Cancer Therapy: Preclinical

Synergistic Anticancer Effects of Pam$_3$CSK$_4$ and Ara-C on B-Cell Lymphoma Cells

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Abstract

**Purpose:** The low immunogenicity of many cancer cells and the immunosuppression by various cancers and anticancer therapies have been an obstacle in the development of efficacious immunotherapies. Our goal was to test whether Toll-like receptor (TLR) agonists and anticancer chemotherapeutic agents synergize in rendering tumor cells more immunogenic.

**Experimental Design:** We treated B-cell lymphoma cells with the TLR1/2 agonist Pam$_3$CSK$_4$ and the genotoxic anticancer agent 1-$\beta$-D-arabinofuranosylcytosine (Ara-C). The effects on the immunogenicity of tumor cells were measured in transfer experiments and in vitro studies.

**Results:** The treatment of B-cell lymphoma cells with the TLR1/2 agonist Pam$_3$CSK$_4$ enhanced the anticancer effects of the genotoxic agent Ara-C. Mice injected with cotreated tumor cells survived longer than mice challenged with Pam$_3$CSK$_4$ or Ara-C–treated cells. Administration of Pam$_3$CSK$_4$ or Ara-C reduced the tumor load of mice injected with tumor cells. Cotreatment had no effect on the rate of apoptosis or proliferation of Ara-C–treated cells, but upregulated the expression of several immunomodulatory molecules. Consistent with an increased immunogenicity of Pam$_3$CSK$_4$ and Ara-C–treated B-cell lymphoma cells, rejection of cotreated tumor cells required natural killer cells and T cells. We demonstrate that the upregulation of immunomodulatory molecules in response to Pam$_3$CSK$_4$ and Ara-C depended in part on NF-$\kappa$B.

**Conclusion:** TLR agonists can increase the efficacy of conventional cancer therapies by altering the immunogenicity of B-cell lymphoma cells. Clin Cancer Res; 20(13); 3485–95. ©2014 AACR.

Introduction

Recent studies suggest that innate and adaptive immune cells play important roles in the antitumor effects of conventional cancer therapies (1, 2). Tumor cells undergo immunogenic cell death in response to certain chemotherapeutic agents such as oxaliplatin and 1-$\beta$-D-arabinofuranosylcytosine (Ara-C; ref. 1). This form of apoptosis enhances tumor-specific immune responses by inducing the release of factors that bind to innate immune receptors on myeloid cells and dendritic cells especially when combined with other immunologically active agents such as Toll-like receptor (TLR) agonists (3).

TLRs play an essential role in immunity through the recognition of pathogen-associated molecular patterns and damage-associated molecular patterns (4). With the exception of TLR3, TLRs depend on the adaptor protein myeloid differentiation primary-response protein 88 (MYD88) for signaling. MYD88-dependent TLR signaling results in the activation of several transcription factors including NF-$\kappa$B, IFN regulatory factors, and activator protein 1. These transcription factors induce the expression of proinflammatory cytokines and costimulatory molecules that are essential for the recruitment and activation of immune cells. Immunomodulatory effects of TLR agonists have been extensively studied in the treatment of cancer (5). TLR9 and TLR7 agonists are being developed and tested as adjuvants for several cancer vaccines (6, 7). TLR agonists were also shown to be effective immunomodulators in the treatment of non–small cell lung cancers, melanoma, and B-cell leukemia (8, 9). Recent studies suggest that TLR2 agonists activate human natural killer (NK) cells, impair the function of regulatory T cells, and enhance the remission of leukemia cells (10, 11). TLR agonists and genotoxic anticancer agents induce the expression of ligands for the activating immune receptors NKG2D and DNAM-1, which are expressed on NK cells and activated T cells (12). Engagement of DNAM-1 and NKG2D by their respective ligands activate NK cells (12). On CD8$^+$ T cells, NKG2D and DNAM-1 trigger costimulatory

