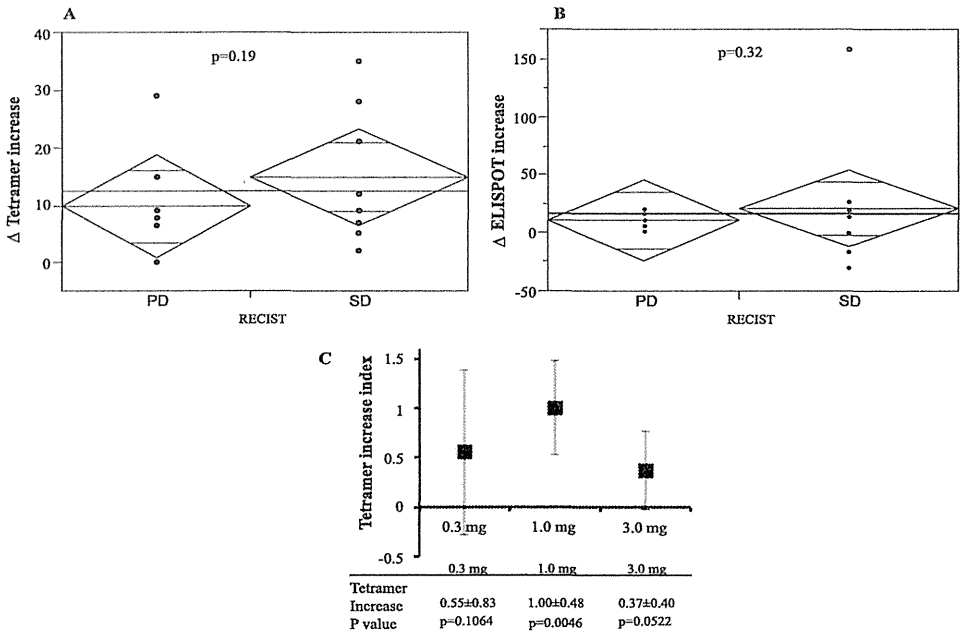

Figure 3: Tetramer assay and ELISPOT assay

(A) The tetramer increase (CTLpost-CTLpre) was calculated from the peptide-specific CTL frequency in PBLs before the first vaccination (CTLpre) and after the fourth vaccination (CTLpost) using the HLA-A24/SVN-2B tetramer. The mean tetramer increases of the PD (non-responders) and SD groups (responders) were compared. (B) The ELISPOT increase was calculated from the numbers of the peptide-specific IFN-γ spots before the first vaccination and after the fourth vaccination. The mean ELISPOT increases of the PD and SD groups were compared. (C) The mean tetramer increase index was calculated according to the following formula: *Tetramer increase index=Log10(1+CTLpost)-Log10(1+CTLpre). The mean tetramer indices of the three groups (0.1mg dose, 1.0mg dose,* and 3.0mg dose) were compared.

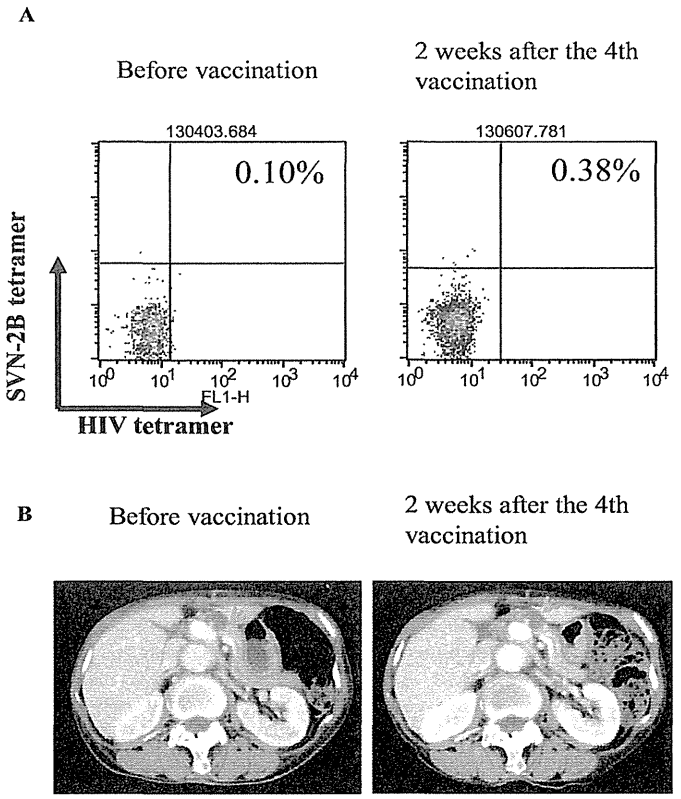

Figure 4: Tetramer assay and CT scan images of the patient with a metastatic pancreatic tumor

An 84-year-old woman with primary colon cancer and metastatic pancreatic tumor. (A) Tetramer assay before vaccination (left panel) and 2 weeks after the 4th vaccination (right panel). (B) CT scan images before vaccination (left panel) and 2 weeks after the 4th vaccination (right panel). The arrowhead indicates the metastatic pancreatic tumor. The tumor grew slightly from 16 mm to 17mm during the 8 weeks of the study.

(p=0.19). To determine the optimal dose of the peptide to induce specific CTLs in patients, the mean tetramer increase indices of the three groups (0.1mg dose, 1.0mg dose, and 3.0mg dose) were compared (Figure 3C). It was found that 1.0mg was the most effective dose for the induction of peptide-specific T cells after the fourth vaccination (p=0.0046).

We also analyzed the peptide-specific IFN-γ responses of CD8-positive T cells by ELISPOT assay. The HIV peptide (RYLRDQQLL) was used as a negative control. The numbers of peptide-specific IFN-γ spots before the first vaccination and after the fourth vaccination were counted, and the ELISPOT increase was calculated (Table 2). There was no significant difference in the mean ELISPOT increase between the SD group and PD group (Figure 3B).

Overall, this study suggests that the immunological response of the vaccine is well represented by tetramer assay rather than ELISPOT assay and that the immunological responses, at least in some patients, appropriately reflect the antitumor responses.

A Case Study

An 84-year-old woman who had primary colon cancer and metastatic liver and pancreatic tumors received the 1.0 mg dose of the SVN-2B vaccine. CT images and tetramer staining data are shown in Figure 4. In this case, the clinical response was defined as SD, and the peptide-specific CTL frequency was increased after the vaccination (Figure 4A). The metastatic pancreatic tumor barely changed from 16 mm to 17 mm during the 8 weeks of the study (Figure 4B). She continued the vaccination after the study. After 6 months, the pancreatic tumor size had increased by 31%, and a new lesion appeared in the caudate lobe of the liver. The time to progression was 267 days. There was no treatment-related AE and she could maintain high quality of daily life for almost one and a half years.

Discussion

The present study demonstrated the safety and clinical efficacy of the survivin-2B peptide vaccine for patients with advanced gastrointestinal cancer. However, the efficacy of vaccination with the SVN-2B peptide plus oil adjuvant Montanide ISA51VG was limited and not sufficient to elicit overt clinical responses. It is obvious that superior clinical and immunological responses are necessary for cancer immunotherapy. It should be considered that vaccination in combination with immunostimulatory adjuvants or cytokines may lead to greater immune and clinical responses. We have reported that type I interferon (IFN) can enhance the antitumor and immunological responses of the peptide vaccine [19,20]. On the basis of the results in this phase I study, we have started a phase II study of the SVN-2B peptide vaccine in combination with IFN-γ.

Immunomonitoring revealed that the tetramer increases were well correlated with antitumor responses as compared with ELISPOT analysis. Therefore, we used the tetramer increase as an index of vaccine-specific immune responses and determined the optimal peptide dose. A significantly higher frequency of tetramer-positive CTLs was induced in the 1mg dose group. However, the optimal dose may vary depending on conditions such as the vaccination interval and combination with distinct adjuvants and/or cytokines, and may have to be reevaluated in combination with IFN. It is enigmatic why the 3mg dose vaccination caused less induction of the peptide-specific CTLs. It was reported previously that persistent vaccine depots could induce sequestration, dysfunction and depletion of antigen-specific CTLs [27]. That may explain, at least in part, the mechanism of the bell-shaped dose effect of the antigenic peptide.

Three patients with a history of immunotherapy such as a dendritic cell vaccine and certain peptide vaccine failed to respond to the SVN-2B peptide vaccine clinically and immunologically. It is possible that their cancers may have had immunoescape phenotypes, thereby maintaining resistance to the vaccine as well as the prior immunotherapy. Alternatively, prior immunotherapy might have affected the immune system, thereby inducing tolerance against the vaccination. In any case, a history of immunotherapy was considered to be a predictive factor for a worse response, and was therefore added to the exclusion criteria in the ongoing phase II clinical study.

In conclusion, we demonstrated the safety and clinical efficacy of the SVN-2B peptide vaccine for patients with advanced gastrointestinal cancer, although clinical interpretation of the results was limited due to this being a phase I study with a small number of patients. At present, a phase II study (UMIN000012146) of the SVN-2B peptide vaccine for advanced pancreatic cancer is ongoing in combination with IFN-γ.

Acknowledgments

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■がんワクチン療法

最新のがんワクチン開発の現状と臨床

免疫力を高めてがんを治療する「がんワクチン」。現在、日本では大学や研究機関でいくつかの臨床開発が行われています。東京大学医科学研究所のグループが取り組んでいる2つのがんワクチンは、すでに効果と安全性の検証段階にあるペプチドワクチンと、難治性の肺がんに対する薬事承認を目指した開発が進んでいます。

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安井 寛

がんワクチン療法の現状と展望

本邦において、がんワクチン療法[1]は、未だ臨床研究の段階を超えず、効果や安全性については、現在、検証中の段階です。つまり、効果が得られることを、科学的に実証されたとは、まだ言えませんし、重篤な副作用が発生する危険性がないとも言えません。科学的に組まれた臨床試験にて効果と安全性を評価する必要があり、さらに、医薬品が保険診療として承認されるためには薬事法に定められた「治験[2]」もしくは「先進医療[3]」により得られた結果を用いて、医薬品医療機器総合機構の審査をうけ、厚生労働省の承認を得る必要があります。

このような前提ではありますが、長年の医科学研究、とくにがん研究、がん免疫学の進歩により、いくつかのがんワクチン療法が臨床開発の段階に至り、活発に研究されておりますので、本稿でご紹介したいと思います。

医師主導の治験が可能に

がん医療においては、基礎医学の進歩、各種治療法の進歩により、がん患者さんの生存期間の延長が認められております。実際に、国立がん研究センターのがん対策情報センターが「地域がん登録」の集計結果をもとに一般の方々へと公開している統計（**表1**）によりますと、1993年から1996年にがんと診断された患者さんの5年相対生存率[4]が53・2％であったのに対して、2003年から2005年に診断された患者さんでは58・6％へと改善しており、緩徐ではありますが着実に、がん患者さんの平均生存は延長してきていることがわかります。

しかしながらその一方で、がんの種類によって5年相対生存率はさまざまです。5年相対生存率が依然、20％台から30％台と低い白血病、多発性骨髄腫、脳・中枢神経系のがん、肺がん、胆のう・胆管がん、肝がん、食道がんはまだまだ治療成績が十分とは言えず、とくに膵臓がんは5年相対生存率が10％未満ときわめて予後不良である現状があります。

