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TLR1/TLR2 Agonist Induces Tumor Regression by Reciprocal Modulation of Effector and Regulatory T Cells

Yi Zhang, Feifei Luo, Yuchan Cai, Nan Liu, Luman Wang, Damo Xu, and Yiwei Chu*

Using TLR agonists in cancer treatment can have either beneficial or detrimental effects. Therefore, it is important to determine their effect on the tumor growth and understand the underlying mechanisms in animal tumor models. In this study, we report a general immunotherapeutic activity of a synthetic bacterial lipoprotein (BLP), a TLR1/TLR2 agonist, on established lung carcinomas, leukemia, and melanoma in mice. Systemic treatment of 3LL tumor-bearing mice with BLP, but not LPS, led to a dose-dependent tumor regression and a long-lasting protective response against tumor rechallenge. The BLP- mediated tumor remission was neither mediated by a direct tumoricidal activity nor by innate immune cells, because it lacked therapeutic effect in immunodeficient SCID mice. Instead, BLP treatment reduced the suppressive function of Foxp3+ regulatory T cells (Tregs) and enhanced the cytotoxicity of tumor-specific CTL in vitro and in vivo. Furthermore, adoptive cotransfer of BLP-pretreated but not untreated CTL and Tregs from wild-type but not from TLR2−/− mice was sufficient to restore antitumor immunity in SCID mice by reciprocally modulating Treg and CTL function. These results demonstrate that the TLR1/TLR2 agonist BLP may have a general tumor therapeutic property involving reciprocal downregulation of Treg and upregulation of CTL function. This property may play an important role in the development of novel antitumor strategies. The Journal of Immunology, 2011, 186: 1963–1969.

Cytotoxic T lymphocytes play a pivotal role in tumor immunosurveillance by specifically recognizing and killing tumor cells and maintaining tumor-specific immune memory (1–3). However, cancer cells are generally poor immunogens and can evade immunosurveillance by a variety of mechanisms including the induction of CD4+CD25+Foxp3+ regulatory T cells (Tregs) (4–6). These Tregs subsequently counteract the initiation and perpetuation of antitumor CTL responses either through cell contact or by secreting suppressive cytokines (5–9). Thus, selective downregulation of Treg function, coupled with the promotion of CTL activity, may represent an attractive strategy for future cancer immunotherapy. Current evidence suggests that certain TLR activations may provide these properties.

TLRs are pathogen pattern recognition receptors that detect different microbial molecular structures and trigger innate immune responses (10, 11). Ten human TLRs have been identified, and although most TLRs act alone, TLR2 forms heterodimers with TLR1 or TLR6, allowing it to recognize a wide range of exogenous or endogenous molecules from pathogen and host, respectively (10, 11). Growing evidence suggests that TLR signaling has a variable but important role in cancer development and treatment (12–16). This is because some TLRs are widely expressed by tumor cells as well as immune cells, and different TLR signals impact differently on all of these cells (12–16). TLR engagement on tumor cells may inhibit, promote, or lack any direct effect on tumorigenicity (12–16). TLR agonists are powerful immune adjuvants for professional APC activation (12, 13). Beside professional APC, T cells also express TLRs (17–20); however, the effect of TLRs on T cell function in cancer immunosurveillance is still not well understood. We have reported that TCR-stimulated human T cells express functional TLR2, but not TLR4, and that TLR2 activation provides costimulatory signals for T cell function (17). This observation has been confirmed and extended by others (18–20). TLRs also influence Treg function. Whereas TLR4 and TLR5 signals directly enhance Treg function (21, 22), TLR8 signals abolish their suppressive activities (23). We and others (24–26) have found that a well-defined TLR1/TLR2 agonist, synthetic bacterial lipoprotein (BLP), was also able to effectively abrogate Treg function by downregulating Foxp3 expression. In contrast, TLR3, TLR7, and TLR9 agonists appear to have no direct effect on the CD4+Foxp3+ Tregs (D. Xu, F. Luo, Y. Zhang, and Y. Chu, unpublished observations). Therefore, it seems that TLR1/TLR2 agonists may have a unique ability to reciprocally regulate both effector and Treg functions and a potential better antitumor effect than other TLR agonists.

