TLR2 Agonist PSK Activates Human NK Cells and Enhances the Antitumor Effect of HER2-Targeted Monoclonal Antibody Therapy

Hailing Lu1, Yi Yang1, Ekram Gad1, Carol Inatsuka1, Cynthia A. Wenner2, Mary L. Disis1, and Leanna J. Standish2

Abstract

Purpose: The therapeutic effect of trastuzumab monoclonal antibody (mAb) therapy has been shown to be partially dependent on functional natural killer (NK) cells. Novel agents that enhance NK cell function could potentially improve the antitumor effect of trastuzumab. We recently identified polysaccharide krestin (PSK), a natural product extracted from medicinal mushroom Trametes versicolor, as a potent toll-like receptor 2 (TLR2) agonist. This study was undertaken to evaluate the effect of PSK on human NK cells and the potential of using PSK to enhance HER2-targeted mAb therapy.

Experimental Design: Human peripheral blood mononuclear cells were stimulated with PSK to evaluate the effect of PSK on NK cell activation, IFN-γ production, cytotoxicity, and trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). Whether the effect of PSK on NK cells is direct or indirect was also investigated. Then, in vivo experiment in neu transgenic (neu-T) mice was carried out to determine the potential of using PSK to augment the antitumor effect of HER2-targeted mAb therapy.

Results: PSK activated human NK cells to produce IFN-γ and to lyse K562 target cells. PSK also enhanced trastuzumab-mediated ADCC against SKBR3 and MDA-MB-231 breast cancer cells. Both direct and interleukin-12–dependent indirect effects seem to be involved in the effect of PSK on NK cells. Oral administration of PSK significantly potentiated the antitumor effect of anti-HER2/neu mAb therapy in neu-T mice.

Conclusion: These results showed that PSK activates human NK cells and potentiates trastuzumab-mediated ADCC. Concurrent treatment with PSK and trastuzumab may be a novel way to augment the antitumor effect of trastuzumab. 

Clin Cancer Res; 17(21); 6742–53. ©2011 AACR.
Translational Relevance

Trastuzumab has become the standard of care for patients with HER2+ breast cancer. Although the mechanism of the antitumor actions of trastuzumab is multifaceted, antibody-dependent cell-mediated cytotoxicity (ADCC) is believed to be one of the major mechanisms. The clinical response to trastuzumab therapy has been shown to be partially associated with the function of natural killer (NK) cells, which are the main mediators of ADCC. Immune response modifiers that can enhance NK cell function and ADCC could potentially improve the clinical response to trastuzumab. In this study, we showed that polysaccharide krestin (PSK), a mushroom extract and a toll-like receptor 2 (TLR2) agonist, can activate human NK cells to secrete IFN-γ and exert enhanced cytolytic activity in lysing K562 target cells and trastuzumab-coated breast cancer cells. Using a mouse model of HER2+ breast cancer, we further showed that concurrent administration of PSK can augment the antitumor effect of anti-HER2/neu mAb therapy. These findings suggest the potential of using PSK, a natural product with potent TLR2 agonist activity, as an adjuvant therapy for patients with breast cancer to improve the therapeutic effect of trastuzumab.

Materials and Methods

Animals

A colony of neu transgenic (neu-T) mice [strain name, FVB/N-TgN (MMTVneu)-202Mul] was established in our animal facilities from breeding pairs obtained from the Jackson Laboratory and maintained as previously described (30). Mice were maintained under strict inbreeding conditions. All of the procedures were conducted in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Human peripheral blood mononuclear cells and cell lines