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Translational Relevance

Immunotherapy has been shown to increase the efficacy of chemotherapy by activating host immune system against cancer cells. Toll-like receptors (TLR) help to initiate immune responses and have been exploited for active immunotherapy against cancer. However, the low immunogenicity of cancer cells has been a major issue in cancer immunotherapy. Here, we show that the TLR1/2 agonist Pam3CSK4 increases the immunogenicity of B-cell lymphoma cells and alters their trafficking pattern when combined with the genotoxic anticancer drug 1-B-D-arabinofuranosylcytosine. Our data suggest that the combination of anticancer chemotherapy with simultaneous TLR activation protocols can potentiate the immunomodulatory effects of TLR agonists and enhance the efficacy of cancer chemotherapies against B-cell lymphomas.

signals, which enhance T-cell responsiveness. DNAM-1 and NKG2D ligands contribute to immune recognition of B-cell tumors in Eμ-Myc mice, a mouse model for human Burkitt lymphoma and non-Hodgkin lymphomas (NHL; refs. 13–15).

To investigate whether the immunotherapeutic effects of TLR1/2 agonists boost the effects of genotoxic anticancer agents, we treated two B-cell lymphoma cell lines derived from Eμ-Myc mice with the TLR1/2 agonist Pam3CSK4 and Ara-C, an alkylating chemotherapeutic agent that induces immunogenic cell death and is commonly used to treat B-cell NHL (16). Here, we show that Pam3CSK4 increases the immunogenicity of Ara-C-treated B-cell lymphoma cells. Combined treatment of B-cell lymphoma cells with Pam3CSK4 and Ara-C enhanced the expression of several immunomodulatory molecules and increased their sensitivity to effector cells of the immune system leading to decreased tumorigenicity of B-cell lymphoma cells.

Materials and Methods

Mice and cells

C57BL/6 mice were purchased from the Centre for Animal Resources at the National University of Singapore (NUS; Singapore). Hemizygous Eμ-Myc mice on a C57BL/6 background were obtained from the Jackson Laboratory. EμM2 lymphoma cells were prepared from a lymph node of 108 days of age Eμ-Myc transgenic C57BL/6 mouse. EμM2 cells were a kind gift by Dr. L.M. Corcoran (WEHI; Australia). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the National University of Singapore.

Treatment of EμM2 and BC2 cells

EμM2 or BC2 cells (1.6 × 10^6/mL) were treated with 0.1% DMSO, 1 μg/mL Pam3CSK4 or 1 μmol/L Ara-C (Sigma) for 16 hours. For the combination treatment, the cells were treated with 1 μg/mL Pam3CSK4 (Invivogen) or endotoxin-free water used for dissolving Pam3CSK4 for 16 hours followed by 1 μmol/L Ara-C or DMSO for 16 hours. Cell-free supernatant was harvested, frozen in liquid nitrogen, and stored at −80°C until measurement. The quantification of IL6, TNFα, CCL5, and MIP-1α (CCL3) in culture supernatants was determined using ELISA Kits (R&D systems or ebioscience) according to the manufacturer’s instructions.

Western blotting

Whole-cell extracts were prepared from EμM2 cells, electrophoresed in 10%, 12%, or 15% SDS-PAGE, and blotted onto nitrocellulose membranes. Antibodies specific for phospho-H2AX-Ser139 (p-H2AX), phospho-p53-Ser18 (p-p53), p53, IkBα, phospho-IKKα/β (p-IKKα/β), IKKγ and IKKβ, phospho-p65 (p-p65), p65 (Cell Signaling Technology), p21 (WAF1/CIP1; Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma), and horseradish peroxidase-coupled second-stage reagents were used to develop the blots (Thermo). Blots were exposed to X-ray films (Fuji). Protein bands were quantitated using Image software (NIH, Bethesda, MD) based on measurement of relative intensity. Values were further normalized to GAPDH levels of respective plots.