Indeed, it has been suggested that TLR2 agonists are potential immune adjuvants for cancer vaccination and may be beneficial in the prevention of cancer relapse during or after chemotherapy (27, 28). Bacillus Calmette-Guérin (BCG) has a long-standing history in treating human bladder cancer, and recently, it was demonstrated that the therapeutic effect is mainly TLR2 mediated (29, 30). Furthermore, TLR2 agonists are potent immunotherapeutic agents in several murine tumor models, and they could also...
promote tumor growth and metastasis in other models (31–36). Therefore, the role of TLR2 in cancer development and treatment, particularly the underlying mechanisms by which they modulate immune responses, are extremely important but not yet fully understood.

Lung cancer is the most common cancer in the world; it is estimated that 1.3 million new cases are diagnosed every year. The current treatment of lung cancer is ineffective, and lung cancer remains the most deadly common cancer (37). New therapeutic strategies for lung cancer are in great need. In this study, we documented that the TLR1/2 agonist BLP exhibits potent antitumor effects in several murine tumor models including the widely used 3LL lung carcinoma model. Systemic BLP, but not LPS, treatment led to complete lung tumor regression and development of a long-lasting protective response against tumor rechallenge. The therapeutic property of BLP was not limited to 3LL tumor; it could also induce rejection of implanted leukemia and delay the growth of implanted melanoma. The mechanism underlying the therapeutic effect of BLP involves directly modulating T cell activities. BLP induced a marked reduction of CD4+Foxp3+ Tregs and diminished the suppressive activity of remaining Tregs in a TLR2-dependent fashion. It also elevated proliferative responses and cytotoxicity of tumor-specific CTL in vivo. The reciprocal effect of BLP on effector and Treg functions unveiled a novel antitumor mechanism of BLP.

Materials and Methods

Mice and cell lines

C57BL/6 mice, SCID mice (C.B-17/ScidCrl-scid-bgBR), and TLR2−/− mice were from the Model Animal Research Center, Nanjing University, People’s Republic of China. All of the animals were maintained in the animal facility of Fudan University under pathogen-free conditions and used at 6–8 wk of age. All animal experiments were carried out in accordance with the experimental animal guidelines.

The 3LL Lewis lung carcinoma tumor cell line and F10 melanoma cell line were kindly provided by Dr. Hong-Ming Hu (Earle A. Chiles Research Institute, Portland, OR). The FBL3 is a murine leukemia cell line that was originally induced with a Friend murine leukemia virus. Tumor cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS and other additives.

T cell isolation and culture

Murine CD4+CD25+ Tregs and CD8+CTLs were purified from spleen and lymph nodes by MACS according to the manufacturer’s instructions (Miltenyi Biotec). The purity of the isolated T cell subpopulations was >95% as assessed by flow cytometry analysis. For proliferation assay, T cells were labeled with 5 μM CFSE (Sigma-Aldrich) and activated with mitomycin C-inactivated 3LL tumor cells for 3 d. The T cell proliferation was measured by flow cytometry analysis.

T- and B-cell suppression assay was performed as described before (25). Briefly, freshly isolated CD8+ T cells were labeled with CFSE as above, and Tregs were pretreated with plate-bound anti-CD3 (5 μg/ml) in the presence or absence of 5 μg/ml BLP (Pam.C3K−, TLR1/TLR2 ligand; Invivogen) for 24 h. The labeled CD8+ T cells (5 × 10^6) were then cultured either alone or together with equal numbers of pretreated Tregs in the presence of soluble anti-CD3 (1 μg/ml) and APC (mitomycin C-inactivated splenocytes) for 3 d. The fluorescence density was analyzed by flow cytometry.

Flow cytometry

3LL tumor cells (1 × 10^6) were stained with directly conjugated Abs: anti-TLR1,-TLR2, and -TLR4 or with appropriate isotype-matched control IgG1 (eBioscience). T cells were stained for surface markers CD4, CD8, CD25 or intracellular marker Foxp3 with fluorescence-conjugated Abs according to the manufacturer’s instructions (eBioscience).