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood or leukapheresis products by centrifugation through a Ficoll-hypaque gradient. Blood or leukapheresis samples were collected from healthy volunteer donors with informed consent using a protocol approved by the Institutional Review Board of University of Washington. NK cells were purified from PBMCs by magnetic negative selection, using Miltenyi NK cell Isolation kit II. NK-92, a cell line that has the characteristics of human NK cells (31), was obtained from American Type Culture Collection (ATCC) and maintained in Alpha Minimum Essential Medium without ribonucleosides and deoxynucleosides but with 2 mmol/L l-glutamine, 0.2 mmol/L inositol, 0.1 mmol/L 2-mercaptoethanol,
0.02 mmol/L folic acid, 100 U/ml interleukin (IL)-2, 12.5% FBS, and 12.5% horse serum. The breast cancer cell lines SKBR3 and MDA-MB-231 were obtained from ATCC and maintained in Dulbecco’s Modified Eagle’s Medium (Cellgro) supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere. The K562 leukemia cell line was also obtained from ATCC and maintained in RPMI (Cellgro) with 10% FBS (Gemini Bioproducts).

**Antibodies and other reagents**
The HER2-specific mAb trastuzumab (Herceptin) was manufactured by Genentech and purchased from the University of Washington Pharmacy. Fluorochrome-conjugated mAbs against CD3, CD56, CD25, CD69, and CD107a were from eBiosciences. Fluorochrome-conjugated mAbs against CD16 and IFN-γ was from Biolegend. Recombinant human IL-12 and anti-human IL-12 neutralizing antibody were purchased from Peprotech. PBS, penicillin–streptomycin, and L-glutamine were obtained from Invitrogen. PSK was purchased from Kureha Corporation. PSK was dissolved in PBS at a stock concentration of 10 mg/mL. Aliquots of 100 μL were stored at −80°C. The frozen aliquots were thawed immediately before use. Anti-rat neu mAb (clone 7.16.4) was produced from a hybridoma cells kindly provided by Dr. Mark Green) by the University of California, San Francisco, Hybridoma and Monoclonal Antibody Core.

**Measurement of human NK cell activation and production of IFN-γ by fluorescence-activated cell sorting**
PBMCs or purified NK cells were cultured in RPMI at the presence of PSK (100 μg/mL) or control PBS for 24 or 48 hours. Brefeldin-A (BFA, 0.5 μg/mL; Sigma–Aldrich), a secretion inhibitor, was included for the last 6 hours of the incubation. At the end of the activation period, the cells were first stained with fluorochrome-conjugated antibodies to surface markers (anti-CD3, anti-CD56, anti-CD25, and anti-CD69). After subsequent fixation and permeabilization, the cells were stained with anti-IFN-γ-PE. In some experiments with PBMCs, the cells were coincubated with anti-IL-12 to determine whether the production of IFN-γ by NK cells is dependent on this cytokine. In experiments with purified NK cells, a suboptimal dose of IL-12 (1 ng/mL) or PSK plus IL-12 was also included. Samples were acquired on FACS Canto II. List mode file was analyzed using FlowJo (Treestar).

**Measurement of CD107a degranulation in NK cells**
The degranulation of NK cells was measured by the expression of CD107a, lysosome-associated membrane protein-1 (LAMP-1). In brief, PBMCs treated with PSK (100 μg/mL, 24 hours) or medium alone were incubated with K562 target cells at effector/target (E:T) ratio of 2:1 for 6 hours. Anti-CD107a-PE antibody was added directly to the cocultures. After 1-hour incubation, BFA was included to the culture and incubated for another 5 hours. Cells were then stained with CD3 and CD56 and analyzed on FACS Canto II.

**Cytotoxicity assay**
A nonradioactive, fluorometric cytotoxicity assay with calcine–acetoxymethyl (calcine AM; ref. 32) was used to measure the lysis of K562 and trastuzumab-mediated ADCC. PBMCs were stimulated with PSK (10 μg/mL) or control PBS for 48 hours before coincubation with target cells. The K562 tumor target cells were loaded with calcine AM (10 μg/mL; Invitrogen) for 1 hour and washed. Labeled target cells were mixed with PBMCs at different E:T ratios (100:1, 50:1, 25:1, and 12.5:1) and plated on 96-well culture plates. After 4-hour incubation at 37°C, the release of calcine into culture medium was measured by a Victor 3 fluorescent plate reader (PerkinElmer). The percentages of specific lysis were calculated according to the formula: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100, where experimental release represents the mean fluorescence for target cells incubated in the presence of effector cells, spontaneous release represents the mean fluorescence for target cells incubated without effector cells, and maximal release represents the mean fluorescence for target cells incubated with Triton X-100. The measurement of trastuzumab-mediated ADCC was done similarly as described earlier for the K562 lysis assay except that the target breast cancer cells SKBR3 and MDA-MB-231 were coated with trastuzumab (5 μg/mL) or control IgG1 for 30 minutes before labeling with calcine AM. The percentages of specific lysis were calculated as earlier. Triplicate wells were set up for each E:T ratio. Results were expressed at mean ± SD of triplicate wells at each E:T ratio.