Flow cytometry

Cells were stained with CD16/CD32-specific antibodies (ebioscience) followed by anti-CD3-PerCP-Cy5.5, anti-NK1.1-APC, anti-CD8-Pacific Blue, anti-CD4-PE, anti-CD40-PE, anti-CD69-FITC, anti-CD80-PE, anti-CD86-APC, anti-CXCR4-APC, anti-H-2Kb-PE, anti-ICAM-1-FITC, anti-NKG2D-PE-CY7, anti-DNAM-1-PE, anti-TLR1-PE, anti-TLR2-FTTC, anti-TLR11a-FTTC (LFA-1), anti-Thy1.1-FTTC, anti-B220-PerCP-Cy5.5, anti-igM-APC (ebioscience), or anti-CD155 (Hycult Biotechnology). Before staining of primary cells, red blood cells were removed using red blood cell lysis buffer (0.15 mol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mol/L Na2EDTA). For the analysis of the hematopoietic precursor population, bone marrow cells were isolated and stained with a biotinylated anti-mouse lineage panel (BD Biosciences), anti-SCA-1-PerCP-Cy5.5, anti-c-KIT-APC (ebioscience), or anti-CD155 (Hycult Biotechnology). Before staining of primary cells, red blood cells were removed using red blood cell lysis buffer (0.15 mol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mol/L Na2EDTA). For the analysis of the hematopoietic precursor population, bone marrow cells were isolated and stained with a biotinylated anti-mouse lineage panel (BD Biosciences), anti-SCA-1-PerCP-Cy5.5, anti-c-KIT-APC-Cy7, anti-AA4-PE, anti-IL7R-APC, anti-ICAM-1-APC, anti-Streptavidin-Pacific Blue, and anti-FLT3-APC (ebioscience). Stained cells were analyzed by multicolor flow cytometry using FACS Calibur, LSRFortessa (BD Biosciences), or CyAn ADP (Beckman Coulter) flow cytometers and FlowJo. 8.8.7. (Tree Star).

For Annexin-V staining, EμM2 cells were treated as indicated above. Treated cells were analyzed using an Annexin V-APC apoptosis detection kit according to the manufacturer’s instructions (ebioscience).

For the analysis of the rate of proliferation, EμM2 cells were labeled with 3 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) according to the manufacturer’s protocol before the treatment of cells with...
Pam$_3$CSK$_4$ and Ara-C. Cells were analyzed by flow cytometry one day later.

Quantitative real-time PCR

RNaseq Mini Kit (Qiagen) was used to isolate total RNA from cells. Reverse transcription was performed using 2 μg of total RNA, random hexamer primer, and moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega). For PCR, amplification mixture consisting of 50 ng of reverse transcribed RNA, 0.8 μmol/L forward primer, 0.8 μmol/L reverse primer, and 12.5 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories) was prepared (25 μL). PCRs were performed in duplicates using the ABI PRISM 7700 Sequence Detection (Applied Biosystems), and the PCR thermocycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute, and 92°C for 1 minute. The expression levels of the genes of interest were normalized to hypoxanthine phosphoribosyltransferase (HPRT). The sequences of primers are as follows:

- **IFNβ**: 
  - Forward: 5'-ttcctcccacgtcaatctttcc-3'
  - Reverse: 5'-tgtcctcatcctggaaggtc-3'

- **IL-1β**: 
  - Forward: 5'-agagcagatagccagagt-3'
  - Reverse: 5'-aatttctccagcactgggtg-3'

- **IL-4**: 
  - Forward: 5'-aatttctccagcactgggtg-3'
  - Reverse: 5'-tgggaggccatcacattgt-3'

- **TGFβ3**: 
  - Forward: 5'-tgggagagccctgttttggc-3'
  - Reverse: 5'-aatgtgggagagcaatgttgc-3'

- **Hprt**: 
  - Forward: 5'-tcacagagccctcagctggttg-3'
  - Reverse: 5'-cttaagtttaatcgttcgtttct-3'

Microarray

Total RNA of treated E62 cells was extracted using RNeasy Kit (Qiagen) according to the manufacturer’s instructions. The quality of total RNA was evaluated using Agilent Bioanalyzer (Agilent Technologies); only samples with a RNA integrity number > 7.5 were used. Gene expression analysis was performed using Illumina mouse version 2 BeadChip arrays (Illumina). cDNA preparation, purification and labeling, array hybridization, and scanning were conducted as per the manufacturer’s instructions. Expression levels were extracted with Affymetrix GeneChip Command Console (AGCC). Arrays were normalized using the robust multiarray average method and properly performing loci on the microarray were filtered using Beeline (Illumina). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2164.

