Peripheral Tolerance to Human Papillomavirus E7 Oncoprotein Occurs by Cross-Tolerization, Is Largely Th-2-independent, and Is Broken by Dendritic Cell Immunization

Tracy Doan, Karen A. Herd, Paul F. Lambert, Germain J. P. Fernando, Michael D. Street, and Robert W. Tindle

Sir Albert Sakzewski Virus Research Centre, Clinical Medical Virology Centre, University of Queensland, Royal Children’s Hospital, Herston, Queensland 4029, Australia [T. D., K. A. H., M. D. S., R. W. T.]; McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706 [P. F. L.]; and Centre for Immunology and Cancer Research University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia [G. J. P. F.]

Abstract

The E7 oncoprotein of human papillomavirus 16 functions as a tumor-specific antigen in transformed epithelial cells of the uterine cervix to which immunotherapeutic strategies aimed at CTL induction may be directed. We previously have shown in mice transgenic for the E7 gene driven off an epithelial specific (keratin-14) promoter, that expression of E7 protein in peripheral epithelium is sufficient to tolerize E7-directed CTL precursors (pCTL; Doan et al., J. Virol., 73: 6166–6170, 1999). Here we show that E7 is presented to T cells for tolerization by cells of bone marrow origin (“cross-tolerization”). We demonstrate that tolerization of E7-directed pCTL occurs within 2 weeks of exposure to E7 in epithelium. It is maintained in the near absence of CD4+ cells and in the absence of the thymus, and is independent of a coexisting E7-directed Th2-type antibody response. Tolerance was broken by immunization with E7 CTL epitope-pulsed dendritic cells. These findings have implications for immunotherapy of patients with human papillomavirus 16-associated cervical carcinoma, whose immune systems may have experienced long-term exposure to E7-expressing epithelial cells.

Introduction

Persistence of the HPV163-derived E7 oncoprotein in transformed cervical epithelium provides a tumor-specific antigen against which CTL-engendering therapeutic vaccine strategies for carcinoma of the cervix may be directed (1). Encouraging results from E7-transfected tumor challenge models in animals (e.g., Ref. 2) have inspired clinical trials of vaccines designed to elicit E7-directed CTL responses (3, 4).

Transgenic mice that express the HPV16 E7 oncogene driven off a K14 promoter only in basal or suprabasal epithelial cells display epithelial hyperplasia and dysplasia with a propensity to papillomavirus and squamous cell carcinoma (5). These mice provide a model for E7-mediated epithelial cell transformation in humans (6). Additionally, they allow investigation of the presentation of the E7 oncoprotein expressed uniquely in epithelium to the immune system, and the subsequent immunological outcome. We have reported that E7 expression in keratinocytes is sufficient to peripherally tolerize the E7-directed pCTL repertoire, measured as diminished CTL responses in vitro following specific immunization (7, 8). Furthermore, specific immunization of E7 transgenic mice failed to prevent or control E7-associated tumor development, as it did in non-E7 transgenic control mice.

On the basis of these observations, the possibility arises that in cervical carcinoma patients, chronic expression of E7 in transformed cervical epithelial cells during the life of the tumor serves to functionally tolerize E7-directed pCTLs. Indeed, patients with HPV16-associated cervical carcinoma make poor E7-directed CTL responses either to endogenously expressed E7 (9) or following E7 immunization (3). Thus, the challenge for immunotherapy for cervical cancer may become how to circumvent tolerance to generate an E7-directed CTL response effective in the control of E7-expressing cells.

In the present study, we use the K14-driven E7 transgenic mouse system to further investigate cognate pCTL tolerance induced by E7 expression in peripheral epithelium. We show that E7 is “cross-presented” to the immune system for specific pCTL tolerization by antigen-presenting cells of bone marrow origin. We also show that pCTL tolerization can be induced within 2 weeks of exposure to E7 in epithelial cells and that it can occur in the presence of a coexisting E7-directed Th2-type antibody response. We demonstrate that E7-directed pCTL peripheral tolerance is largely independent of CD4+ T cells and does not require the persistence of the thymus. We show that immunization with peptide-pulsed DCs is sufficient to overcome tolerance and generate E7-directed CTL responses in E7 transgenic mice equivalent to those seen in non-E7 transgenic controls. These results are relevant to the understanding of the immunological consequences of epithelial E7 expression and have implications for the formulation of therapeutic vaccine strategies for cervical dysplasia/neoplasia that target the E7 oncoprotein.