Tumor model and treatment

We used s.c. tumor transplantation models to enable easy tumor measurement (38). Briefly, C57BL/6 or SCID mice were injected s.c. with 3LL (5 × 10^4/mouse), FBL3 (5 × 10^4/mouse), or F10 (1 × 10^4/mouse) tumor cells in 100 μl PBS in the flank. Tumor growth and mortality was monitored every 2 d. The tumor area was measured with a digital caliper and calculated using the formula: largest diameter × smallest diameter. For treatment, 5 d after tumor cell inoculation, mice were administered with different doses of BLP (5–50 μg/mouse), LPS (50 μg/mouse), or PBS as indicated, and then repeated three times on days 10, 15, and 20. Both BLP and LPS were from Invivogen. In the CD8-depletion experiment, tumor-bearing mice were injected with 200 μg/mouse/dose anti-CD8 Ab or control IgG (eBioscience) i.p. every 3 d from day 0–23 post tumor challenge for a total of nine injections. The CD8+ cell populations were reduced by >90% as measured by flow cytometry analysis.

T cell engulfment of SCID mice

SCID mice were inoculated with 3LL tumor cells (5 × 10^4/mouse) s.c. in the right flank. Five days later, the mice were engrafted i.v. with purified CD8+ T cells (2 × 10^6) and/or Tregs (2 × 10^6) as indicated. These CD8+ T cells and CD4+CD25+ Tregs were sorted from untreated 3LL-bearing wild-type (WT) C57BL/6 or TLR2−/− mice. In SCID mice engrafting experiments, prior to the engraftment, CD8+ T cells were reactivated with inactive 3LL cells and BLP (5 μg/ml) for 48 h in vitro. Tregs were activated with plate-bound anti-CD3 with or without BLP (5 μg/ml) for 24 h (25).

Cytotoxicity assays

For in vitro assays, splenocytes were isolated from 3LL-bearing mice, and the cells (2 × 10^7 cells/well) were cultured with mitomycin C-inactivated 3LL cells (2 × 10^6/well) as Ag in complete medium with 50 U/ml rIL-2 for 17 d. Target 3LL cells were labeled with 5 μM CFSE. For cytotoxicity assays, the Ag-specific effector cells were mixed with CFSE-labeled target cells at different ratios and cultured for 4 h. Immediately before flow cytometry analysis, propidium iodide (1 μg/ml) was added to each well. The in vivo cytotoxicity assays were performed as described (39). Briefly, C57BL/6 mice were inoculated with or without 3LL tumor cells and then treated with or without BLP as above. On day 20, target splenocytes (5 × 10^7) from normal C57BL/6 mice were pulsed with 3LL-specific peptide Ag MUT1 (FEQNTNFAQ, 10 μg/ml) (38) at 37°C for 1 h and then labeled with 20 μM CFSE (CFSE 10^6/mouse) or left un pulsed but labeled with 0.5 μM CFSE (CFSE 10^6/mouse). The CFSEhigh and CFSElow cells were mixed in a 1:1 ratio, and a total of 1 × 10^6 cells were injected i.v. into naive or tumor-bearing C57BL/6 mice after BLP treatment. Three days post-transfer, total splenocytes were harvested, and the CFSEhigh to CFSElow cell ratio was analyzed by flow cytometry. The specific cell lysis was calculated as described (39).

Cytokine ELISA

The cytokine concentrations in serum and culture supernatant of tumor, draining lymph node (DLN), or spleen cells were quantified by ELISA following the manufacturer’s instructions (eBioscience).

Statistical analysis

ANOVA followed by Student t test was applied in vitro studies. Analysis between in vivo groups was examined by Mann–Whitney U test or ANOVA. A p value <0.05 was considered significant.

Results

BLP treatment induces a dose- and time-dependent tumor remission and host survival

To examine the role of TLR1/TLR2 in cancer, we initially tested their effect on a lung carcinoma model in vivo (38). Groups of C57BL/6 mice were implanted with syngeneic 3LL carcinoma s.c.; on day 5 post tumor inoculation when tumors were palpable, mice were treated with i.p. BLP injections and repeated three times on days 10, 15, and 20 (Fig. 1). Tumor size and survival in these mice were monitored regularly. We first examined the effect of different doses of BLP on tumor growth and found that the therapeutic effect of BLP was dose dependent; lower doses of BLP were less effective, whereas complete tumor regression can be observed with 50 μg BLP (Fig. 1A). The maximum therapeutic effect required multiple administration of BLP at indicated time points as described above; one or two injections of BLP at earlier time points did not have significant antitumor effect (Fig. 1B).

