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A Chemically Defined TLR3 Agonist with Anticancer Activity

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ABSTRACT
Toll-like receptor 3 (TLR3) agonists such as polyinosinic:polycytidylic acid (poly(I:C)) have immunostimulatory effects that can be taken advantage of to induce anticancer immune responses in preclinical models. In addition, poly(I:C) has been introduced into clinical trials to demonstrate its efficacy as an adjuvant and to enhance the immunogenicity of locally injected tumors, thus reverting resistance to PD-L1 blockade in melanoma patients. Here, we report the pharmacokinetic, pharmacodynamic, mechanistic and toxicological profile of a novel TLR3 agonist, TL-532, a chemically synthesized double-stranded RNA that is composed by blocks of poly(I:C) and poly(A:U) (polyadenylic – polyuridylic acid). In preclinical models, we show that TL-532 is bioavailable after parenteral injection, has an acceptable toxicological profile, and stimulates the production of multiple chemokines and interleukins that constitute pharmacodynamic markers of its immunostimulatory action. When given at a high dose, TL-532 monotherapy reduced the growth of bladder cancers growing on mice. In addition, in immunodeficient mice lacking formylpeptide receptor-1 (FPR1), TL-532 was able to restore the response of orthotopic subcutaneous fibrosarcoma to immunogenic chemotherapy. Altogether, these findings may encourage further development of TL-532 as an immunotherapeutic anticancer agent.

Introduction

Toll-like receptor 3 (TLR3) agonists, most prominently, poly-inosinic:polycytidylic acid (poly(I:C)), have been extensively characterized in preclinical cancer models to understand their antineoplastic potential. Thus, poly(I:C) has been found to induce potent anticancer effects if injected locally into tumors or systemically, usually in combination with other treatments such as CpG-containing oligodeoxynucleotides (which stimulates TLR9)[1], tumor vaccines[2,3], type-1 interferon[4], Bacillus Calmette-Guérin immunotherapy[5], blockade of the programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) interaction[6,7], immunogenic chemotherapy[8] and stimulator of interferon genes (STING) agonists[9]. Such poly(I:C) effects have been attributed to TLR3-mediated killing of cancer cells[10] or the activation of immune effectors including dendritic cells (DC)[11], natural killer (NK) cells[12] and macrophages[13] or the depletion of myeloid-derived suppressor cells[14]. Of note, direct effects of poly(I:C) on TLR3-expressing cancer cells may indirectly enhance anticancer immune responses, for instance by eliciting the secretion of immunostimulatory type-1 interferons[15] and chemokines[16], as well as by inducing immunogenic cell death[7]. Indeed, the knockout of TLR3 in cancer cells may facilitate their evasion from immunosurveillance[17,18]. Of note, retrospective analyses suggest that polymorphisms in the human *TLR3* gene may affect risk, prognosis and therapeutic outcome of patients with various malignancies including mammary carcinoma[19], colorectal cancer[20], stomach adenocarcinoma[21] and hepatitis C-induced liver cancer[22,23].

Spurred by these preclinical results, poly(I:C) has been introduced into clinical trials as an adjuvant for eliciting anticancer immune responses by vaccination[24–26]. In addition, poly(I:C) has been evaluated for its capacity to treat solid tumors by intra-tumor injections, alone[27] or in combination with recombinant fms-like tyrosine kinase 3 (FLT3) protein and radiotherapy[28]. Encouraging results have been obtained in anti-PD-1 refractory melanoma patients, in which the intralesional injection of BO-112, a nanoplexed form of poly(I:C), reverted anti-PD-1 resistance[29]. Moreover, multiple clinical trials are underway to evaluate poly(I:C) (which is provided by some companies in specific galenic formulations) or other TLR3 agonists for its anticancer activity[30,31].

Encouraged by these findings, attempts are on the way to create new TLR3 ligands that are chemically distinct from poly(I:C). Similar to poly(I:C) (high molecular weight (HMW) 1500–8000 bp), TL-532 is a fully synthetic double stranded

RNA (HMW 70 bp; Kd <5 nM)[32]. However, at difference with poly(I:C), it is composed by blocks of poly(A:U) (polyadenylic – polyuridylic acid) and poly(I:C) thus limiting off-target effects by overcoming the structural homogeneity of other TLR3 agonists. Here, we report the preclinical characterization of TL-532 at the pharmacokinetic, pharmacodynamic, mechanistic and toxicological levels. In addition, we provide evidence that TL-532 has anticancer effect in mouse models.

Results

Pharmacokinetic characteristics of TL-532

To determine the pharmacokinetic characteristics of TL-532, we parenterally administered the drug once into male swiss mice via different routes (i.e., intraperitoneal [i.p.], intravenous [i.v.] or subcutaneous [s.c.] injection at 5 mg/kg, 2 mg/kg, and 10 mg/kg, respectively) and then determined TL-532 plasma concentrations by high-performance liquid chromatography (HPLC) coupled to UV absorbance measurements (Figure 1A–C). Plasma TL-532 peaked 0, ~30 and ~60 min post i.v., s.c. and i.p. injection, respectively, and exhibited a rapid decline, suggesting that the half-life of the product is in the range of 1–2 h (Figure 1A–C, Table S1). Next, we determined the biodistribution of TL-532 in BALB/c mice bearing clearly discernible subcutaneous MBT2 bladder carcinomas by i.v. injecting them with TL-532 conjugated to the fluorochrome cyanine-5 (TL-532-Cy5). We then determined the abundance of TL-532-Cy5 in different tissues including liver, tumor and the tumor-draining lymph node by fluorescence-based tomography (Figure 1D, Fig. S1). The distribution of TL-532-Cy5 at the site of the tumor represents ~ 4% of the injected dose and ~ 10% compared to the whole body. When reported on a per/weight basis as shown in Figure 1E, the amount of TL-532-Cy5 slightly undercut the one found in the liver (by a factor of ~ 2). The intratumoral peak of TL-532-Cy5 was attained ~60 min post-injection and declined with a half-life of ~1 h. In conclusion, TL-532 has an in vivo half-life in the range of 1–2 h in organs and tumor, as well as in plasma (post s. c. and i.p. injection).