Materials and Methods

Mice. KA (E7+ A2.1Kb+) and FA (E7+ A2.1Kb+) F1 mice have been described (8). KC57 (E7+ A2.1Kb+) F1 mice were derived by crossing K14 HPV16E7 males (5) with C57Bl/6 females. Mice were housed under specific pathogen-free conditions, and genetic authenticity was tested at intervals. Mice were used at 6–15 weeks of age, but within a given experiment were littermates or closely age- and sex-matched.

Radiation Chimeras. Radiation chimeras were made as described (8). To derive mice that expressed E7 in peripheral epithelium and A*0201 only in cells of bone marrow origin, KC57 mice were immunologically ablated by thymectomy, lethal irradiation, and administration of Thy1-depleting antibody. These mice were reconstituted with bone marrow cells depleted of CD4+, CD8+, and Thy1+ cells from KA(E7+) mouse donors, which were made to mature through a FA(E7−) neonatal thymus implant under the kidney capsule. Hematopoietic reconstitution with cells containing the A2.1Kb transgene was confirmed 12–15 weeks later by PCR of DNA derived from spleen cells using A*0201 (A2.1)-specific primers HLA A Ex2 5'-TGGGATAGACGGAAGGAC-3' and HLA A Ex3 5'-CCAAGAGCGCAGTCCCTC-3', CD4+ cell and CD8+ cell reconstitution were confirmed as described (8). These mice were denoted KA->KC57(FA) mice. Control chimeras that expressed A*0201 in both epithelium and the bone marrow compartment were made by recon-
stituting immunologically ablated KA mice with KA-derived bone marrow matured through a FA thymus implant [denoted KA→KA(FA) mice]. KA-, FA-, and KC57- “sham” control mice underwent surgical manipulation without cell/organ transfers.

KA→KA(FA) mice have the phenotype E7+ive epithelium, A*0201+ive epithelium, A*0201+ive bone marrow-derived cells. KA→KC57(FA) mice have the phenotype E7+ive epithelium, A*0201+ive epithelium, A*0201+ive bone marrow-derived cells.

**Cells.** EL4.A2 cells are susceptible to specific CTL lysis through both H-2b and A*0201 pathways and were maintained as described (8).

**Peptides and Epitopes.** Peptides containing H-2k and HLA A*0201-restricted CTL epitopes of HPV16 E7 (EGPFLVTL)(11) and E5LLMTGVGLV(10), respectively, and HLA A*0201-restricted CTL epitope of influenza matrix protein (58 GILGFVFTL66; Ref. 12) were synthesized and used as described (8).

**Immunizations.** For induction of Th1 responses (CTL and IFN-γ secretion), groups of three mice were immunized once with 50 μg of an equimolar peptide mixture of CTL epitopes, 10 μg of Quil A adjuvant, and 2.5 μg of tetanus toxoid (CSL, Melbourne, Australia) as a source of T-helper epitopes in 100 μl of PBS, s.c. at the base of the tail. Ten days later, splenocytes were harvested and either restimulated in vitro for 6 days in the presence of 1 μg/ml of individual peptide prior to inclusion in CTL assays or set up in ELISPOT assays. For induction of Th2-type antibody responses, groups of three mice were immunized s.c. with 100 μg of HPV16 E7-GST fusion protein in algamulin adjuvant.

**CTL and ELISA Assays.** Chromium-release CTL assays and antibody ELISA assays were conducted as described previously (13).

**IFN-γ ELISPOT.** Cells secreting IFN-γ in an antigen-specific manner were detected using the ELISPOT assay (14). In brief, splenocytes from immunized mice were incubated overnight in the presence of cognate peptide, in nitrocellulose microtiter plate wells coated with anti-IFN-γ antibody (PharMingen). After removal of cells and extensive washing, the wells were reacted with biotin-conjugated anti-IFN-γ detection antibody (PharMingen) and developed with avidin-horseradish peroxidase (Sigma) and metal-enhanced diaminobenzidine substrate (Sigma). Controls included spleen cells from irrelevantly immunized mice, and incubation in the absence of cognate peptide.