このため、手術困難例、再発例、標準治療で十分な効果を得られないがん患者さんに対してより良い治療

(1)がんワクチン療法
免疫細胞ががん細胞を攻撃する「目印」を注射や点滴などで投与して、がん細胞を攻撃する免疫細胞を増やして、がんを抑えようとする治療方法。

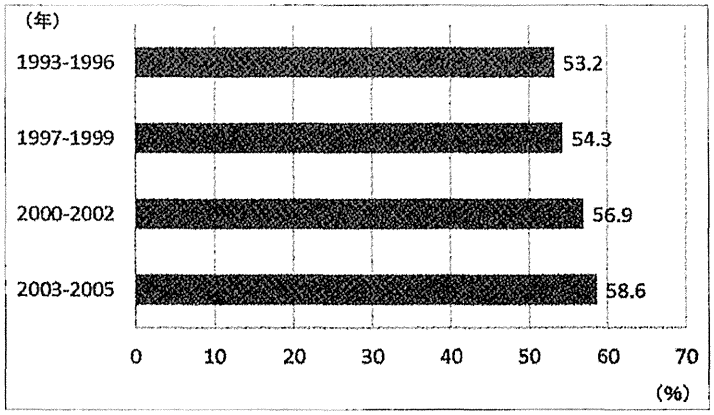
(2)治験
国から医薬品として承認を受けるために実施する臨床試験のこと。

(3)先進医療
通常、保険診療を行う病院では、研究段階の治療方法は受けることができない。先進医療は、研究段階ではあるが、高度な医療として国に認められ、例外的に、保険診療を行う病院でも受けることができる治療方法。

(4)5年相対生存率
がんと診断された場合に、治療でどのくらい生命を救えるかを示す指標。あるがんと診断された人のうち5年後に

表1　がんと診断されてからの生存率（5年相対生存率）の推移

地域がん登録の統計をもとに、1993年から1996年、1997年から1999年、2000年から2002年、2003から2005年に、がんと診断された患者さんの5年相対生存率の推移を示しております。なおこの集計では、上皮内がん（大腸の粘膜がんを含む）を除いて解析しております。



法が開発されることが必要と考えられますし、新しく開発されつつある治療法の候補が、速やかに実用化へと向かうことが求められております。そのためには、大学などでのがん研究がより活発に行われるとともに、研究成果を新薬候補として薬事承認へと向けるための体制を産官学が連携して整備する必要があります。

わが国でも、大学などによる基礎研究の成果を新薬の臨床開発へとつなげるため、製薬企業にのみ認められた治験を薬事法改正により医師主導で行えるようになりました（図1）。

近年、がんワクチンの分野でも、このような医師主導治験[5]が行われるようになってきており、本稿でもご説明したいと思います。

がんペプチドワクチン療法とは

がん免疫と、がんペプチドワクチン療法につきましては、河上先生、中面先生、笹田先生の稿でもご説明があるかと思いますが、本稿でも簡単にご説明したいと思います。

免疫は、ウイルスや細菌などに対する生体の防御反応として知られています。同時に、正常な細胞とは異なるがん細胞に対しても、免疫は異常な細胞を識別して死滅されることにより体を守る作用があると考えられております。一方、がん細胞には、正常細胞に比べて過剰に発現したり、がん細胞のみに発現する遺伝子や蛋白質があり、「がん抗原」と呼ばれております。

免疫細胞は、がん抗原を目印としてがん細胞を識別し、がん細胞を死滅させます。がんワクチン療法は、さまざまな種類のがん細胞に存在するがん抗原タンパク質を小さく断片化した分子（ペプチド）を合成して投与することにより、樹状細胞という抗原提示細胞にがん抗原の情報を伝えます。

ペプチドは、樹状細胞表面にあるヒト白血球抗原（human leukocyte antigen：HLA）[6]に提示され、その結果、がん細胞だけを認識して破壊するリンパ球（細胞傷害性T細胞（cytotoxic T Lymphocyte：CTL））が誘導され、がん治療に利用されます。

臨床開発を進めているがんワクチン

現在、本邦では、大学やがん研究センターなどの研究機関にて、いくつかのがんワクチンの臨床開発が行われております。本稿では、わたしたちが東京大学医科学研究所にて、臨床開発に携わっている2つのがんワクチンについて、ご紹介したいと思います。

1つ目は、「サバイビン」という分子を標的としたがんペプチドワクチンです。札幌医科大学において、

生存している人の割合が、日本人全体で5年後に生存している人の割合に比べてどのくらい低いかで表します。100％に近いほど治療で生命を救えるがん、0％に近いほど治療で生命を救い難いがんであることを意味します。

(5)医師主導治験
医師主導治験は、医師自らが治験を企画・立案し、治験計画届を提出して行う治験です。従来の治験は製薬企業の主導で行われてきましたが、平成15年の薬事法改正により、医師主導治験が可能となりました。改正以前は、医薬品としては有望であっても、開発費用に見合うだけの市場性やリスク面が整わない場合には、製薬企業などが治験に乗り出せない場合がありました。このため、大学などの研究機関が、医療現場でニーズが高い医薬品や医療機器を研究開発した場合でも、企業にとって採算が取れない製品の開発は進みづらい状況にありました。医師自らが治験を実施することを制度上可能とすることで、企業のみでは臨床開発が進みづらい医薬品や医療機器でも、薬事承認取得を目指すことが可能となりました。

(6)ヒト白血球抗原（human leukocyte antigen：HLA）
HLAは、ペプチドを載せることができるお皿のような形を持った分子であり、この分子の形は人によって異なり、A24型、A02型といったような遺伝子型によって規定されます。日本人

20年のがん免疫研究によって開発されたものです。このワクチンは、「がん幹細胞」に多く発現するがん抗原を標的としております。がん幹細胞は、がん再発と転移の根幹をなす細胞と推察されており、がん幹細胞を標的とする治療は、治療抵抗性のがんにも有効な治療法になるのではと期待されております。実際にサバイビンは、がん細胞以外にも精巣、胸腺、胎盤といった正常な組織の細胞にも存在しておりますが、がん幹細胞において正常な細胞に比べて数多く存在していることがわかっております。

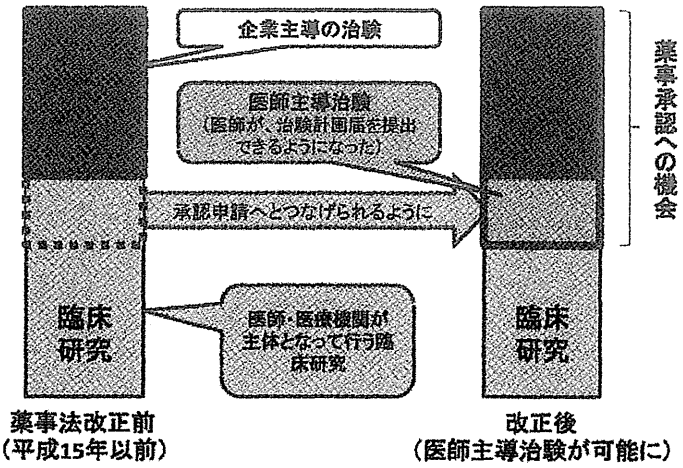


図1　医師主導治験導入による治験の範囲拡大
薬事法改正により、企業主導のみならず、医師主導での治験実 施が可能となり、薬事承認を目指した臨床開発の機会が増えました。医師主導治験は、医師自らが治験を企画・立案し、治験計画届を提出して行う治験です。従来の治験は製薬企業の主導で行われてきましたが、平成15年の薬事法改正により、可能となりました。改正以前は、医薬品としては有望であっても、開発費用に見合うだけの市場性やリスク面が整わない場合には、製薬企業などが治験に乗り出せない場合がありました。このため、大学などの研究機関が、医療現場でニーズが高い医薬品や医療機器を研究開発した場合でも、企業にとって採算が取れない製品の開発は進みづらい状況にありました。医師自らが治験を実施することを制度上可能とすることで、企業のみでは臨床開発が進みづらい医薬品や医療機器でも、薬事承認取得を目指すことが可能となりました。

このワクチンは、日本人に最も多いとされるＨＬＡのＡ24型と結合しやすいことから、ＨＬＡ－Ａ24のＨＬＡ型を持っている患者さんが対象となります。これまで、札幌医科大学にて、大腸がん・膵臓がん・乳がん・口腔がん・膀胱がんを対象とした自主臨床研究が行われ、ペプチドワクチンを反復して皮下注射した結果、患者さんの体内でリンパ球が刺激され、サバイビン分子が存在するがん細胞を攻撃して死滅させる能力を持ったリンパ球（細胞傷害性Ｔリンパ球）が誘導され、一部の患者さんでは病状が安定する方もおられました。

これらの臨床研究の結果をふまえて、薬事承認(7)（医薬品としての厚労省の認可）に向けた検討として、進行消化器がんを対象とした第1相試験が医師主導治験として行われ、安全性と、免疫学的な有効性とが確認されております。現在、進行膵臓がんを対象とした第2相試験の医師主導治験を準備しており、開始されております。このたびは、ワクチンのみならず、腫瘍免疫を高めるとされるインターフェロンとの併用についても、効果と安全性を検証する予定です。

難治がんである肺がんに対するがんワクチンについては、東京大学医科学研究所と滋賀医科大学で開発が進められています。１０００症例以上の肺がん検体を用いた網羅的な遺伝子・タンパク質の発現解析を通じて40種類以上のがん抗原が発見され、ＣＴＬを活性化できるがんペプチドが複数作製されています。このようにして開発されたペプチドワクチンを利用し、肺がんに対する効果を統計学的かつ免疫学的に評価する

の6割がＡ24型のＨＬＡ分子を有していることから、国内でのがんワクチンの臨床試験では、ＨＬＡ Ａ24型に対するがん抗原ペプチドが使用されることが多いです。

(7)薬事承認
医薬品として国から承認を受けること。

ために、滋賀医科大学附属病院において、標準的な抗がん剤治療が無効になった進行肺がんの患者さんを対象に臨床試験を行ってきました。現在、国内の4大学の附属病院で標準的な治療が効かなくなった進行非小細胞肺がんの患者さんを対象に、薬事承認を目指した医師主導治験を実施しています（第2相臨床試験として実施。被験者（治験参加者）の登録は終了）。

基礎医学の成果を新薬として薬事承認へとつなげるためには、10年以上の研究継続が必要とされます。がんワクチンにおいても、同様であり、多くの研究者が長年かけて研究してきた成果が、薬事承認を目指した臨床試験として、科学的に検証されつつあります。私たちは、膵臓がん、肺がんにおいて、薬事承認を目指して、がんワクチン療法の臨床開発を進めるとともに、さらに幅広いがん腫を対象としたペプチドワクチン療法の開発に取り組んでいきたいと思います。

