LETTERS

Adaptive immunity maintains occult cancer in an equilibrium state

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The capacity of immunity to control and shape cancer, that is, cancer immunoediting, is the result of three processes[1–8] that function either independently or in sequence[9]: elimination (cancer immunosurveillance, in which immunity functions as an extrinsic tumour suppressor in naive hosts); equilibrium (expansion of transformed cells is held in check by immunity); and escape (tumour cell variants with dampened immunogenicity or the capacity to attenuate immune responses grow into clinically apparent cancers). Extensive experimental support now exists for the elimination and escape processes because immunodeficient mice develop more carcinogen-induced and spontaneous cancers than wild-type mice, and tumour cells from immunodeficient mice are more immunogenic than those from immunocompetent mice. In contrast, the equilibrium process was inferred largely from clinical observations, including reports of transplantation of undetected (occult) cancer from organ donor into immunosuppressed recipients[10]. Herein we use a mouse model of primary chemical carcinogenesis and demonstrate that equilibrium occurs, is mechanistically distinguishable from elimination and escape, and that neoplastic cells in equilibrium are transformed but proliferate poorly *in vivo*. We also show that tumour cells in equilibrium are unedited but become edited when they spontaneously escape immune control and grow into clinically apparent tumours. These results reveal that, in addition to destroying tumour cells and sculpting tumour immunogenicity, the immune system of a naive mouse can also restrain cancer growth for extended time periods.

To assess whether an equilibrium state can develop during primary tumourigenesis, groups of sex- and age-matched wild-type C57BL/6 or 129/SvEv mice were injected with a single low dose of the chemical carcinogen 3′-methylcholanthrene (MCA) (Fig. 1a) and monitored for the appearance of progressively growing sarcomas for the next 200 or 230 days, after which new tumour formation decreases precipitously in C57BL/6 and 129/SvEv mice, respectively (Supplementary Fig. 1). Mice developing progressively growing sarcomas during the aforementioned times were removed from the experiments. The remaining mice, which often displayed small stable masses at the site of MCA injection, were placed on weekly injections of either control monoclonal antibody or monoclonal antibodies that deplete or block specific immunological components, and thereafter monitored for the appearance of progressively growing tumours.

When two cohorts of wild-type C57BL/6 mice were treated with 25 μg MCA, 3/16 (19% cohort 1, Fig. 1b) and 4/19 (21%, cohort 2, Fig. 1c) developed progressively growing sarcomas by day 200 and were removed from the experiment. When the remaining mice in cohort 1 were treated with control immunoglobulin, none developed additional tumours (Fig. 1b). In contrast, when the remaining mice in cohort 2 were treated with a mixture of monoclonal antibodies that deplete $CD4^+$ and $CD8^+$ cells and neutralize interferon-gamma (IFNγ), 60% (9/15) developed progressively growing sarcomas at the MCA injection site (Fig. 1c).

Variations of this experiment were repeated several times in two laboratories, using mice with either homogeneous C57BL/6 or 129/SvEv backgrounds. The results were in complete agreement and revealed that anti-CD4/CD8, anti-IFNγ, or anti-IL-12p40 (which is critical for IFNγ production) were equally effective in inducing sarcoma outgrowth and were not more effective when used in combination (Fig. 1d, and Supplementary Fig. 2). In total, of 187 mice treated with low-dose MCA, 86 (46%) developed progressively growing sarcomas following depletion of CD4/CD8 cells, IFNγ and/or IL-12—components that participate in adaptive immunity. In contrast, tumour outgrowth did not occur when monoclonal antibodies were used that deplete natural killer cells (anti-NK1.1), block natural killer cell recognition (anti-NKG2D) or inhibit one natural killer cell effector function (anti-TNF-related apoptosis inducing ligand (TRAIL)) ($n = 55$) (Fig. 1d). These data suggest that adaptive immunity plays a particularly important part in preventing late MCA-induced sarcoma outgrowth.

