Chemotherapy Enhances Cross-Presentation of Nuclear Tumor Antigens

Chidozie C. Anyaegbu1,2, Richard A. Lake1,2, Kathy Heel3, Bruce W. Robinson1,2, Scott A. Fisher1,2

1 National Centre for Asbestos Related Diseases (NCARD), QEII Medical Centre, Nedlands, Western Australia, 2 School of Medicine and Pharmacology, University of Western Australia, QEII Medical Centre, Nedlands, Western Australia, 3 School of Pathology and Laboratory Medicine, University of Western Australia, QEII Medical Centre, Nedlands, Western Australia

Abstract

Cross-presentation of tumor antigen is essential for efficient priming of naive CD8+ T lymphocytes and induction of effective anti-tumor immunity. We hypothesized that the subcellular location of a tumor antigen could affect the efficiency of cross-presentation, and hence the outcome of anti-tumor responses to that antigen. We compared cross-presentation of a nominal antigen expressed in the nuclear, secretory, or cytoplasmic compartments of B16 melanoma tumors. All tumors expressed similar levels of the antigen. The antigen was cross-presented from all compartments but when the concentration was low, nuclear antigen was less efficiently cross-presented than antigen from other cellular locations. The efficiency of cross-presentation of the nuclear antigen was improved following chemotherapy-induced tumor cell apoptosis and this correlated with an increase in the proportion of effector CTL. These data demonstrate that chemotherapy improves nuclear tumor antigen cross-presentation and could be important for anti-cancer immunotherapies that target nuclear antigens.

Introduction

Recognition of tumor antigen by specific T cells is a necessary prerequisite for the induction of effective anti-tumor immune responses [1]. This is initiated by cross-presentation, a phenomenon where professional antigen presenting cells (APC) such as dendritic cells (DC) capture, process, and present exogenous antigens through the class I pathway [2]. Cross-presentation of cancer antigens is an important step in tumor-specific immune responses as it allows for the presentation of tumor-specific antigens to T cells, and the production of tumor-specific CTL. The efficiency of cross-presentation has been shown to be influenced by the level of APC activation and maturation status [4], as well as the properties of the tumor cell and model antigen. Factors such as antigen dose [5], type (modified self-antigen or mutated tumor neoantigen) [6], location of the antigen in the tumor cell [7,8], and subcellular location within tumor cells [10,11] can affect cross-presentation efficiency. With respect to location, while some studies have shown that the cytoplasm is the main source of antigen, they have compared the secretory, cell-membrane, and cytoplasmic compartments, but did not assess the contribution of nuclear antigen. Many of the tumor-associated antigen (TAA) targets are located in the nucleus, such as survivin, MAGE-A10, and WT1, and are predominantly expressed in the nucleus [12–14]. Moreover, several clinical studies have correlated the nuclear localization of different TAs with poor clinical outcome in a variety of cancer types [15–20]. Therefore, it is important to understand the relative availability of cross-presentation of antigens in the nuclear compartment compared to other cellular compartments. We hypothesized that nuclear localization of an antigen would limit cross-presentation and hence cross priming of naïve CD8+ T cells because APCs would not be able to access nuclear antigen as easily as antigen from other cellular locations. Furthermore, since apoptosis is known to cause nuclear degradation [21,22] and extracellular release of nuclear contents [23], we reasoned that apoptosis-inducing chemotherapy would improve the efficiency of cross-presentation of nuclear antigens.

To address these questions, we developed a murine tumor model of cross presentation in which MHC mismatch between the tumor cell and model antigen precludes direct antigen presentation. We engineered B16 tumors to differentially express a model tumor antigen (influenza HA H2-Kb) to improve cross-presentation. We then investigated the capacity of cross-presentation of tumor-specific epitopes expressed in frame with EGFP in the secretory, cytoplasmic, or nuclear compartments, and compared their potential to induce proliferation of H-2Kb−/− restricted CL4-specific CD8+ T cells in vivo as a measure of cross presentation. We then investigated the capacity of the tumor antigen-presenting tumor cell and its ability to induce apoptosis, and Finally, we showed that nuclear antigen is not cross-presented as efficiently as its cytoplasmic and secreted counterparts, and that gemcitabine-induced tumor cell apoptosis could reverse this, unlocking nuclear antigen cross-presentation to equivalent levels in vivo.
vitro, which correlated with an increase in the proportion of effector CL4-specific CTL.

