggesting the existence of ss and ds forms of PV-derived RNAs in PV-infected cells (Supplementary Fig. S2).

A functional screening for PV-derived ssRNA and dsRNA was performed using an IFN-β promoter reporter assay to determine the RNA structure recognized by TLR3. Approximately 540–920 nts contiguous sense (PV1–10) or complementary (cPV1–10) RNAs and their dsRNA forms (dsPV1–10) were transcribed *in vitro* using PV-cDNA as a template (Fig. 1c and Supplementary Table S1), and their TLR3-activating ability was assessed. HEK293 cells transfected with the TLR3-expression vector or empty vector, together with the IFN-β promoter reporter plasmid, were stimulated with PV-RNAs in FCS-free or FCS-containing media. All PV-derived dsRNAs induced TLR3-dependent IFN-β promoter activation similar to poly(I:C) (Fig. 1d, left panel). Interestingly, several PV-ssRNAs, either sense or complementary RNA, also activated the IFN-β promoter through TLR3 in FCS-free medium (Fig. 1d, centre and right panels). We further examined the effect of FCS on the IFN-β-inducing abilities of PV-RNAs using PV5. PV5 retained TLR3-activating ability, even in the FCS-containing medium (Fig. 1e).

To explore the relationship between TLR3-activating capacity and the degradability of PV-RNAs, PV-RNAs were incubated in FCS(−) or FCS(+) medium for 30 min at 37 °C and then subjected to agarose gel electrophoresis. Non-functional PV-RNAs were readily degraded in the FCS(−) or FCS(+) medium, whereas functional RNAs, including high-potential PV5 and cPV5 and low-potential PV6, were stable before and after incubation in the FCS(+) medium (Fig. 2a). Correspondingly, dose-dependent TLR3 activation was observed with PV5 and PV6 but not with others including cPV1 (Fig. 2b). Notably, these PV-RNAs stimulated the cytosolic RNA sensors when transfected into cells (Supplementary Fig. S3a). TLR3-mediated IFN-β promoter activation was also observed with PV5- or cPV5-containing long-size PV-RNAs, although the activity was less than that of PV5 (Supplementary Fig. S3b and Supplementary Table S1). In addition, segmentation of PV5 into three ~200-nt RNAs (PV5 a, b and c) destroyed the activity of intact PV5, despite the fact that these RNA segments were resistant to degradation (Supplementary Fig. S3c and Supplementary Table S1). These results suggested that an appropriate length of PV-RNAs with a stable structure activate endosomal TLR3, inducing IFN-β promoter activation.

PV-RNAs induce IFN-β production by human fibroblasts. We next examined whether PV-RNAs induced IFN-β production from human fibroblasts and epithelial cells that endogenously expressed TLR3. MRC5 cells were stimulated with cPV1, PV5, PV6 or dsPV5 extracellulary, and IFN-β mRNA was assessed using quantitative PCR (qPCR). PV5 and PV6 induced IFN-β mRNA expression in MRC5 cells similar to dsPV5 stimulation