**Analysis of TLR2 mRNA expression using real-time reverse transcriptase PCR**
To analyze TLR2 expression on purified CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells, NK cells were first enriched with PBMCs by magnetic negative selection using Miltenyi NK cell isolation kit II. Then, the enriched NK cells were stained with anti-CD3, CD56, and CD16 to sort for CD56<sup>bright</sup>CD16<sup>−/low</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cells using BD FACS Aria sorter. The sorted populations had more than 99% purity. CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells, and CD11c<sup>+</sup> DC were also sorted from PBMCs as controls. RNA was isolated from fluorescence-activated cell-sorted cells or whole PBMCs using RNAqueous4PCR kit (Ambion). cDNAs were prepared using Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was carried out using TaqMan primer and probe from Applied Biosystems in 384-well plates using an ABI 7900 (Applied Biosystems). Cycling conditions were similar as previously described (30). The expression of TLR2 mRNA was normalized to hypoxanthine ribonucleotransferase using the ΔC<sub>T</sub> method (30).

**Measurement of cytokine and chemokine secretion from PSK-stimulated PBMCs or purified NK cells using Luminex analysis**
PBMCs or MACS-purified NK cells (200,000 per well) were plated in 96-well round-bottom culture plates and treated with serial dilutions of PSK (25–400 μg/mL) for 24
or 48 hours. The supernatants were harvested and levels of various cytokines and chemokines [IL-12p40, IL-12p70, TNF-α, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, IL-1α, and IL-1β] were measured using a Luminex kit purchased from Millipore following the manufacturer’s instruction.

**Treatment of tumor-bearing mice with PSK and anti-HER2/neu mAb (7.16.4)**

neu-T mice received subcutaneous implant of 1 million MMC cells, a cell line derived from a syngeneic spontaneous breast cancer in these mice (33). At 2 weeks after implantation (average tumor size = 50 mm³), mice were randomly assigned to receive treatment with 7.16.4 alone (15 mg/kg, tail vein injection, 3 times per week), PSK alone (100 mg/kg, oral gavage, 3 times per week), 7.16.4 plus PSK, or PSK plus a control irrelevant IgG of the same isotype. Mice in the 7.16.4-alone group received oral gavage of PBS of the same volume. To determine the role of immune cells in the antitumor effect of PSK and 7.16.4, some mice received depletion of CD4, CD8 T cells, or NK cells using the monoclonal antibodies (clone GK1.5 for CD4, clone 2.43 for CD8, and clone PK136 for NK) at 1 week before and during PSK and 7.16.4 treatment, using similar
protocol as previously described (34). Tumors were measured every other day with Vernier calipers and tumor volume was calculated as the product of length × width × height × 0.5236. In vivo data are presented as mean ± SD of each treatment group.

Statistical analysis
Statistical analysis was conducted using GraphPad Prism (GraphPad Software). Data were analyzed using the Student t test, ANOVA, or the Mann–Whitney U test when Guassian distribution cannot be assumed. A value of $P < 0.05$ was considered statistically significant.