Materials and Methods

Mice

Female, C57BL/6 (H-2b) x BALB/c (H-2b) F1 mice (H-2b\(\text{\textit{nu}}\)), and Clone 4 (CLA) TCR transgenic mice, whose CD8 T cells express TCRs that specifically recognize the H-2b restricted ‘CLA’ epitope (IYSTVASSL; residues 518–526) of influenza haemagglutinin (HA) protein [25], were obtained from the Animal Resource Centre (Canning Vale, Perth, Australia). All mice were used at seven to ten weeks of age, and maintained under standard conditions (M-block Animal Facility, Queen Elizabeth II Medical Centre, The University of Western Australia; UWA, Perth, Australia). Animal experiments were approved by The University of Western Australia Animal Ethics Committee (RA/3/100/1016), and conducted in accordance with the Australian National Health and Medical Research Council’s Code of Practice for the Use of Animals for Scientific Purposes.

Tumor cell line and subcutaneous transplantation

B16.F10 (H-2b) melanoma tumor cell line (ATCC, Manassas, VA; CRL-6475) was used in all experiments. Tumor cells were maintained in RPMI-1640 (Invitrogen Life Technologies, Mulgrave, Australia) supplemented with 10% Fetal Calf Serum (FCS; Invitrogen Life Technologies), 50 µg/mL gentamicin (David Bull Labs, Kewdale, Australia), 60 µg/mL penicillin (CSL, Melbourne, Australia) 20 mM HEPEs (Sigma Aldrich, Sydney, Australia) and 0.05 mM 2-mercaptoethanol (% pH 7.2; Merck, Kilsyth, Australia). 5×10^6 viable B16 cells in 100 µL PBS were inoculated subcutaneously (s.c.) into the lower right flank of recipient F1 mice. Tumor size was measured across two dimensions at least three times weekly with microcalipers. Mice were euthanized via Penthrane inhalation (Abbot Laboratories, USA) and death confirmed by cervical dislocation when tumors reached 100 mm^3 as per animal ethics approval.

Generation of gene constructs targeting model antigen to distinct subcellular compartments

The secretory gene construct (sec-EGFP-CL4) was generated by attaching the H-2K\(\text{\textit{b}}\)-derived endoplasmic reticulum (ER) signal peptide (MVPCTLLLLLAAALAPTQTRAV) to the amino-terminus of enhanced Green Fluorescent Protein (EGFP). A truncated version of the ER signal peptide (underlined sequence) was used to generate the cytoplasmic construct (cyto-EGFP-CL4), while the Simian Virus 40 T-antigen (SV40 T-Ag) nuclear localisation signal (MPKKKRRKV), was used to generate the nuclear construct (nuc-EGFP-CL4). The following amino acid sequence QVG MVPCTLLLLLAAALAPTQTRAV was fused to the carboxy-terminus of EGFP in all three constructs. This sequence encodes the CL4 epitope (underlined; the MHC Class I restricted region of Influenza haemagglutinin protein) preceded, and followed by, short spacer sequences to ensure that antigen presentation required cytosolic processing. All gene constructs were generated using PCR primers. Resulting PCR products were cloned into pCR4Blunt-TOPO (Zero Blunt PCR Cloning Kit; Invitrogen Life Technologies), and subcloned into pLent6/V5-GW.blasticidin lentiviral vector, using BamHI/XhoI cohesive ligation, for introduction into B16 cells. Sequence integrity of all clones was confirmed by DNA sequencing.

