

RESEARCH ARTICLE

Peptidoglycans Promotes Human Leukemic THP-1 Cell Apoptosis and Differentiation

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Abstract

The innate immune system coordinates the inflammatory response to pathogens. To do so, its cells must discriminate self from non-self utilizing receptors that identify molecules synthesized exclusively by microbes. Toll-like receptors have a crucial role in the detection of microbial infection in mammals and insects. In mammals, they have evolved to recognize conserved products unique to microbial metabolism. These include lipopolysaccharide (LPS), lipoteichoic acids, and peptidoglycans (PGN). We show here that TLRs, including TLR2, are expressed on the THP-1 human leukemia cell line. Activation of TLR2 signaling in THP-1 by PGN induces the synthesis of various soluble factors and proteins including interleukin-1 β , interleukin-8 and TNF- α and apoptosis of THP-1 with PGN dose and time dependence. Moreover, in this study we show that PGN induces apoptosis of THP-1 cells in a TNF- α -dependent manner. These findings indicate that TLR2 signaling results in a cascade leading to tumor apoptosis and differentiation, which may suggest new clinical prospects using TLR2 agonists as cytotoxic agents in certain cancers.

Keywords: TLR2 - PGN - apoptosis - differentiation - leukemia cell line

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Introduction

Many microbial constituents are recognized by Toll-like receptors (TLRs) expressed on macrophages or dendritic cells. TLR engagement can trigger immune responses. Peptidoglycan (PGN), and lipoteichoic acid act as ligands of TLR2 (Li et al., 2012). Double stranded RNA (poly(I:C) RNA), LPS, flagellin, and the CpG motif of unmethylated DNA (CpG DNA) act as ligands of TLR3, TLR4, TLR5, and TLR9, respectively (Bunting et al., 2011; Zoglmeier et al., 2011; Rakhesh et al., 2012; Wang et al., 2012).

In response to TLR ligands, macrophages and dendritic cells produce several inflammatory cytokines such as TNF- α , IL-6, IFN- γ , and IL-12 to activate immune responses (Aderem, 2001). In addition, TLR stimulation by diverse microbial products directly induces the maturation of dendritic cells, which is an essential step for subsequent adaptive immune responses (Roses et al., 2008). Presently, we found that TLRs including TLR2 are expressed on tumor cells from a wide variety of tissues. Several TLR agonists have been developed as anticancer drugs. The TLR7 agonist imiquimod, for example, has been used to treat superficial basal cell carcinoma (Stockfleth et al., 2003), while the TLR9 agonist CpGODN B type is being evaluated in clinical trials in patients with

melanoma and lymphoma (Jahrsdorfer et al., 2005). TLR4 agonists, including monophosphoryl lipid, have been used as adjuvant for vaccines against HBV and other pathogens (Baldrige et al., 2004).

Several mechanisms have been proposed to explain the apparent adjuvant effects of TLR agonists on antitumor immunity. First, TLRs trigger the secretion of critical cytokines such as IL-1, IL-6 and IL-12 by DCs, which are important for T-cell differentiation and the induction of potent adaptive immunity. Several groups have shown that conjugation of certain TLR ligands (that is, for TLR2, TLR4, TLR7 and TLR9) to peptides or proteins significantly enhances CD4⁺ and CD8⁺ T-cell responses compared with administration of TLR ligands or a peptide/protein mixture alone (Wille-Reece et al., 2005; Blander et al., 2008). Second, TLRs can directly stimulate the proliferation of CD4⁺ and CD8⁺ T cells as well as reverse the suppressive function of Treg cells (Peng et al., 2005). Finally, TLR9 and TLR3 agonists may also induce apoptosis of TLR-expressing tumor cells (Salaun et al., 2006).

Based on the accumulating evidence for the involvement of the ligands of TLRs in the therapy of human malignant tumors, we tested whether PGN as the ligand of TLR2 might induce apoptosis and differentiation of human leukemia cells.