Constructs and transduction

MSCV-IRES-Thy1.1 DEST (#17442), pBABE-puro-1KBo-super repressor (#15291), pBABE-puro, pSicoR p53 shRNA (#12090), and pSicoR (#11579) plasmids were obtained from Addgene. Transduction of cells was performed as previously described (17).

Adoptive transfers studies

Before in vivo injections of E62 cells, dead cells were removed by Ficoll gradient centrifugation (1700 rpm, 20 minutes, 18°C). After washing three times with RPMI, 5 × 10^5 E62 cells were resuspended in 100 μL of PBS and intravenously injected into mice. Eleven days postinjection, spleenocytes, blood, and bone marrow cells were stained and analyzed by flow cytometry. For in vivo blocking studies, mice received intraperitoneally 500 μg anti-NK1.1 (PK136, ATCC), 500 μg anti-CD4 (GK1.5 a kind gift of Dr. J. Wong; NUS), 250 μg anti-CD8 (Clone #2.43, ATCC), or equivalent amounts of isotype control antibodies on days 1, 4, and 9 after injection of tumor cells. Mice were euthanized at the terminal stage of disease when weight loss was more than 15% or when ruffled hair coat, reduced locomotor activity, or a hunched back was observed.

To test the effect of TLR stimulation and chemotherapy on tumor cells in vivo, female C57BL/6 mice were injected intravenously with 5 × 10^3 Thy1.1+E62 cells in 150 μL PBS. The next day, mice received 100 μL Pam3CSK4 (1 μg/μL) or endotoxin-free water followed by 10 mg Ara-C or 100 μL PBS 16 hours later, both administered intraperitoneally. At 12 days postinjection of tumor cells, blood, spleen, and bone marrow were analyzed for the percentage of Thy1.1+ B220+ cells.

Statistical analysis

Mean% tumor load between groups was compared using two-tailed unpaired t-tests (Prism, 5.0c, GraphPad). Survival was represented by Kaplan–Meier curves and statistical analysis of survival was performed with the log-rank Mantel-Cox Test. P < 0.05 denotes significance.

Results

Ara-C and Pam3CSK4 treatment reduces the tumorigenicity of E62 cells

Synergy of TLR agonists with chemotherapeutic anticancer agents and radiation has been shown in murine models of sarcoma, mammary carcinoma, and lymphoma (5). It is believed that TLR agonists enhance the efficacy of conventional cancer treatment by promoting the ability of dendritic cells to induce tumor-specific immune responses (5). We previously found that genotoxic anticancer agents induce the expression of immunomodulatory molecules including ligands for NKG2D, DNAM-1, and LFA-1 (12). Similarly, TLR agonists induce the expression of immunomodulatory molecules, some of which overlap with molecules induced in response to genotoxic agents (4, 18, 19). To investigate whether TLR agonists and genotoxic agents synergize in rendering tumor cells more immunogenic, we treated E62 cells, a lymphoma cell line derived from EμM2 cells, with DNA-damaging agents Ara-C and the TLR1/2 agonist Pam3CSK4 (20, 21). E62 cells expressing the congenic marker Thy1.1 were treated with Ara-C and Pam3CSK4 and adoptively transferred to C57BL/6 mice. As a control, E62 cells were treated with Ara-C, Pam3CSK4, or DMSO before injection. The survival of mice that received Pam3CSK4 and Ara-C–treated E62 cells was increased when compared with mice that received control-treated E62 cells suggesting that cotreatment reduced the tumorigenicity of E62 cells (Fig. 1A). In agreement with this observation, the tumor load in the spleen (Fig. 1B) and...
Cotreatment reduced the percentage of Thy1.1* cells one day postinjection of tumor cells. Encouragingly, blood (Fig. 1C) was lower in mice injected with Pam3CSK4 and Ara-C–treated EμM2 cells when compared with the tumor load in control mice. To test whether administration of Pam3CSK4 and Ara-C also reduced tumorigenicity of EμM2 cells, mice were injected with Pam3CSK4 and Ara-C one day postinjection of tumor cells. Encouragingly, cotreatment reduced the percentage of Thy1.1*B220* EμM2 cells suggesting that administration of Pam3CSK4 also enhances the anticancer effects of Ara-C in vivo (Fig. 1D). In contrast, treatment of mice with Pam3CSK4 increased the tumor load in the blood in 2 out of 5 mice indicating that Pam3CSK4 may also induce effects that support tumor proliferation when injected without Ara-C.