Immunological and anticancer effects of monotherapy with high-dose TL-532

We next determined some pharmacodynamic aspects of TL-532. After 10 repeated daily i.p. injections into tumor-free mice, high-dose TL-532 (380 mg/kg/day) induced several chemokines (CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL10/IP10) and interleukins (IL2, IL6, IL10, IL17a, TNF), as determined by multiplexed ELISA (Figure 2A). Of note, this regimen did not cause any increase in C-reactive protein (CRP), which is a well-established marker of systemic inflammation (Figure 2B). Repeated s.c. injections of TL-532 at the same dose and frequency induced a similar pattern of cytokines as did i.p. injection, though with a more important induction of CXCL9 (Figure 2C, Fig. S2A). In contrast, administration via the i.v. route was less effective in inducing chemokines and interleukins than i.p. or s.c. injections (Figure 2C, Fig. S2B), correlating with the half-life of TL-532 and perhaps reflecting the accelerated clearance of TL-532 (Figure 1A–C). Neither s.c. nor i.v. administration of TL-532 (380 mg/kg/day for 10 days) caused an increase in CRP (Figure 2B).
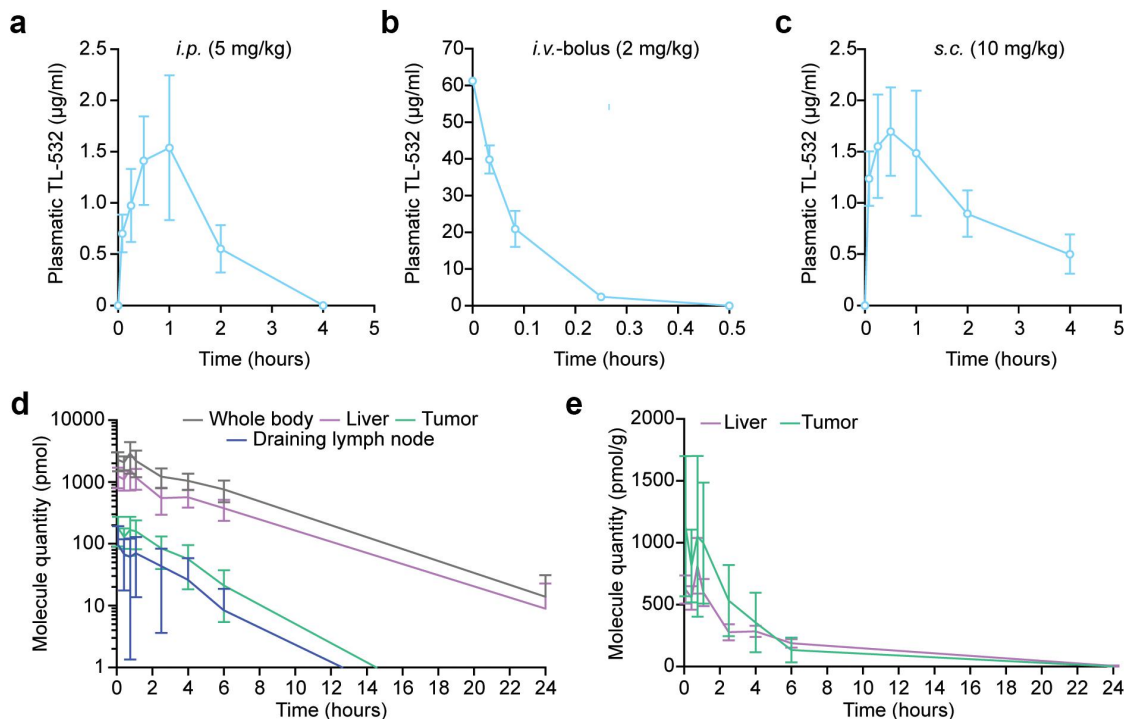
Figure 1. Pharmacokinetics and biodistribution after TL-532 administration in mice. TL-532 pharmacokinetics were evaluated in 12 male swiss mice by i.P. (a), i.V. bolus (b) and s.C. (c) injection at the following concentrations of 5 mg/kg, 2 mg/kg, and 10 mg/kg, respectively. Biodistribution of Cyanine-5 conjugated TL-532 injected intravenously in four BALB/c-nude mice bearing MBT2 tumors was performed using quantitative fluorescent molecular tomography (d and e). Global quantifications over time are shown for the whole-body, liver, tumor and draining lymph-nodes (d), and expressed relatively to the organ weight for both liver and the tumor (e).
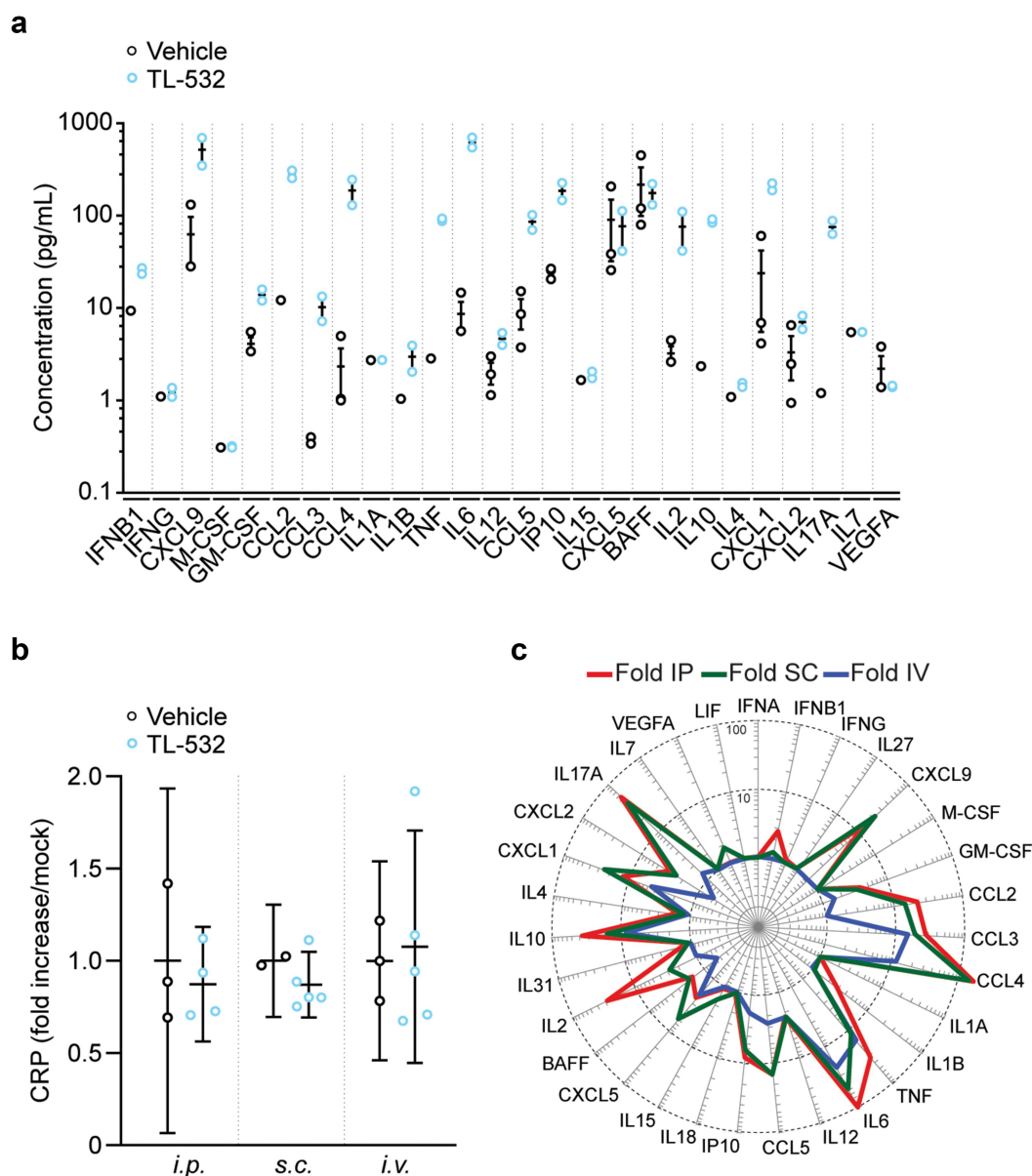
Figure 2. Cytokine, chemokine and C reactive protein (CRP) production after i.P. administration of TL-532. After 10 treatments with either TL-532 (380 mg/kg) or vehicle (0.9% NaCl), cytokine/chemokine (a) and CRP (b) production were evaluated by ELISA 3 h after the tenth i.P. administration of TL-532 (380 mg/kg) or vehicle (0.9% NaCl). Plasmatic soluble factor secretion signature for each route of administration (i.P. i.V. or s.C.) is shown as the fold change of median values normalized to the vehicle controls (c) (n = 2–5).