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Table 1. Prime for this Study

Gene	sense	Anti-sense
GAPDH	AATCCCATCACCATCTTCCA	CCTGCTTACCACCTTCTTG
TLR1	TAATGCATTTGATGCCCTGC	CCTTCAAGGAAGTGCAGAAAT
TLR2	TTCTTTTCTTCCCTGGGCA	TGAATTTGTTTACCAGTGGGA
TLR3	AAAACCTTTGCTTCTGACAC	GGAATCGTTACCAACCACATT
TLR4	TGTGGCTCAACAATCTTATCCA	CTAAATGTTGCCATCCGAAA
TLR5	GCTTTTCAGGGACTGTTCAT	CAAAACCATGTGAAAGATCCA
TLR6	TCTTGGGATTGAGTGCTATGA	CGGTGTACAAAGCTGTCTGTG
TLR7	GCCAACTTCACTTGAATCTCC	GCTGACAAATTTGGAGTTGCT
TLR8	GCTGACAAATTTGGAGTTGCT	AAATGCAATGCCCGTAGAGA
TLR9	TGAGGACCTGGCCAATCTGA	AAGGCCCTGAAGATGCCGA
TLR10	CAAAATGCACAAATGCCACAC	AAAATCCAGATGGGCTGA
CD14	TGTGAGCTGGACGATGAAGA	AGGCATGGTCCCGGTTAT
CD11b	GTGACATTCCTTCTTGATTG	TGACATCCTCATATCCCAAGG
CD11c	CCAAAAGATAACAGCTGCCA	GCCTGAATTCCTCGAAAGTGA
TNF- α	AAGCCTGTAGCCATGTTGT	AGTCGGTACCCTTCTCCA
TNFR1	TTCACCGCTCAGAAAACCA	GGGATAAAAGGCAAAGACCA
TNFR2	TGACCAGACAGCTCAGATGTG	ACTGCATCCATGCTTGATT
CD86	ATATGGGCCGCAACAAGTTT	AAGTGAAGATAAAAGCCGC
CD40	ATGGTCTGCTGCTCTGCA	ACACATGGAGAAAGCCGA
CD80	ATGATGTCTGGGACATGAA	TGGGAGCAGGTTATCAGGAAA
IL-1 α	TGGCTTTTGGGTATCTCAGGCA	TTCAAGGAGAGCATGGTGGT
IL-1 β	GACGGACCCAAAAGATGAA	CAGCCACGAGGCTTTTGT
IL-6	TGTGGGTGAGGACACGATAGT	ACTCCAGAAGACCAGAGGAAA
IL-8	TGGCAGCTTCTGATT	TCAAAAACCTTCTCCAACCC
IL-12	CCCCTGAAGAAGATGGTATCA	TGATGAAGAAGCTGCTGGTGT
IFN α	CGTCATGAAAATTTGGTGT	ATAATCCTGGATCACAGGCGT
IFN β	TCTCCTCAAATGCTCTCTCT	TACTCCTTGGCCTTCAGTAA
IFN γ	TGCAGGTCAATTCAGATGTAGC	ATGCTCTTCGACCTCGAAACA

Materials and Methods

THP-1 cell culture

Human leukemia cell THP1 were cultured in RPMI media, supplemented with FCS (10% v/v), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 μ g/ml), and maintained at 37°C and 5% CO₂. During routine culture, cell viability was assessed by trypan blue exclusion and was always found to be 90%.

RT-PCR

Total RNA was extracted from 5 \times 10⁶ cells using Trizol (Invitrogen) as described by the manufacturer. mRNA was reverse transcribed with RevertAid (Invitrogen) at 42°C for 60 minutes, and the resulted cDNA was subjected to PCR (95°C for 1 minutes followed by 25-35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 68 for 1.5 minute, and an extension for 10 minutes at 68°C). The primer is used in this study and presented in Table 1.

Elisa

Production of TNF- α by THP-1 was assessed in culture supernatants with the DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. The results are expressed as the means \pm SD of four cultures. For statistical analysis of the results, groups were compared by use of Student's t test.

Flow cytometry

THP-1 were grown to subconfluency, detached with cold Dulbecco's PBS (5 mmol/L EDTA), and washed with fluorescence-activated cell sorting buffer (5 mmol/L EDTA, 0.1% NaN₃, and 1% FCS, in Dulbecco's PBS). After incubation with a monoclonal antibody against human TLR2 and CD14 (R&D Systems) for 30 minutes on ice, the cells were stained with a FITC-labeled secondary antibody and examined for CXCR4 expression by flow cytometry (BD Bioscience, San Jose, CA).