独立行政法人国立がん研究センターがん研究開発費「地域がん登録精度向上と活用に関する研究」平成22年度報告書

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札幌医科大学内科教授、同学長・理事長を歴任。この間、米国NIH国際博士研究員、英国ケンブリッジ大学MRC／LMBで、ミルンコタイン教授（ノーベル賞受賞者）に師事。2010年より東京大学教授、同医科学研究所病院長を歴任。現在、東京大学医科学研究所医療イノベーション室特任教授。腫瘍免疫学や、がんの分子診断・治療を一貫して研究。紫綬、褒章など多数受賞。

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1999年札幌医科大学医学部卒業。日本学術振興会特別研究員、ハーバード大学タフツファーバーがん研究所博士研究員、札幌医科大学第一内科を経て、2013年より東京大学医科学研究所附属病院特任講師。文部科学省橋渡し研究加速ネットワークプログラム事業に従事し、一貫して基礎医学と臨床医学とを両輪としたがん研究を志している。

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Defined TLR3-specific adjuvant that induces NK and CTL activation without significant cytokine production *in vivo*

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Ligand stimulation of the Toll-like receptors (TLRs) triggers innate immune response, cytokine production and cellular immune activation in dendritic cells. However, most TLR ligands are microbial constituents, which cause inflammation and toxicity. Toxic response could be reduced for secure immunotherapy through the use of chemically synthesized ligands with defined functions. Here we create an RNA ligand for TLR3 with no ability to activate the RIG-I/MDA5 pathway. This TLR3 ligand is a chimeric molecule consisting of phosphorothioate ODN-guided dsRNA (sODN-dsRNA), which elicits far less cytokine production than poly(I:C) *in vitro* and *in vivo*. The activation of TLR3/TICAM-1 pathway by sODN-dsRNA effectively induces natural killer and cytotoxic T cells in tumour-loaded mice, thereby establishing antitumour immunity. Systemic cytokinemia does not occur following subcutaneous or even intraperitoneal administration of sODN-dsRNA, indicating that TICAM-1 signalling with minute local cytokines sufficiently activate dendritic cells to prime tumoricidal effectors *in vivo*.

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Double-stranded (ds) RNA is often a signature of viral infection, which induces production of type I interferon (IFN) and inflammatory cytokines[1,2]. Its putative analogue polyinosinic:polycytidylic acid (poly(I:C)) exhibits both strong antiviral and anticancer potential[3,4]. Poly(I:C) has been considered a promising adjuvant for cancer immunotherapy for several decades[4–6]. In mouse models, growth retardation of syngenic implant tumours is observed following administration of poly(I:C)[7,8], which is due to dendritic cell (DC)-derived natural killer (NK) and cytotoxic T-cell (CTL) activity[9,10]. Nevertheless, it has not been successfully used therapeutically in patients with cancer[5,6]. The amount of poly(I:C) required for an adequate therapeutic response causes side effects, including arthlargia, fever, erythema and sometimes life-threatening endotoxin-like shock[5,6], which have prevented application of this dsRNA analogue from the clinical use. These side effects may be related to cytokine storm induced by dsRNA, although the situation is somewhat alleviated when minimal poly(I:C)-LC (poly-L-lysine and methylcellulose) is used instead of effector-inducible doses of mere poly(I:C) alone[5,6,11].

According to recent understanding on pattern recognition of innate immunity, poly(I:C) is a ligand for multiple pattern recognition receptors (PRRs), including protein kinase R, retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and Toll-like receptor (TLR) 3 (refs 1,4,12). Virus replication usually produces dsRNA within the cytoplasm of infected cells and stimulates the cytoplasmic RNA sensors[12,13]. In contrast, TLR3 is activated when dsRNA liberated from virus-infected cells is internalized into the endosome of non-infected phagocytes[4,14], such as DCs and macrophages. Type I IFN and DC-mediated immune responses are evoked to suppress virus replication. Physiologically, these responses occur in a complex manner, therefore, what happens *in vivo* when only a single receptor is stimulated remains to be elucidated, whereas what happens *in vivo* when a single gene is disrupted has been reported in knockout (KO) mouse studies[1]. It is therefore crucial in drug design to create PRR ligands specific for each PRR for the development of immune adjuvant.

Regression of tumour with a lesser major histocompatibility complex expression[15] is caused by reciprocal activation of NK cells by poly(I:C)-stimulated DC[9,16]. However, in antitumour immunity, constitutive proliferation of antitumour CTL is important and antigen (Ag)-presenting DC must capture not only innate patterns but also tumour-associated Ag (TAA) for their cross-priming[10,17]. CD8α$^+$ DC in mouse lymphoid tissue[10,18,19] and CD103$^+$ and CD141$^+$ DCs in humans[18–20] are representative subsets that express TLR3 and induce efficient Ag cross-presentation in response to dsRNA enabling presentation of Ags to CD8$^+$ T cells on their major histocompatibility complex class I proteins. In contrast, interleukin (IL)-12, IL-6, tumour necrosis factor (TNF)-α and IFN-α/β are the main mediators released in the serum secondary to exogenously administered poly(I:C)[4,20,21]. Studies in KO mice suggested that TLR3 has a pivotal role in inducing cross-presentation[10,17], but its role is marginal in systemic cytokine/IFN production *in vivo*[21]. Most cytokines (except IL-12 p40) and type I IFN detected in serum are attributable to poly(I:C)'s stimulation of RIG-I and/or MDA5, that is, the mitochondrial antiviral-signalling protein (MAVS) pathway[12,21]. CTL/NK cell activation and robust cytokine production can be assigned, although partly overlapping, to the TLR3/Toll-IL-1 receptor domain-containing adaptor molecule (TICAM)-1 or MAVS pathway, respectively.

Here we generated synthetic dsRNA derivatives expected to specifically act on TLR3, but not on RIG-I/MDA5. These ligands exhibited strong activity in inducing antitumour CTL and NK cells and caused marked regression of tumours without off-target effects including significant increases of serum cytokine/IFN levels in mouse models.

Results

Design of novel TLR3 agonist. What we experienced in developing an RNA adjuvant was that: very little *in vitro* transcribed dsRNAs entered the human cells[22], whereas poly(I:C) as well as CpG or control GpC phosphorothioate oligodeoxynucleotides (sODNs) reached the endosome in human myeloid DCs and epithelial cells. Poly(I:C) and sODNs appeared to share the uptake receptor[23]. To deliver dsRNA to endosome TLR3, we have connected sODN to 5′ sense RNA and annealed it with antisense RNA (Fig. 1a) to guide dsRNA internalization into TLR3-positive endosomes. The RNA source was chosen from a vaccine strain of measles virus (MV), as children around the world undergo MV vaccination without severe adverse events. Because >40 bp dsRNA may be the minimal length for activation of TLR3 (ref. 24), we selected the region of defective interference RNA in the vaccine MV that causes no RNA interference[25].

Because direct chemical synthesis of long sequences of RNA was unfeasible in our laboratory until recently, we first made the RNA duplex structures by *in vitro* transcription and annealing. sODN was connected via a linker DNA to dsRNA, so the three-chain structures were first designed (Fig. 1b) to carry forward by many trial-and-error tests for a specific TLR3 agonist. A GpC-type sODN cap cM362 (Fig. 1a,b) facilitates targeting to TLR3-positive endosomes, does not activate TLR9 and blocks dsRNA-mediated RIG-I/MDA5 activation; therefore it meets our criteria for a single PRR agonist.

Testing function of *in vitro* transcribed sODN-dsRNAs. Various kinds of sODN-dsRNA hybrid molecules were prepared by *in vitro* transcription and annealing (Supplementary Fig. 1a, Supplementary Tables 1 and 2). In preliminary experiments to screen for a preferential sODN-dsRNA, we tested reporter gene (Luc125 for IFN-β promoter) activation in HEK293 cells expressing human TLR3.

sODN-dsRNAs with dsRNA of >99 bp in length induced TLR3-dependent IFN-β-promoter activation similar to that induced by poly(I:C) in the presence or absence of fetal calf serum (FCS), whereas sODN-dsRNAs with dsRNA of <79 bp induced hardly any activation of TLR3 (Supplementary Fig. 1b,c). Notably, none of the sODN-dsRNAs examined were able to activate cytoplasmic RNA sensors when transfected into HEK293 cells (Supplementary Fig. 2). We examined whether GpC motif or the length of sODN influenced the uptake of sODN-dsRNA. TLR3-mediated IFN-β promoter activation by sODN-dsRNA was independent of the presence of a GpC motif of sODN but dependent on the length; almost >20-mer of sODN is required for full activation of endosomal TLR3 (Supplementary Fig. 3). 139 bp dsRNA and control B-type (c2006) or C-type (cM362) sODN were good candidates for activation of endosomal TLR3 with no TLR9 activation.

We next examined the internalization of Cy3-labelled cM362-dsRNA (cM362-79, cM362-99 and cM362-139) in HeLa cells. cM362-dsRNAs were all similarly bound to the cell surface at 4 °C, but dsRNA73 and dsRNA139 without cM362 could not bind (Supplementary Fig. 4). When cells were incubated at 37 °C, cM362-99 and cM362-139 both entered the cells more quickly than cM362 and cM362-79, localized in the early endosome after 15 min incubation and were retained for up to 120 min, whereas cM362 and cM362-79 co-localized with EEA1 at a later time point (30 min) and quickly moved to the lysosomes. Localization of cM362-139 in the lysosomes was observed after 60 min