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The therapeutic effect of BLP was long-lasting, no tumor relapse and metastases were detected in the mice, and all treated mice survived for >3 mo without further BLP treatment (Fig. 1C,1D). In contrast, TLR4 agonist LPS treatment under the same conditions had no significant effect on both tumor growth and survival (Supplemental Fig. 1A,1B). We further tested the therapeutic effect of BLP in leukemia (FBL3) and melanoma (F10) models. As shown in Fig. 1, BLP treatment effectively rejected implanted FBL3 tumors and delayed the growth of F10 melanoma. In both tumor models, BLP treatment also prolonged survival of mice in a dose-dependent fashion. Our results demonstrate that BLP but not LPS is capable of rejecting several tumors in our experimental setting.

The antitumor effect of BLP is mediated by neither its direct tumoricidal activity nor innate immune cell activation

We next investigated the mechanism by which BLP induced tumor remission in the 3LL tumor model. One possibility was that BLP may kill tumor cells by direct tumoricidal activity as observed using TLR3 and TLR9 agonists (40, 41). We therefore studied the expression and function of TLRs on 3LL tumors. 3LL cells expressed moderate levels of surface TLR1, TLR2, and TLR4 as determined by flow cytometry analysis (Supplemental Fig. 2A). However, BLP or LPS treatment of 3LL tumor cells neither affect their proliferation nor induced apoptosis (Supplemental Fig. 2B,2C). In addition, TNF-α and IL-6 production or MHC expression by 3LL tumor cells were also not altered by BLP or LPS treatment (data not shown). Similar results were also observed in freshly isolated 3LL tumor cells from syngeneic mice and FBL3 and F10 tumors (data not shown). This result suggests that BLP-induced tumor remission in vivo is unlikely to be due to a direct tumoricidal effect or direct modulation of immunogenicity of 3LL tumor.

Innate immune cells such as NK cells also express TLR2 and are involved in tumor killing (1, 13). We investigated whether innate cells were responsible for BLP-induced tumor remission in vivo. Immunodeficient SCID mice that have an intact innate system but lack of T or B cells were used in this experiment. The mice were implanted with 3LL tumor cells and treated with PBS or BLP as in Fig. 1C. SCID mice developed similar levels of skin tumor as normal mice, and BLP lost its therapeutic effect on the tumor development in the SCID mice (Supplemental Fig. 2D). In addition, BLP had no significant influence on the growth of the same tumor in TLR2−/− mice (Supplemental Fig. 2E). These results suggest that adaptive but not innate immune cells play a major role in BLP-mediated tumor remission.

BLP mediates an antitumor effect by enhancing a tumor-specific CTL response

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BLP mediates an antitumor effect by enhancing a tumor-specific CTL response

We next investigated whether BLP treatment enhanced CTL function in the context of a lung cancer model. The mice were
implanted with 3LL tumor cells and treated with or without BLP as in Fig. 1C. Twenty days later, mice were sacrificed, and sera, DLN, as well as spleens were harvested. We first determined the effect of BLP on the T cell differentiation ex vivo. CD8+ T cells were isolated from the DLN and spleens, labeled with CFSE, and stimulated with inactivated 3LL tumor cells. T cell proliferation as indicated by CFSE dilution was measured by flow cytometry analysis. CD8+ T cells isolated from BLP-treated tumor-bearing mice exhibited vigorous proliferation upon in vitro restimulation with 3LL tumor cells, whereas CD8+ T cells isolated from PBS-treated mice failed to do so (Fig. 2A). Compared with the control, BLP also dramatically enhanced the Ag-specific tumor killing as measured by in vitro cytototoxicity assay (Fig. 2B). We further determined BLP-induced peptide-specific cytotoxicity in tumor-bearing mice in vivo and found that BLP-treated mice enhanced specific lysis of target cells loaded with MUT-1 peptide (97.05%) compared with that in PBS-treated (34.71%) and in the control naive mice. This was evidenced by their different ability to lyse 3LL-Ag–pulsed (CFSEhigh) and unpulsed (CFSElow) target cells.