To address potential explanations for the decreased tumorigenicity, we compared the rate of cell death/apoptosis of EμM2 cells in response to the different treatments. Cotreatment of EμM2 cells with Pam3CSK4 and Ara-C did not significantly change the rate of apoptosis when compared with cells treated with Ara-C (Fig. 1E). Similarly, Pam3CSK4 did not influence the rate of proliferation of Ara-C–treated EμM2 cells (Fig. 1F). These data support the conclusion that the decreased tumorigenicity of Pam3CSK4...
The immune system plays an important role in the suppression of tumors (22). To test whether the reduced tumorigenicity of cotreated cells depended on the rejection by immune cells, we depleted different immune cell subset that have been implicated in tumor surveillance before injection of treated EμM2 cells (13). Simultaneous depletion of CD4⁺ and CD8⁺ cells increased the tumor load in the blood and spleen at 11 days postinjection (Fig. 2A and B). Depletion of NK cells together with CD4⁺ or CD8⁺ cells also increased the tumor load, but to a lesser degree than in mice depleted of CD4⁺ and CD8⁺ cells (Fig. 2A and B). In contrast, single depletion of NK1.1⁺, CD4⁺, or CD8⁺ cells had no significant effect on the tumor load in the blood or spleen at 11 days postinjection (Fig. 2A and B). Hence, T cells and NK cells contribute to the rejection of Pam3CSK4 and Ara-C–treated EμM2 cells.

Synergistic upregulation of immunomodulatory molecules in EμM2 and BC2 cells in response to treatment with Pam3CSK4 and Ara-C

The depletion experiments suggested that the immunogenicity of EμM2 cells was increased in response to Pam3CSK4 and Ara-C treatment. To identify immunomodulatory genes, which are specifically upregulated in response to Pam3CSK4 and Ara-C, we compared Pam3CSK4 and Ara-C–treated EμM2 cells was not due to changes in the rate of intrinsic apoptosis or proliferation.

**Decreased tumorigenicity of Pam3CSK4 and Ara-C–treated EμM2 cells depends on NK and T cells**

The immune system plays an important role in the suppression of tumors (22). To test whether the reduced tumorigenicity of cotreated cells depended on the rejection by immune cells, we depleted different immune cell subset that have been implicated in tumor surveillance before injection of treated EμM2 cells (13). Simultaneous depletion of CD4⁺ and CD8⁺ cells increased the tumor load in the blood and spleen at 11 days postinjection (Fig. 2A and B). Depletion of NK cells together with CD4⁺ or CD8⁺ cells also increased the tumor load, but to a lesser degree than in
and/or Ara-C–treated EμM2 cells to DMSO–treated cells using DNA microarrays (Fig. 3A). Verification of changes in expression by flow cytometry confirmed that the cell surface molecules CD40, CD69, CD80, CD86, ICAM-1, TLR1, TLR2, RAE-1β8, DNAM-1, and H-2Kβ are specifically upregulated on EμM2 and BC2 cells, another cell line derived from Eμ-Myc mice, in response to Pam3CSK4 and Ara-C (Fig. 3B and Supplementary Fig. S1). In contrast, the expression of the chemokine receptor CXCR4, which mediates migration of B-cell lymphomas to bone marrow of mice injected with Pam3CSK4 and Ara-C–treatment are shown. B, Pam3CSK4 and/or Ara-C–treated EμM2 cells were assessed for cell surface expression levels of CD40, CD69, CD80, CD86, CD115/ICAM-1, MHC Class I (H-2Kβ), and CXCR4 by flow cytometry (black line). Staining was compared with the respective isotype controls (filled histograms). C, supernatants of Pam3CSK4 and/or Ara-C–treated EμM2 cells were analyzed for the amounts of IL6, TNF-α, CCL5, and MIP-1α by ELISA. The data represent mean ± SD of three independent experiments. D, levels of Il-1β, Il4, Tgfβ3, and Il-4 transcripts in Pam3CSK4 and/or Ara-C–treated EμM2 cells were determined by real-time RT-PCR. Data show mean ± SD of three independent experiments.