Two possibilities could explain the late tumour outgrowth we observed on ablating adaptive immunity: either the treatment permitted delayed *de novo* transformation or it facilitated expansion of pre-formed occult cancer cells. To explore the former and further substantiate the selective importance of adaptive immunity in preventing late tumour outgrowth, experiments were conducted using mice lacking either recombination-activating gene (*Rag*)-*1* or *Rag2*, which possess an intact innate immune compartment but lack lymphocytes that mediate adaptive immunity (that is, T cells, natural killer T cells and B cells). In stark contrast to the results obtained with wild-type mice, very few late-forming tumours were observed in $Rag1^{-/-}$ C57BL/6 mice exposed to 5 μg MCA (a dose that induces tumour outgrowth comparable to that in wild-type mice exposed to 25 μg MCA) and subsequently treated on day 200 with control immunoglobulin, anti-CD4/-CD8/-IFNγ (Fig. 2a, b), or anti-NK1.1 (Fig. 2c). Very few late-forming tumors were also observed in $Rag2^{-/-}$ 129/SvEv mice treated with 25 μg MCA (so as to provide a carcinogenic insult comparable to that used for wild-type mice) and subsequently treated on day 230 with control immunoglobulin or anti-IFNγ (to assess the effects of natural-killer-cell-produced IFNγ on cells of innate immunity) (Fig. 2d). These results show that sarcoma formation in immunodeficient $Rag^{-/-}$ mice was essentially

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complete within 200 days of MCA exposure. In addition, the mean time to tumour formation in MCA-treated *Rag1*$^{-/-}$ C57BL/6 mice (105 ± 5 (± s.e.m.) days) differed strikingly from that in MCA-treated wild-type mice rendered immunodeficient at day 200 using the anti-CD4/-CD8/-IFNγ mixture (25 ± 6 days) (Fig. 2e). Similar findings were made using 129/SvEv strain mice: 186 ± 7 days for MCA-treated *Rag2*$^{-/-}$ mice versus 38 ± 6 days for MCA-treated wild-type mice immunodepleted at day 230 (Fig. 2f). Taken together, these data argue strongly against continuous *de novo* transformation as the mechanism underlying the late tumor outgrowth in wild-type mice after immunodepletion.

We therefore considered the possibility that at least some MCA-treated wild-type mice that remained free of progressively growing tumours harboured fully transformed sarcoma cells, the outgrowth of which was immunologically restrained. In the MCA model,