Western blot analysis

For Western blot analysis, THP-1 was cultured for indicated periods in the presence of PGN (10 μ g/ml or 20 μ g/ml). Cells were then washed with ice-cold PBS and lysed in sample buffer (62.5mM Tris-HCl (pH 6.8), 2% SDS, 10% Glycerol, 50 mM DTT, and 0.1% bromophenol blue). Cell lysates were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore) and probed with primary antibodies. Anti-Actin antibody (Cell Signaling) was used as a loading control.

Adhesion assay

THP-1 cells in 6-well culture plates were cultured with different concentration of PGN (0 μ g/ml, 0.5 μ g/ml, 1 μ g/ml, 5 μ g/ml, 10 μ g/ml and 20 μ g/ml respectively) for 24 hours, then were washed with PBS to remove the cells that didn't adhere to the culture well. Cells were visualized by microscopic examination.

Statistical Analysis

All statistical analyses were done using the SPSS 10.0 software package (SPSS Inc., Chicago, USA). Differences between groups were compared using Student's t-test. All tests were two-tailed and p<0.05 were considered significant different.

Results

Toll-like receptors are expressed on THP-1

In this study, we found that THP-1 expressed almost TLRs except for TLR3 by RT-PCR compared to PBMC which expressed all TLRs, The broad expression of TLRs on THP-1 suggest that TLRs may have a previously unrecognized in human leukemia cell biology (Figure 1A).

THP-1 respond to PGN through TLR2

We next asked if TLRs were functional in THP-1. To activate TLR2 in THP-1 cells, we incubated the cells with PGN, the natural ligand for TLR2. After incubation of THP-1 cells with PGN, by fluorescence-activated cell sorting (FACS) analysis, we found that

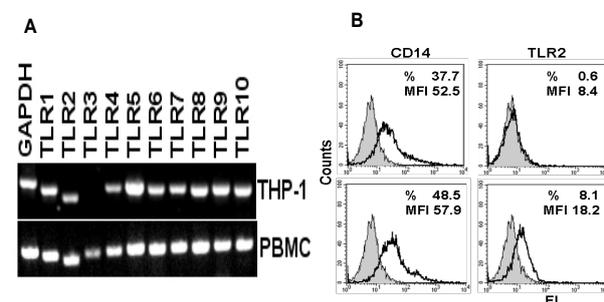


Figure 1. The Expression of TLRs in Human Leukemic THP-1. (A) mRNA expression of TLRs in THP-1. THP-1 cells cultured on six-well plates were harvested. Total RNA was extracted and RT-PCR was performed to detect TLR1-10 gene expression. TLR1-10 gene in PBMC were measured as controls. (B) Protein expression of TLR2 in THP-1 cells. THP-1 cells cultured on six-well plates were harvested or treated with PGN. Fixed and stained with mouse anti-TLR2 and CD14 antibody followed by incubation with FITC-labeled rabbit anti-mouse IgG. TLR2 and CD14 expression was detected by FACS

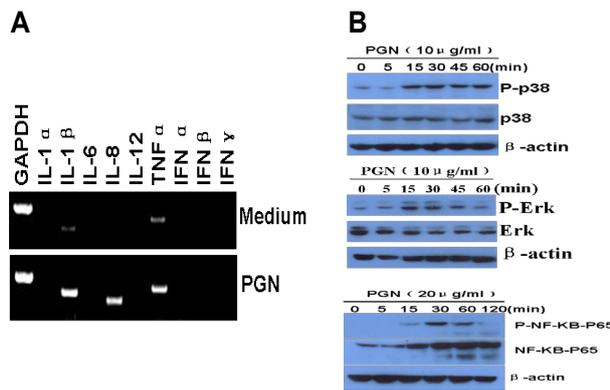


Figure 2. PGN can Activate TLR2 in THP-1. (A) RT-PCR results show that PGN up-regulated the gene express of cytokines including IL-1 β , IL-8, TNF- α . (B) Western blot results show that PGN induced the phosphorylation of the P38, Erk, NF-KB p65 subunit