Reduced loss of common lymphoid progenitors in the bone marrow of mice injected with Pam3CSK4 and Ara-C–treated EμM2 cells

Leukemia is often associated with bone marrow disorders (24). Transplanted Eμ-Myc B-cell lymphomas were shown to infiltrate the bone marrow leading to abnormal bone marrow function (25–27). Furthermore, EμM2 expressed CXCR4, which mediates migration of B-cell lymphomas to the bone marrow (28). We therefore investigated the effects...
of cotreatment on the number of bone marrow cells after adoptive transfer of Pam₃CSK₄ and Ara-C–treated EμM₂ cells. We observed a 7.5-fold reduction in the number of common lymphoid progenitor (CLP; Lin⁻IL7Rα⁻SCA-1low-C-KITlowAA4⁻FLT3high) after injecting EμM₂ cells treated with DMSO, Pam₃CSK₄, or Ara-C when compared with unchallenged mice (Fig. 4A). In contrast, CLP numbers decreased by only 1.6 times in mice that received Pam₃CSK₄ and Ara-C when compared with Ara-C–treated EμM₂ cells (Fig. 4A). No changes in other precursor subsets were observed after injection of EμM₂ cells.

CLPs give rise to NK and T cells among other immune cells (29). Consistent with the loss of CLPs, the percentage of peripheral blood NK cells, CD4⁺, and CD8⁺ T cells decreased in mice that received EμM₂ cells. In mice that were injected with cotreated EμM₂ cells, the loss of splenic T cells was less pronounced, possibly due to the reduced loss of CLPs (Fig. 4B).

**Pam₃CSK₄ enhances Ara-C–induced NF-κB activation**

To investigate the molecular mechanisms responsible for synergy of Pam₃CSK₄ and Ara-C cotreatment, we first tested whether Pam₃CSK₄ enhances the DNA-damaging effects of Ara-C. Analysis of the phosphorylation of H₂AX, a marker of DNA damage, and the phosphorylation of p53, an important mediator of the DNA damage response, by Western blot analysis showed no significant increase of γ-H₂AX-p-Ser13 or p53-p-Ser15 expression in EμM₂ cells cotreated with Pam₃CSK₄ and Ara-C when compared with Ara-C–treated cells (Fig. 5A; ref. 30).

Pam₃CSK₄ activates NF-κB through MYD88 while the DNA damage-responsive kinase ataxia telangiectasia mutated (ATM) associates with the NF-κB essential modulator (NEMO) in response to genotoxic agents such as Ara-C (31, 32). To determine whether cotreatment of cells with Pam₃CSK₄ enhances Ara-C–mediated activation of NF-κB, we analyzed the expression levels of IKKα-p-Ser176/180, IKKβ-p-Ser177/181, p65-p-Ser536, and IκBα, an inhibitor of NF-κB. Treatment of EμM₂ cells with Pam₃CSK₄ enhanced and prolonged Ara-C–induced phosphorylation of IκBα and p65 (Fig. 5B). In accordance with this observation, IκBα was degraded faster in cotreated cells.

**NF-κB mediates the effects of Pam₃CSK₄ and Ara-C cotreatment**

To determine whether p53 or NF-κB plays a role in the observed synergism between Pam₃CSK₄ and Ara-C, we transduced EμM₂ cells with p53 shRNA or an IκBα super repressor and assessed their effects on the expression of cell surface molecules (Supplementary Fig. S2). Inhibition of
p53 had no effect on Pam\textsubscript{3}CSK\textsubscript{4} and Ara-C–induced expression of the tested cell surface molecules (Fig. 6A). In contrast, inhibition of NF-κB abrogated the upregulation of the NF-κB target genes CD69, CD80, CD86, ICAM-1, and H-2K\textsuperscript{b} in response to Pam3CSK4 and Ara-C (Fig. 6B). Expression of the IκB target genes CD69, CD80, CD86, ICAM-1, and H-2K\textsuperscript{b} was significantly reduced by Pam3CSK4 and Ara-C, and lysis of E\textsubscript{μ}M2 cells by NK cells and T cells.