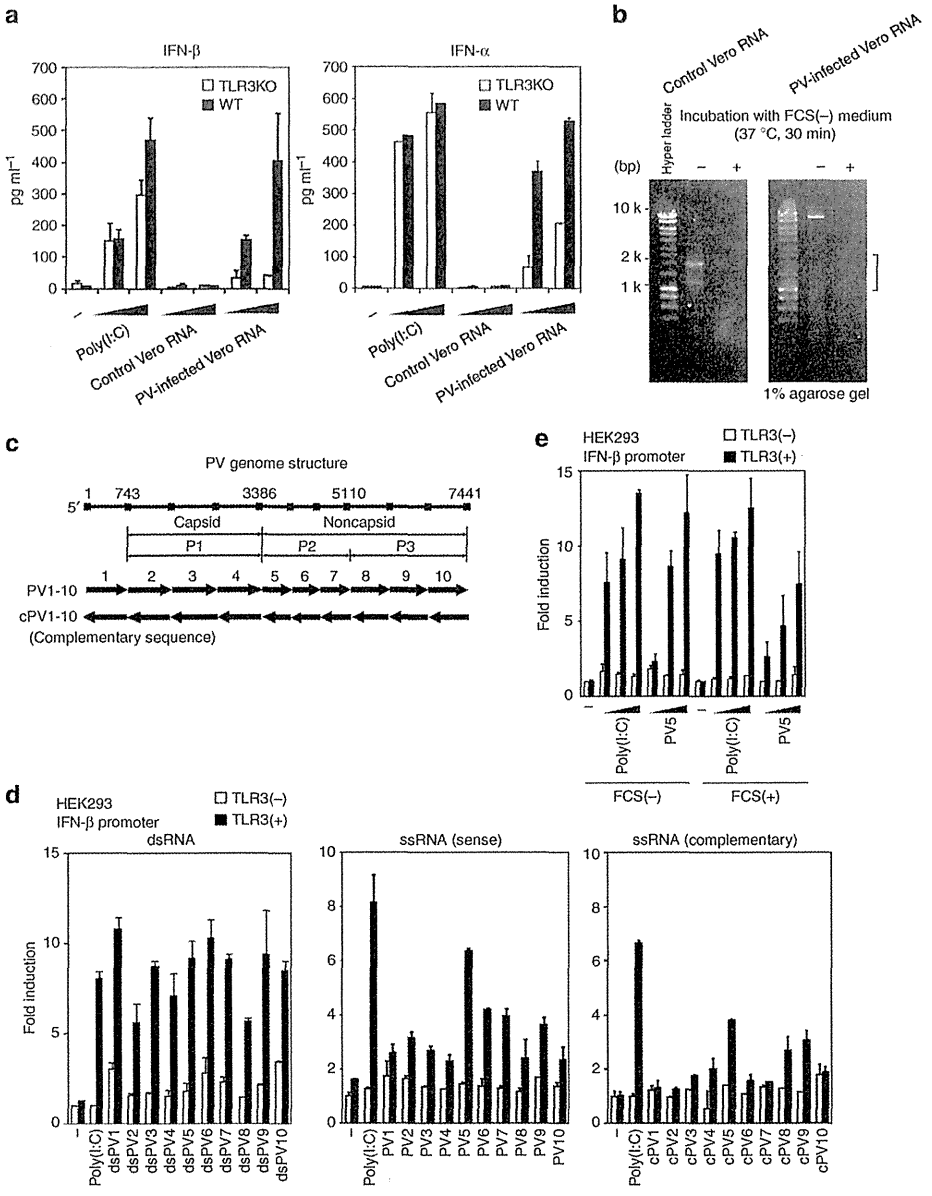

Figure 1 | Extracellular PV-RNAs induce TLR3-mediated type I IFN production. (**a**) Mouse splenic CD11c$^+$ DCs from WT or TLR3$^{-/-}$ mice (1×10^6 per ml) were stimulated with medium alone (−), poly(I:C), total RNA from PV-infected Vero cells or total RNA from uninfected Vero cells (25, 100 μg ml^{-1}) for 24 h in FCS(−) AIM-V medium. RNAs were pre-treated with polymyxin B (5 μg ml^{-1}) for 1 h before addition. IFN-α/β levels in culture supernatants were measured using ELISA. Representative data from three independent experiments are shown (mean ± s.d.). (**b**) The RNA extracted from PV-infected or uninfected Vero cells was incubated in FCS(−) AIM-V medium for 30 min and then electrophoresed on a 1% agarose gel. The PV-RNAs were segmented into approximately 1,000–2,000-nt RNAs in FCS-free medium (right panel, square bracket). (**c**) Constructs of *in vitro*-transcribed PV-RNAs. Diagram of PV genome is shown (Top). Coding regions for viral capsid proteins (P1) and noncapsid proteins (P2 and P3) are indicated. Positions of the sense RNAs (PV1–10) and the complementary RNAs (cPV1–10) corresponding to the PV genome are shown as arrows. Each starting and ending position and length are described in Supplementary Table S1. (**d**) PV-RNA-induced TLR3-mediated IFN-β promoter activation. HEK293 cells were transfected with an empty vector (−) or expression plasmid for TLR3, together with the IFN-β promoter reporter and phRL-TK. Twenty-four hours after transfection, culture media was removed and 10 μg ml^{-1} poly(I:C), PV-derived dsRNAs, dsPV1–dsPV10 (left panel), sense RNAs, PV1–PV10 (middle panel) or complementary RNAs, cPV1–cPV10 (right panel), were added with FCS-free medium. Luciferase activity was measured 6 h after stimulation and shown as mean fold index induction compared with non-stimulated cells. Representative data from three independent experiments are shown (mean ± s.d.). (**e**) Effect of FCS on the PV5-induced TLR3-mediated IFN-β promoter activation. Cells were stimulated with increasing amounts of poly(I:C) or PV5 (2.5, 10 or 25 μg ml^{-1}) in the condition of FCS-free or -containing medium.