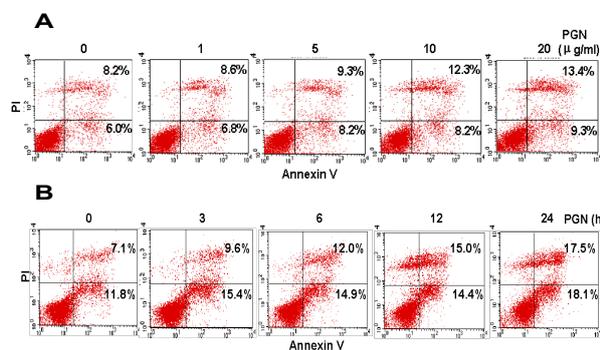


Figure 3. PGN can Induces THP-1 Apoptosis. (A) PGN can induce THP-1 time-dependent apoptosis. (B) PGN can induce THP-1 dose-dependent apoptosis

THP-1 celled expressed CD14, TLR2 on protein level and expressed increasingly after incubation of PGN (Figure 1B). Interestingly, we found that THP-1 cells constitutively express IL-1 β , IL-8, TNF- α and IL-1 β , IL-8 and TNF- α production was strongly induced upon PGN treatment (Figure 2A). Furthermore, we confirmed the activation of the TLR2 signal pathway by analyzing the phosphorylation of downstream signaling molecules. The kinetics of phosphorylation of downstream p38, Erk, NF-kB in THP-1 cells after PGN stimulation was analyzed by immunoblotting with phosphorylation-specific and control antibodies (Figure 2B).

TLR2 agonists can directly trigger THP-1 cells apoptosis, dependently of TNF- α

To investigate the role of TLR2 agonists on THP-1, human leukemia cells THP-1 were cultured with 0 μ g/ml, 1 μ g/ml, 5 μ g/ml, 10 μ g/ml, 20 μ g/ml PGN for 24 hours. Surprisingly we found that PGN can strongly induce dose-dependent apoptosis of THP-1 cells, starting by a 14.2% apoptosis rate and reaching a level of 22.7% apoptosis rate after 20 μ g/ml PGN stimulation (Figure 3A). Importantly, THP-1 cells exposed for 24 h to 20 μ g/ml PGN almost underwent a 1.5-fold increase in apoptosis as illustrated FACS. Furthermore, we incubated the THP-1 cells with 20 μ g/ml PGN after 0h, 3h, 6h, 12h, 24h and found that PGN induced THP-1 cells significant time-dependent apoptosis. Interestingly, the apoptosis rate of THP-1 cells exposed for 24h to 20 μ g/ml PGN increased two times less than that

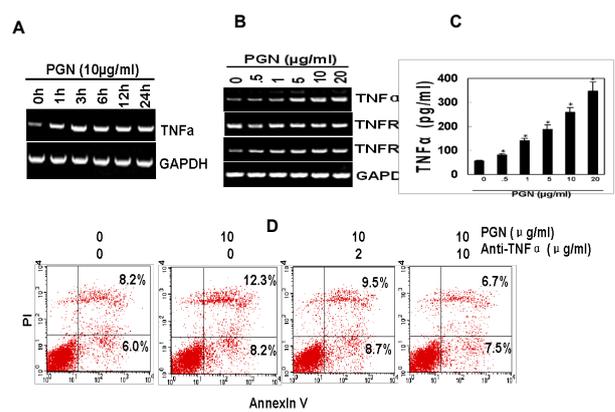


Figure 4. PGN Induces TNF- α -dependent Apoptosis in THP-1. (A) RT-PCR results show that PGN up-regulated the gene express of TNF- α time-dependently. (B) RT-PCR results show that PGN up-regulated the gene express of TNF- α but not TNFR1 and TNFR2 dose-dependently. (C) ELISA results show that PGN up-regulated the protein express of TNF- α in dose-dependent. * p <0.05 compared to untreated group. (D) TNF- α neutralization can inhibit PGN-induced cell apoptosis in THP-1