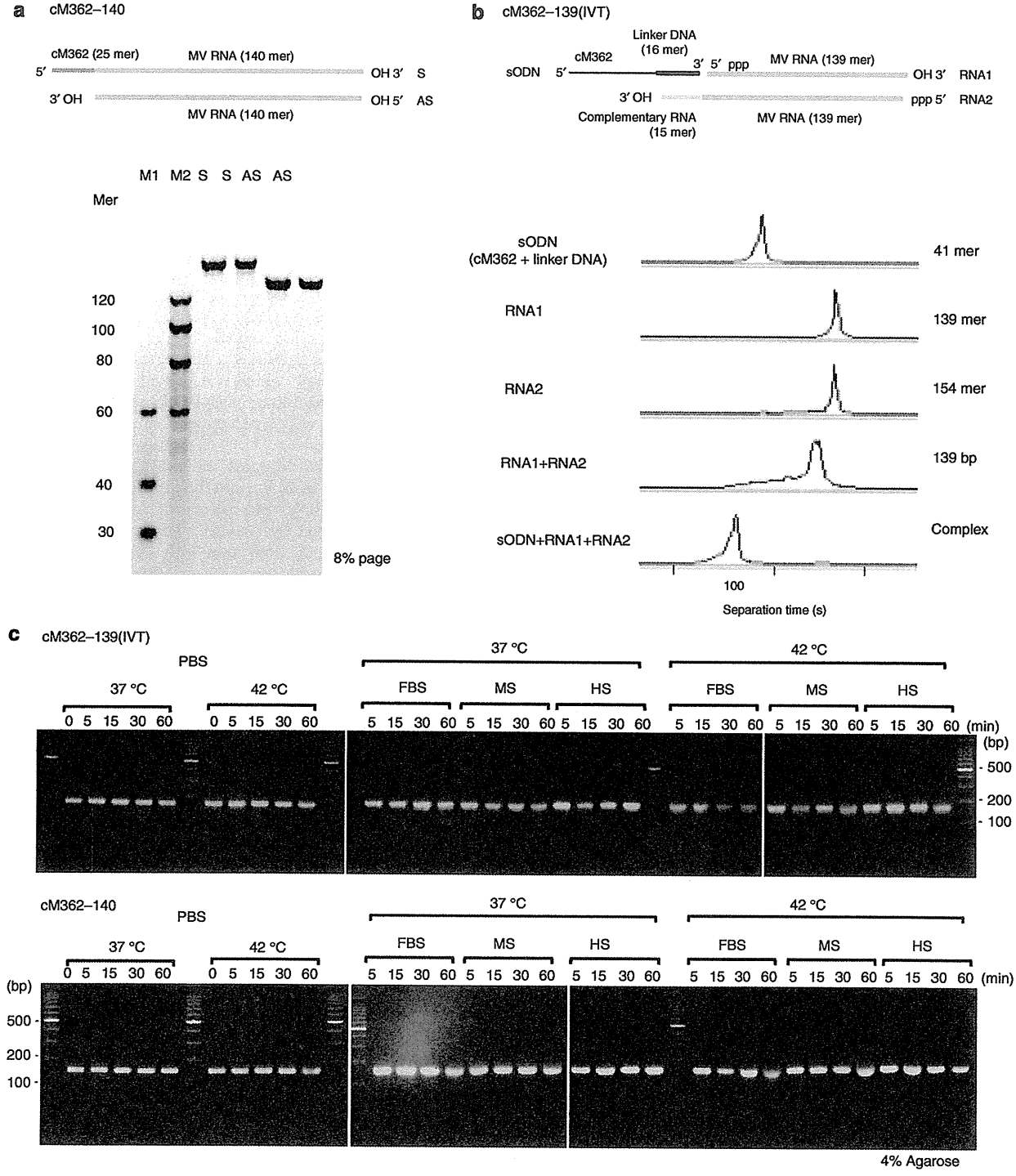

Figure 1 | Preparation of cM362-139 and cM362-140. (**a**) Schematic diagram of cM362-140. cM362-140 consists of chemically synthesized two nucleotide strands. The sense strand (S) is a 140 mer RNA capped with cM362 (25 mer) at 5′ site. The antisense strand (AS) is the complementary 140 mer RNA. Five pmol of S and AS RNAs were analysed on 8% PAGE containing 7M urea. M1 and M2, RNA size markers. (**b**) Schematic diagram of cM362-139(IVT) and its electropherograms. cM362-139(IVT) consists of three nucleotide strands, sODN (cM362 + linker DNA; 41 mer), *in vitro* transcribed sense RNA strand (RNA1; 139 mer) and antisense RNA strand (RNA2; 154 mer). All sequences of DNA and RNAs are described in Supplementary Tables. sODN, RNA1, RNA2, dsRNA (RNA1 + RNA2) and complex cM362-139 (sODN + RNA1 + RNA2) were analysed using multi-channel microchip electrophoresis. (**c**) Stability of cM362-139(IVT) and cM362-140. cM362-139(IVT) and cM362-140 were incubated in PBS with or without 10% heat-inactivated FBS, mouse serum (MS) or human serum (HS) at 37 °C or 42 °C for indicated time points. Aliquots containing 0.1 μg of treated cM362-dsRNAs were loaded onto a 4% agarose gel.

incubation, which was relatively slow compared with cM362-99 (Supplementary Fig. 4). These results suggested that the length of dsRNA together with the presence of cM362 influences the internalization speed and retention time in the TLR3-situated early endosome.

The abilities of cM362-dsRNAs to induce cytokine production were then examined using splenic DCs from wild-type and TLR3-deficient mice. cM362-79 and -139 induced a slight increase in TNF-α and IL-6 production by splenic CD11c$^+$ DCs in a TLR3-dependent manner (Supplementary Fig. 5a). Again, cM362-dsRNAs did not activate cytoplasmic RNA sensors in mouse splenic CD11c$^+$ DCs (Supplementary Fig. 5b). Thus, the chimeric compound cM362-139 appears to possess novel features that enable it to be quickly delivered to TLR3-positive endosomes, retained for a long period in the endosomes, and activate TLR3 but not RIG-like receptor (RLR).

As a TLR3-specific agonist with high activity, we selected cM362-139 for further *in vivo* studies with tumour-loaded mice.

Chemical synthesis of cM362-140. As chemical synthesis of sODN-dsRNA is indispensable for complying with good manufacturing practice (GMP) criteria, we started trials for chemical synthesis of cM362-140 (Fig. 1a), and its activity was analysed in comparison with *in vitro* transcribed cM362-139 (Fig. 1a,b). A synthesized 165 mer sense cM362-RNA hybrid and a 140mer antisense RNA, both of which consisted of single bands on 8% polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography/mass spectrometry analysis were annealed to make cM362-140 (Fig. 1a, lower panel). First, the degradability of cM362-139(IVT) and chemically synthesized cM362-140 were tested under different conditions. Both compounds were stable during incubation in PBS with or without 10% FBS, mouse serum or human serum at 37 °C for 60 min. cM362-139(IVT) was slightly degraded in PBS containing FBS or mouse serum but not human serum during incubation at 42 °C (Fig. 1c). Notably, cM362-139(IVT) was susceptible to degradation by nucleases during incubation in RNase-free water containing 10% FBS or mouse serum, but relatively resistant to human serum (Supplementary Fig. 6). In contrast, cM362-140 was quite stable under all these conditions. Thus, synthetic cM362-140 consisting of the cM362-capped RNA strand and antisense RNA strand was found to be more resistant to serum nucleases than cM362-139(IVT) consisting of three nucleotide strands.

cM362-140 activates TLR3 but not cytoplasmic RNA sensors. cM362-140 efficiently induced TLR3-dependent IFN-β promoter activation similar to cM362-139(IVT), when it was used to stimulate HEK293 cells expressing human TLR3 by simple addition or endosomal delivery (Fig. 2a, left and centre panels). Activation of cytoplasmic RNA/DNA sensors by cM362-140 was hardly observed in HEK293 cells similar to cM362-139(IVT) (Fig. 2a, right panel). To address the potential of cM362-140 for cytokine induction, splenic DCs from wild-type, *Tlr3*$^{-/-}$ or *Mavs*$^{-/-}$ mice were stimulated with poly(I:C), cM362-139(IVT), control synthetic dsRNA140 or cM362-140, either alone or complexed with N-(1-(2,3-Dioleoyloxy)propyl)-N, N, N-trimethylammonium methyl-sulfate) (DOTAP) to deliver them to endosomes, or complexed with Lipofectamine to deliver them to cytoplasm. Extracellular addition of cM362-139(IVT) and cM362-140 to splenic DCs induced a subtle increase in TNF-α, IL-6 and IFN-β production compared with poly(I:C) treatment, whereas synthetic dsRNA140 (with no GpC) did not induce any cytokine over the detection limits (Fig. 2b, left panels). Endosomal delivery of cM362-139(IVT) or cM362-140 with DOTAP also induced minimal levels of TNF-α, IL-6 and IFN-β dependent upon TLR3 (Fig. 2b, centre panels). When the compounds were transfected into cytoplasm with Lipofectamine, MAVS-dependent cytokine production was barely observed with cM362-140, whereas only low levels of IL-6 and IFN-β were induced with cM362-139 in TLR3 KO DC (Fig. 2b, right panel). This MAVS activity may reflect the exposure of a few 5′-triphosphated species of cM362-139(IVT) due to minor RNA degradation. These results indicate that cM362-140 targets endosomal TLR3 and activate the TICAM-1 pathway in both human and mouse cells.

***In vivo* cytokine induction by cM362-140.** Injection of poly(I:C) into mouse peritoneal cavity strongly induced proinflammatory cytokine production in a TLR3-independent manner and high level of TNF-α and IL-6 were detected in sera at 3 h after injection (Fig. 3a). In contrast, both cM362-140 and cM362-139(IVT) hardly induced cytokine production and serum TNF-α, IL-6 and IL-10 levels were very low, which is mediated by TLR3 (Fig. 3a). Unlike poly(I:C), cM362-140 or -139(IVT) induced undetectable levels of IFN-β in wild-type mouse sera (Supplementary Fig. 7).

A subcutaneous (s.c.) injection of cM362-140 induced the mRNAs of IFN-β and IL-6, but not TNF-α, in the inguinal and axillary lymph nodes (LNs) and spleen; the expression level was lower than that induced by poly(I:C) (Fig. 3b). These results suggest that TLR3-specific activation with cM362-140 results in low levels of cytokine production *in vivo* either by intraperitoneal (i.p.) or s.c. administration.

EG7 tumour regression by CTL induced by cM362-139/140. The next question was whether cM362-139(IVT) causes tumour growth retardation as observed with poly(I:C). EG7 cells (a lymphoma cell line containing ovalbumin, OVA) were inoculated into the back of wild-type (WT) C57BL/6 mice, and the indicated materials were injected s.c. around the EG7 tumour that developed (Fig. 4). Tumour growth was mildly retarded by treatment with poly(I:C) or cM362-139(IVT) alone (Fig. 4a). Combination therapy of OVA and poly(I:C) or cM362-139(IVT) resulted in complete remission of EG7 tumour >12 days after the treatment (Fig. 4a). The results infer that the combination of RNA adjuvant + tumour Ag exerts antitumour immune effect in spite of the low induction of proinflammatory cytokines.

We next tested whether s.c. injection of cM362-139(IVT) plus OVA induced CTL proliferation. The OVA tetramer assay and IFN-γ production were employed to evaluate OVA-specific CD8$^+$ T-cell activation. Combination therapy of cM362-139(IVT) with OVA exhibited an increase in the frequency of Ag-specific CD8$^+$ T cells comparable to poly(I:C) with OVA (Fig. 4b). Ag-specific CD8$^+$ T cells clonally proliferated against EG7 as *in vitro* cytotoxicity was directed exclusively to EG7 in mice stimulated with cM362-139(IVT) with OVA (Fig. 4c) as well as poly(I:C) with OVA[9]. EG7 growth retardation by cM362-139(IVT) with OVA was largely abrogated in TLR3 KO mice (Supplementary Fig. 8), suggesting that cM362-139(IVT) acts on host TLR3 *in vivo*. However, mild tumour growth retardation was still observed with cM362-139(IVT) with OVA in TLR3 KO mice (Supplementary Fig. 8), implying minor involvement of EG7 cell TLR3 or other host RNA sensors, such as RLR[26] and DEAD-box helicases[27], in *in vivo* tumour regression. Yet, tumor cell's TLR3 signaling and chemokine induction might affect tumor remission (refs 28–30).