In the next experiment, we administered high-dose TL-532 to BALB/c mice that were inoculated *s.c.* with syngeneic MBT-2 bladder carcinomas. TL-532 was either injected three times per week up to 10 times (380 mg/kg; *i.p.* or *s.c*) or provided as a continuous *i.v.* infusion (47.3 g/L; 200 µL/h for 1 h). Compared to vehicle-treated controls, TL-532 led to a significant inhibition of tumor growth, irrespective of the route of administration (*i.p.* in Figure 3A, Fig. S3A, *s.c.* in Figure 3C, Fig. S3C and *i.v.* in Figure 3E, Fig. S3E). The weight of the animals was monitored as well. While *i.p.*-injected mice did manifest a minor weight loss (Figure 3B, Fig. S3B), the *s.c.* and *i.v.* administration of TL-532 did not cause any significant weight loss (Figure 3D,F, Fig. S3D,F).

We conclude that repeated administration of TL-532 induces tonic activation of immune system, as reflected by an increase in the circulating levels of multiple chemokines and interleukins. In addition, monotherapy with high-dose TL-532 has anticancer effects.

On-target effects of TL-532 on mouse dendritic cells in vitro via TLR3

We determined whether TL-532 would act on-target (via TLR3) or might also have off-target effects in a sort of mini-immune system composed by professional antigen-presenting cells and antigen-specific major histocompatibility
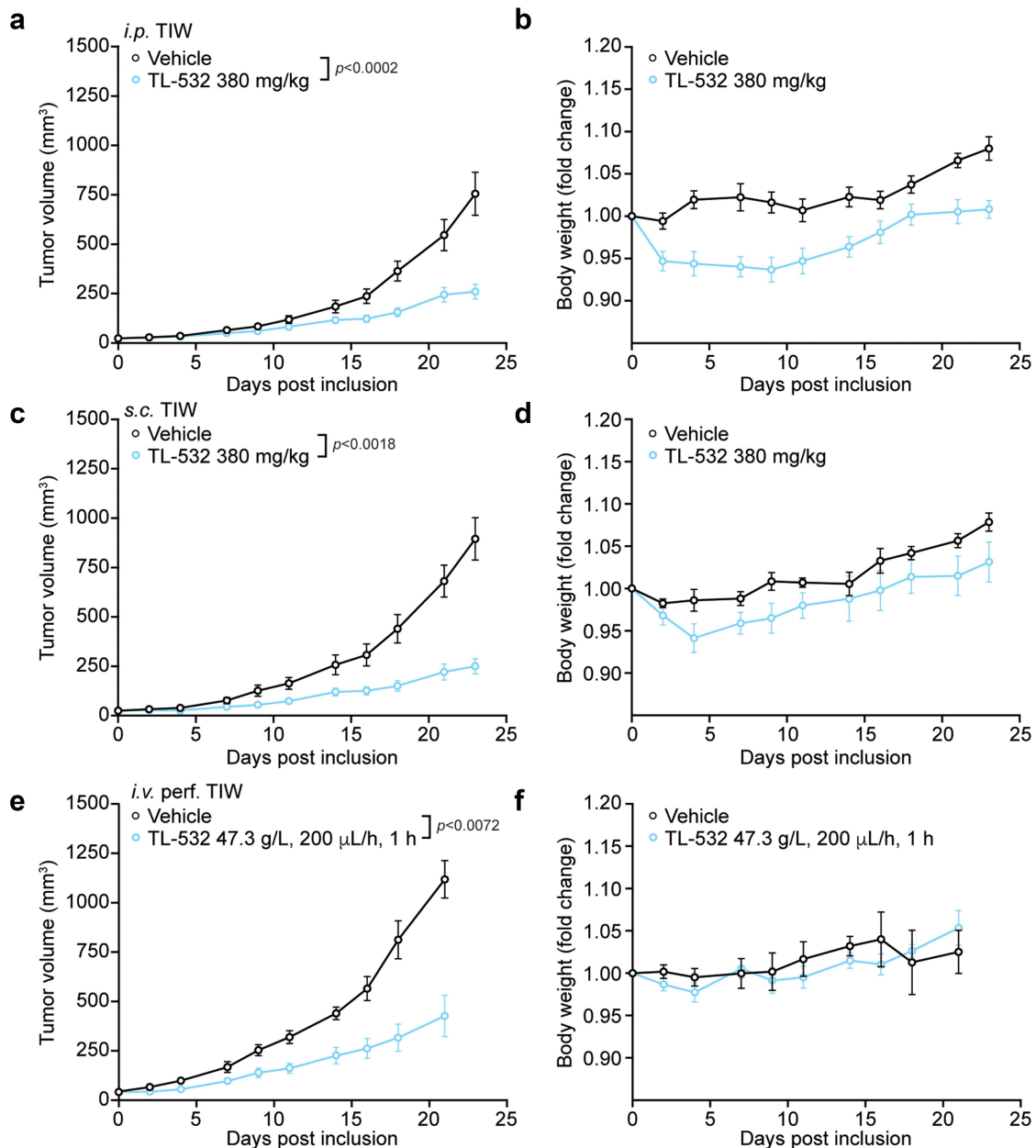
Figure 3. Effects of i.P., s.C. and i.V. TL-532 in MBT2-bearing mice. Murine MBT-2 bladder cancer cells were inoculated s.c. into immunocompetent C3H/HeN mice. TL-532 was administered i.P. (380 mg/kg) (a and b), s.c. (380 mg/kg) (c and d) and i.V. (47.3 g/L; 200 µL/h for 1 h) (e and f), thrice a week, up to 10 treatments. Tumor volume (a, c and e) and body weight loss (b, c and f) were routinely assessed.