**Adoptive Transfer of Spleen Cells.** Recipient mice (three per group) were whole-body lethally irradiated (1000 rad) and reconstituted the same day with 5 × 10^7 splenocytes from donor mice, injected into the surgically exposed spleen. Sham control mice were not irradiated and underwent surgical procedure without splenocyte transfer. Fourteen days later, the mice were immunized for specific CTL response induction with a peptide mixture containing E7/A*0201, E7/H-2b, and influenza matrix/A*0201 epitopes. Splenocytes from immunized mice were restimulated in vitro 10 days later with individual peptides, and CTL assays were conducted as described.

**CD4+/ive T-Cell Depletion.** Mice were depleted of CD4+ T cells as described (8). This regimen provided >95% CD4+ T cell depletion from at least day 15 (the earliest time point tested) until the end of the experiment as confirmed by fluorescence-activated cell-sorting analysis on peripheral blood mononuclear cells conducted at intervals (8).

**Immunization with DCs.** DCs were prepared from KA(E7+) mouse bone marrow (femur and tibia). Briefly, erythrocytes were removed by lysis with ammonium chloride, and then lymphocytes, granulocytes, and Ia- cells were depleted with monoclonal antibodies and a magnetic cell sorter (MACS), according to manufacturer’s instructions. The monoclonal antibodies were as follows: 2.43, anti-CD8; GK1.5, anti-CD4; RA3/3A1/6.1, anti-B220/CD45R; and B21-2, anti-Ia. DC progenitors were cultured to maturity with granulocyte macrophage colony-stimulating factor and interleukin-4 (10 ng/ml each) in DMEM containing 10% FBS for 8–10 days. DCs were washed and resuspended in DMEM containing 10% FBS (2.5 × 10^7/ml) containing 1 μg of peptide (an equimolar mix of E7/A*0201 and E7/H-2b epitopes) and incubated at 37°C for 60 min. (We determined that a sample of washed, E7 peptide-pulsed DCs did in fact bear E7 peptide on their surface by showing that they could be specifically lysed by splenocytes from E7 peptide-immunized mice.)

Experimental mice were then immunized i.v. with 5 × 10^6 peptide-pulsed or nonpulsed DCs. After 3 weeks, spleens were removed. Splenocytes were depleted of APCs as described (15). APC-depleted splenocytes (2 × 10^7/ml) were restimulated with E7-expressing EL4.E7 cells (2 × 10^5/ml; Ref. 8) in 6-well culture plates for 6 days, and then used in CTL assay. (This procedure was used in preference to peptide stimulation in the presence of APCs because the latter might recall FBS-directed CTls primed by powerful presentation of the injected DCs that had been grown in FBS-containing medium.)

**Results.**

E7-directed pCTls Are Cross-Tolerized by Presentation of E7 by Bone Marrow-derived Cells. To inquire whether E7 is sampled from epithelial cells by “professional” APCs for cross-presentation to E7-directed pCTls, we constucted bone marrow radiation chimeras from E7 transgenic mice with differential expression of A*0201. KA→KC57(FA) mice, which express A*0201 in bone marrow-derived cells but not in epithelium, and KA→KA(FA) mice, which express A*0201 in bone marrow-derived cells and epithelium (Fig. 1A), were immunized twice with an equimolar mix of peptides containing E7/A*0201, influenza/A*0201, and E7/H-2b peptide epitopes. Ten days later, E7-directed activated T-cells were detected in a IFN-γ release assay (ELISPOT; Fig. 1B) and quantified by calculation of frequency of IFN-γ-secreting cells. KA→KC57(FA) mice were adequately reconstituted for A*0201-restricted responses as evidenced by their capacity to mount influenza-specific responses comparable to FA(E7−) and KA(E7+) control mice (Fig. 1B, photographs 1, 4, and...
The expected down-regulated CTL responses to both A*0201- and H-2β-restricted E7 CTL epitopes (but not the “irrelevant” influenza matrix epitope; Refs. 7, 8) were observed in KA(E7+) sham control mice compared with E7-directed CTL responses in FA(E7−) sham mice (Fig. 2, IB and IC). The E7-directed CTL responses in CD4-depleted KA(E7+) mice were down-regulated and comparable to those seen in KA(E7+) sham mice (Fig. 2, IA and IB). This result indicates that the presence of a major peripheral CD4 T-cell population is not required for the persistence of E7-directed CTL tolerance observed in mice that express E7 in epithelium.