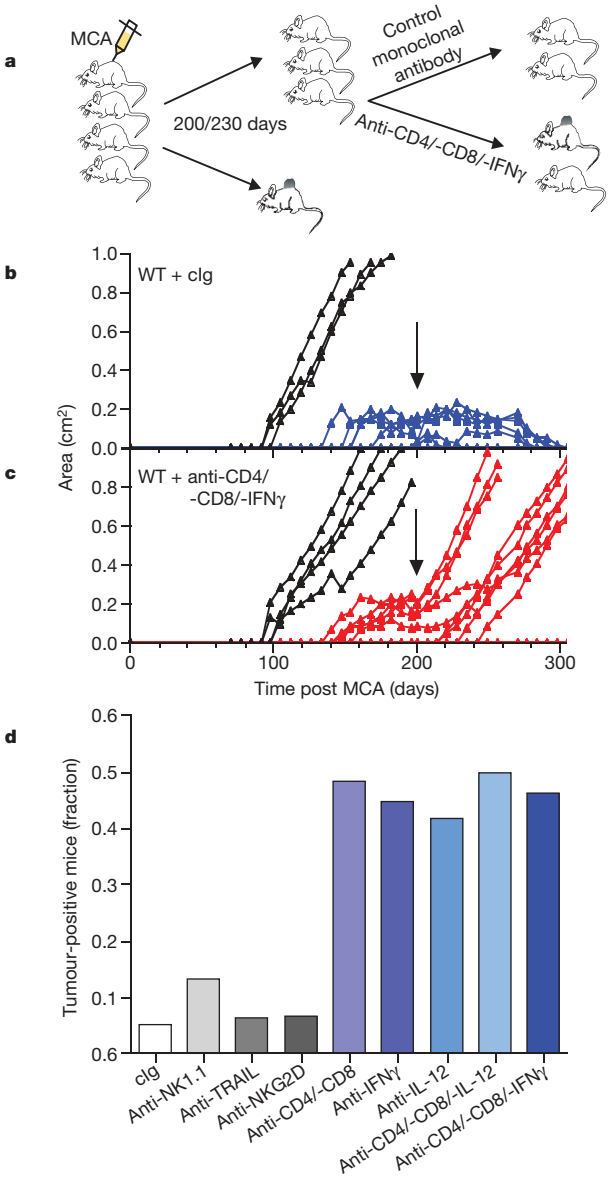

Figure 1 | The adaptive immune system promotes an equilibrium state in primary MCA-induced sarcomas. **a**, Protocol to test for the existence of an equilibrium state during MCA tumourigenesis. Details are described in the text and Methods. **b**, Sixteen wild-type (WT) C57BL/6 mice were treated with 25 μg MCA. At 200 days, the 13 tumour-free mice were treated weekly with control immunoglobulin (cIg) and monitored for the appearance of late-forming tumours. **c**, Nineteen WT C57BL/6 mice were treated as in **b**. At 200 days, the 15 tumour-free mice were injected weekly with anti-CD4/-CD8/-IFNγ ($P = 0.0008$ Fisher's exact test between anti-CD4/-CD8/-IFNγ and cIg). **d**, Fraction of WT C57BL/6 mice treated with 25 μg MCA that develop late-forming tumours after treatment with the following monoclonal antibodies starting at day 200: cIg ($n = 76$), anti-NK1.1 ($n = 30$), anti-TRAIL ($n = 16$), anti-NKG2D ($n = 15$), anti-CD4/-CD8 ($n = 29$), anti-IFNγ ($n = 29$), anti-IL-12p40 ($n = 12$), anti-CD4/-CD8/-IL-12 ($n = 12$) and anti-CD4/-CD8/-IFNγ ($n = 13$).

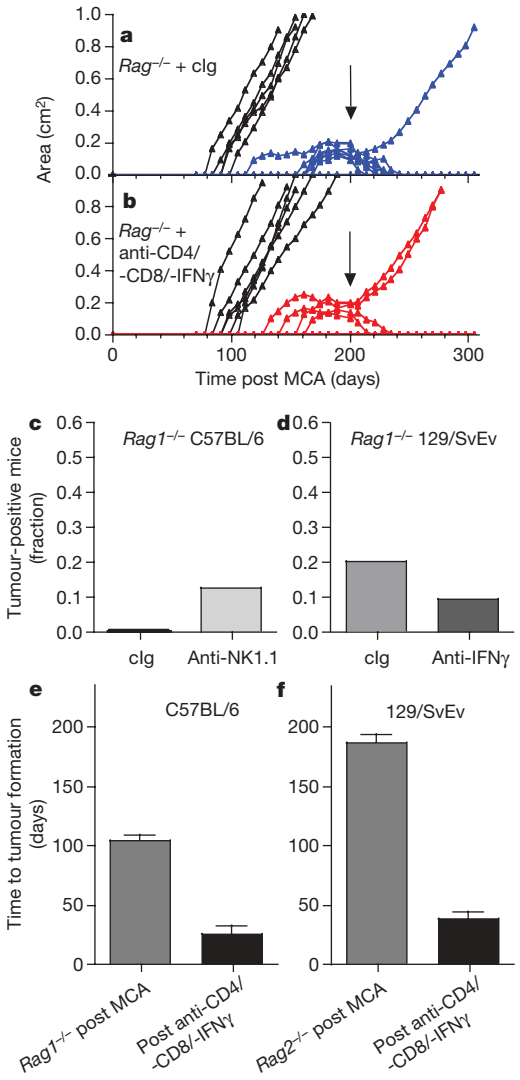

Figure 2 | Antibody-induced equilibrium disruption does not occur as a result of prolonged *de novo* transformation. *Rag1*$^{-/-}$ C57BL/6 mice were injected with 5 μg MCA and, at day 200, 21 tumour-free mice were treated weekly with cIg (**a**), whereas 20 other tumour-free mice were treated with anti-CD4/-CD8/-IFNγ (**b**). Mice were monitored for the appearance of late-forming sarcomas. The difference between anti-CD4/-CD8/-IFNγ and cIg groups is not significant ($P = 0.616$, Fisher's exact test). **c**, Thirty-two tumour-free *Rag1*$^{-/-}$ C57BL/6 mice, injected initially with 5 μg MCA, were treated weekly with either cIg ($n = 16$) or anti-NK1.1 ($n = 16$), starting at day 200 and monitored for sarcoma appearance. The difference between anti-NK1.1 and cIg groups is not significant ($P = 0.484$, Fisher's exact test). **d**, Twenty-seven tumour-free *Rag2*$^{-/-}$ 129/SvEv mice, injected initially with 25 μg MCA, were treated weekly with either cIg ($n = 15$) or anti-IFNγ ($n = 12$) starting at day 230 and followed for sarcoma appearance. The difference between anti-IFNγ and cIg groups is not significant ($P = 0.605$, Fisher's exact test). **e**, Mean time to tumour formation ± s.e.m. for *Rag1*$^{-/-}$ C57BL/6 mice treated with 25 μg MCA ($n = 11$) or tumour-free WT C57BL/6 mice treated initially with 25 μg MCA and subsequently with anti-CD4/-CD8/-IFNγ starting at day 200 ($n = 8$) ($P = 0.0003$, Mann–Whitney rank sum test). **f**, Mean time to tumour formation ± s.e.m. for *Rag2*$^{-/-}$ 129/SvEv mice treated with 25 μg MCA ($n = 30$) or tumour-free WT 129/SvEv mice treated initially with 25 μg MCA and subsequently with anti-CD4/-CD8/-IFNγ starting at day 230 ($n = 30$) ($P < 0.0001$, Mann–Whitney rank sum test).