complex class I (MHC-I) T cells. For this, we took advantage of a peculiar dendritic cell preparation that is generated from immortalized precursors in the form of de-inhibited/de-immortalized DCs (de-iniDCs), as explained in the Materials & Methods. When pulsed with ovalbumin (OVA) protein, such de-iniDCs can present the OVA-derived SIINFEKL peptide bound to K^b MHC-I to SIINFEKL/K^b-specific B3Z T cell hybridoma cells, stimulating their IL2 secretion[33]. The de-iniDCs were either wild type (WT) or had been subjected to the CRISP/Cas9-mediated knockout of *Fpr1* and *Tlr3*, alone or in combination[8]. Poly(I:C), poly(A: U) and TL-532 indistinguishably enhanced antigen presentation by WT (genotype: *Fpr1*^{+/+} *Tlr3*^{+/+}) and DCs lacking *Fpr1* only (*Fpr1*^{−/−} *Tlr3*^{+/+}), but failed to act on *Fpr1*^{+/+} *Tlr3*^{−/−} or *Fpr1*^{−/−} *Tlr3*^{−/−} DCs (Figure 4).

These findings indicate that, similarly to poly(I:C) and poly (A:U), TL-532 acts on-target, on TLR3, to stimulate immune responses.

TL-532 compensates deficient immunosurveillance in *Fpr1*^{−/−} mice

Formyl peptide receptor 1 (FPR1) is necessary for DC homing on cancer cells that release the FPR1 ligand annexin A1 (ANXA1), which allows further cellular engagement and cross-presentation of tumor-associated antigens[8,34]. *Fpr1* deficiency entails an immune defect that leads to the early manifestation of some cancers (such as luminal B breast cancer in patients and hormone-induced breast cancer in mice)[8], as well as to resistance to immunogenic chemotherapy[34]. The TLR3 ligand
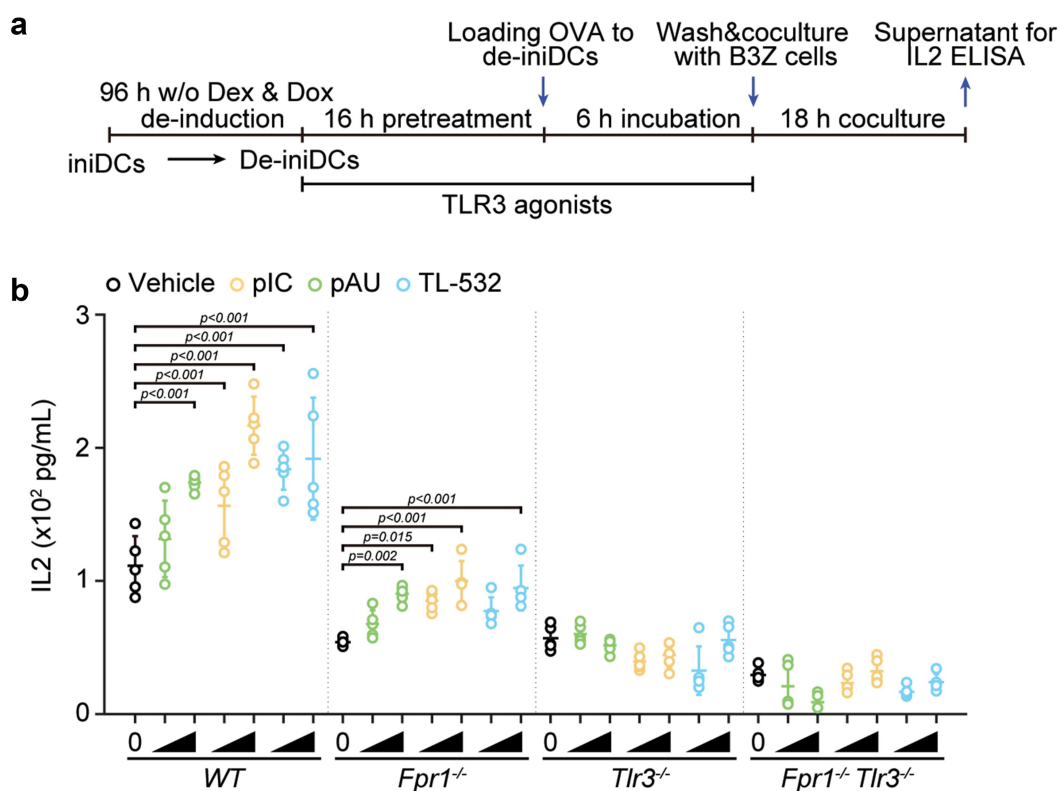
Figure 4. In vitro effects of TLR3 agonists on the antigen cross-presentation by de-iniDcs of different genotypes. (a) Scheme of the in vitro antigen cross-presentation assay. Inducible immortalized dendritic cell (iniDC) precursors were differentiated to DCs (de-iniDC) in the absence of dexamethasone or doxycycline (Dex/Dox) for 3 days. De-iniDcs were pretreated with the indicated TLR3 agonists (poly(I:C) (pIC) and poly(A:U) (pAU) at 2 and 20 µg/mL, and TL-532 at 20 and 200 µg/mL.) for 16 h. For measuring antigen cross-presentation, soluble ovalbumin (OVA) was added to the de-iniDC culture and the de-iniDcs were incubated for additional 6 h before wash and co-culture with B3Z hybridoma T cells. Eighteen hours later, the co-culture supernatants were collected for the quantification of IL2 by ELISA. (b) IL2 concentrations were calculated based on standard curves and normalized to untreated wild-type controls. One representative experiment out of three replicates is reported as aligned dot plots (mean ± SD, n = 5). Statistical significance was calculated by means of two-way ANOVA with Dunnett's multiple comparisons test.