We finally tested whether chemically synthesized cM362-140 harbours the ability to retard EG7 growth in the same model. The synthetic cM362-140 showed ~80% activity for IFN-β reporter activation compared with cM362-139(IVT) and a single

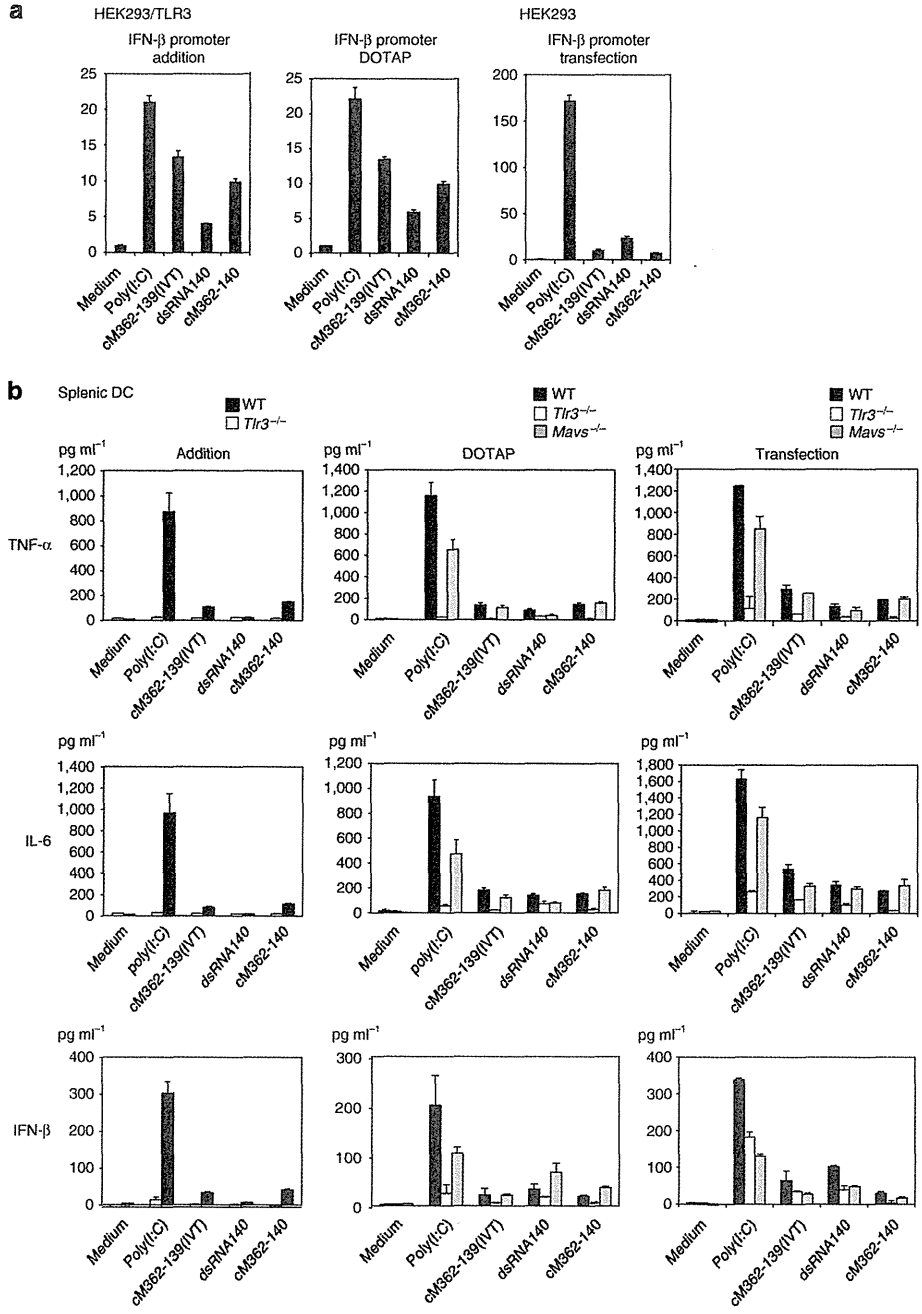

Figure 2 | TLR3-activating ability of cM362-140 in human and mouse cells. (**a**) TLR3-dependent IFN-β promoter activation by cM362-140. HEK293 cells were transiently transfected with IFN-β reporter and phRL-TK together with (left and middle panels) or without the expression plasmid for hTLR3 (right panel). Twenty-four hours after transfection, culture medium was removed and 10 μg ml^{-1} poly(I:C), cM362-139(IVT), dsRNA140 or cM362-140 in fresh medium (left panel), or the same compounds complexed with DOTAP liposomal reagent (middle panel), or with Lipofectamine 2000 (right panel) were added to cells. Luciferase activity was measured 6 h (left and middle panel) or 24 h (right panel) after stimulation, and expressed as fold induction relative to the activity of non-stimulated cells. Representative data from three independent experiments, each performed in triplicate, are shown (mean ± s.d.). (**b**) Splenic CD11c$^+$ DCs (1.0×10^6 per ml) isolated from *Tlr3*$^{-/-}$, *Mavs*$^{-/-}$ or WT mice were stimulated with 10 μg ml^{-1} untreated (left panels), DOTAP liposomal reagent-conjugated (middle panels) or Lipofectamine 2000-conjugated (right panel) nucleic acids as indicated. Twenty-four hours after stimulation, IFN-β in the culture supernatants was quantified using ELISA. TNF-α and IL-6 levels were measured using CBA. Representative data from three to five independent experiments are shown (mean ± s.d.).

treatment with cM362-140 caused barely any regression of EG7 tumours in this model (Fig. 5a). Nevertheless, combination therapy with OVA + cM362-140 still induced tumour growth retardation (Fig. 5a). OVA-specific CD8$^+$ T cells proliferated and activated in the mice stimulated with OVA + cM362-140, as assessed by tetramer assay (Fig. 5b) and IFN-γ production (Fig. 5c). We confirmed that the induction of OVA-specific CD8$^+$ T-cell activation by cM362-140 + OVA largely depends

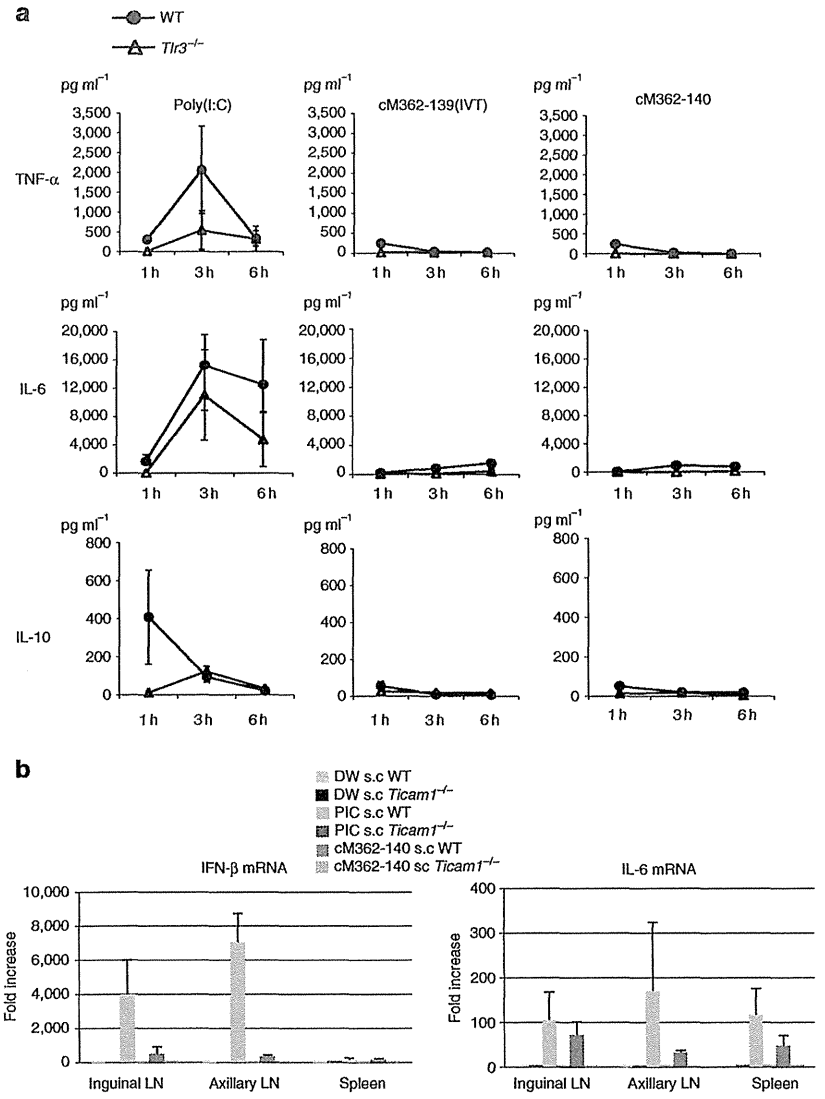

Figure 3 | cM362-139(IVT) and cM362-140 do not induce inflammatory cytokines. (**a**) Wild-type or *Tlr3*$^{-/-}$ female mice (9 week) were injected i.p. with 50 μg poly(I:C), cM362-139(IVT) or cM362-140 in RNase-free water. At timed intervals, blood was collected from the tail vein and TNF-α, IL-6 and IL-10 levels in each sample were measured using a CBA. Data are shown as the mean ± s.e.; $n=3$ mice per group. (**b**) Wild-type or *Ticam*$^{-/-}$ female mice were injected s.c. with distilled water (DW), poly(I:C) or cM362-140 in RNase-free water. After 6 h, spleen, inguinal and axillary LNs were harvested and *IFN-β* and *IL-6* mRNA expressions were quantified by qPCR. Data are expressed as the fold induction relative to the expression in DW-injected mice and shown as the mean ± s.e.; $n=3$ mice per group.

on TLR3/TICAM-1 using KO mice (Supplementary Fig. 9). Although polyI:C induces RIP1/3-mediated necroptosis via TICAM-1 in some tumour lines, cM362-140 was not the case in EG7 tumour (Supplementary Fig. 10). Hence, TLR3 has an important role in inducing cM362-140-mediated immune response and tumour growth retardation in the s.c. setting we employed in this study.

Antigen-specific CD8$^+$ T-cell priming by cM362-139/140. The Ag-specific CD8$^+$ T-cell priming ability of cM362-139/140 in tumour-free settings was next examined using spleen and inguinal LN cells. Wild-type mice were injected s.c. with OVA with or without RNA adjuvants twice per week. Since OVA-specific CD8$^+$ T cells most proliferated in spleen or inguinal LN 4 days after the last injection of OVA + poly(I:C) (Fig. 6a), spleen and LN cells were harvested from mice 4 days after the last adjuvant injection. cM362-139/140 significantly induced OVA-specific CD8$^+$ T-cell proliferation in the inguinal LN and spleen compared with poly(I:C) (Fig. 6b). OVA-specific IFN-γ production in spleen cells was also efficiently induced by cM362-139(IVT) and cM362-140 (Fig. 6c). The TICAM-1 pathway was mainly involved in Ag-specific CD8$^+$ T-cell activation induced by cM362-140 (Fig. 6d).