**Discussion**

Here, we show that the TLR1/2 agonist Pam\textsubscript{3}CSK\textsubscript{4} enhances the anticancer effects of the chemotherapeutic drug Ara-C. Cell intrinsic tumor-suppressor mechanisms are likely to contribute to the reduced tumorigenicity of cotreated tumor cells although pretreatment of E\textsubscript{μ}M2 cells with Pam\textsubscript{3}CSK\textsubscript{4} and Ara-C did not significantly affect the rate of apoptosis or proliferation as compared with Ara-C treatment alone. The anticancer effects of Pam\textsubscript{3}CSK\textsubscript{4} and Ara-C were severely impaired in NK cells and T cell-depleted mice suggesting that the effects of the cotreatment are mainly mediated by extrinsic pathways. Surprisingly, we found that CD4\textsuperscript{+} T cells contribute to the protective effects of Pam\textsubscript{3}CSK\textsubscript{4} and Ara-C treatment. CD8\textsuperscript{+} T cells are critical for immunosurveillance, but recent evidence has also uncovered an important role for CD4\textsuperscript{+} T cells in anticancer immune responses (33–37). Consistent with a role for CD4\textsuperscript{+} T cells in the rejection of cotreated E\textsubscript{μ}M2 cells, we have previously found that CD4\textsuperscript{+} T cells in collaboration with CD8\textsuperscript{+} T cell and NK cells mediate antitumor responses in E\textsubscript{μ}-Myc mice (13). It was suggested that CD4\textsuperscript{+} T cells are required for optimal tumor-specific CD8\textsuperscript{+} T-cell responses.

![Figure 5.](image-url) Pam\textsubscript{3}CSK\textsubscript{4} enhances Ara-C–induced NF-κB activation. A, immunoblot analysis of E\textsubscript{μ}M2 cells treated with DMSO (white column), 1 μmol/L Ara-C (light grey column), 1 μg/mL of Pam\textsubscript{3}CSK\textsubscript{4} (dark grey column) for 16 hours or 1 μg/mL Pam\textsubscript{3}CSK\textsubscript{4} (16 hours) followed by 1 μmol/L Ara-C for 16 hours (black column) and probed with antibodies for the DNA damage marker p-H2AX (Ser139) and the DNA damage response marker p-p53 (Ser18). Representative immunoblot images are shown (left). Protein band densities of three independent experiments were quantitated and normalized to GAPDH (right). B, immunoblot analysis of E\textsubscript{μ}M2 cells treated with 1 μg/mL Pam\textsubscript{3}CSK\textsubscript{4} (right panel and black column) or vehicle (left panel and light grey column; 16 hours) followed by 1 μmol/L Ara-C for the indicated times. Blots were probed for phospho-IKK, IKKα, IKKβ, IκBα, phospho-p65, and p65 using specific antibodies. Representative immunoblot images are shown (top). The densities of phosphorylated p65 bands of three independent experiments were quantified and normalized to total levels of p65 protein and GAPDH at the beginning of Ara-C treatment. IκBα levels were quantified and normalized to GAPDH (bottom) at the beginning of Ara-C treatment. *, P < 0.05; **, P < 0.01.
by providing help in the form of cytokines and costimulatory signals (38).