poly(I:C) is able to overcome this immune defect when injected into tumor-bearing $Fpr1^{-/-}$ mice[8]. We therefore decided to perform a side-by-side comparison of TL-532, poly(I:C) and poly(A:U) with respect to their capacity to reestablish deficient chemotherapy responses in $Fpr1^{-/-}$ mice. For this, we designed in experiment (Figure 5A) in which WT C57BL/6 mice or isogenic $Fpr1^{-/-}$ mice were s.c. inoculated with syngeneic MCA205 cutaneous fibrosarcomas. In addition, WT C57BL/6 mice were inoculated with genetically modified MCA205 cells lacking the FPR1 ligand annexin A1 (genotype: $Anxa1^{-/-}$). Then, tumor-bearing mice (i.e., WT mice with WT MCA205 tumors, left panels in Figure 5B), (WT mice with $Anxa1^{-/-}$ MCA205 tumors, middle panels in Figure 5B) and $Fpr1^{-/-}$ mice with WT tumors, right panels in Fig. 5B) were treated with chemotherapy (via i.p. injection of the immunogenic cell death inducers mitoxantrone, MTX, and cyclophosphamide, CTX) or vehicle (PBS), followed by i.p. injections of TL-532 (used at two doses: dose 1:200 µg/mouse or dose 2:2 mg/ mouse), TL-532, poly(I:C), poly(A:U) or vehicle (Figure 5A). In mice without prior chemotherapy, TL-532, poly(I:C) and poly(A:U) failed to reduce tumor growth (upper panels in Figure 5B). Chemotherapy with MTX/CTX reduced tumor growth significantly in WT mice bearing WT tumors, but failed to yield significant effects when either FPR1 (on the host) or its ligand ANXA1 (on tumor cells) were absent from the system (compare black and red lines in the middle and

lower panels in Figure 5B). TL-532, poly(I:C) and poly(A:U) did not improve outcome of MTX/CTC chemotherapy against WT tumors growing on WT hosts. However, if combined with MTX/CTX chemotherapy, TL-532, poly(I:C) or poly(A:U) conferred significant tumor growth reduction, even if the tumors lacked ANXA1 or the host was deficient for FPR1 (middle and lower panels in Figure 5B, Fig. S4).

In conclusion, it appears that, like poly(I:C) (and poly(A:U)), TL-532 is able to restore chemotherapy-induced immunosurveillance in spite of defective FPR1/ANXA1 signaling.

Toxicity and dose finding in cynomolgus monkeys

TL-532 was i.v. injected in cynomolgus monkeys (one group of one male and one female) on days 1, 7, 13 and 19 at ascending concentrations of 35, 70, 140 and 280 mg/ kg (dose volume of 2 mL/kg into the cephalic vein). Apart from erythema at the injection site, there were no major signs of toxiciy apart from an increase in alanine aminotransferase and aspartate aminotransferase activity at day 20, for the male only. The potential toxicity of TL-532 was then evaluated in three groups of monkeys (one male and one female) following three repeated i.v. (days 1, 5 and 9) injections of 70, 140 and 280 mg/kg in a dose volume of 2 mL/kg. TL-532 administered at 70 mg/kg induced no signs of toxicity in the male and female cynomolgus monkeys.
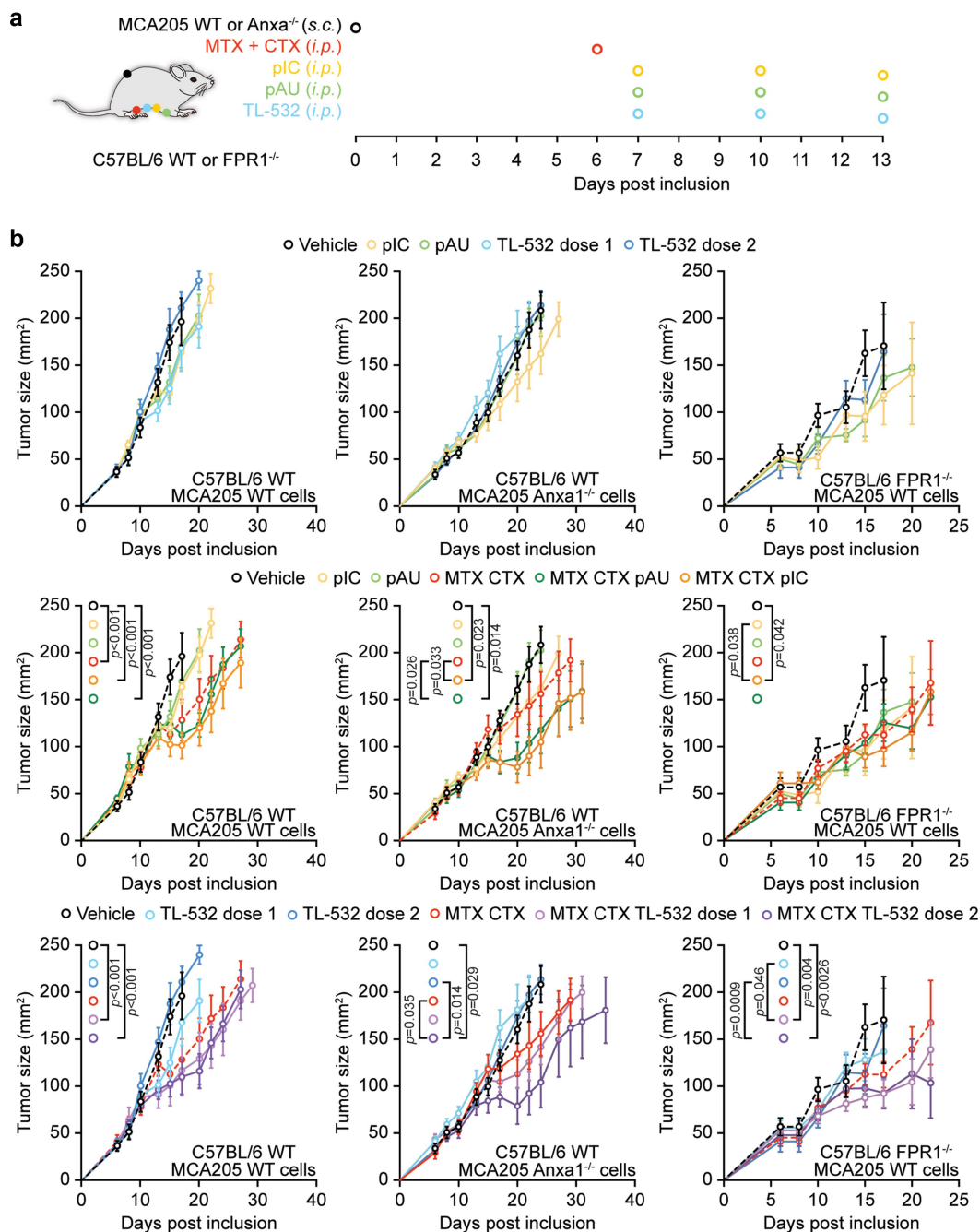
Figure 5. TLR3 agonists restore the efficacy of chemotherapy in defective Anxa1/Fpr1 signaling in fibrosarcoma-bearing mice. (a) Scheme of the in vivo tumor growth experiment. (b) Murine MCA205 cells WT or Anxa1$^{-/-}$ were inoculated s.c. into immunocompetent C57BL/6 WT or Fpr1$^{-/-}$ mice. When tumor became palpable (with a size inferior to 80 mm^2) mice received 5.17 mg/kg i.P. mitoxantrone (MTX), 50 mg/kg i.P. cyclophosphamide (CTX), 50 μg/mouse, i.P. poly(I:C) (pIC), 150 μg/mouse i.P. poly(A:U) pAU, and 200 μg/mouse or 2 mg/mouse i.P. TL-532 (injected at days 1, 4, and 7 post treatment), or an equivalent volume of NaCl 0.9%. Tumor growth was routinely assessed using a digital caliper. Of note, data from the right panel represent results of two experiments yielding similar results.