NK cell-mediated B16 tumour regression by cM362-139/140. Using a C57BL/6-B16 syngeneic NK-sensitive tumour-implant model[9], we evaluated NK-dependent antitumour activity of cM362-139(IVT) injected s.c. around the pre-formed tumour (Fig. 7a). Suppression of tumour growth, determined as reported previously[9], was observed in the group that received cM362-139(IVT) compared with the water-treated group. The retardation of B16 tumour growth appeared to depend on TLR3

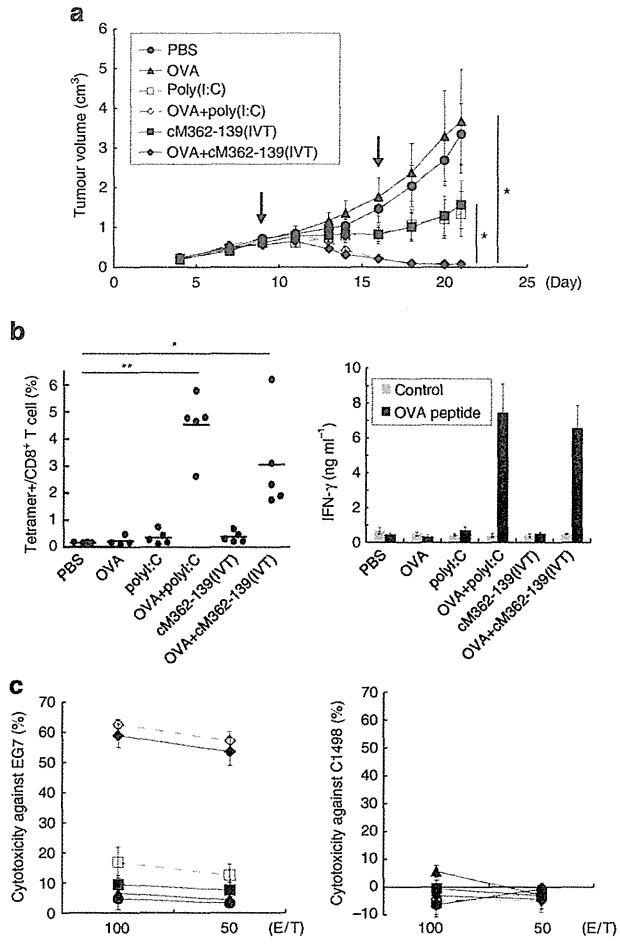

Figure 4 | cM362-139(IVT) induces CTL-mediated tumour regression. (**a**) Antitumour effect of cM362-139(IVT). WT mice were challenged with EG7 cells, and 7 and 14 days later they were s.c. injected with control PBS (●), OVA (▲), polyI:C (□) and OVA + polyI:C (◇) or cM362-139(IVT) (■) or OVA + cM362-139 (◆). Tumour size was evaluated in each group. All error bars used in this figure show ± s.e.m. Data are representative of two independent experiments. Each group consisted of five mice. $*P<0.05$ (ANOVA with Bonferroni's test). (**b**) OVA-specific CTL induction by cM362-139(IVT). Left panel: spleen cells were harvested at day 21 (7 days after 2nd therapy) and the proportion of tetramer-positive cells/CD8$^+$ T cells was evaluated. $*P<0.05$, $**P<0.01$. Right panel: spleen cells were harvested at day 21 as for the left panel. The cells were stimulated with OVA peptide for 3 days and the level of IFN-γ in the culture supernatant was measured. (**c**) Ag-specific cytotoxicity induced by cM362-139(IVT). Splenocytes collected from tumour-bearing mice at day 21 were cultured in the presence of immorbilized EG7 for 5 days. Then, the cytotoxicity against EG7 (left panel) or C1498 (control, right panel) was measured by ^{51}Cr release assay.

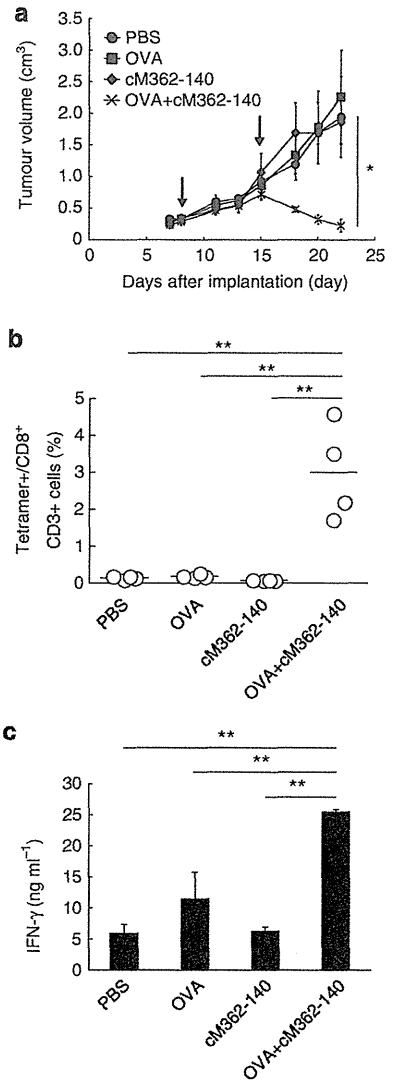

Figure 5 | cM362-140 induces EG7 tumour growth retardation. (**a**) Antitumour effect of cM362-140. As in Fig. 4, tumour-bearing mice were s.c. injected with PBS, OVA, cM362-140 and OVA + cM362-140 at timed intervals (days 8 and 15). Tumour size was evaluated in each group. (**b**) OVA-specific CTL induction by cM362-140. The proportion of tetramer-positive cells/CD8$^+$ T cells in spleen was evaluated at day 22 (7 days after 2nd therapy). (**c**) Ag-specific IFN-γ production induced by cM362-140. Splenocytes were harvested at day 22 and incubated with OVA peptides for 3 days. The level of IFN-γ in the supernatant was measured by ELISA. $*P<0.05$, $**P<0.01$ (ANOVA with Bonferroni's test).

and TICAM-1 (Fig. 7a,b). NK1.1$^+$ cells were involved in this tumour growth retardation (Fig. 7c), consistent with the NK-sensitive properties of B16 cells. No direct tumour cytolysis by macrophages[31] was associated with B16 growth retardation in the cM362-139(IVT) therapy. Splenocytes from the cM362-139(IVT)-treated group exerted higher cytotoxicity than those from the control group *in vitro* (Fig. 7d).

The chemically synthesized TLR3 ligand cM362-140 expressed a similar tumour-suppressing activity against B16 implant melanoma in the same model (Fig. 7e). This cM362-140-mediated NK-tumoricidal activity on B16 tumours was abrogated in *Ticam1*$^{-/-}$ mice (Fig. 7e). Thus, cM362-140 suppresses NK-sensitive tumours *in vivo* via TLR3 by acting as an NK-inducing adjuvant.

Discussion

Cancer immunotherapy relies on suitable adjuvants. Many TAA peptides have been synthesized, but the lack of an appropriate adjuvant to induce an immune response against the peptides has hampered progress in peptide vaccine therapy. Although many candidates, most of which were retrospectively recognized as TLR

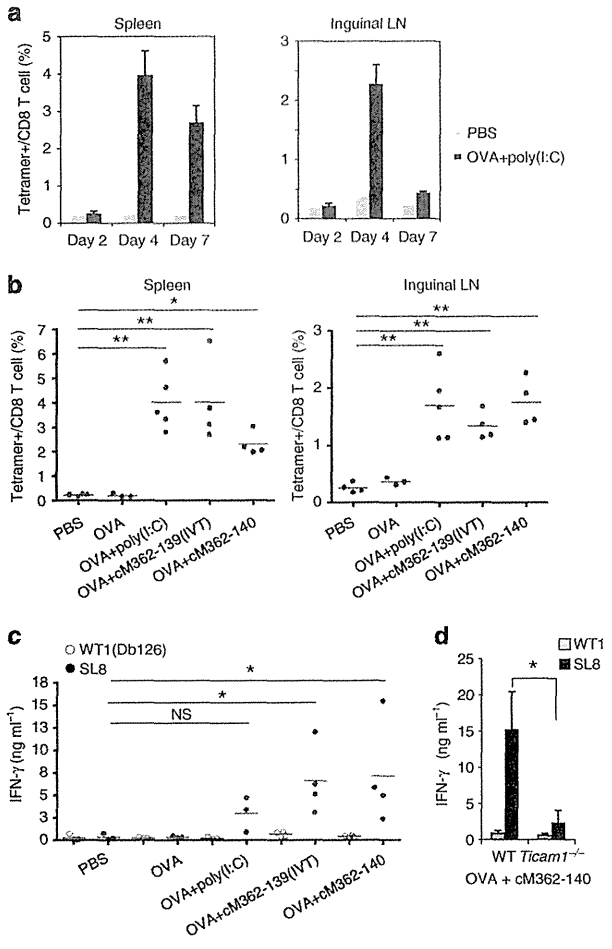

Figure 6 | cM362-139/140-induced antigen-specific CD8 T-cell activation in tumour-free settings. (**a**) Time-course experiments of poly(I:C)-induced antigen-specific CD8 T-cell activation in spleen and inguinal LN. WT mice were injected s.c. with 100 μl PBS or 50 μg poly(I:C) + 100 ng OVA twice per week. Spleen and inguinal LN cells were harvested at 2, 4 or 7 days after the last adjuvant injection and an increase of OVA-specific CD8 T-cell proliferation was evaluated with tetramer assay. (**b**,**c**) WT mice were injected s.c. with PBS, OVA, OVA + 50 μg poly(I:C), OVA + 50 μg cM362-139(IVT) or OVA + 70 μg cM362-140 twice per week. Spleen and inguinal LN cells were harvested 4 days after the last adjuvant injection and an increase of OVA-specific CD8 T-cell proliferation (**b**) and IFN-γ production (**c**) were then evaluated. (**b**) Proportion of tetramer-positive cells/CD8 T cells in spleen and inguinal LN. (**c**) Spleen cells were stimulated with OVA (SL8) or WT1 (Db126) peptide for 3 days and the level of IFN-γ in the culture supernatant was measured using a CBA. NS, no significant (>0.05), $*P<0.05$, $**P<0.01$, compared with PBS control (ANOVA with Dunnett's test). (**d**) cM362-140 + OVA induced Ag-specific CTL activation via the TICAM-1 pathway. Wild-type or *Ticam1*$^{-/-}$ mice were injected s.c. with PBS, OVA or OVA + cM362-140 as described above. Spleen cells were harvested 4 days after the last adjuvant injection and OVA-specific IFN-γ productions were assessed. PBS or OVA injection did not induce IFN-γ production from spleen cells. The data from OVA + cM362-140 injection are shown. $*P<0.05$ (Student's *t*-test).

agonists, have been tested in humans[1,6], they have not yet been clinically approved because of their undesired effects.