Cotreatment of cells with Pam3CSK4 and Ara-C induced the expression of a number of several immunomodulatory molecules including cell surface proteins, cytokines, and chemokines. The costimulatory molecules CD80, CD155, and RAE-1 play a critical role in antileukemia immune responses mediated by NK cells and T cells (12, 39). Transduction of acute myelogenous leukemia (AML) cells with a lentivirus encoding CD80 and IL2 increased their sensitivity to NK cell and T-cell cytotoxicity (40). We recently showed that CD155 expression on tumor cells in Eμ-Myc mice is important for immune recognition of tumor cells by NK and T cells (13). NKG2D ligands also play an important role in antitumor immunosurveillance in Eμ-Myc mice (14). Hence, it is likely that the upregulated expression of RAE-1 and the constitutive expression of CD155 in EμM2 cells contribute to the enhanced immunogenicity after cotreatment. Treatment of EμM2 and BC2 cells with Pam3CSK4 and Ara-C also upregulated the expression of IFN-β, CCL5, and MIP-1α that have been implicated in tumor surveillance. IFNβ induces apoptosis of tumor cells at high doses and enhances immune responses by activating multiple cell types, including CD8+ T cells and NK cells (41). CCL5 and MIP-1α were shown to attract NK and T cells to tumor cells (42). Cotreatment also enhanced the expression of cytokines, which can have opposing effects on tumorigenesis including IL1, IL4, IL6, CD40, TNFα, and TGFβ3. IL1 and IL6 potentiate the proliferation, differentiation, and activation of several immune cell subsets involved in antitumor responses (43). However, IL1 was also found to promote tumor invasiveness and angiogenesis (44), whereas IL6 can promote or prevent lymphoma development by...
acting on cells at distinct stages of hematopoietic development (45, 46). CD40, TNFα, and IL4 can induce B-cell proliferation, while also targeting cells for apoptosis (47). TGFβ3 suppresses the proliferation of premalignant cells, but enhances the invasion and metastasis of more advanced tumor cells (48). Despite the opposing effects of many cytokines and chemokines expressed in response to Pam1, CSK4, and Ara-C, the decreased tumorigenicity of cotreated EμM2 cells suggests that the overall tumor suppressing effects dominate in vivo.

CXCR4, which binds the chemokine CXCL12, is upregulated in various late-stage cancers including EμM2 and BC2 cells used in this study (49). Strikingly, the expression of CXCR4 was downregulated in response to Pam1, CSK4 and Ara-C cotreatment. CXCL12 secretion by stromal cell of the bone marrow was shown to attract cancer cells including acute lymphoblastic leukemia and AML (49). Interestingly, pre-B cells associate with CXCL12-abundant reticular (CAR) cells in the bone marrow, and ablation of CAR cells is associated with a loss of CLPs (50, 51). Hence, the reduced loss of CLPs in the bone marrow of mice injected with cotreated tumor cells may be, in part, due to the lower CXCR4-mediated infiltration of the bone marrow by EμM2 cells and the reduced competition of pre-B cell-like EμM2 and CLPs for CAR cells (50, 51). Loss of CLPs and peripheral T cells may contribute to impaired immunosurveillance of tumor cells.

Our data suggest that the synergistic effect of Pam1, CSK4 and Ara-C treatment is mediated by NF-κB. TLR2 forms a heterodimer with TLR1 in response to Pam1, CSK4 and Ara-C cotreatment. Pam1 activates the adaptor proteins MAL and MYD88 and the activation of IL1 receptor-associated kinase 4 (IRAK4), IRAK1, and TNF receptor-associated factor 6 (TRAF6; ref. 52). Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TGFβ-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), and TAB2 leading activation of NF-κB. Interestingly, DNA lesions in response to DNA-damaging agents activate NF-κB via ATM-dependent phosphorylation and ubiquitylation of NEMO (53). Similar to TLR2 signals, ATM-driven NF-κB activation depends on TRAF6, TAK1, and TAB2. It is therefore possible that Pam1, CSK4 and Ara-C signals synergize at the level of TAK1/TAB-mediated degradation of the components of the IKK complex. In summary, our data highlight the possibility that in addition to the previously known effects of TLR2 agonists on immune cells, the mechanism of action of TLR1/2 agonists includes changes in immunogenicity and trafficking of tumor cells. In conclusion, our data suggest that TLR1/2 agonists not only act as potent adjuvants in cancer immunotherapy, but also render TLR1/2-expressing tumor cells more immunogenic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S.K. Lee, S. Gasser
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Lee, J.Y. Chwee, C. Ma, N.L. Bert, S. Gasser
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References


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