TL-532 administered three times *i.v.* at 280 mg/kg, 4 days apart, induced changes in hematology parameters and bilateral pyelonephritis in the male and female cynomolgus monkeys (determined on day 10). This dose level was considered to be above the maximum tolerated dose. When administered three times at 140 mg/kg, changes induced were limited to minor clinical signs in females (piloerection, abnormal appearance of fur and/or alopecia) after the last dosing associated with body weight losses (−8%) and changes in hematology parameters in both genders (increase in total circulating leukocytes neutrophils,

decrease in red blood cell count, hemoglobin and hematocrit).

Hence, repeated dosing of 70 mg/kg TL-532 can be considered as safe in this non-human primate.

Discussion

In this paper, we report on the pharmacokinetic, pharmacodynamic, mechanistic and toxicological characterization of TL-532, which, in molecular terms, is a hybrid molecule encompassing features of two widely used TLR3 ligands, poly(I:C)

and poly(A:U). Unlike poly(A:U) as well as poly(I:C) and its derivatives which have an enzymatic manufacturing process leading to a wide range in product size thus inducing batch-dependent variations in activity, TL-532 is a defined chemically synthetized dsRNA sequence that can be further bio-conjugated to improve target delivery[32].

We found that TL-532 can be easily detected post-injection as a native molecule by HPLC. Pharmacokinetic measurement of the tissue redistribution of native TL-532 and its fluorescent derivative TL-532-Cy5 revealed similar half-lives, suggesting that labeling with cyanine-5 did not distort the pharmacokinetic features of TL-532. The use of TL-532-Cy5 allowed to demonstrate that the molecule effectively distributes into cancers, though with a relatively short half-life (in organs, tumors and plasma after *s.c.* and *i.p.* injection) of approximately 1–2 h, with an increased clearance in plasma after *i.v.* injection. We found that administration of TL-532 by *i.p.* or *s.c.* injection (but less as an *i.v.* bolus injection) led to an increase of multiple chemokines and interleukins in the plasma, reflecting a systemic immune activation. High-dose administration of TL-532 (by *i.p.* or *s.c.* injection as well as by continuous *i.v.* infusion) inhibited the growth of tumors in syngeneic mice as a monotherapy. Moreover, low-dose administration of TL-532, which failed to affect the growth of tumors as a monotherapy, synergized with chemotherapy to reduce the growth of cancers in mice in which the ANXA1-FPR1 signaling pathway was interrupted due to knockout of *Anxa1* in cancer cells or that of *Fpr1* in the host. In this respect, TL-532 exhibited similar effects as established TLR3 agonists such as poly(I:C) and poly(A:U). Reassuringly, we found that the immunostimulatory effects of TL-532 on an in vitro mini-immune system composed by DCs and MHC-I-restricted T cells were lost when *TLR3* was knocked out in DCs, meaning that this agent acts on-target. Finally, we obtained evidence in favor of the notion that TL-532 can be safely administered to non-human primates.

Altogether, the aforementioned results support the continued development of TL-532. Pending further toxicological examination of a GMP product, it will be important to study the possibility to select patients that have a high chance to respond to TLR3 agonists based on biomarkers. Not surprisingly, the clinical effects of poly(A:U) against mammary carcinoma turned out to be more pronounced against breast cancers expressing TLR3 than against TLR3-negative tumors[35]. Hence, the presence of TLR3 on tumor cells or on other cells in the tumor microenvironment might guide the inclusion of patients in clinical trials. In addition, based on the preclinical experiments involving *Fpr1*^{-/-} mice, a specific segment of immuno-deficient patients bearing the *FPR1* rs867228 loss-of-function allele might constitute an ideal target population for testing TLR3 agonists in the context of chemotherapy. Although it can be argued that human FPR1 and mouse Fpr1 share limited homology[36], it appears that the migration-deficient phenotype of human dendritic cells expressing *FPR1* rs867228 and mouse dendritic cells lacking *Fpr1* is similar[34,37], supporting the idea that *Fpr1*^{-/-} mice constitute a valid model for the immunodeficiency associated with *FPR1* rs867228.