Stable lentiviral transduction of B16 tumor cells with gene constructs

To generate lentivirus for integrating gene constructs into B16 target cells, 293FT lentiviral producer cells (Invitrogen Life Technologies) were co-transfected, using Lipofectamine™ 2000 transfection reagent (Invitrogen Life Technologies), with pHIV-PV-SVG lentiviral packaging plasmid and pLent6/V5-GW.blasticidin expression vector containing sec-EGFP-CL4, cyto-EGFP-CL4, or nuc-EGFP-CL4 gene constructs. Transfected 293FT cells were cultured in DMEM (Invitrogen Life Technologies) containing FCS (10%), L-glutamine (2 mM), non-essential amino acids (NEAA, 0.1 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Lentivirus-containing supernatant was harvested after a 72-hour incubation, and added neat to a fresh culture of B16 cells. Stably transduced B16 cells were generated using blasticidin antibiotic selection (10 µg/mL in RPMI-1640, Invitrogen Life Technologies, for 10–12 days). Stable EGFP positive cells were sorted to a purity of >98% using BD Influx™ cell sorter (Becton Dickinson, San Jose, USA), and clonal populations expressing EGFP-CL4 protein in secretory (B16.Sec), cytoplasmic (B16.Cyto), or nuclear (B16.Nuc) subcellular compartments isolated by limiting dilution assay.

Preparation of whole cell lysates and secretion fractions for EGFP ELISA assay

Whole cell lysates were extracted from each cell line by resuspending 5×10^6 tumor cells in 500 µL of radio immunoprecipitation assay (RIPA) lysis buffer (150 mM sodium chloride; 1% Triton X-100; 0.1% sodium dodecyl sulphate; 50 mM Tris, pH 8.0; 1/100 dilution Protease Inhibitor Cocktail (P8340, Sigma Aldrich) and 0.5% sodium deoxycholate) and incubating for 30 minutes at 4°C. Cell debris was removed by centrifugation (5000 g for 10 mins) and supernatants containing whole cell extracts gently transferred to a fresh tube. To generate secretion fractions, each cell line was seeded at 1×10^6 cells/10 cm cell-culture dish. Following a 24-hour incubation, supernatant containing secretory fraction was harvested and concentrated using the Amicon Ultra-15 centrifugal 10 kDa filter device (Merck Millipore, Massachussets, USA). Protein concentrations were determined by Bradford assay. EGFP-CL4 levels in cell lysates and fractions were then quantified using GFP ELISA assay kit (Cell Biolabs Inc. San Diego, USA), as per manufacturer’s instructions.

Confocal microscopy

EGFP expressing B16 cells were cultured on glass coverslips and fixed with 4% paraformaldehyde. Cell nuclei were counterstained with Hoechst 33342 and confocal microscopy performed using a Nikon A1Si spectral detector confocal system (Nikon, Melville, USA). Imaging was performed with a 60× oil immersion objective lens. For each cell line, at least 10 single plane images of representative cells (optical slice < 0.125 µm) were recorded. Images were analysed using the NIS-C Elements software (Nikon).

Acquisition and analysis of ImageStream imaging flow cytometry data

The ImageStreamX imaging flow cytometer (Amnis, Seattle, USA) was used to confirm the differential expression of EGFP-CL4 antigen in respective B16 tumor cell lines. Briefly, each cell line was counter-stained with Hoechst 33342 nuclear marker and 10,000 cells acquired for analysis. Cell populations were hierarchically gated for single cells that expressed both EGFP and Hoechst 33342 as previously described [26]. Following acquisition,
the relationship between EGFP and nuclear images was analysed using the “Similarity” feature of the IDEAS software (Amnis) [26].

The similarity score provides a measure of the degree of nuclear localisation of EGFP by comparing a log-transformed Pearson’s correlation coefficient between the pixel values of two image pairs. Cells with a high similarity score exhibit strong correlation between images (i.e. EGFP is predominantly nuclear located), while cells with a low similarity score exhibit no correlation between images (i.e. EGFP is predominantly located within the cytoplasm).