E7-directed CTL Tolerance Occurs in the Presence of a Coexistent Th2-Type Antibody Response to E7. To inquire whether E7-directed pCTL tolerance is influenced by the presence of a concomitant Th2-type E7 antibody response, groups of KA(E7+) mice were immunized for antibody with E7 in algamugin adjuvant before (Fig. 2, IIA), concurrent with (Fig. 2, IIB), or after (Fig. 2, IIC) immunization for CTLs with E7 minimal CTL epitope RAHYNNIVTF in Quil A adjuvant. [In general, immunization with protein/peptide antigen in algamugin induces an antibody response with a predominant Th2 (IgG1) component but no measurable CTLs].

Regardless of whether KA(E7+) mice had an E7 antibody response or not at the time of immunization for CTLs, they maintained a down-regulated E7-directed CTL response (Fig. 2, IIA) compared with FA(E7−) mice (Fig. 2, IIB). Similarly, KA(E7+) mice immunized for E7 antibody at the same time as immunization for E7 CTLs displayed down-regulated E7 CTL responses (Fig. 2, IIB), as did KA(E7+) mice immunized first for E7 CTL induction and later for E7 antibody (Fig. 2, IIC).

Together these data indicate that maintenance of E7-directed pCTL tolerance occurs in the presence of a coexisting Th2-type antibody response to E7.

Thymus Is Not Necessary for Maintenance of E7-directed CTL Tolerance. We have excluded the possibility that E7-directed pCTL tolerance is due to “central” (i.e., thymus-mediated) rather than “peripheral” (i.e., skin epithelium-mediated) tolerization (7). It has been suggested, however, that the distinction between central (thymic) T-cell tolerance and peripheral T-cell tolerance is not absolute and that intrathymic generation and release to the periphery of cognate immunoregulatory T cells during adult life may contribute to the maintenance of self-tolerance to peripheral antigens (16). We wished to ask whether the maintenance (in contrast to the induction) of peripheral tolerance to E7 antigen in KA(E7+) mice was dependent on the continued presence of the thymus.

Neonatally thymectomized KA(E7+) mice and controls were immunized at 8–10 weeks for CTL response induction with a mixture of peptides containing the E7/A*0201, H-2β, and influenza matrix/A*0201 CTL epitopes. These mice displayed down-regulation of E7-directed CTL responses similar to that in sham-thymectomized KA(E7+) controls and unlike the nontolerized E7-directed responses displayed by non-E7 transgenic thymectomized or sham-thymectomized FA(E7−) mice (Fig. 2II). Responses to the irrelevant influenza epitope were comparable in all groups of mice, indicating that neonatal thymectomy did not adversely affect genetically the capacity to mount CTL responses. These data indicate that the presence of

### Table 1. CTL lysis by splenocytes from irradiated recipient mice after adoptive transfer of donor mouse splenocytes

<table>
<thead>
<tr>
<th>Epitope/Restriction</th>
<th>CTL lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FA(d)−irradFA(r)</td>
</tr>
<tr>
<td>E7/H-2β</td>
<td>7.1 ± 1.1</td>
</tr>
<tr>
<td>E7/A*0201</td>
<td>10.5 ± 1.1</td>
</tr>
<tr>
<td>Influenza matrix/A*0201</td>
<td>50.7 ± 0.2</td>
</tr>
</tbody>
</table>

a: T cell ratio of 10:1 after background (target cells without sensitizing peptide) subtraction, ± SD.
b: d, donor; r, recipient.
a thymus in adult life is not necessary for the maintenance of E7-directed pCTL tolerance in mice expressing E7 in peripheral epithelium.