Currently, several trials are evaluating the antineoplastic effects of several TLR3 agonists. Such trials use ampligene (which is a modified poly(I:C) into which uridylic acid

molecules have been introduced at specific intervals) against breast or ovarian cancer (NCT03734692, NCT04081389), BO-112 (which is poly(I:C) stabilized with poly-L-lysine) against melanoma (NCT04570332) and poly-ICLC (which is poly(I:C) acid stabilized with poly-L-lysine carboxymethyl cellulose) against melanoma (NCT04364230), mostly in combination with a range of distinct immunotherapies (inhibition of PD-1, vaccination against tumor antigens, administration of recombinant IFN-α2b2) or together with chemotherapy[31]. However, none of these trials targets a patient subpopulation that would be selected based on biomarkers rather than on purely clinical criteria. Nonetheless, future retrospective analyses of these clinical trials might confirm the potential utility of candidate biomarkers such as TLR3 expression in the tumor and polymorphisms affecting *TLR3* or *FPR1*.

In sum, the present paper provides information on the preclinical characterization of TL-532 as a novel TLR3 agonist endowed with anticancer activity. Future studies must determine the clinical utility of TL-532 or similar TLR3 agonists.

Material and methods

Chemicals

Unless otherwise indicated, media and supplements for cell culture were obtained from Gibco-Invitrogen Life Technologies Inc. (Carlsbad, CA, USA). Plasticware was purchased from Corning B.V. Life Sciences (Amsterdam, The Netherlands). Mitoxantrone dihydrochloride (MTX), cyclophosphamide (CTX), sodium chloride 0.9%, polyadenylic:polyuridylic acid (poly(A:U)) and polyinosinic:polycytidylic acid (poly(I:C)) were provided by Sigma Aldrich (St. Louis, MO, USA). Synthesis of TL-532 and TL-532-Cy5 was performed by Horizon Discovery (Waterbeach, UK) and NittoAVECIA (Milford, MA, USA), respectively. Lyophilized powders were resuspended in apyrogenic, nuclease-free, sterile 0.9% NaCl (InvivoGen, San Diego, CA, USA). 0.9% NaCl was used as a mock condition for all experiments unless otherwise stated.

Mouse experiments

Mice were bred and maintained in the animal facilities of the Centre de Recherche des Cordeliers, Centre Leon Berard (P-PAC, Plateforme du Petit Animal du CRCL – Agreement n° D 69 388 0202) and Université Claude Bernard Lyon-I (SCAR, Service Commun des Animaleries de Rockefeller – Agreement n° C69388 10 01), in specific pathogen – free conditions in a temperature-controlled environment with 12 h light/12 h dark cycles and received food and water *ad libitum*. Animal experiments followed the Federation of European Laboratory Animal Science Association (FELASA) guidelines and followed EU Directive 63/2010. Protocol #34928–2022012015416724-v7 was approved by the Ethical Committee of the CRC (registered C2EA–05 at the French Ministry of Research). Protocols #37310–2022041314222803-V2 was approved by ACCeS (registered C2EA–10 at the French Ministry of Research), and Projects #23845–201906281645171-V10 and #31779–20210220151652226-V4 were approved by the ethical

committee CECCAPP (registered C2EA–15 at the French Ministry of Research). Six- to 7-week-old female wild-type C57Bl/6 mice were obtained from Envigo (Indianapolis, IN, USA). Eight-week-old Balbc-nude-mice, eight-week-old C3H/HeN mice and 11-week-old C3H/HeN jugular vein catheterized mice were obtained from Charles River (Wilmington, MA, USA). Pharmacokinetics were evaluated in 12 male swiss mice from Janvier Labs, Le Genest-Saint-Isle, France, with a weight ≥35 g.

Pharmacokinetic experiments

TL-532 pharmacokinetics were evaluated by Eurofins Scientific (Luxembourg), in 9 weeks male Swiss mice from Janvier Labs with a weight at least equal to 35 g, for *i.v.* bolus, *i.p.* and *s.c.* administration routes at the respective doses of 2 mg/kg, 5 mg/kg and 10 mg/kg. Three mice per administration routes were used. Plasma samples were collected at indicated time before TL-532 quantification by HPLC-UV method from Eurofins BIOANALYSES.

Dose ranging and maximum-tolerated dose

Dose range finding (DRF) and maximum-tolerated dose (MTD) studies were established in cynomolgus monkeys (Macaca fascicularis) by the European Research Biology Center (ERBC, Baugy, France). To determine the MTD, one male and one female received four increasing doses (35, 70, 140 and 280 mg/kg) of TL-532 by bolus *i.v.* injection at a dose volume of 2 mL. Each dose was given every 6 days at day 1, 7, 13 and 19, and animal was sacrificed at day 20. To establish suitable DRF, three groups of one male and one female received three repeated doses of TL-532 at 70, 140 and 280 mg/kg, respectively. TL-532 was administered every 4 days on day 1, 5 and day 9 by bolus *i.v.* injection at a dose volume of 2 mL/kg and animals were sacrificed at day 10. For both MTD and DRF studies, parameters monitored included daily mortality checks, clinical examination, changes in body weight and blood samples were taken for hematology, coagulation and serum biochemical tests before first dosing and the day after last administration at day 20 and day 10, respectively.

TL-532 biodistribution

TL-532 biodistribution was determined on BALB/c-nude mice bearing ectopic tumor. Briefly, mice were grafted ectopically by subcutaneous injection of 1×10^6 MBT2 cells (in 50 μL sterile RPMI medium without FBS or antibiotic) into the right flank. When tumor reached about 150 mm^3, tumor vascularization was both determined by echo-doppler and contrast echography using the Vevo LAZR Photoacoustic Imaging System (FUJIFILM Visualsonics, Amsterdam, The Netherlands). The four most representative mice in terms of tumor volume and vascularization were injected into the caudal vein with 5 nmol of TL-532 conjugated with cyanine-5 (TL-532-Cy5), before being analyzed by using FMT-4000 Quantitative Fluorescent Molecular Tomography (Perkin Elmer, Waltham, MA, USA), at the time points: 5 min, 25 min, 45 min, 1 h 5 min, 2 h 25 min, 4 h, 6 h and 24 h. TL-532-Cy5 quantities, expressed in pmol,

were determined in the whole body, liver, tumor and draining lymph-nodes as region of interest (ROI).