To determine whether BLP treatment was also able to directly abolish the Treg-mediated suppression on tumor-specific CTL, we pretreated the purified Tregs with BLP or PBS for 24 h, a condition that abolishes Treg function in vitro, before using the cells in a standard suppressive assay (25). The concentrations of IFN-γ and IL-2 were determined by ELISA.

**Optimal antitumor effect of BLP requires TLR2 expression on Tregs**

To determine the significance and relative importance of TLR2 expression on Tregs and CTLs in BLP-triggered antitumor immunity in vivo, SCID mice were implanted with tumor cells 5 d before adoptive transfer of tumor Ag-stimulated CTL and Tregs.
from WT or TLR2^{-/-} mice as indicated (Fig. 4A). All of these cells were isolated and stimulated with inactivated tumor cells and BLP as described in Fig. 3C and in the Materials and Methods section. Tumor-specific cytotoxicity of the CTL and suppressive function of the Tregs were confirmed in vitro as described in Figs. 2B and 3B (data not shown). The SCID mice engrafted with the cultured CTL and Tregs both from TLR2^{-/-} mice had similar tumor burdens as the unengrafted mice (Fig. 4A). In contrast, the SCID mice engrafted with the BLP-treated cells both from WT mice effectively rejected their skin tumors (Fig. 4A). Interestingly, a similar antitumor effect was observed in mice that received cotransfer of BLP-treated WT Tregs with either BLP-treated TLR2^{-/-} or WT CTLs, suggesting that the expression of TLR2 by CTL was dispensable in the BLP-mediated antitumor immunity (Fig. 4A). In contrast, cotransfer of BLP-treated TLR2^{-/-} Tregs with WT CTLs failed to suppress tumor growth. The number of Treg found in DLNs on day 23 post tumor inoculation did not differ among all treatment groups, suggesting TLR2^{-/-} or WT Tregs had a similar capacity to proliferate and survive in SCID mice after BLP treatment (Fig. 4B). However, BLP-treated Tregs from WT but not TLR2^{-/-} mice lost their suppressive function, as demonstrated indirectly by the increased CTL numbers in the DLNs and the higher levels of serum IL-2 and IFN-γ found in mice receiving BLP-treated WT as compared with TLR2^{-/-} Tregs, regardless of whether the cotransferred CTL were from either WT or TLR2^{-/-} mice (Fig. 4B, 4C). These results showed that the BLP-mediated antitumor immunity required the expression of TLR2 on Tregs but not CTLs. The direct blockade of Treg...
induction and function by BLP could lead to indirectly augmenting the CTL response to tumor Ags.

**BLP-treated mice develop long-lasting antitumor immunity**

To test whether BLP can induce long-term antitumor immunity against parent tumor rechallenge, we injected 3LL tumor cells into the right flank of the BLP-cured mice (Fig. 1C), which were free of tumors for 100 d. As a control, the mice were also challenged with FBL3 tumor cells in the same way but on the left flank. A control group of naive mice was also included, which was inoculated with both 3LL and FBL3 tumor as with the BLP-cured mice. As shown in Fig. 5A, naive mice were susceptible to both 3LL and FBL3 tumors. However, the BLP-cured 3LL mice only developed FBL3 but not 3LL tumors 25 d post tumor implantation without further BLP treatment (Fig. 5B). These mice remained free of 3LL tumor until they were sacrificed due to the development of FBL3 tumors (data not shown). No tumor metastases in the lung tissue were detected (data not shown). These results indicated that BLP-treated mice developed Ag-specific memory immune response, which can protect against tumor relapse and rechallenge.