**MTT assay**

The metabolic activity (sensitivity) of B16 tumors after exposure to gemcitabine (Gem) was determined using the MTT [3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (Sigma-Aldrich), according to manufacturer’s protocol. Briefly, 24 h after seeding tumor cells to 96-well flat-bottomed
Chemotherapy Enhances Cross-Presentation of Nuclear Tumor Antigen

To study the effect of cellular localization on tumor-specific CD8\(^+\) T cell responses, we modified B16 tumor cells to express the MHC class I K\(^d\) restricted immunodominant epitope of the influenza virus HA protein, CL4, fused with EGFP, as a quantifiable model tumor antigen in nuclear (B16.Nuc), secretory (B16.Sec), or cytoplasmic (B16.Cyto) compartments (Fig. 1A). The dense nuclear expression of EGFP-CL4 in B16.Nuc, but diffuse dense nuclear expression of EGFP in B16.Cyto and B16.Sec tumors, demonstrated that antigen was successfully targeted to the intended cellular locations (Fig. 1B). These data were quantitated by imaging flow cytometry, a technique that combines the statistical power of flow cytometry with the spatial resolution of confocal imaging flow cytometry, a technique that combines the statistical power of flow cytometry with the spatial resolution of confocal microscopy. Based on the IDEAS algorithm [26], where similarity scores above 2 and below 1 indicate a high, or low degree of EGFP/nuclear co-localisation respectively, B16.Nuc had the highest EGFP/nuclear co-localisation score of 2.317 (Fig. 1C). Due to the low error associated with analysing large numbers of cells (>10,000 per sample), we detected a statistically significant EGFP/nuclear co-localization.
in B16.Sec relative to B16.Cyto (Fig. 1C). As expected, B16.Sec secreted the highest concentration of EGFP-CL4 protein into culture supernatant (Fig. 1D).

Importantly, all three tumor cell lines expressed a similar steady-state whole cell level of EGFP-CL4 protein at approximately 6000 pg per 10⁶ cells, or 3.33 × 10⁻¹² nmol of EGFP-CL4 per cell (Fig. 1D). These results were confirmed by the similar live-cell EGFP fluorescence intensities of respective tumors (Fig. 1E). This also confirmed stability of EGFP-CL4 fusion protein as the fluorescence intensities remained unchanged in long-term culture, without additional antibiotic selection, for over 50 passages (Fig. 1E). Although B16 parental tumor showed a slightly faster in vivo outgrowth, subsequent comparative studies confirmed that all three antigen-bearing tumors displayed a similar in vivo growth pattern (Fig. 1F). Taken together, these data demonstrate that B16.Nuc, B16.Cyto, and B16.Sec tumors targeted antigen to their respective cellular locations, and expressed similar levels of EGFP-CL4 fusion protein as well as in vivo growth pattern.

Nuclear localized tumor antigen is not cross-presented as efficiently as cytoplasmic and secretory antigen

Having generated the B16 tumors with differentially localized antigen, we proceeded to assess the impact of cellular antigen location on cross-presentation. Because our B16 tumors (H-2b) lacked the relevant restriction element (H-2d) for recognition by the CL4 TCR, we could rule out direct presentation of the antigen by the tumors [33] or by MHC-peptide exchange; a phenomenon described as “cross dressing” [20]. Thus in tumor-bearing C57BL/6 x BALB/C F1 mice (H-2 bxd), the proliferation of adoptively transferred H-2d restricted CL4-TCR transgenic CD8 T cells could only be induced when F1 host APCs cross-presented CL4 antigen. In order to better define the threshold level of antigen that we previously reported to be required for cross-presentation [30,31] and determine whether this threshold level differed depending on the cellular location of antigen, we inoculated F1 mice with B16 parental cells that had been mixed with either B16.Nuc, B16.Cyto, or B16.Sec tumor cells in different proportions, diluting the concentration of antigen to the doses shown in Fig. 2A. These experiments demonstrated that nuclear antigen required a twofold higher threshold concentration for cross-presentation relative to the cytoplasmic and secretory antigen; 194 nmol versus 97 nmol respectively (Fig. 2B).