In this study, we designed many nucleotide adjuvants and tested their functional properties. Our approach is timely since most dsRNA receptors have been identified in the mouse and

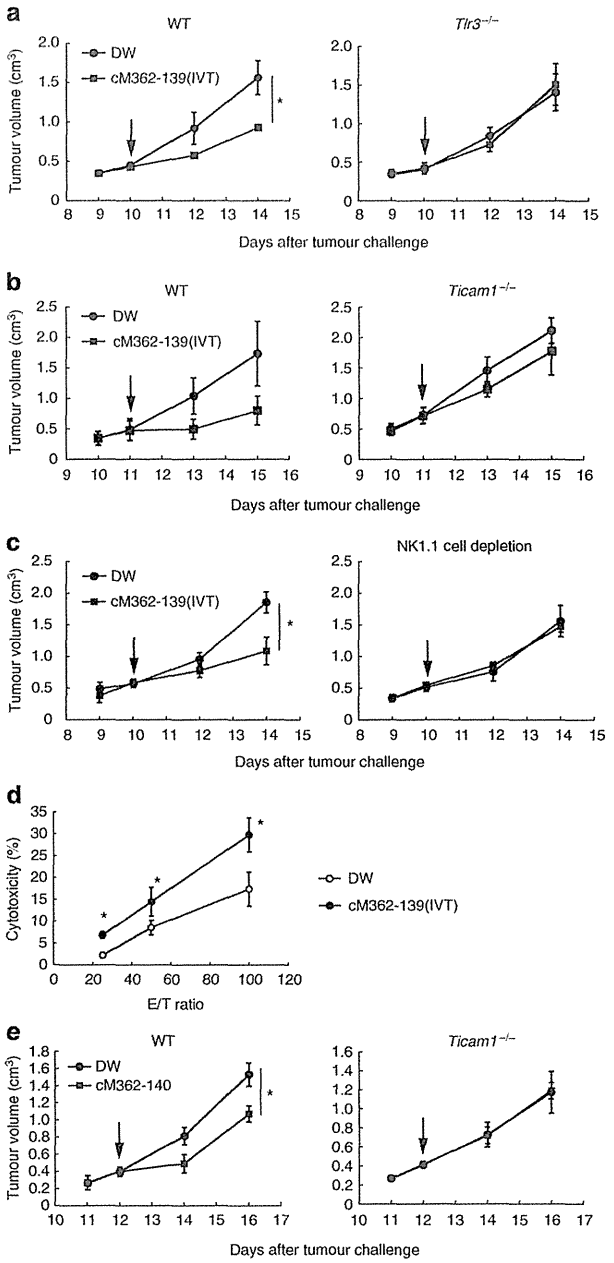

Figure 7 | Therapeutic effects of sODN-dsRNA in B16 tumour-implant model. (**a**,**b**) B16 tumour growth in mice after cM362-139(IVT) treatment. B16 melanoma cells were s.c. implanted into B6 WT mice (**a** and **b**), *Tlr3*$^{-/-}$ mice (**a**), or *Ticam1*$^{-/-}$ mice (**b**). cM362-139(IVT) or distilled water was injected s.c. around the tumour on the day indicated by arrow and then tumour volume was measured. Data are shown as tumour average volume ± s.e.; $n=3$ mice per group. $*P<0.05$. (**c**) Effect of NK cell depletion on cM362-139(IVT) treatment. Tumour-bearing mice were injected with anti-NK1.1 antibody to deplete NK cells. After 24 h, cM362-139(IVT) or distilled water was injected into the mice as described in **a**. (**d**) Cytotoxic activity of DX5^{+} NK cells isolated from cM362-139(IVT)-treated mice. DX5^{+} cells were isolated from B16 tumour-bearing mice treated with cM362-139(IVT) or distilled water for 18 h. Cytotoxic activity of DX5^{+} cells against B16 target cells was measured by ^{51}Cr release assay. (**e**) B16 tumour growth in mice after cM362-140 treatment. Tumour-bearing mice were treated with cM362-140 as described in **a**.

human[1–4], and their structures and properties have been known for a decade[32–34]. Based on our current understanding of the dsRNA response, poly(I:C) activates both TICAM-1 (ref. 35) and MAVS pathways[36] resulting in systemic cytokinemia in mice[21,37]. Viral replication intermediates, double-stranded RNA and 5′-triphosphate RNA cause activation of the RLR pathway and robust cytokine production[38,39]. Here we chemically synthesized and tested a synthetic compound, cM362-140, an sODN-dsRNA that entered the endosomes and activated the TLR3/TICAM-1 pathway *in vivo*. Specifically, cM362-140 activated TLR3 in myeloid cells, including DCs in draining LNs, and induced activation of NK cells and proliferation of CTL. There was no systemically significant production of cytokines, including IFNs, after treatment with cM362-140. Our study establishes the proof-of-concept that modified or complexed RNA can regulate the immune response through TLR3 (refs 37,40), and that cM362-140 performs this function *in vivo*.

In this study, a systemic increase in the cytokine levels was not required for the induction of an antitumour immune response. Instead, a basal cytokine level effectively primed DCs to activate tumoricidal NK cells and CTL response. Subcutaneous or i.p. injection of cM362-140 barely activated the RLR pathway, whereas poly(I:C) activated this pathway to induce systemic cytokinemia. cM362-140 activated TICAM-1 for DC maturation, but barely induced chemokine production or necroptosis in tumour cells (Supplementary Fig. 10), which were reported as a direct action of poly(I:C)[29,30]. Therefore, cM362-140 eliminates major inflammatory responses caused by poly(I:C).

The 5′-sODN sequence of cM362-140 targeted the dsRNA to the endosome and evaded recognition by RIG-I/MDA5. A 140-bp stretch of dsRNA was required for TLR3 multimerization and TICAM-1 activation, but unsatisfactory for endosome targeting[40,41]. The 5′-sODN sequence of cM362 does not contain a CpG motif, which stimulates cytokine production via TLR9 in plasmacytoid DC. To prevent Dicer-mediated RNA interference, RNA sequences specific to human mRNAs were not employed. In addition, the antisense 5′-end of cM362-140 was OH, neither phosphorylated nor capped, unlike viral or host RNA products. Although cM362-140 was an artificial compound to circumvent host innate sensors, its constituents are native and modifiable to maximize antitumour response. Although the nucleic acid-sensing system differs somewhat between mouse and human[2,4]: generally, human BDCA3$^+$ DCs express a high level of TLR3 but no TLR9 (ref. 20), whereas mouse CD8α$^+$ DCs express TLR3 and TLR9 (refs 2,18,41), extrapolating results from the mouse and applying them to the human will be crucial for establishing human immunotherapy in the future.

Type I IFNs have remarkable antiviral and antitumour properties, but sometimes elicit severe side effects during treatment in patients. For example, s.c. injection of poly(I:C) induces inflammation, erythema and fever[42]. In clinical trials, cancer patients cannot tolerate high doses of poly(I:C), even if administered via s.c. injection[11,42–44]. Poly(I:C) consists of polyI and polyC chains of variable lengths that differ in function from one batch to the next, and, for unknown reasons, exogenous poly(I:C) activates cytoplasmic RNA sensors[36]. By contrast, cM362-140 is uniform. It binds TLR3 and fails to activate cytoplasmic RNA sensors, indicating that immune modulation by RNA occurs only in the draining LNs and tumour microenvironment[45]. Thus, cM362-140 enables us with a 'defined' immunotherapy to patients without systemic cytokine response or inflammation.

Several compounds that activate the TICAM-1 pathway are clinically available. Monophosphoryl lipid A is a TLR4 adjuvant that activates the TICAM-2/TICAM-1 cascade via TLR4/MD-2 (ref. 46). However, monophosphoryl lipid A still retains the TLR4-mediated MyD88 activation, which conceptually different from sODN-dsRNA that activates a single cascade of PRR. Although poly(A:U) mildly activates the TLR3 pathway and induces type I IFN in humans[47], it has far less adjuvancy than poly(I:C)[6,28,48]. In a short-term clinical trial, poly(I:C$_{12}$U), also known as ampligen, has been shown to be less toxic than other immunotherapies[49,50]. Unfortunately, data on its uniformity and TLR3-specificity as an adjuvant are scant. Poly(I:C)LC, another antitumour adjuvant, has shown clinical promise, but it causes cytokine toxicity, thereby precluding its further development[5,6,42–44,51]. Type I IFN induction and the output of IFNAR activation appear to be predominant in low-dose poly(I:C) administration in human volunteers[42]. In another clinical study, a low dose (1.4 mg per body) of poly(I:C)LC in combination with NY-Eso-1, which contains TAA epitopes, has been shown to mount a tumour-specific T-cell response[11]. Although low doses of poly(I:C)LC induce type I IFN, it only insufficiently stimulates T-cell proliferation *in vivo*[11]. cM362-140 is advantageous over poly(I:C)LC in that a high dose can be used to specifically activate TICAM-1, but not MAVS *in vivo*. This is the first report of the successful chemical creation of a long, sequence-defined and bioactive TLR3-specific ligand.

The targeting of programmed cell death-1 and cytotoxic T-lymphocyte-associated protein-4 with monoclonal antibodies in patients with progressive-staged cancer may provide another immunotherapy breakthrough[52]. Except for an alum adjuvant that can induce a Th2 response[2,53], there is no suitable adjuvant-TAA combination for immunotherapy. Immune enhancing by adjuvant would be required since tumour cells can undergo mutations and survive to circumvent immune attack. A main problem is that current adjuvant candidates are all inflammatory, facilitating formation of tumour-supporting microenvironment, that accelerates genome instability, tumour growth and progression[45]. Here, sODN-dsRNA is a non-inflammatory adjuvant sustaining DC-mediated NK/CTL activation, its combining with TAAs will bring a therapeutic benefit to a number of patients with intractable tumours.

Methods

Cell culture, reagents and plasmid. HEK293 cells were maintained in Dulbecco's Modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated FCS (Invitrogen) and antibiotics. HeLa cells were kindly provided by Dr T. Fujita (Kyoto University) and maintained in Eagle's minimal essential medium (Nissui,) supplemented with 1% L-glutamine and 5% FCS. B16D8 melanoma cells were cultured at 37 °C under 5% CO_2 in RPMI containing 10% FCS, penicillin and streptomycin. EG7 and C1498 cells were purchased from ATCC and cultured in RPMI-1640 supplemented with 10% FCS, 55 μM 2-mercaptoethanol (2-ME) and 1 mM sodium pyruvate, and RPMI-1640 supplemented with 10% FCS and 25 ng ml^{-1} 2-ME, respectively. Poly(I:C) was purchased from Amersham Biosciences. Endotoxin-free ovalbumin was purchased from Hyglos. $OVA_{257-264}$ peptide (SL8), control WT1 peptide (Db126) and OVA ($H2K^b$-SL8) tetramer were from MBL. Human serum type AB was from Lonza. ODNs were synthesized by GeneDesign. Following antibodies were used in this study: Alexa Fluor-568-conjugated secondary antibody (Invitrogen), FITC-CD8α (53-6.7) and APC-CD3ε (145-2C11) (BioLegend), and PerCP/Cy5.5-7AAD (BD Biosciences). The human TLR3 expression plasmid was constructed in our laboratory[33].

Preparation of *in vitro* transcribed sODN-dsRNAs. The leader-trailer sequence of a MV laboratory-adapted strain of Edmonston was used as the dsRNA template[25]. DNA fragments covering this region of the MV genome and the T7 promoter sequence were amplified using PCR with specific primers and the plasmid pCR-T7 MV as a template. Sense and antisense MV RNAs from the PCR products were *in vitro* transcribed using an AmpliScribe T7 transcription kit (Epicentre Technologies) according to the manufacturer's protocol. The transcribed products were purified by 8% PAGE containing 7 M urea. After visualization by ultraviolet illumination, the appropriate bands were excised and eluted with 0.3 M sodium acetate. The eluted RNAs were ethanol precipitated and resuspended in RNase-free water. For large-scale preparation of RNAs, electro-elutions were performed using D-Tube Dialyzer Maxi (Novagen) and eluted RNAs were dialyzed, concentrated and precipitated with ethanol. The concentration of RNA was determined by measuring the absorbance at 260 nm in a

spectrophotometer. To generate sODN-dsRNA, sODN + linker DNA, sense and antisense RNA were mixed and annealed. sODNs, MV RNA sequences in sODN-dsRNA and PCR primers used in this study are described in Supplementary Tables 1–3.