(Fig. 2c). mRNA encoding the proinflammatory cytokines, IL-6 and TNF-α, were also induced by PV5, PV6 or dsPV5. Similarly, HeLa cells greatly increased IFN-β mRNA expression in response to PV5, PV6 or dsPV5 (Fig. 2d). In contrast, cPV1 did not induce any cytokine mRNA transcription from MRC5 and HeLa cells.

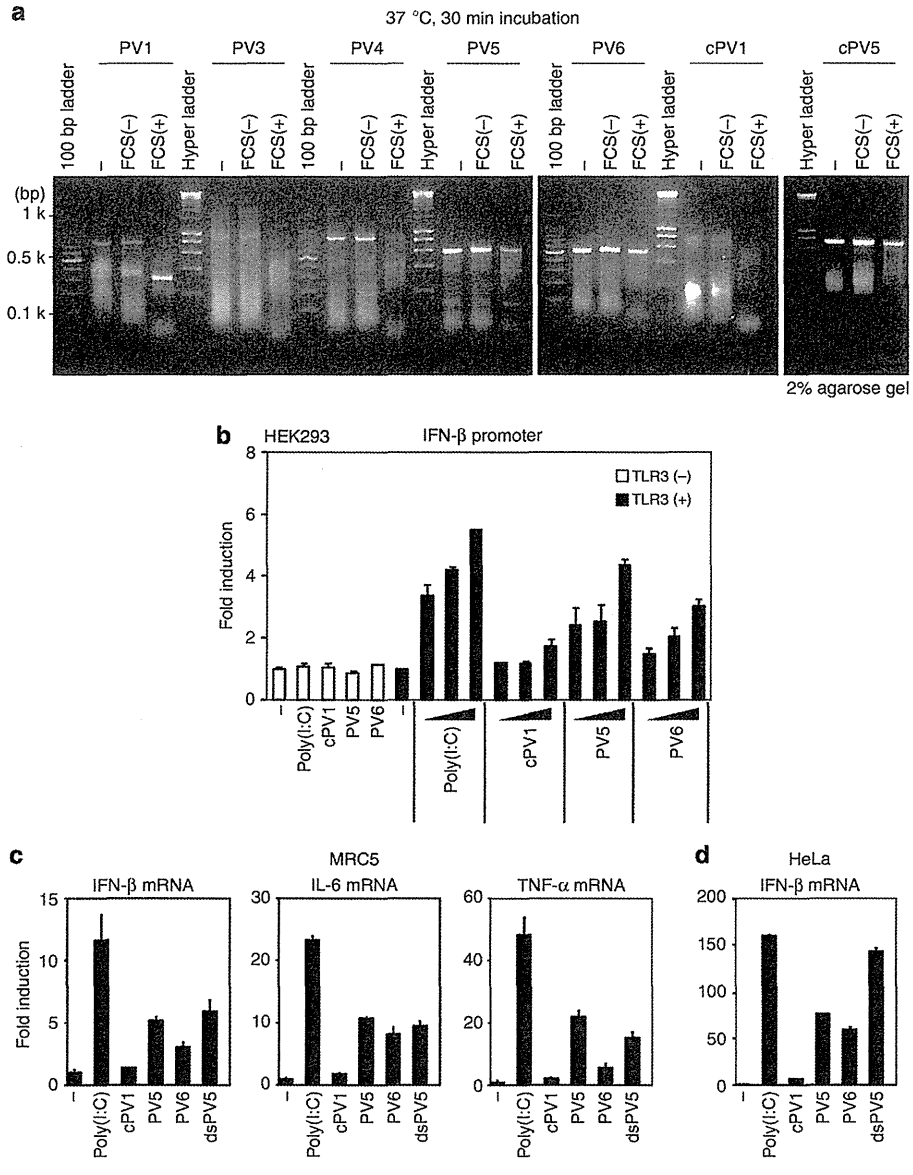

Figure 2 | PV-ssRNA-induced TLR3 activation is correlated with the degradability of RNAs. (**a**) PV-RNAs were incubated in cell-free FCS-free or -containing medium at 37 °C. Non-treated RNA or RNA incubated for 30 min were loaded onto a 2% agarose gel. (**b**) HEK293 cells transiently expressing TLR3 were stimulated with increasing amounts of poly(I:C), cPV1, PV5 or PV6 (2.5, 10 or 25 μg ml^{-1}) for 6 h. HEK293 cells transfected with empty vector were stimulated with 25 μg ml^{-1} poly(I:C) or indicated PV-RNA. IFN-β promoter activation is shown as mean fold index induction compared with non-stimulated cells. Representative data from three independent experiments are shown. (**c**,**d**) IFN-β mRNA expression induced by PV-RNAs in human cells expressing TLR3. MRC5 cells (**c**) or HeLa cells (**d**) in FCS(–) AIM-V medium were stimulated with 20 μg ml^{-1} poly(I:C) or PV-derived RNAs for 3 h. Total RNA was extracted and quantitative PCR was performed using primers for the respective genes. Expression of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA expression. Data are shown as the mean ± s.d. Representative data from three independent experiments are shown.

TLR3 recognizes PV-RNA through the dsRNA-binding sites. Biochemical studies using *in vitro*-transcribed dsRNAs have shown that relatively long stretches of dsRNA (>90 bp in length) are required for TLR3-mediated IFN-β promoter activation in HEK293/TLR3 cells and cytokine production from mouse myeloid DCs[11,27]. To determine the structural features of TLR3-activating or non-activating RNAs, the secondary structure of PV-RNAs was predicted using the mfold WebServer program, which calculates the minimum free-energy secondary structure[28]. Each PV-RNA contains ds (<11 bp in length) and ss regions, and, intriguingly, the percentages of stem/loop structure were almost the same in each PV-RNA (Fig. 3a). However, the secondary structure models of cPV1, PV5 and PV6 have clearly showed that cPV1 contains multiple branched stems and large loop structures that may be targeted by RNases, whereas functional PV5 and PV6 possess tandemly arranged ds regions, which were segmented with bulge or internal loops. Thus, RNAs that we prepared are not a typical ds structure of the TLR3 ligand. The topology of multiple dsRNA regions and the overall secondary and tertiary structures of PV-RNAs appear to influence the stability and TLR3-activating ability.