Gemcitabine induces apoptosis-mediated cell death of B16 tumor cells

Since our data suggested that nuclear antigens are relatively inefficiently cross presented compared to other subcellular compartments, we hypothesised that this situation could be altered...
by gemcitabine, an immunogenic chemotherapy agent that we previously reported to increase the availability of antigen for cross-presentation by inducing apoptotic tumor cell death [24]. Importantly, B16 parental, B16.Nuc, B16.Cyto, and B16.Sec tumors were equally sensitive to gemcitabine with an IC50 between 3 and 4 ng/ml as determined by MTT assay (Fig. 3A). This sensitivity was also observed in vivo, as a single i.p. injection of gemcitabine (240 µg/g) significantly retarded the outgrowth of all B16 tumors compared to saline treated mice (Fig. 3B). Thus, gemcitabine induces similar levels of apoptosis-mediated cell death on all of the B16 tumors in this model.

Gemcitabine improves cross-presentation efficiency of nuclear antigen in a dose dependent manner

After establishing the comparable sensitivity of B16 tumors to gemcitabine-mediated apoptosis, we proceeded to test whether gemcitabine could augment the cross-presentation efficiency of nuclear localized antigen and enhance cross presentation to a level similar to that seen with cytoplasmic and secreted tumor antigens. As all three tumors were similar in (i) their antigen content (Fig. 1E), (ii) were from the same parental line, (iii) grew at similar rates in vivo and (iv) exhibited comparable degrees of apoptosis-induced cell death, we can reasonably assume a similar amount of tumor debris following chemotherapy. Therefore, to determine if there was any change in the cross presentation of nuclear antigens following gemcitabine induced immunogenic cell death, F1 mice were inoculated with B16 parental cells mixed with either no antigen, or 19.4, 97, or 194 nmol of CL4 antigen-containing B16.Nuc, B16.Cyto, or B16.Sec tumor cells. Tumor bearing mice were treated with gemcitabine or saline and the proliferation of adoptively transferred CL4-specific CD8\(^+\) T cells in tumor draining LNs of mice assessed. Compared to saline, gemcitabine doubled the cross-presentation efficiency of nuclear antigen to levels comparable to the cytoplasmic and secretory antigen, both of which demonstrated only marginal increases relative to saline treated controls, thus abrogating the difference in cross-presentation efficiency between antigens in different cellular locations (Fig. 4A). Consequently, the threshold amount of antigen required for nuclear localized antigen to be cross-presented was lowered two-fold from 194 (with saline) to 97 nmol (Fig. 4A). Interestingly, while gemcitabine doubled the cross-presentation efficiency of nuclear antigen, the proportion of CL4-specific CD8 T cells expressing IFN\(\gamma\) remained unchanged (Fig. 4B).

Discussion

Cross presentation is essential for the generation of effective anti-tumor immunity [3]. Tumor-specific CTL, with the capacity to destroy tumor cells, are only generated following effective cross-priming of naïve CD8\(^+\) T cells by professional APC. To evaluate the efficiency of this process for different tumor compartments, we developed a murine model of tumor antigen cross-presentation, in which tumor cells differentially expressed a model antigen in either the secretory, cytoplasmic or nuclear compartments and compared their ability to induce proliferation of antigen-specific CD8\(^+\) T cells as a read out of cross-presentation. We observed a significant difference in EGFP/nuclear localisation between secretory and cytoplasmic located tumor antigen. This is consistent with expression in the secretory pathway of secreted tumor antigen as secreted proteins show more nuclear co-localization than cytoplasmic proteins due to their transport through the endoplasmic reticulum, which borders the nuclear envelope [32,33]. Despite this, all tumors expressed similar levels of tumor antigen in their respective cellular compartment and grew at comparable rates in vivo, indicating we had developed a robust model suitable for comparing the efficacy of nuclear antigen cross-presentation versus other compartments.
Nuclear antigen is not cross-presented as efficiently as cytoplasmic or secreted antigen, but is restored following treatment with gemcitabine