cellular transformation and tumour development occur exclusively at the site of carcinogen injection. Examination of the injection site in 129/SvEv mice treated with 25 μg MCA revealed the presence of small 2–8 mm masses that became palpable within 150 days of MCA injection but did not change in size during an additional 150 days. When sectioned and analysed by haematoxylin and eosin staining, 47% (8/17) of the stable masses contained clusters of large cells showing variable degrees of atypia, with enlarged vesicular nuclei, prominent nucleoli and heterogeneous morphologies (Fig. 3a, c), and which also expressed vimentin (Fig. 3c, inset). These features mirrored the immuno-cytological spectrum observed in progressively growing primary MCA sarcomas (Fig. 3b, d). The remaining masses from MCA-treated wild-type mice showed variable degrees of inflammation associated with areas of dense fibrosis and lipid droplets (the oil diluent of the MCA) often surrounded by multinucleated giant cells (Supplementary Fig. 3a, c). The MCA injection site in *Rag2*$^{-/-}$ mice also contained fibrotic areas and lipid droplets but lacked both atypical cells and multinucleated giant cells (Supplementary Fig. 3b, d). Immunohistochemical staining of stable masses containing atypical cells from MCA-treated wild-type mice and growing primary MCA sarcomas from wild-type mice revealed the presence of CD3^{+} T cells, B220^{+} cells and F4/80^{+} mononuclear phagocytes infiltrating into regions containing atypical cells (Supplementary Fig. 4). When stable masses containing atypical cells were transiently cultured, a population of atypical fibroblast-like cells grew out that formed progressively growing tumours when transplanted into immunodeficient *Rag2*$^{-/-}$ mice (Fig. 3i). In contrast, normal skin fibroblasts from either MCA-treated mice (Fig. 3i) or untreated wild-type mice (data not shown) did not form tumours in *Rag2*$^{-/-}$ mice. Thus, atypical cells from stable masses that formed in MCA-treated, wild-type mice were fully transformed.