**Immunization with E7 Peptide-pulsed DCs Overcomes E7-directed CTL Tolerance.** In view of the potent antigen-presenting capacities of DCs, we asked whether immunization of KA(E7+) mice with DCs pulsed with E7 CTL peptides would overcome the down-regulated E7-directed CTL responses observed when KA(E7+) mice were immunized with peptide plus adjuvant. Restimulated splenocytes from KA(E7+) mice immunized with E7 peptide-pulsed DCs, but not DCs alone, specifically killed EL4.E7 target cells with comparable efficiency to splenocytes from identically immunized FA(E7) mice (Fig. 3, A and B) and also to splenocytes from FA(E7) mice immunized with E7 peptides in Quil A adjuvant (Fig. 3, B and C).

These data indicate that E7 CTL responses similar to those seen in immunized non-E7 transgenic (i.e., not tolerant to E7) mice can be induced in mice expressing E7 in skin (i.e., tolerant to E7), by immunization with DCs presenting E7 CTL epitope.
Discussion

Transgenic mouse models in which E7 is driven off skin-specific (keratin) promoters have been used to mimic E7 expression in human cervical epithelium and to investigate immunological outcomes of E7 expression (17, 18). We have reported that naïve E7 pCTLs are down-regulated (i.e., tolerized) in mice in which E7 is expressed solely in keratinocytes of peripheral squamous epithelium, as measured by the greatly reduced capacity to make E7-directed CTL responses after specific immunization compared with non-E7 transgenic but otherwise syngeneic mice (7, 8). In the present study, we use the E7 transgenic mouse model to immunologically characterize E7-directed pCTL tolerance.

We report that E7 pCTLs recognizing an A*0201-restricted CTL epitope were down-regulated in mice in which A*0201 was expressed solely on cells of bone marrow origin and not on E7-expressing keratinocytes. Because bone marrow-derived cells do not express E7 in this K14 promoter-driven system (reverse transcription-PCR; Ref. 8) and therefore cannot present putative endogenous E7 to pCTLs (7), the data in the present study indicate that E7 in keratinocytes was accessed by bone marrow-derived APCs for processing and presentation to bone marrow-derived E7-directed pCTLs through the A*0201 pathway. These data are in agreement with a prevailing view that peripherally expressed antigens are “sampled” from the periphery by APCs that present processed antigen to specific T cells in the lymph node for activation (i.e., cross-priming) or tolerization (i.e., cross-tolerance; Ref. 19). Although the determinants of whether pCTLs are tolerized, (as reported here), remain ignorant (naïve), or are activated in response to neo-antigen are not fully understood, the amount of antigen and its location clearly play significant roles. We previously have argued that the E7-directed peripheral CTL tolerance seen in KA(E7+) mice but not in other lines of transgenic mice expressing E7 in squamous epithelium (e.g., Ref. 18) is likely due to higher levels of E7 expression in KA(E7+) mice (7). Our data do not preclude a role for direct presentation of E7 to the immune system by keratinocytes in addition to the presentation by cells of bone marrow derivation reported here. Processed self-peptides presented at the keratinocyte surface in the context of MHC class I but in the absence of appropriate second signal molecules (B7.1/CD28, CTLA) may anergize cognate T cells (20).

Our results indicate that tolerization of naïve E7-directed pCTLs occurs within 14 days of exposure to the E7-expressing epithelial environment, in agreement with findings for epitHELially expressed antigens in other systems (21). We report that E7 pCTL tolerance persisted after a >95% reduction in CD4+ cells. Our data thus suggest that maintenance of tolerance is independent of the availability of CD4+ cognate help. The possibility remains that CD4+ T-cell depletion removed tolerance but did not return the E7-directed CTL response to normal levels if stimulation (as opposed to tolerization) of E7-specific CTLs required CD4+ T-cell help. This is unlikely, however, because it has been shown that peptide-generated E7-specific CTLs are CD4+ T-cell help independent (i.e., CD4+ cells neither enhanced nor suppressed anti-E7 CTL activity in nontolerized mice; Ref. 22). Kurts et al. (23) have shown that provision of OVA-specific CD4+ helper T-cells impaired peripheral CTL tolerance by preventing OVA-specific CD8+ CTL deletion in mice expressing an OVA transgene as a cell membrane-associated protein in pancreatic islets. This suggests a regulatory role for CD4+ cells. The location (pancreas versus epithelium), antigen availability (membrane-bound versus nuclear-cytoplasmic), and numbers of cognate T cells (high versus low) are likely to play roles in the apparent difference between the OVA transgenic system and ours with regard to the effect of CD4+ cells on the maintenance of CTL tolerance.