Throughout this study, all tumor antigens were efficiently cross-presented when antigen concentration was sufficiently high. However, when lower tumor antigen concentrations were studied, we observed that nuclear tumor antigen was not as efficiently cross-presented, requiring at least a two-fold higher concentration relative to secreted or cytoplasmic tumor antigen to achieve similar level of CD8+ T cell proliferation. We concluded that the reduced efficiency of cross-presentation was likely due to the distinct nuclear localization of tumor antigen, since all other factors, including cellular tumor antigen levels and the in vivo growth rates of each tumor type, were similar. However, treatment with the apoptosis inducing nucleoside analogue gemcitabine was able to restore nuclear antigen cross-presentation to levels equivalent to secreted or cytoplasmic compartments. The antigen-dose dependent nature of this improvement suggested that treatment with gemcitabine boosted the amount of antigen available for cross-presentation and thus for cross priming. While the F1 cross-presentation model precludes any assessment of tumor regression due to the inability of direct antigen presentation, the increase in the proportion of CL4 CD8+ T cells associated with gemcitabine treatment and their ability to express IFNγ correlates to our earlier studies in which these CL4 cells drive an anti-tumour response against CL4 bearing tumours [25,31]. It is therefore interesting to consider whether the reduced efficacy of nuclear tumor antigen cross-presentation before gemcitabine treatment might play a crucial role in tumor development, given that tumor antigen detection is critical for effective immune surveillance that protects the host during cancer development [34]. One possible interpretation is that the relatively reduced level of nuclear antigen cross-presentation might correlate with less effective immunosurveillance, limiting recognition of nuclear antigen bearing tumors by the host immune system. This hypothesis fits with the observation that cancer patients with tumor antigen localized in the nucleus have a more aggressive disease progression than patients with the same antigen expressed in the cytoplasm, or other tumor cell compartments [15–19]. Alternatively, it might be that after gemcitabine treatment, newly synthesized antigen may not localize correctly to the nucleus, resulting in increased cytosolic antigen that led to the observed increase in cross-presentation. Importantly, the enhanced expression of nuclear tumor antigen following gemcitabine treatment has been observed in human cancers [14,35] and while the exact mechanism involved remains uncertain, it has been suggested that gemcitabine induced apoptosis promotes nuclear fragmentation [21] and such fragments may lead to the exposure of nuclear contents [23] and an increased availability of nuclear-bound tumor antigen for cross-presentation. Taken together, these data suggest that the relatively reduced cross-presentation of nuclear localized antigen can be improved by a gemcitabine-mediated, tumor apoptosis-induced, nuclear degradation and this increases the availability of these nuclear antigen for cross-presentation. Future studies will assess the key questions of antigen localization following gemcitabine treatment and also determine how effective other chemotherapy drugs are in ‘exposing’ nuclear tumor antigens to T cells.

Harnessing nuclear antigens to improve cancer immunotherapies

Combination chemo-immunotherapy has recently been shown to be a powerful anti-cancer strategy, provided the right combination of chemotherapy drugs and immunotherapeutic agents are used [5,36]. Therefore, the data from studies like ours have the potential to inform the planning and development of future clinical protocols. For example, since TAAs like survivin and MAGE-A10 are nuclear localized in a variety of cancer types [12,13], chemotherapy that augments the cross presentation of these antigens might boost the efficacy of survivin or MAGE-A10 vaccines. Furthermore, given the crucial role that cross-presentation also plays in thymic development of tolerance to TAAs [37], the reduced cross-presentation of nuclear localized antigen relative to cytoplasmic and secretory antigen, may result in a peripheral T cell repertoire of nuclear-TAA reactive T cells that is less tolerant than those T cells reactive to antigen from other compartments. Thus nuclear TAAs might conceivably be better targets for cancer immunotherapy. In this regard, it may be feasible to use the increased availability of nuclear antigen after chemotherapy (Fig. 4A) to ‘turn tumors into their own vaccines’ [reviewed in (38)]. This, in turn, has the potential to enhance newly emerging immunotherapies such as anti-CTLA-4/anti-PD-1/L1 immune checkpoint blockade [39,40].

In summary, nuclear tumor antigen are relatively less well cross-presented to anti-tumor T cells than those from the cytoplasmic and secretory compartments, but this situation can be reversed by an apoptosis-inducing chemotherapy.