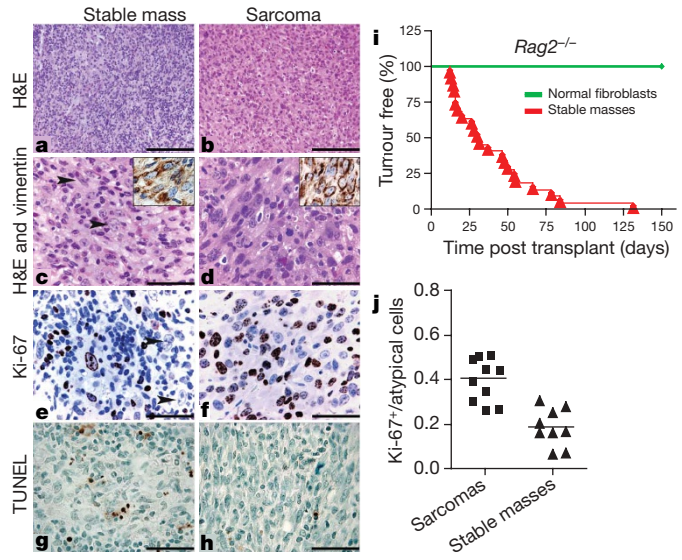

Figure 3 | Demonstration of occult cancer in immunocompetent mice in the equilibrium phase of cancer immunoediting. Sections from representative stable masses isolated from WT 129/SvEv mice treated with 25 μg MCA (**a**, **c**, **e**, **g**) and progressively growing sarcomas from MCA-treated WT 129/SvEv mice (**b**, **d**, **f**, **h**) stained for haematoxylin and eosin (H&E) (**a**, **b**, **c**, **d**), vimentin (inset in **c** and **d**), the proliferation marker Ki-67 (**e**, **f**) and the apoptosis marker TUNEL (**g**, **h**). In **c** and **e**, arrowheads indicate atypical cells. Magnification: 200× ; scale bars, 100 μm (**a** and **b**), 600× ; scale bars, 33 μm (**c**–**f**) and 1,000× ; scale bars, 20 μm (**g** and **h**). **i**, Fraction of tumour-free *Rag2*$^{-/-}$ 129/SvEv mice following subcutaneous injection with 10^6 normal skin fibroblasts ($n = 1$, diamonds) or 10^6 cells isolated from long-term stable masses from MCA-treated WT 129/SvEv mice ($n = 5$, triangles). Each cell line was injected into four *Rag2*$^{-/-}$ recipients. **j**, Proportion of atypical cells staining positive for Ki-67 in formalin-fixed tissue from growing primary MCA-sarcomas from WT mice ($n = 10$, squares) or stable masses from MCA-treated WT 129/SvEv mice ($n = 9$, triangles) ($P = 0.0003$ Mann–Whitney ranked sum test, difference between proliferation indices of tumour cells in stable masses versus growing primary MCA sarcomas).

These results contradict an earlier report stating that MCA treatment of wild-type mice leads to encapsulation of MCA crystals without apparent cellular transformation[11]. However, the former study failed to characterize 'cells with altered morphology' that were noted in the MCA granulomas and that may have been identical to the transformed atypical cells that we describe herein. Moreover, MCA crystals are not observed in our mice, indicating that the MCA might not have been fully dissolved in the earlier study and therefore may have achieved extremely high *in vivo* concentrations resulting in rapid cellular transformation and tumour outgrowth, thereby obscuring detection of transformed cells, the growth of which was under immunologic restraint.

The paradox that stable masses from our MCA-treated immunocompetent mice often contained transformed cells but did not increase in size *in vivo* suggested that net tumour cell expansion was being immunologically restrained. This possibility was explored by comparing sections of stable masses containing atypical cells or progressively growing primary sarcomas for expression of markers of proliferation (Ki-67)[12,13] (Fig. 3e, f) or apoptosis (TUNEL) (Fig. 3g, h). Analysis of 10 progressively growing MCA sarcomas revealed strong nuclear reactivity for Ki-67 in a high proportion of tumour cells (mean proliferation index = 40%) (Fig. 3f, j), and detectable levels of cellular apoptosis (Fig. 3h). This result is consistent with the high histological grade of these lesions shown by cellular pleiomorphism, numerous visible mitoses and abundant necrosis. In contrast, only a limited percentage of atypical cells in nine stable masses stained positively for Ki-67 (mean proliferation index = 18%) (Fig. 3e, j) and these samples also showed 1.7-fold more TUNEL staining than growing sarcomas (Fig. 3g). Of note, both sample sets contained populations of proliferating, Ki-67^{+} leukocytes. Thus, stable masses are characterized by a combination of increased apoptosis and decreased tumour cell proliferation.

The concept that the immune system could maintain tumours in a 'dormant' state originally stemmed from experiments involving transplantation of certain tumour cell lines into pre-immunized mice[14,15]. In the current study, we show that immunity can restrain the outgrowth of occult tumour cells during primary tumour induction in naive mice and additionally demonstrate that the lack of net expansion of 'dormant' tumour cells in MCA-treated immunocompetent mice is most probably the result of a continuously ongoing combination of cytostatic and cytolytic immune effects. Therefore the term 'equilibrium' best describes the persistence of cancer that we observe in wild-type mice exposed to low-dose MCA.