**Discussion**

We report in this study a long-lasting immunotherapeutic property of a TLR1/TLR2 agonist on established murine tumor models. More importantly, we uncovered a novel mechanism by which BLP mediates tumor regression through reciprocal modulation of effector and Treg function. Our studies further determined the requirement of expression of TLR2 on Tregs but not effector CTLs for BLP to exhibit its effect in an adoptive transfer model. To our knowledge, this reciprocal effect on Tregs and CTLs is unique to TLR1/2 signaling, because signaling through other TLRs has not been demonstrated to have such effect. This may help to explain its general therapeutic effect on all tested tumor models in this study and the failure of TLR4 agonist LPS in this cancer context. It may also explain the disappointing results from clinical trials using other TLR ligands (14, 42, 43). From these observations, we believe that an ideal therapeutic TLR agonist must possess the capacity to: 1) enhance effector and memory CTL activity; 2) reduce Treg function; and 3) not promote tumorigenesis. As such, our study suggests that TLR1/2 agonists may be exceptional candidates to be included in cancer immunotherapy clinical trials.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** BLP treatment induces long-lasting memory antitumor immunity. A, Naive mice are equally susceptible to syngeneic 3LL and FBL3 tumor. Naive mice (n = 5) were inoculated with 3LL on the right and FBL3 on the left flank s.c. without BLP treatment and tumor growth was measured as indicated. B, BLP-cured 3LL mice develop memory antitumor immunity. Mice (n = 5) were implanted with 3LL cells and treated with BLP as Fig. 1C. These BLP-cured 3LL mice that were tumor free for 100 d were rechallenged with the same number of 3LL tumor on the right and FBL3 on the left side of flank as control. The mice received no further BLP treatment and tumor development was monitored regularly as before. Data are mean ± SEM. Representative results of one of three independent experiments. ***p < 0.001 compared with controls in all figures as indicated.

The inability of BLP therapy in SCID mice tumors suggests that innate immune cells, by themselves, could not contribute to the BLP-mediated therapeutic antitumor effect without adaptive immune responses. Our results do not exclude their potential role in various stages of CTL expansion and differentiation. For example, BLP can also promote the maturation and activation of Ag-presentation of APCs, in particular dendritic cells (13). APCs will then initiate T cell activation and induce surface TLR expressions via TCR and costimulatory signals (17, 25). This allows T cells to directly respond to TLR stimulation and enhance their proliferation, activation, or survival (17–21). Furthermore, tumor-specific CTL can also activate innate cells, including APC and NK cells, by production of IFN-γ and IL-2 to further amplify the antitumor effect in an autocrine manner (1, 13).

It is unclear why 3LL tumor cells express TLR1 and TLR2 but are apparently unable to respond to TLR1/TLR2 agonist (Supplemental Fig. 2). However, this observation agrees with a previous report using other tumor cells, which suggests that this lack of response may be a general phenomenon in cancers, and it may be worthwhile to investigate the biological significance of TLR unresponsiveness in cancer development and immunosurveillance (44).

We noted that although BLP is able to control the growth of all three tested tumors in vivo, the susceptibility of these tumors to BLP treatment seems variable. Like other immune modulating agents, BLP was more effective in immunogenic tumors such as FBL3 or 3LL and less effective in mice bearing poorly immunogenic F10 melanoma.

A recent report by Kim et al. (35) showed that TLR2 signals may also stimulate metastasis of 3LL cells, the same tumor cells we have used. 3LL-secreted factors promote tumor metastasis indirectly by triggering myeloid cell TNF-α production via TLR2/6 (35). However, our results clearly demonstrate that TLR1/2 ligand, BLP, successfully rejects established 3LL tumor and prevents metastasis. These findings have raised an intriguing question and suggest that TLR2 signals may be able to drive opposite outcomes in cancer, depending on the different combination of TLR2 with its coreceptors in vivo. This may also provide a possible explanation for the result showing that Listeria infection enhances H22 tumor growth via TLR2 (36). Therefore, the precise signaling pathways derived from TLR2 and coreceptors need further investigation.

Given BCG mainly signals via TLR1/TLR2, and BCG therapy is beneficial in human bladder cancer, TLR1/TLR2 agonists may be important for human cancer. Despite the fact that BCG is widely used in treatment of bladder cancer, the role of TLR1/2-mediated therapeutic effect in the clinical setting of cancer is still obscure. Furthermore, recent clinical studies indicate that high levels of tumor infiltration by activated CTL combined with a low number of Tregs is a significant prognostic factor for patient survival in cancers (45–48). The clinical impact of our finding on cancers may also be highlighted by the close association of TLR1/TLR2 and coreceptor polymorphisms with progression and clinical outcomes in certain human malignancies (49, 50).

However, the potential application of TLR agonists in the control of malignancy has complicated their dual role in cancer promotion and suppression. Therefore, carefully examining their role in immune and cancer cells and tailoring their effect in individual cancers will aid the development of more effective immune-based therapeutic interventions against cancers.

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