We asked whether E7-directed pCTL tolerance was influenced by the presence of a concomitant E7-directed antibody response. We show that induction of a Th2-type antibody response either before, concurrent with, or after immunization for CTLs did not modify the down-regulated state of E7 pCTLs in mice expressing E7 constitutively in epithelium.

We have shown in our previous studies (7) that the induction of E7-directed CTL tolerance observed in KA mice is not due to central tolerization by the concomitant expression of E7 in thymic cortical epithelium that occurs in these mice. (Expression of antigen in thymic cortical epithelium transmits a positive, rather than a negative, T-cell selection signal; Ref. 24). In the present study, we demonstrate that E7-directed pCTL tolerance persisted in adult KA(E7+) mice thymectomized at birth, indicating that the continued presence of a thymus in adult life is not necessary for the maintenance of E7-directed CTL tolerance. These data suggest that intrathymic generation and release to the periphery of cognate immunoregulatory T-cells as reported in other systems (e.g., Ref. 16) does not contribute substantially to the maintenance of peripheral tolerance in mice expressing E7 in the skin. It also suggests that a putative accessing of E7 in thymic cortical epithelium by E7-directed pCTLs or APCs in adult life does not contribute to the maintenance of peripheral tolerance.

By immunizing E7-tolerant (E7 transgenic) mice with DCs pulsed with E7 peptide, we generated CTL responses approaching in magnitude those seen in nontolerant (non-E7 transgenic) mice. Reversal of tolerance to a tumor antigen by DC immunization has been observed in MUC-1 carcinoma transgenic mice (25). It is not clear from our studies why presentation of epitopes derived from endogenous E7 in skin induces CTL tolerance, whereas presentation of the same epitopes by pulsed DCs used for immunization induces CTL activa-
tion. Toes et al. (26) have reported that administration of a given peptide in adjuvant to the immune system may be tolerogenic, whereas administration of the same peptide pulsed onto DCs is stimulatory; together with our result, this emphasizes the importance of the mode of epitope delivery in determining the balance of T-cell tolerization and activation. Reduction in growth of a challenge dose of E7-expressing tumor, but not control tumor, obtained by prior immunization of mice with DCs pulsed with the E7 CTL epitope has been reported (27).

We argue from our animal models that the chronic expression of E7 in transformed epithelial cells of women with progressive HPV-associated cervical disease may serve to functionally tolerate E7-directed pCTLs in the periphery. The challenge for immunotherapy for cervical cancer becomes how to vaccinate to circumvent E7-directed CTL tolerance. Vaccines comprising DCs presenting cognate antigen for cervical cancer have been efficacious in eliciting CTL responses in human clinical trials. Induction of HPV16 E7-specific, A*0201-restricted CTLs by E7-pulsed autologous DCs in patients with HPV-associated cervical cancer recently has been demonstrated (28), suggesting the validity of the DC approach to the derivation of a therapeutic vaccine for cervical cancer.

Other vaccine strategies known to enhance E7-directed CTLs, e.g., intracutaneous injection of E7 into the MHC class I1 compartment using E7-DNA vaccine encoding the lysosomal-associated membrane protein sorting signal, may be similarly effective in surmounting E7-directed tolerance (29, 30). DCs expressing Sig/E7/lysosomal-associated membrane protein induce E7-directed CTLs in immunized mice (30).

Acknowledgments

Dr. Simone Zehnter provided help with the preparation of DCs. We thank members of the Center for Immunology and Cancer Research for helpful discussion, and Donna West and her staff for animal husbandry.

References

Peripheral Tolerance to Human Papillomavirus E7 Oncoprotein Occurs by Cross-Tolerization, Is Largely Th-2-independent, and Is Broken by Dendritic Cell Immunization

Tracy Doan, Karen A. Herd, Paul F. Lambert, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/11/2810

Cited articles This article cites 29 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/11/2810.full.html#ref-list-1

Citing articles This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/60/11/2810.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.