Throughout these experiments we noticed that a small percentage of low-dose MCA-challenged wild-type mice, either left untreated, or treated with control monoclonal antibody, went on to develop late-appearing sarcomas (Fig. 1d, Supplementary Fig. 2). We therefore asked whether sarcoma cells that spontaneously escape equilibrium could be distinguished on the basis of immunogenicity from those remaining in equilibrium. As controls, we also confirmed the immunogenicities of MCA sarcoma cell lines generated in the current study that grew out with normal kinetics from MCA-treated wild-type mice (edited tumour cells) and *Rag2*$^{-/-}$ mice (unedited tumour cells). In agreement with previous reports[1], edited MCA-sarcoma cell lines from wild-type mice displayed dampened immunogenicity because they formed progressively growing tumours in either *Rag2*$^{-/-}$ or naive wild-type recipients (Fig. 4a, b, respectively), and unedited MCA sarcoma cell lines from *Rag2*$^{-/-}$ mice were highly immunogenic because, although they grew in *Rag2*$^{-/-}$ recipients (Fig. 4a), 40% were rejected in naive wild-type mice (Fig. 4b). Notably, tumour cells that had been maintained in equilibrium (that is, derived either directly from stable masses or from MCA-treated, wild-type mice that had undergone anti-CD4/-CD8/-IFNγ-mediated equilibrium disruption) formed progressively growing tumours in

Rag2$^{-/-}$ mice (Fig. 3i and 4a, respectively) but were highly immunogenic in wild-type mice because 51% and 31%, respectively, were rejected (Fig. 4b). In contrast, cell lines produced from late-forming sarcomas, which grew out spontaneously from mice in equilibrium, formed tumours when transplanted into either *Rag2*$^{-/-}$ mice or naive wild-type recipients (Fig. 4a, b, respectively). Thus, tumour cells held in equilibrium have an unedited phenotype, whereas those that spontaneously progress to the escape phase are edited. We therefore conclude that at least some tumour cell editing occurs at the temporal interface between equilibrium and escape.

In summary, in addition to the immune system's capacity to destroy and shape cancer, this study shows that immunity can also control cancer for long time periods by a process called equilibrium. We show that equilibrium is indeed a component of cancer immunoediting because tumour cells in equilibrium are highly immunogenic (unedited), whereas those spontaneously exiting equilibrium that become growing tumours have attenuated immunogenicity (edited)—results that place this process temporally between elimination and escape. We demonstrate that elimination and equilibrium can be mechanistically distinguished because, although the former requires the actions of both innate and adaptive immunity[8], equilibrium is maintained solely by adaptive immunity. Similarly, we show that equilibrium and escape are distinct because, whereas equilibrium represents a time of tumour cell persistence without expansion, escape is characterized by progressive tumour growth. We do not envisage that every tumour cell must pass through an elimination process before it enters equilibrium, nor do we hold that every progressively growing tumour must transit through an equilibrium process. However, we predict that many clinically apparent tumours may progress through a linear 'elimination→equilibrium→escape' continuum, and ongoing work is focused on obtaining molecular signatures of tumour cells in each step of this process. Nevertheless, our results confirm an important, but heretofore untested, prediction of the cancer immunoediting hypothesis—the existence of an equilibrium state—thereby providing additional support to the central premise of cancer immunoediting: that immunity can influence cancer development both quantitatively and qualitatively. Our findings in this mouse model also have potential relevance to human cancers. First, they indicate that maintaining cancer in an equilibrium state may represent a relevant goal of cancer immunotherapy in which augmentation of adaptive tumour immunity could result in improved tumour control. Second, they provide mechanistic underpinnings for the recent findings that the quality and quantity of the immune reaction within certain tumour types (for example, colorectal and ovarian cancers) are reliable prognostic indicators of cancer patient survival[16–18]. Third, they explain how occult cancer can be transplanted from organ donor to recipient[10], because tumour cells held in equilibrium in the donor may grow in a recipient who is naive to the antigens of the transplanted tumour cells and is immunosuppressed. Fourth, they provide a mechanism that can explain the presence of occult tumour cells in organs—for example, the prostate—of individuals lacking clinical symptoms of disease[19–22]. Finally, our study raises the possibility that at least some of the proposed tumour-promoting actions of chronic inflammation may be a result of interfering with adaptive immunity's capacity to hold occult cancers in equilibrium[23,24]. Our results thus provide a foundation for future work to define the molecular mechanisms by which adaptive immunity maintains cancer in an equilibrium/dormant/persistent state, perhaps paving the way for development of new therapeutic modalities to convert cancer into a controllable chronic disease.