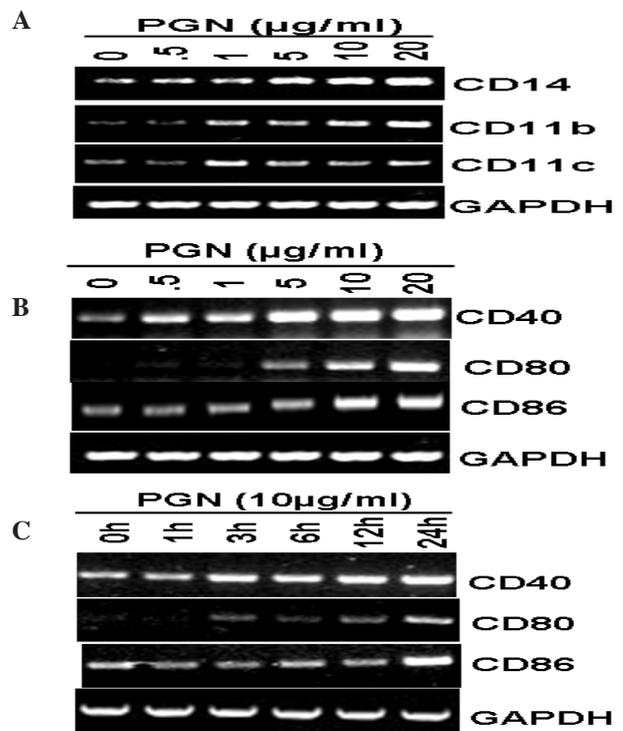


Figure 5. PGN can Induce the Express of Differentiation Markers in THP-1. (A) RT-PCR results show that PGN up-regulated the gene express of CD14, CD11b, CD11c, by dose-dependently. (B) RT-PCR results show that PGN up-regulated the gene express of CD40, CD80, CD86 dose-dependently. (C) RT-PCR results show that PGN up-regulated the gene express of CD40, CD80, CD86 time-dependently

exposed for 0h to 20 μ g/ml PGN (Figure 3B). To analyse the reason of apoptosis that PGN induced THP-1 cells, we found that THP-1 cells highly expressed TNFR1 and TNFR2 that are natural receptors of TNF- α and PGN can stimulate THP-1 cells to up-regulate expression of TNF- α in a concentration-dependent and time-dependent manner by RT-PCR (Figure 4A-B). The protein levels of TNF- α increased very rapidly in the cells culture supernatant and reached the highest level of concentration after incubation

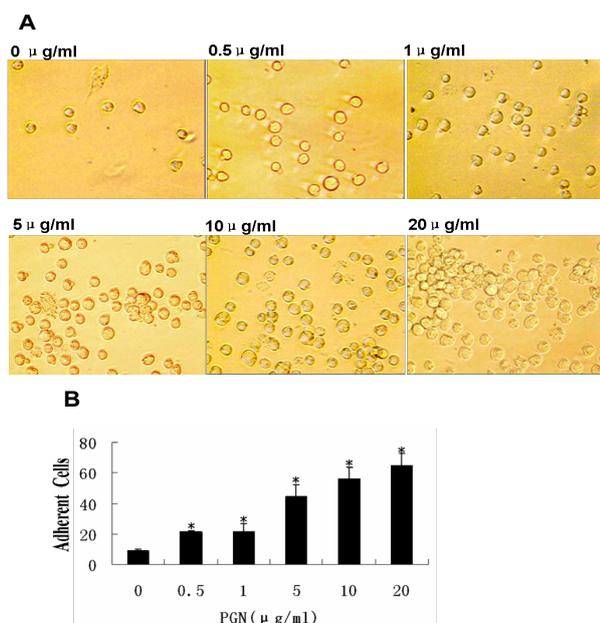


Figure 6. PGN can Induce the Adhesion of THP-1 Cells to Six-well Plates. (A) THP-1 cells were treated with 0, 0.5, 1, 5, 10 and 20 µg/ml of PGN for 24h. Then, the cells that adhere to the well were fixed and photographed. (B) The adhesive cells were counted. The adhesive ability of cells were expressed as the mean number of cells that adhered to the well of plates. * $p < 0.05$ compared to untreated group

with 20µg/ml PGN (Figure 4C).