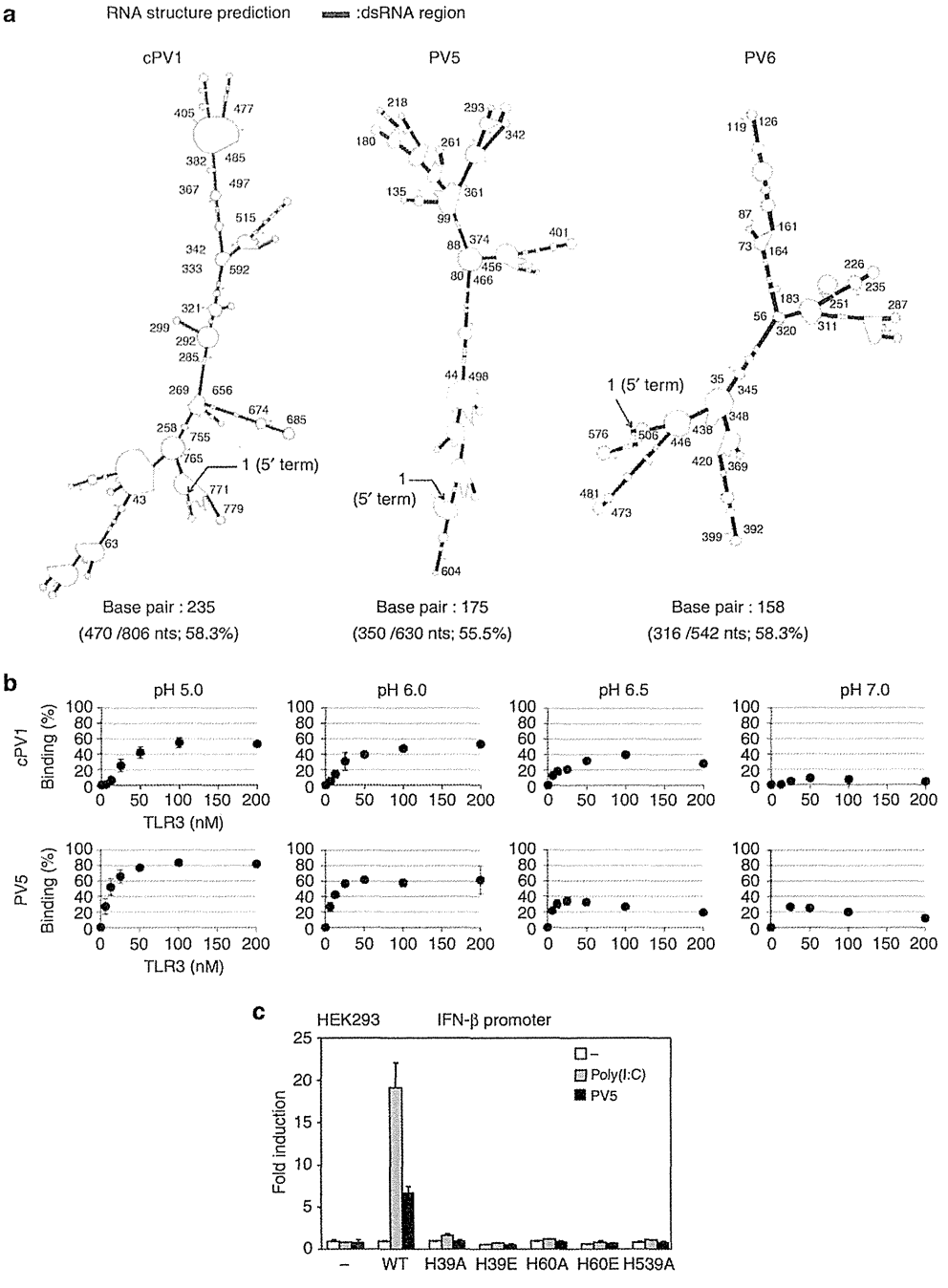

Figure 3 | Both N- and C-terminal dsRNA-binding sites of TLR3 are required for PV-RNA-induced TLR3 activation. (**a**) Secondary structure of PV-RNAs (cPV1, PV5 and PV6) predicted by the mfold software. Thick lines indicate dsRNA regions (1–11 bp). The starting and ending positions of ds regions are shown with the nt number. A total number of base pair and the number of nts involved in base pairing in cPV1, PV5 and PV6 are described under the secondary-structure model. (**b**) Binding affinity of PV-RNAs to TLR3 ECD under different pH conditions. ^{32}P-labelled PV-RNAs (cPV1 and PV5) were mixed with varying concentrations of hTLR3 ECD protein and passed through a nitrocellulose filter. After washing, bound radioactivity was measured, and binding activities were calculated as a percentage of input RNA before filtration. The apparent dissociation constant (Kd) values calculated for cPV1 and PV5 were 39 ± 16 and 10 ± 3 nM (pH 5.0), and 31 ± 13 and 7 ± 3 nM (pH 6.0), respectively. (**c**) HEK293 cells were transfected with an empty vector or expression plasmid for WT TLR3 or each TLR3 mutant (H39A, H39E, H60A, H60E and H539A), together with the IFN-β promoter reporter plasmid and phRL-TK. Twenty-four hours post transfection, cells were stimulated with 10 μg ml^{-1} poly(I:C) or PV5 in FCS-free medium. Luciferase activity was measured 6 h after stimulation. Representative data from three independent experiments are shown.