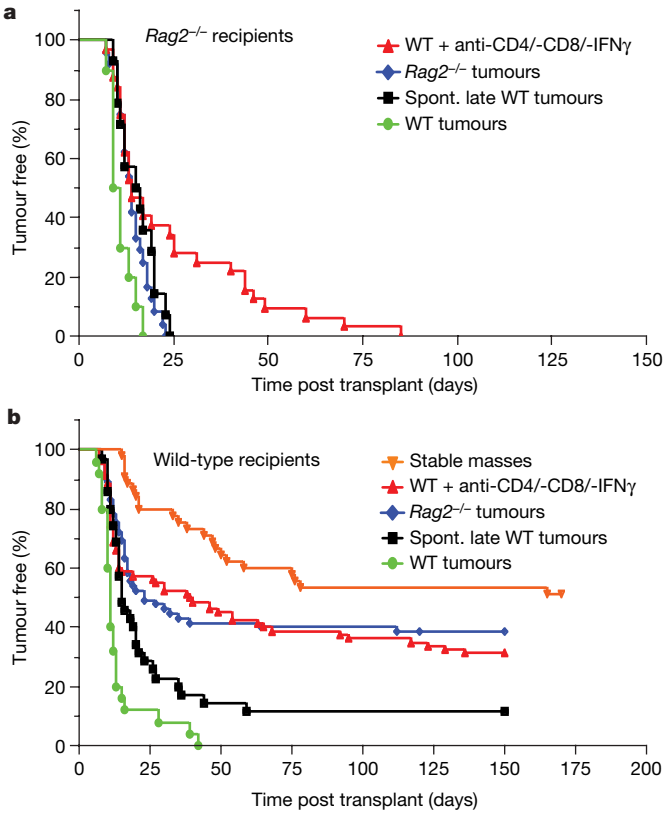

Figure 4 | Sarcoma cells in equilibrium show high immunogenicity, whereas those spontaneously exiting equilibrium have attenuated immunogenicity. a, Kaplan–Meier survival analysis of *Rag2*$^{-/-}$ 129/SvEv mice injected with sarcoma cell lines from classical progressively growing MCA-sarcomas isolated from WT ($n = 5$, circles) or *Rag2*$^{-/-}$ 129/SvEv mice ($n = 13$, diamonds) or isolated from tumours forming in MCA-treated WT 129/SvEv mice after anti-CD4/-CD8/-IFNγ-mediated equilibrium disruption ($n = 16$, triangles) or isolated from late-appearing tumours from MCA-treated WT 129/SvEv mice that spontaneously progress from equilibrium to escape ($n = 7$, squares). Each cell line was injected at 10^6 cells per mouse into two mice each. **b**, Kaplan–Meier survival analysis as in **a** except that 5-member groups of naive WT 129/SvEv mice were used as the recipients for each cell line. Also tested were sarcoma cell lines grown directly out of stable masses from MCA-treated WT mice in equilibrium ($n = 5$, inverted triangles).

METHODS SUMMARY

Wild-type or *Rag*-null mice were injected subcutaneously with low doses of MCA (Sigma Fine Chemicals), as described[1,25], and monitored for tumour development. After 200 days (C57BL/6) or 230 days (129/SvEv), MCA-treated mice bearing progressively growing tumours were removed from the experiment and the remaining mice were treated weekly with either control immunoglobulin or monoclonal antibodies that deplete or block specific immune components and were monitored for tumour development for the next 100 days. Tumour transplantation experiments were performed, as described previously[1,25]. For morphologic and immunohistologic evaluation of primary growing sarcomas and stable masses, sections of formalin-fixed, paraffin-embedded tissue samples were evaluated after staining with haemotoxylin and eosin or monoclonal antibodies specific for either cell surface markers or the Ki-67 protein. Apoptosis was determined by TUNEL staining. The proliferation index is defined as the percentage of large, atypical, cells showing enlarged nuclei with prominent nucleoli that stained positively for Ki-67 in multiple high power (600×) fields.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Mice. Wild-type C57BL/6 mice were purchased from the Walter and Eliza Hall Institute of Medical Research and *Rag1*$^{-/-}$ C57BL/6 mice were bred at the Peter MacCallum Cancer Centre. Wild-type and *Rag2*$^{-/-}$ 129/SvEv mice were purchased from Taconic Farms and entered into the experiments at 8 to 12 weeks old. Mice were housed according to the American Association for Laboratory Animal Science conditions in specific pathogen-free facilities at the Washington University School of Medicine, St. Louis, Missouri and the Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.