So far we have shown that PGN induces TNF- α production and the apoptosis in THP-1 cells. PGN-induced secretion of TNF- α and apoptosis occurred almost simultaneously. Therefore, we wanted to determine if the apoptosis of THP-1 cells stimulated by PGN is related to TNF- α secreted by THP-1 cells. For these experiment, we added different concentration specific mAb of TNF- α to the culture supernatant of THP-1, surprisingly, neutralization of TNF- α with specific mAb significantly reduced PGN-induced apoptosis, demonstrating that type TNF- α were necessary for TLR2-mediated cell death. Taken together, these data demonstrate that PGN induces the apoptosis of human leukemic THP-1 cells in a TNF- α -dependent way (Figure 4D).

TLR2 agonists can directly induce THP-1 cells differentiation

To examine the effects of TLR2 stimulation on THP-1 cells differentiation, THP-1 cells were stimulated with PGN and we found that PGN can induce the express of CD11b, CD11c, CD40, CD80, CD86 that those are differentiation markers of the process of THP-1 cells maturation (Figure 5A-B-C). Through adhesion test, we surprisingly found that PGN can promote THP-1 cell adhere to the wall of culture container in a dose-dependent way as is characteristic of mature monocytes (Figure 6A).

Discussion

Although involvement of TLR2 agonists in apoptosis and differentiation has recently been suggested (Bsibsi et al., 2012), direct demonstration of the participation of this

TLR2 agonist in cancer cell apoptosis and differentiation is lacking. The present work demonstrates that the agonist of TLRs can trigger the apoptosis and differentiation of cancer cells.

Toll-like receptors (TLRs) have emerged as sensors that can detect a variety of invading pathogens and malignant cells, thus serving as a first line of defense against infectious diseases and cancer. Ligand recognition by TLRs triggers dendritic cells (DCs) and other antigen-presenting activates intracellular signaling pathways through NF- κ B, mitogen-activated protein kinases and interferon regulatory factors 3 (Takaoka et al., 2005), leading to the production of pro-inflammatory cytokines including TNF- α , IL-1, IL-6, IL-8 and so on (Zughaier et al., 2011). In the study, we tested that THP-1 expressed almost all TLRs except for TLR3 by RT-PCR and PGN can induce the express of TLR2 in protein level by FACS with TLR2 antibody. We showed that PGN can significantly induce NF- κ B, ERK activation, and promote THP-1 to secrete IL-1 β , IL-8, TNF- α , which confirmed that PGN can activate THP-1 by TLR2 and play the important biologic effect..

The reports have previously demonstrated that ligand recognition by TLRs triggers human prostate cancer cells and glioma cells apoptosis through TLR3 and TLR9 respectively (Paone et al., 2008). Recently, we also demonstrated that PGN, the ligand of TLR2 induced THP-1 apoptosis by time- and dose-dependent manner. We further studied the mechanism how PGN induces THP-1 apoptosis. We showed that in THP-1 supernatant adding in with specific mAb of TNF- α , the apoptosis effect of THP-1 was decreased after PGN stimulation, these data demonstrate that PGN induces the apoptosis of human leukemia THP-1 cells in a TNF- α -dependent way, and, there are reports demonstrated that activation of TLRs induced tumor cells apoptosis by other stimulating mechanism (Paone et al., 2008). However, some reports demonstrated that activation of TLRs can trigger proliferation and survival of multiple cancer cells.

In addition, TLR stimulation by diverse microbial products directly induces the maturation of immunologic cells, which is an essential step for subsequent adaptive immune responses (Negishi et al., 2012). THP-1 is called human monocytic leukemia cell that is a type immature immunologic cell. We showed that PGN can stimulate the expression of CD11b, CD11c, CD40, CD80, CD86 which are differentiation markers of monocytes in the course of maturation. Several reports indicate that activation of TLRs can inhibit certain leukemia cell differentiation.

In conclusion, we demonstrated that TLR2 is expressed in human leukemia cell THP-1, and that PGN can induce the apoptosis of THP-1 in a TNF- α -dependent way and differentiation of THP-1. Our results also indicate that PGN maybe stimulate immunologic cells maturation in vitro (Hermans et al., 2007). Collectively, these results open a new range of therapeutic applications for TLR2 agonists as adjuvants in leukemia and raise the exciting concept of multifunctional adjuvants that are able to both directly kill the tumor and enhance the host's immune response against it.

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