Preparation of cM362-140. The chemically synthesized long RNAs as an alternative to *in vitro* transcribed RNAs were completed by a ligation reaction mediated by splint DNA[54] with slight modification. The outline of chemical synthesis was described below.

To prepare the sense strand of cM362-140, the ligation reactions were performed in two steps. First, S2 RNA (40 nmol), S3 RNA (40 nmol) and specific splint DNA (40–48 nmol) were mixed, heated at 90 °C for 5 min and slowly cooled to 4 °C (Supplementary Table 4A,B). Following hybridization, T4 DNA ligase (Takara) was added and incubated at room temperature for 16–22 h. The ligation reaction mixtures comprised 15.4 μM annealed complex, 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP and ~31 U μl^{-1} T4 DNA ligase. As the second ligation, S1 cM362-RNA (40 nmol) and the specific splint DNA (40–48 nmol) for the second ligation site were added into the first ligation reaction mixture, hybridized and T4 DNA ligase was added. The mixture was incubated at room temperature for 16–22 h. The derived full-length 165 mer sense strand was isolated by 8% PAGE containing 7 M urea and electro-elution. The subsequent procedure was the same as described in the section describing purification of *in vitro* transcribed RNAs. Overall yield was 8–10%.

To prepare the antisense RNA of cM362-140, three fragment RNAs, AS1, AS2 and AS3 (33 nmol each) and the related splint DNAs (33 nmol each) were mixed, hybridized and then T4 DNA ligase was added. The mixture was incubated at room temperature for 16–22 h. The following procedure was the same as described above. Overall yield was 15–22%. To generate cM362-140, sense- and antisense-RNAs were annealed.

Analysis with microchip electrophoresis. To analyse the cM362-139 complex, we used a microchip electrophoresis instrument (model SV1210; Hitachi Electronics Engineering Co. Ltd.). The standard procedure for electrophoresis using 2-mercaptoethanol (ME) has been previously described[55,56]. sODN or RNAs were adjusted to 0.2 μM with water. The sODN/RNA1/RNA2 complex (designated *cM362-139*) and dsRNA (RNA1 + RNA2) were prepared by mixing DNA or RNAs and hybridizing in water (final concentration 0.4 μM). The sample solution (10 μl) was applied to the sample well of the microchip device and the programme was run at 600 V for 120 s (injection time), then at 1,100 V for 180 s (separation time) under 350 V of return voltage at 20 °C. During the electric separation, DNA or RNA peaks were detected by laser induced fluorescence and analysed.

Agarose gel electrophoresis. cM362-139(IVT) and cM362-140 were incubated in PBS or in RNase-free water with or without serum, for 60 min at 37 °C or 42 °C. Aliquots containing 0.1–0.2 μg of incubated sODN-dsRNAs were then mixed with 10 × loading dye (Takara Bio Inc.) and loaded onto 3 or 4% agarose gel (Nusieve 3:1 Agarose, Lonza) containing ethidium bromide. After electric separation, nucleic complexes were visualized using ultraviolet transilluminator (FAS-III, Toyobo).

Reporter gene assay. HEK293 cells (8×10^5 cells per well) were cultured in six-well plates and transfected with the TLR3 expression vector or empty vector (400 ng per well) together with the reporter plasmid (400 ng per well) and an internal control vector, phRL-TK (Promega; 20 ng per well) using Lipofectamine 2000 (Invitrogen). The p-125 luc reporter containing the human IFN-β promoter region (−125 to +19) was provided by Dr T. Taniguchi (University of Tokyo). Twenty-four hours after transfection, cells were collected and resusupended in medium with or without FCS. Then, cells were seeded into 96-well plates and stimulated with the indicated RNAs for 6 h. The *Firefly* and *Renilla* luciferase activities were determined using a dual-luciferase reporter assay kit (Promega). The *Firefly* luciferase activity was normalized to the *Renilla* luciferase activity and was expressed as the fold induction relative to the activity in unstimulated vector-transfected cells. All assays were performed in triplicate.

Cytokine assay. Splenic CD11c$^+$ DCs from wild-type, *Tlr3*$^{-/-}$ or *Mavs*$^{-/-}$ mice were prepared as described previously[9,31]. Cells were suspended in RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated FCS and antibiotics and stimulated with the indicated RNAs. Twenty-four hours after stimulation, culture supernatants were collected and analysed for cytokine levels by ELISA or Cytometric Bead Array (CBA). ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories. CBA flex sets for mouse IL-6 and TNF-α were purchased from BD Bioscience. Experiments were performed according to the manufacturer's instructions and samples were analysed using the Facs Aria (BD Bioscience).

Confocal microscopy. HeLa cells (1.0×10^5 cells per well) were cultured on microcover glasses (Matsunami Glass Ind., Ltd) in a 12-well plate. Three hours after seeding, cells were transfected with GFP-fused Rab5a or Lamp1 using BacMam systems (Cell Light Early Endosomes-GFP BacMam 2.0 or Cell Light Lysosomes-GFP BacMam 2.0, Life technologies) or left untreated. Sixteen hours after transfection, cells were incubated with 15 μg ml^{-1} Cy3-labelled cM362, cM362-dsRNAs or dsRNAs for 30 min at 4 °C. Cells were washed twice and further incubated at 37 °C. And then, cells were fixed with 4% paraformaldehyde at the time points indicated. The coverslips were mounted onto slide glass with Prolong Gold with DAPI for nuclei staining. Cells were visualized at a × 63 magnification using a Zeiss LSM520 META microscope (Carl Zeiss Microscopy GmbH).

Quantitative PCR (qPCR). Total RNA was extracted using the Trizol reagent (Qiagen) and reverse-transcribed using a high-capacity cDNA Reverse Transcripition kit (Applied Biosystems) and random primers according to the manufacturer's instructions. QPCR was performed using specific primers for mouse *GAPDH*, *IFN-β*, *IL-6* and *TNF-α* (Supplementary Table 5) and the Step One Real-time PCR system (Applied Biosystems).

Mice. *Ticam1*$^{-/-}$ and *Mavs*$^{-/-}$ mice were made in our laboratory and back-crossed more than eight times to adapt C57BL/6 background[9,10]. Inbred C57BL/6 WT mice were purchased from CLEA Japan. *Tlr3*$^{-/-}$ mice were kindly provided by Dr S. Akira (Osaka University). Mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Female mice 6–12 weeks of age were used in all experiments, all of which were performed according to the guidelines issued by the Hokkaido University Animal Care and Use Committee.

***In vivo* mouse cytokine assay.** Wild-type and *Tlr3*$^{-/-}$ mice (9 weeks) were injected i.p. with 50 μl (50 μg) poly(I:C), cM362-139 (IVT) or cM362-140, and blood was collected from the tail vein at timed intervals. Cytokine levels in sera were measured using a CBA. In some cases, wild-type and *Ticam1*$^{-/-}$ mice were s.c. injected with 75 μl (75 μg) poly(I:C) or cM362-140. After 6 h, mice were killed and draining inguinal LN, axillary LN and spleen were harvested[10]. *IFN-β*, *IL-6* or *TNF-α* mRNA expression in these lymphoid organs was measured by qPCR.

Tumour challenge and sODN-dsRNA treatment. Mice 6–10 weeks of age were used in all experiments. Mice were shaved at the back and injected s.c. with 200 μl of 6×10^5 B16D8 cells in PBS (−). Tumour volumes were measured at regular intervals using a caliper[9]. Tumour volume was calculated using the following formula: tumour volume (cm^3) = (long diameter) × (short diameter)^2 × 0.4. 75 μl (75 μg) sODN-dsRNAs or distilled water with no detectable LPS was mixed with *in vivo*-JetPEI (Polyplus), a polymer-based transfection reagent, according to the manual and then injected s.c. around the tumour. Treatment was started when the average tumour volume of 0.4–0.6 cm^3 was reached. To deplete NK cells, we injected titrated anti-NK1.1 ascites (PK136) i.p. in tumour-bearing mice the day before sODN-dsRNA treatment. Depletion of NK1.1$^+$ cells was verified by flow cytometory.

In the case of EG7 cell challenge, mice were injected s.c. with 200 μl of 2×10^6 syngenic EG7 cells in PBS (−). When the average tumour volumes reached ~0.6 cm^3, 50 μl of 100 μg endotoxin-free ovalbumin in PBS (−) with or without 50 μl of 50 μg poly(I:C) or sODN-dsRNA was injected s.c. around the tumour. PBS (−) (100 μl) was used as a control. Treatments were performed twice per week.

CTL activity in tumour-bearing mice after adjuvant therapy. Female mice 6–10 weeks of age were used for this study. Splenocytes were harvested from tumour-bearing mice at 7 days after the last adjuvant treatment. In the case of tumour-free settings, spleen and inguinal LN cells were harvested from wild-type or *Ticam1*$^{-/-}$ mice 4 days after the last adjuvant injection. The cells were stained with FITC-CD8α (1:200), PerCP/Cy5.5-7AAD (1:200), APC-CD3ε (1:200) and PE-OVA-tetramer (1:50) to detect Ag-specific CD8$^+$ T cells. To evaluate cytokine production, splenocytes (2×10^6 per 200 μl per well) were cultured for 3 days in the presence of 100 nM OVA peptide (SL8: SIINFEKL) or control WT1 peptide (Db126: RMFPNAPYL) and IFN-γ production was analysed with CBA or ELISA. To assess the cytotoxic activity of CTLs, splenocytes (1×10^6 per ml) were co-cultured with mitomycin C-treated EG7 cells (5×10^5 per ml) in the presence of 10 U ml^{-1} IL-2 for 5 days. Then, the cells were incubated with ^{51}Cr-labelled EG7 or C1498 cells for 4 h and determined cytotoxic activity[10]. The cytotoxicity was calculated by this formula: Cytotoxicity (%) = [(experimental release − spontaneous release)/(total release—spontaneous release)] × 100.

Cytotoxic activity assay of NK cells. Mice bearing B16 tumour were injected s.c. with cM362-139(IVT) mixed with *in vivo*-JetPEI. After 18 h, mice were killed and DX5$^+$ NK cells were isolated from spleen using DX5-positive selection microbeads (Miltenyi) according to the manual[9]. B16 cells were labelled with ^{51}Cr for 1 h and then washed three times with medium. DX5$^+$ cells and ^{51}Cr-labelled B16 cells were co-cultured at the indicated ratio[31]. After 4 h, supernatants were harvested and ^{51}Cr release was measured in each sample. Specific lysis was calculated by the following formula: cytotoxicity (%) = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

Statistical analysis. The significance of differences between groups was determined by the Student's *t*-test. In tumour-implant or -free mouse experiments, one-way analysis of variance with Bonferroni's multiple-comparison test or Dunnett's test was performed to analyse statistical significance.

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Author contributions

M.M. and T.S. conceived and designed the experiments. M.T., F.N., M.A., N.I., A.M.-S. and H.S. performed the experiments. M.M. conducted the project. M.M. and T.S. analysed data and wrote the paper.

Additional information

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