Results

**PSK stimulates human CD56\textsuperscript{bright} NK cells to produce IFN-\textgamma**

PSK induces IFN-\textgamma secretion from PBMCs in a dose-dependent manner (Fig. 1A). Intracellular staining showed that IFN-\textgamma is mainly produced by CD56\textsuperscript{bright} NK cells, although there is a slight induction of IFN-\textgamma in CD56\textsuperscript{dim} NK cells (Fig. 1B). The percentages of cells that are positive for IFN-\textgamma are 1.0 ± 0.2% in control CD56\textsuperscript{dim} cells and 1.6 ± 0.6% in PSK-treated CD56\textsuperscript{dim} NK cells ($P = 0.3$), and are 1.8 ± 0.5% in control CD56\textsuperscript{bright} cells and 5.9 ± 1.1% in PSK-stimulated CD56\textsuperscript{bright} NK cells ($P = 0.009$, Fig. 1C). To determine whether both CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells are activated, we measured the expression of activation markers, CD25 and CD69, on NK cells after PSK treatment. As shown in Fig. 1D, PSK upregulates the expression of CD25 and CD69 in both CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells.

**PSK stimulates the cytolytic function of human NK cells and augments trastuzumab-mediated ADCC**

Expression of CD107a in the presence of K562, a MHC-I-devoid leukemia cell line, has been used as a marker of NK cell cytotoxicity (35). Fluorescence-activated cell-sorting (FACS) analysis showed that NK cells in PSK-treated PBMCs have higher expression of CD107a (8.0 ± 0.2%) than that present in the control group (4.2 ± 1.0%, $P = 0.02$ between PBS and PSK, Fig. 2A and B). The specific lysis of K562, as measured by calcein AM release assay, was also significantly enhanced in PSK-stimulated PBMCs. As shown in Fig. 2C, the percentage of specific lysis was approximately 2-fold higher in PSK-stimulated PBMCs than in unstimulated PBMCs at different E:T ratios ($P < 0.0001$). We next measured the potential of PSK to augment trastuzumab-mediated ADCC against 2 breast cancer cell lines, SKBR3 and MDA-MB-231. As shown in Fig. 3A, SKBR3 expresses high levels of HER2 and MDA-MB-231 expresses low levels of HER2. Pretreatment of PBMCs with PSK (10 \textmu g/mL, 72 hour) resulted in significantly enhanced ADCC against both cancer cell lines (Fig. 3B). Similar results were obtained using PBMCs from 5 different donors as summarized in Supplementary Table. Measurement of IFN-\textgamma in ADCC supernatant showed that pretreatment with PSK results in significantly enhanced IFN-\textgamma production in response to trastuzumab-coated cancer cells (Fig. 3C).

**PSK has both direct and IL-12–dependent indirect effects on NK cells**

There are controversial reports as to whether NK cells are activated by TLR2 agonists directly or indirectly via accessory cells (21–23). To determine whether the effect of PSK on NK cells is direct or indirect, we first used IL-12 blockade by including anti-IL-12 antibody during PSK treatment of PBMCs. As shown in Fig. 4A–C, IL-12 blockade did not
affect PSK-induced upregulation of CD25 on NK cells but significantly decreased PSK-induced IFN-γ production, as shown by decreased levels of IFN-γ among CD56bright NK cells (Fig. 4B) and decreased levels of IFN-γ in culture supernatant from PBMCs (Fig. 4C). It is noted that there is residual amount of IFN-γ production even when IL-12 is blocked, suggesting that IL-12 independent induction of IFN-γ by PSK may also exist (Fig. 4B and C). In contrast, PSK-induced TNF-α production by PBMCs was not decreased in the presence of anti-IL-12 antibody (Supplementary Fig. S1). This could be due to the fact that monocytes and DCs can produce large amounts of TNF-α in addition to NK cells. It also suggests that TNF-α production by NK cells could be regulated differently than IFN-γ production by NK cells and is independent of IL-12. To confirm that PSK-stimulated IFN-γ production by NK cells is dependent on IL-12, we treated MACS-purified NK cells with PSK, a suboptimal dose of IL-12 (1 ng/mL, as determined by dose titration experiment shown in Supplementary Fig. S2), or PSK plus IL-12. Results showed that PSK by itself significantly induced the expression of CD25 on purified NK cells (Fig. 4D), but the effect on IFN-γ production is moderate (not significant by intracellular staining but significant by ELISA measurement, Fig. 4E and F). In the presence of IL-12, PSK...
resulted in enhanced IFN-γ production, which is significantly higher than either PSK or IL-12 treatment alone (Fig. 4E and F). These data suggest that PSK-induced IFN-γ production but not CD25 upregulation is dependent on IL-12. Experiments using NK-92 cells yielded results consistent with those from purified NK cells showing that the production of IFN-γ in response to PSK is dependent on IL-12 (Supplementary Fig. S3). We also measured the expression of TLR2 on NK cells by FACS analysis and real-time PCR. FACS analysis showed that TLR2 is detectable in CD56bright NK cells, but the expression level is much lower than that on B cells or DCs (Supplementary Fig. S4A). Reverse transcriptase-PCR analysis using fluorescence-activated cell-sorted cells confirmed that CD56bright NK cells express more TLR2 mRNA than CD56dim NK cells and T cells, although there is inconsistency between mRNA and FACS data on the relative expression levels of TLR2 on CD56bright NK cells as compared with that on B cell or DCs (Supplementary Fig. S4B). TLR2 expression was not induced upon PSK treatment (data not shown).