References

1. Steer HJ, Lake RA, Nowak AK, Robinson BW (2010) Harnessing the immune system to treat cancer. Oncogene. 2. Segura E, Villadangos JA (2011) A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells. Traffic 12: 1677–1685. 3. Kurts C, Robinson BW, Knolle PA (2010) Cross-presentation in health and disease. Nat Rev Immunol 10: 403–414. 4. Nierkens S, Tel J, Jansen E, Adema GJ (2013) Antigen cross-presentation by dendritic cells. Traffic 12: 1677–1685. 5. Lake RA, Robinson BW (2005) Immunotherapy and chemotherapy—a practical partnership. Nat Rev Cancer 5: 397–405. 6. Lemmerz V, Fatho M, Gentilini C, Frye RA, Lille A, et al. (2005) The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A 102: 16015–16018.

Acknowledgments

The authors would also like to acknowledge the facilities, and the scientific and technical assistance of staff at the UWA Animal Care Services and the National Imaging Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments.

Author Contributions

Conceived and designed the experiments: CA RAL SAF BWR KH. Performed the experiments: CA SAF KH. Analyzed the data: CA RAL SAF KH BWR. Contributed reagents/materials/analysis tools: CA RAl SAF KH BWR. Wrote the paper: CA RAL SAF BWR KH.

PLOS ONE | www.plosone.org 7 September 2014 | Volume 9 | Issue 9 | e107894

7. Brusa D, Garetto S, Chiogno G, Scatolini M, Migliore E, et al. (2000) Post-apoptotic tumors are more palatable to dendritic cells and enhance their antigen cross-presentation activity. Vaccine 26: 6422–6432. 8. Matheoud D, Prile L, Hoefll G, Vimeux L, Parent L, et al. (2010) Cross-presentation by dendritic cells from live cells induces protective immune responses in vivo. Blood 115: 4412–4420. 9. Wolkers MC, Bouwenstijn N, Bakker AH, Toebes M, Schumacher TN (2004) Antigen bias in T cell cross-presentation. Science 304: 1314–1317. 10. Shen L, Rock KL (2004) Cellular protein is the source of cross-priming antigen in vivo. Proc Natl Acad Sci U S A 101: 3035–3040. 11. Zeelenberg IS, van Maren WW, Boissima A, Van Hout-Kuijer MA, Den Brok MH, et al. (2011) Antigen localization controls T cell-mediated tumor immunity. J Immunol 187: 1281–1288. 12. Schultz-Thater E, Ficuciello S, Iszii G, Le Maguer C, Zajac P, et al. (2011) MAGE-A10 is a nuclear protein frequently expressed in high percentages of
tumor cells in lung, skin and urothelial malignancies. Int J Cancer 129: 1137–1148.

13. Andersen MH, Svane IM, Becker JC, Straten PT (2007) The universal character of the tumor-associated antigen survivin. Clin Cancer Res 13: 5991–5994.

14. Takahara A, Koido S, Ito M, Nagasaki E, Sagawa Y, et al. (2011) Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response. Cancer Immunol Immunother 60: 1289–1297.

15. Ingberstrøm BA, Boye K, Tilde C, Neeland JM, Fløtmark K, et al. (2012) B7-H3 expression in colorectal cancer: nuclear localization strongly predicts poor outcome in colon cancer. Int J Cancer 131: 2528–2536.

16. Lo HW, Xia W, Wei Y, Ali-Seyed M, Huang SF, et al. (2005) Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer. Cancer Res 65: 338–348.

17. Tinguely M, Jenni B, Knights A, Lopes B, Korol D, et al. (2008) MAGE-C1/CT-7 expression in plasma cell myeloma: subcellular localization impacts on clinical outcome. Cancer Sci 99: 720–725.

18. Xia W, Wei Y, Du Y, Liu J, Chang B, et al. (2009) Nuclear expression of epidermal growth factor receptor is a novel prognostic value in patients with ovarian cancer. Mol Carcinog 48: 610–617.