We then tested the binding ability of intact PV5 and cPV1 to the recombinant TLR3 ectodomain (ECD) protein under the various pH conditions using a filter binding assay[29]. The ability of PV5 to bind TLR3 was higher than cPV1 at low concentrations of TLR3 ECD in an acidic environment (pH 5.0 and 6.0) (Fig. 3b). Even in a neutral pH (7.0), PV5 weakly bound to TLR3 ECD,

whereas cPV1 remained unbound (Fig. 3b). The binding of PV-RNAs to TLR3 ECD was specific because unlabelled PV5 inhibited the ^{32}P-labelled PV5 binding dose-dependently. In addition, dsDNA (200 bp in length) hardly bound to TLR3 ECD under any pH condition (Supplementary Fig. S4).

TLR3 ECD is composed of 23 leucine-rich repeats (LRRs) and the N- and C-terminal flanking regions[30]. Structural analysis of mouse TLR3 ECD–dsRNA complex revealed that TLR3 ECD dimerized on 46-bp dsRNA[31]. dsRNA interacted with both an N- and C-terminal-binding site on the glycan-free surface of each TLR3 ECD, which are on opposite sides of the dsRNA. The point mutation analyses of human/mouse TLR3 demonstrated that H539 and N541 located in LRR20, H39 in LRR-NT and H60 in LRR1 form the C- and N-terminal-binding site, and are essential for dsRNA-induced TLR3-mediated signalling[29,32]. We therefore investigated whether PV5, like dsRNA, activates TLR3 through interaction with the N- and C-terminal-binding sites of TLR3. When TLR3 mutants, in which H39, H60 or H539 was substituted with Ala or Glu, were expressed in HEK293 cells, PV5 failed to induce IFN-β promoter activation, similar to poly(I:C) (Fig. 3c). Thus, PV5 appears to interact with the N- and C-terminal dsRNA-binding sites of TLR3 and oligomerize the receptor, leading to the activation of downstream signalling molecules. However, we cannot rule out the possibility that amino acids of the TLR3 ECD, irrelevant to the direct dsRNA binding, participate in the interaction with PV-RNAs.

PV5 is internalized and colocalizes with endosomal TLR3. TLR3 activation by poly(I:C) requires clathrin-mediated internalization of extracellular poly(I:C)[33]. In human myeloid DCs and epithelial cells such as HeLa cells, the cytoplasmic raft protein, raftlin, induces poly(I:C) uptake through interaction with clathrin–AP-2 complex[34,35]. To understand how extracellular PV-RNAs activate endosomal TLR3, we examined the requirement of raftlin in PV-RNA-induced TLR3 activation by gene silencing of raftlin with small interfering RNA (siRNA). PV-ssRNA or -dsRNA-induced IFN-β promoter activation was greatly decreased when raftlin was knocked down in HEK293 cells, similar to what was observed with poly(I:C) stimulation (Fig. 4a). Furthermore, raftlin-knockdown HeLa cells were also impaired in their ability to induce the expression of IFN-β mRNA in response to PV5 (Fig. 4b). Again, TICAM-1 was essential for PV-RNA-induced signalling (Fig. 4a).

Immunofluorescent analyses demonstrated that surface-bound Cy3-labelled PV5 was internalized within a 5-min incubation at 37 °C. PV5 colocalized with TLR3 and the early endosome marker, EEA1, after 15 min, and this was sustained for up to 60 min (Fig. 4c). In contrast, co-localization of Cy3-PV5 with LAMP1, a late endosome/lysosome marker, was observed after incubation for 30 min (Fig. 4c). The internalization of PV5 was similar to that of poly(I:C)[35]. Indeed, PV5 activity was inhibited by the pre-treatment of cells with B-type CpG oligodeoxynucleotide (ODN), which shares an uptake receptor with poly(I:C) (Supplementary Fig. S5). Taken together, these results strongly suggest that functional PV-RNAs use the poly(I:C)/ODN-uptake receptor for raftlin-mediated endocytosis and that long-term retention of PV5 in the early endosome allows TLR3 to oligomerize for IFN-β production in human cells.