PSK stimulates the production of IL-12 and other proinflammatory cytokines and chemokines from PBMCs

Culture supernatant from PSK-treated PBMC was collected for Luminex analysis of IL-12 and other cytokines...
and chemokines. As shown in Fig. 5A, PSK significantly induced the production of IL-12p40. PSK also significantly induced other proinflammatory cytokines and chemokines (TNF-α, IL-6, IL-8, MIP-1α, MIP-1β, IL-1α, and IL-1β) from PBMCs. The level of cytokine/chemokine induction is similar to our previous observation on the effect of PSK on mouse splenocytes (25). Interestingly, this panel of cytokines and chemokines (except IL-12) was also induced in the cytolytic assay when PSK-stimulated PBMCs were cocultivated with K562 (Supplementary Fig. S5), indicating that PSK stimulates the cytokine-secreting and cytolytic activity of NK cells simultaneously.

Luminex analysis was also conducted to measure the potential secretion of cytokine/chemokines by PSK-treated purified NK cells. As shown in Fig. 5B, PSK induced the production of TNF-α, MIP-1α, and MIP-1β by NK cells. Interestingly, the pattern of induction for these cytokine/chemokines seems to be different from that for IFN-γ (Fig. 4F) and seems to be independent of IL-12 (Fig. 5B). The time course of cytokine/chemokine induction in 1 of the 5 donors tested in Fig. 5B was shown in Fig. 5C.

Combination of PSK and anti-HER2/neu mAb (7.16.4) has enhanced antitumor effect in a mouse model of breast cancer

To evaluate the potential synergistic antitumor effect between PSK and anti-HER2 mAb therapy, we treated neu-T mice bearing HER2/neu+ breast tumors with 7.16.4, an anti-ErbB2 mAb, alone or in combination with oral PSK. The mechanisms of action of 7.16.4 remain...
mean (SD), n = 5 per group. The difference between no-depletion group (PSK + 7.16.4) and depletion group was calculated using 2-tailed Student t test. *P < 0.001, **P < 0.0001.

Discussion

Enhancing NK cell function is important to improve the clinical response to trastuzumab and other mAb therapy. In this study, we have shown that in vitro treatment with TLR2 agonist PSK can activate human NK cells and augment trastuzumab-mediated ADCC. In a mouse model of HER2+ breast cancer, orally administered PSK augments the antitumor effect of anti-HER2/neu mAb therapy. These findings indicate the potential of using a natural product as an adjuvant to improve the clinical response to trastuzumab.