Reagents. Anti-CD4 (GK1.5)[26], anti-CD8 (YTS-169.4)[27] anti-IFNγ (H22)[28], anti-NKG2D (C7)[29], anti-NK1.1 (PK136)[30], anti-TRAIL (N2B2)[31], anti-IL-12p40 (C17.8)[32] and control immunoglobulin (the PIP monoclonal antibody specific for glutathione *S*-transferase[33]) were generated from spent culture supernatant or hybridoma ascites and purified in aggregate-, endotoxin-, and protein A-free form. Antibodies were injected at an initial dose of 250–750 μg each with a weekly (CD4, CD8, NK1.1, IFNγ) or biweekly (NKG2D, TRAIL, IL-12p40) maintenance dose of 250 μg of each.

MCA tumour induction. Groups of wild-type and *Rag1*$^{-/-}$ C57BL/6 mice were injected subcutaneously in the hind flank with 5 or 25 μg of MCA in 0.10 ml of corn oil, as described[34]. Mice were monitored every 7 days for tumour development from 70 days after MCA treatment. Tumours $>0.5\ cm^2$ in area and demonstrating progressive growth were recorded as 'tumour positive'. Wild-type and *Rag2*$^{-/-}$ 129/SvEv mice were injected subcutaneously in the flank with 25 μg MCA (Sigma Fine Chemicals) dissolved in 0.15 ml of peanut oil (with gentle heating), as described[35]. Mice were monitored every 7 days for tumour development from 90 days after MCA treatment. 129/SvEv mice were considered tumour positive when masses reached an average diameter of 9 mm and continued to grow progressively.

Cell lines. Cell lines were created by mechanical disruption of tissue, followed by a 1 h treatment with collagenase Type IA ($0.5\ mg\ ml^{-1}$) and culture in endotoxin-low RPMI medium ($<0.01\ EU\ ml^{-1}$) supplemented with 10% fetal calf serum ($0.25\ EU\ ml^{-1}$).

Tumour transplantation. Before use, a vial of frozen sarcoma cells was thawed and cultured *in vitro* in RPMI medium supplemented with 10% fetal calf serum for two passages. Tumour cells were collected by incubation in 0.25% trypsin, washed two times in RPMI medium and one time in endotoxin-free PBS and then injected subcutaneously in a volume of 0.15 ml PBS into the shaved flanks of mice. All injected cell lines were more than 90% viable, as determined by trypan blue exclusion. Tumour growth was monitored by measuring two perpendicular diameters.

Histology, proliferation index and immunohistochemistry. Sections were obtained from formalin-fixed, paraffin-embedded tissue samples. For morphological evaluation, sections were stained in haemotoxylin and eosin. The proliferation index was calculated from tissue sections stained using an indirect immunoperoxidase technique that employed as primary antibody the clone Tec-3 (DakoCytomation, 1:25 dilution), which is specific for the Ki-67 protein, a marker of proliferating cells, and a biotinylated rabbit anti-rat IgG secondary antibody (Vector Laboratories, 1:200 dilution). At least 200 atypical/neoplastic cells were counted and scored per sample. All other immunohistochemical stains were performed on formalin-fixed, paraffin-embedded 4 μm tissue sections using standard techniques. The following primary antibodies were used: anti-vimentin (Abcam, ab28028, 1:100 dilution following proteinase K digestion for 15 min.), anti-CD3 (Cell Marque, CMC363, 1:1,500 dilution), anti-CD45R/B220 (BD Pharmingen, no. 550286, 1:200 dilution), and anti-F4/80 antigen (Serotec, MCA497, 1:5,000 dilution following proteinase K digestion for 10 min.). Chromogenic terminal-deoxynucleotidyl-transferase-mediated nick-end labelling (TUNEL) staining with methyl green counterstaining was done as per the manufacturer's instructions (ApopTag Peroxidase In situ Kit, no. S7100, Chemicon International).

Statistical analysis. Fisher's exact test was used to determine the significance of the association between two variables (tumour development and antibody treatment) in a 2×2 contingency table. The Mann–Whitney rank sum test was used to assess whether two samples of observations (time to tumour formation and proliferation index) come from the same distribution, without assuming equal variances between the two populations.

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