Multiparameter ELISA

Mouse tumor interstitial fluids and plasma were analyzed using multiplexed ELISA mouse ProcartaPlex 31-Plex Immunoassays (Thermo Fischer Scientific, Waltham, MA, USA, #PPX-31-MXD5Y2R). Beads corresponding to kit analytes were read on a dual-laser flow-based detection instrument (Luminex Bioplex MAGPIX, Bio-Rad, Hercules, CA, USA). Data were analyzed using xPONENT (Bio-Rad), Bio-Plex Manager 6.1 (Biorad), and ProcartaPlex Analysis App (Thermo Fischer Scientific) software. C-reactive protein (CRP) ELISA (Thermo Fischer Scientific, EM20RB) was performed on plasma and analyzed using SparkControl Magellan 3.1 (Tecan, Männedorf, Switzerland) software.

Cell lines and culture conditions

Bladder cancer cell-line MBT2 cells, syngeneic to C3H/HeN strain mice, kindly provided by Pr. Yves Fradet's laboratory (Laboratoire d'Uro-Oncologie Expérimentale, Université Laval, Québec, QC, Canada) and cultured in RPMI-1640 (Gibco, Thermo Fischer Scientific, #21875–034) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, #15140–122). Murine fibrosarcoma MCA205 cells (class I MHC haplotype H-2b, syngeneic for C57BL/6 mice) were cultured in Roswell Park Memorial Institute medium (RPMI)-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/mL, penicillin G sodium salt, 100 μg/mL streptomycin sulfate and 1 mM HEPES buffer.

TL-532 used as monotherapy by systemic administration

Eight-week-old C3H/HeN mice were grafted ectopically by subcutaneous injection of 1×10^6 MBT2 cells (in 50 μL sterile RPMI medium without FBS or antibiotic) into the right flank. Mice were treated when the tumors reached a mean volume of 30 mm^3, using TL-532 at 380 mg/kg, thrice a week, up to 10 treatments, administered either by *i.p.*, *s.c.*, or *i.v.* infusion route at 8 mL/kg/h flow rate. Vehicle (0.9%-NaCl) was administered to control mice at the same volume and frequency. Tumor development was monitored three times a week, with tumor volume determined by the formula (L × W × H) x 3,14159/6 where L is tumor length, W is tumor width and H is tumor height. Mice were sacrificed 3 h after the last treatment at day-23 for plasma and tumor interstitial fluids soluble factors quantification.

IniDC cell culture

The murine inducible immortalized dendritic cell (iniDC) precursors and murine B3Z hybridoma T cells were maintained as previously described[33]. Both cell lines were cultured in basic DC medium (RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL pen/strep, and 50 μM beta-mercaptoethanol). B3Z T cells do not need any supplementation of cytokines. To maintain the expression of the large T antigen for the immortalization and to repress

activation of iniDCs, basic DC medium was complemented with 100 μM of dexamethasone plus 1 μg/mL of doxycycline (Dex/Dox) and 10 ng/mL recombinant GM-CSF, respectively. Dex/Dox supplement removal and an increase in the concentration of recombinant GM-CSF for 3~4 days let to the de-immortalize and differentiation of iniDCs into de-iniDCs with the characteristic immunophenotype of primary dendritic cells.

In vitro antigen cross-presentation assay

De-iniDCs were seeded at a concentration of 1×10^6 cells/mL in basic DC medium containing 20 ng/mL recombinant GM-CSF in 96-well tissue culture U-bottom plates. The cells were pretreated with TLR3 agonists for 16 h. Then, OVA was added at a final concentration of 1 μg/mL, gently agitated and then incubated for 6 h in the cell culture incubator. Then, the plates were centrifuged at 500 g for 5 min and the supernatant containing OVA was removed. The cells were washed with 200 μL of RPMI 1640 medium before centrifugation at 500 g for 5 min. This washing step was repeated once more before adding the 1×10^5 B3Z cells/well in basic DC medium containing 20 ng/mL recombinant GM-CSF. The coculture was then incubated in an incubator for 18 h before collecting the supernatant for ELISA. IL2 concentration was measured with the ELISA MAX™ Deluxe Set Mouse IL-2 (Biolegend, Ref 431,004) following the manufacturer's protocol.

Tumor chemotherapy model

For the establishment of syngeneic solid tumors, 3×10^5 wild-type (WT) or $Anxa1^{-/-}$ murine fibrosarcoma MCA205 cells were inoculated s.c. (near the thigh) into WT, Formyl peptide receptor 1 mutated homozygous ($Fpr1^{-/-}$) or Fpr1 heterozygous ($Fpr1^{\pm}$) C57BL/6 (H-2b) mice, and tumor surface (longest dimension × perpendicular dimension) was routinely monitored using a digital caliper. When the tumor surface reached 35–60 mm², mice received 5.17 mg/kg i.p. MTX in 200 μl PBS and 50 mg/kg i.p. CTX in 200 μl PBS or an equivalent volume of vehicle (PBS). When appropriate, mice also received 50 μg/mice i.p. poly(I:C) or 150 μg/mice i.p. poly(A:U) or 200 μg/mice i.p. TL-532 or 2 mg/mice i.p. TL-532 in 50 μl of PBS (injected at day 1, 4 and 7 after the other treatments). All experiments contained at least five mice per group.

Statistical analysis of in vivo experiments

Longitudinal analysis of tumor growth data was performed over the entire time course by linear mixed-effect modeling. Wald test was used to compute p values by testing jointly that both tumor growth slopes and intercepts (on a log scale) were the same between treatment groups of interest (https://kroemerlab.shinyapps.io/TumGrowth/")[38]. Tumor growth curves are represented either as: individual curves from all measurements of each mouse and group-averaged tumor size alongside its SEM computed at each time point. For mice euthanized before the selected sampling point, the last measure was retained.

Abbreviations

CTX	Cyclophosphamide
DC	dendritic cell
iniDC	inducible immortalized dendritic cell
i.p.	intraperitoneally
i.t.	intratumorally
i.v.	intravenously
MHC-I	major histocompatibility complex class I
MTX	mitoxantrone
OVA	ovalbumin
pAU	polyadenylic:polyuridylic acid
pIC	polyinosinic:polycytidylic acid
ROI	region of interest
s.c.	subcutaneously
WT	wildtype

Author contributions

J.L.N, S.T., S.A.S, M. B-J., P.L, M.B., Y.P., C.P., L.Z., M.M., C.R., M. P-L, and B.M. performed experiments; O.K., G.K. and B.W. conceived the study and wrote the paper.

Data availability statement

All data are available from the corresponding authors upon reasonable request.

Research and Personalized Medicine (CARPEM). This study contributes to the IdEx Université de Paris ANR-18-IDEX-0001.

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