NK cells are the major mediator of ADCC, and the function of NK cells has been shown to impact the treatment outcomes of trastuzumab, rituximab, and cetuximab (8). NK cell function is frequently impaired in patients with cancer and improving NK cell function via cytokines or TLR agonists has shown promise to augment ADCC (38–42). For example, IL-2 ex vivo treatment of NK cells was shown to restore the impairment of trastuzumab-mediated ADCC in the patients with gastric cancer (11). IL-12 has also been shown to augment trastuzumab-mediated ADCC and enhance the antitumor actions of trastuzumab via NK IFN-γ secretion (41, 42). Multiple TLR agonists, especially the agonists of TLR9 and TLR7/8, have shown potential to augment NK cell function (24, 40, 43, 44). For example, CpG ODN has been reported to increase IFN-γ production by NK cells and enhance trastuzumab-mediated lysis of breast cancer cells (17). TLR7 and 8 agonists have also been shown to induce IFN-γ production by NK cells (15, 18). Our study shows the potential of using a natural product with TLR2 agonist activity to augment NK cell function. The concentration of PSK used in our study (10–100 μg/mL) has been reported to be achievable in the blood of patients with cancer who received standard oral administration of the drug (3 g daily; ref. 45).

Whether TLR2-mediated activation of NK cells is direct or indirect remains controversial in the literature (21–23). Our results showed that TLR2 is expressed on NK cells, although the level is significantly lower than that on
agonists that are currently evaluated in clinical trials, such as CpG, imiquimod, or poly(I):poly(C), is known safety profile. PSK is a mushroom extract that has been widely used in Asian countries for its immune potentiating and antitumor effects. A meta-analysis of data from 3 randomized clinical trials in Japan in 1,094 patients with colorectal cancer showed that PSK significantly increased both overall survival and disease-free survival of patients with curatively resected colorectal cancer (29). The antitumor effect of PSK has also been shown in other types of cancer, including stomach cancer (27, 47) and lung cancer (48). To our knowledge, our study represents the first report on the potential of PSK to augment trastuzumab-mediated ADCC and the synergistic antitumor effect between PSK and HER2-targeted mAb therapy in a preclinical model. This provides rationale for future clinical trials testing the adjuvant effect of PSK when administered concurrently with trastuzumab. Although the study reported in this article used only PBMCs from normal healthy donors, a recently finished clinical study in patients with breast cancer conducted by Standish and colleagues (manuscript in preparation) also showed a trend toward increased NK cell cytolytic activity after oral administration of Turkey tail mushroom extract, the same species of mushroom from which PSK is extracted. Whether PSK can enhance the therapeutic effect of trastuzumab and a HER2-targeted vaccine in patients with breast cancer will be tested in our group. Because our previous studies have shown that the potential of PSK to stimulate NK cells is dependent on TLR2 (25), we speculate that the clinical response to combination therapy with PSK and trastuzumab may also be dependent on TLR2 and may be impacted by functional TLR2 gene polymorphism that have been reported (49). Hopefully, these questions can be addressed in clinical trials in the future. NK cell function impacts the clinical response not only to trastuzumab but also to other mAb therapies, such as rituximab for lymphoma and cetuximab for head and neck cancer (8). Thus, results from the current study could potentially be expanded to other types of cancer. Our study highlights the potential of combining complementary and alternative medicine therapy to mainstream cancer therapy for enhanced therapeutic effect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by NIH grants R01CA138547 (to H. Lu), R01AT004314 (to M.L. Disis), U19AT006028 (to L.J. Standish), and a gift to the Tumor Vaccine Group.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 29, 2011; revised August 31, 2011; accepted September 1, 2011; published OnlineFirst September 14, 2011.
References


TLR2 Agonist PSK Activates Human NK Cells and Enhances the Antitumor Effect of HER2-Targeted Monoclonal Antibody Therapy

Hailing Lu, Yi Yang, Ekram Gad, et al.

Clin Cancer Res 2011;17:6742-6753. Published OnlineFirst September 14, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-1142

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/09/14/1078-0432.CCR-11-1142.DC1

Cited articles
This article cites 48 articles, 27 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/17/21/6742.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/17/21/6742.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.