19. Ralhan R, Cao J, Lim T, Macmillan C, Freeman JL, et al. (2010) EpCAM nuclear localization identifies aggressive thyroid cancer and is a marker for poor prognosis. BMC Cancer 10: 331.

20. Kitamura H, Torigoe T, Hirohashi Y, Asanuma H, Inoue R, et al. (2013) Nuclear, but not cytoplasmic, localization of survivin as a negative prognostic factor for survival in upper urinary tract urothelial carcinoma. Virchows Arch 462: 101–107.

21. Okada H, Mak TW (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer 4: 592–603.

22. Kramer A, Liashkovich I, Oberleithner H, Ludwig S, Mazur I, et al. (2008) Apoptosis leads to a degradation of vital components of active nuclear transport and a dissociation of the nuclear lamina. Proc Natl Acad Sci U S A 105: 11236–11241.

23. Andrà R, Crisol L, Prado R, Boyano MD, Arluzea J, et al. (2010) Plasma membrane and nuclear envelope integrity during the blebbing stage of apoptosis: a time-lapse study. Biol Cell 102: 25–35.

24. Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, et al. (2003) Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host-tumor-specific CD8 T cells. J Immunol 170: 4903–4913.

25. Marzo AL, Lake RA, Robinson BW, Scott B (1999) T-cell receptor transgenic analysis of tumor-specific CD8 and CD4 responses in the eradication of solid tumors. Cancer Res 59: 1071–1079.

26. George TG, Fanning SL, Fitzgerald-Bocarsly P, Medeiros RB, Highfill S, et al. (2006) Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. J Immunol Methods 311: 117–129.

27. Lyons AR, Parish CR (1994) Determination of lymphocyte division by flow cytometry. J Immunol Methods 171: 131–137.

28. Van den Broeck W, Derore A, Simoons P (2006) Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. J Immunol Methods 312: 12–19.

29. Quah IJ, Parish CR (2010) The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. J Vis Exp.

30. Robinson BW, Lake RA, Nelson DJ, Scott RA, Marzo AL (1999) Cross-presentation of tumour antigens: evaluation of threshold, duration, distribution and regulation. Immunol Cell Biol 77: 532–538.

31. Marzo AL, Lake RA, Lo D, Sherman L, McWilliam A, et al. (1999) Tumor antigens are constitutively presented in the draining lymph nodes. J Immunol 162: 3830–3845.

32. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, et al. (2002) Molecular Biology of the Cell. New York: Garland Science.

33. Presley JF (2005) Imaging the secretory pathway: the past and future impact of live cell optical techniques. Biochim Biophys Acta 1744: 259–272.

34. DuPage M, Mazumdar C, Schmidt LM, Cheung AF, Jacks T (2012) Expression of tumour-specific antigens underlies cancer immunoeediting. Nature 492: 405–409.

35. Pareli M, Bellati F, Videtta M, Focaccetti C, Mancone C, et al. (2013) Discovery of chemotherapy-associated ovarian cancer antigens by interrogating memory T cells. Int J Cancer.

36. Lesterhuis WJ, Salmons J, Nowak AK, Rozali EN, Khong A, et al. (2013) Synergistic effect of CTLA-4 blockade and cancer chemotherapy in the induction of anti-tumor immunity. PLoS One 8: e61895.

37. Adamopoulou E, Tenzer S, Hillen N, Klug P, Rota IA, et al. (2013) Exploring the MHC-peptide matrix of central tolerance in the human thymus. Nat Commun 4: 2039.

38. van der Most RG, Currie A, Robinson BW, Lake RA (2006) Cranking the immunologic engine with chemotherapy: using context to drive tumor antigen cross-presentation towards useful antitumor immunity. Cancer Res 66: 601–604.

39. Lesterhuis WJ, Haanen JB, Punt CJ (2011) Cancer immunotherapy–revisited. Nat Rev Drug Discov 10: 591–600.

40. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ (2012) Programmed death ligand 2 in cancer-induced immune suppression. Cln Dev Immunol 2012: 656340.