PV-RNAs induce TLR3-dependent IFN production by mouse DCs. To show the TLR3 dependency in PV-RNA-induced cellular responses, mouse macrophages and DCs from WT or TLR3-deficient mice were used for cytokine assay. When bone marrow-derived macrophages from WT mice were stimulated with cPV1, PV5, PV6 or dsPV5 in the FCS-containing medium, they produced IFN-β in response to PV5, PV6 or dsPV5 (Supplementary Fig. S6). Similarly, splenic CD11c$^+$ DCs produced significant amounts of IFN-α/β, TNF-α and IL-6 in response to PV5, PV6 or dsPV5 in the FCS-containing medium (Fig. 5a). The activity of PV-RNAs were augmented in FCS-free medium (Supplementary Fig. S7). Unlike human fibroblasts and epithelial cells, mouse CD11c$^+$ DCs produced cytokines in response to cPV1 with or without FCS in the media, although the levels were relatively low compared with those induced by exposure to PV5, PV6 or dsPV5. Notably, PV-RNA-induced cytokine production was absent from TLR3-deficient DCs (Fig. 5a). Remarkably, cPV1 induced substantial production of proinflmmatory cytokines in FCS-free medium by mouse splenic CD8α$^+$ DCs, expressing a high level of TLR3 in a TLR3-dependent manner, similar to cells cultured with PV5, PV6 or dsPV5 (Supplementary Fig. S8). One possible interpretation of these results is that intact cPV1 possesses TLR3-activating ability and that CD11c$^+$ or CD8α$^+$ DCs promptly take up intact cPV1 before being degraded in FCS-free or -containing medium and deliver it to the endosomal compartment where TLR3 resides. Indeed, intact cPV1 bound to the TLR3 ECD under acidic conditions, though with lower affinity than PV5 (Fig. 3b). When PV5 was pre-treated with RNaseIII, a dsRNA-specific RNase, cytokine production by splenic CD11c$^+$ DCs was abrogated, indicating that RNA duplex is required for PV5-induced TLR3 activation (Fig. 5b).

The estimated secondary-structure model of PV5. To clarify the secondary structure of PV5, we performed the partial digestion of PV5 using MazF at room temperature. MazF is an ACA-specific endoribonuclease that specifically cleaves ssRNA regions at the 5′-end of the ACA sequence, but not dsRNA regions[36]. Cleavage occurs at the 5′ end of the first A residue in an ACA sequence, which produces 2′,3′-cyclic phosphate and the other 5′ OH group[37]. There are 12 ACA sites in PV5. Therefore, to estimate the size of cleaved fragments, PV5-deletion mutants were also digested with MazF (Fig. 6a). Comparison of the cleaved fragments with the mutant RNAs clearly demonstrated that the 124 ACA site in PV5, which was expected to be a dsRNA region, was resistant to cleavage (Fig. 6b). At approximately the 40-mer RNA position, two fragments were detected in PV5(456)/MazF and PV5/MazF. The slow-migration band intensity increased in a time-dependent manner and the fast-migration band intensity gradually decreased. This indicated that the former may be the final digested fragment and the latter was at an intermediate stage of digestion. This was confirmed by additional digestion analyses (Supplementary Fig. S9). We compared our data with several secondary-structure models (RNAfold, mfold and centroidfold) of PV5 and determined that the mfold secondary-structure model was most fitted among them (Fig. 6b and Supplementary Fig. S10).

The core RNA structure required for TLR3 activation. Based on the mfold model, we attempted to identify the core RNA structure required for TLR3 activation in PV5. PV5-derived RNAs partly having PV5 secondary structure (PV5-D1–PV5-D5) were made by *in vitro* transcription (Fig. 7a), and their stability and TLR3-activating ability were assessed. PV5-D1, D3 and D5 were resistant to degradation, and extracellularly activate TLR3 similar to PV5, leading to IFN-β promoter activation in HEK293 cells and cytokine production in mouse splenic DCs or bone marrow-derived macrophages (Fig. 7b–d). In contrast, PV5-D2 and -D4, which were resistant to degradation in FCS-free medium but were readily degraded in FCS-containing medium,