Dissecting the immune microenvironment of breast cancer

Ciampricotti, M.

Chapter 2

Development of metastatic HER2+ breast cancer is independent of the adaptive immune system

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Abstract

The tumour-modulating effects of the endogenous adaptive immune system are rather paradoxical. Whereas some clinical and experimental observations offer compelling evidence for the existence of immunosurveillance, other studies have revealed promoting effects of the adaptive immune system on primary cancer development and metastatic disease. We examined the functional significance of the adaptive immune system as a regulator of spontaneous HER2+ breast tumourigenesis and pulmonary metastasis formation using the MMTV-NeuT mouse model in which mammary carcinogenesis is induced by transgenic expression of the activated HER2/neu oncogene. Although T and B lymphocytes infiltrate human and experimental HER2+ breast tumours, genetic elimination of the adaptive immune system does not affect development of premalignant hyperplasias or primary breast cancers. In addition, we demonstrate that pulmonary metastasis formation in MMTV-NeuT mice is not dependent on the adaptive immune system. Thus, our findings reveal that spontaneous HER2 driven mammary tumourigenesis and metastasis formation are neither suppressed or altered by immunosurveillance mechanisms, nor promoted by the adaptive immune system.

Keywords

Breast cancer, Metastasis, Her2/neu, Adaptive immune system, Innate immune system, Immunosurveillance

Introduction

It has become generally accepted that chronic activation of innate immune cells contributes to cancer development and/or progression, however, the role of adaptive immune cells is still a matter of debate [1-3]. For decades, it was believed that the adaptive immune system protects organisms from tumour development, a process referred to as immunosurveillance [1,4]. This hypothesis is supported by epidemiological studies showing increased incidence of pathogen-associated cancers in immunocompromised patients [3]. In addition, the concept of tumour suppression and tumour editing by the adaptive immune system has been supported by studies in a chemically induced mouse sarcoma model [5-6]. However, the role of the adaptive immune system during development of pathogen and chemical unrelated solid cancers are less clear. In fact, recent studies using genetically engineered mouse (GEM) tumour models do reveal a more controversial function of the adaptive immune system during tumourigenesis. Whereas some studies using GEM models for spontaneous tumourigenesis did not reveal any modulating role for the adaptive immune system in tumour formation or progression [2,7], other experimental studies revealed an unexpected tumour-promoting role for certain components of the adaptive immune system [8-11]. For example, genetic elimination of the adaptive immune system in a transgenic mouse model for multistage skin carcinogenesis protected against spontaneous tumour formation [8,11]. Likewise, lymphocytes were shown to promote induction of chronic hepatitis and subsequent hepatocellular carcinoma development in lymphotoxin transgenic mice [9]. Thus, in addition to the concept of immunoediting in which the adaptive immune system “sculpts” developing tumours, these studies indicate existence of alternative pathways, in which a spontaneously developing tumour avoids or even harnesses components of the adaptive immune system to its own advantage. However, the degree to which these pathways are tissue-, organ-, cell type-, or oncogene-specific remains to be evaluated.

Breast cancer is a heterogeneous disease. The recent availability of advanced molecular technologies has led to the characterization of different molecular portraits of breast cancer, which can roughly be divided into five distinct breast cancer subtypes: “luminal-A”, “luminal-B”, “basal”, “HER2 positive” and “normal breast-like” [12-13]. These distinct subtypes of breast cancer are dependent on different oncogenic pathways, are characterized by unique gene expression patterns, have different prognostic

characteristics, and display differential sensitivity to anti-cancer drugs [14-15], and are thus also likely to be differentially regulated by the adaptive immune system. It has recently been shown that mammary tumour metastasis formation in the MMTV-PyMT breast cancer mouse model is dependent on $CD4^+$ T cells [10]; however, it is unknown whether this mechanism accounts for other breast cancer types.

Between 15 and 20 percent of invasive breast cancers are HER2+ [16]. Overexpression of HER2 is an adverse prognostic factor associated with poorly differentiated, high-grade tumours, metastasis formation, relative resistance to certain chemotherapy regimens and greater risk of recurrence [14,16]. Prominent lymphocytic infiltrates and high expression of lymphocyte-associated genes in human HER2+ breast cancers have been reported to correlate with lower recurrence rates [17]. Likewise, a recent clinical study demonstrated that a T cell metagene could be used as an independent predictor of favourable prognosis in patients with HER2+ breast cancer [18]. These studies indicate that the intra-tumoural presence of lymphocytes is beneficial for breast cancer patients. However, from these clinical observations it is unclear whether the presence of lymphocytes *causes* a favourable prognosis, or whether the lymphocytes are present as *a consequence* of a distinct natural history of the good prognosis subtype of HER2+ breast cancers, and thus represent a biological marker instead of a biological anti-cancer weapon. Importantly, immunocompromised patients are not at increased risk of developing breast cancer [3,19-20], suggesting that human breast cancer formation might not be suppressed by immunosurveillance mechanisms.

The aim of this study was to dissect the functional role of the adaptive immune system during *de novo* HER2+ breast cancer development and pulmonary metastasis formation, using the MMTV-NeuT mouse model in which mammary tumourigenesis is induced by transgenic expression of the activated HER2/neu oncogene driven by the MMTV promotor [21-22].

Materials and Methods

Mice

MMTV-NeuT mice (Balb/c F>12) [22] were purchased from Charles River, and maintained by mating MMTV-NeuT males with Balb/c females. MMTV-NeuT mice were intercrossed with RAG-2$^{-/-}$ mice [23] on the Balb/c background (F>10) [24] to generate breeding colonies of NeuT/RAG-2$^{+/-}$ and NeuT/RAG-2$^{-/-}$ mice and wild type littermate control RAG-2$^{+/-}$ and RAG-2$^{-/-}$ mice. Genotyping was performed by PCR analysis on tail tip DNA as described previously [22-23]. Transgene positive female animals were monitored weekly by palpation for mammary tumour development. Once palpable tumours were present, tumour size was measured twice a week using a caliper. Ninety minutes before sacrifice, mice were injected i.p. with 50 mg/kg bodyweight bromodeoxyuridine (BrdU) (Sigma, Zwijndrecht, the Netherlands). All mice were kept in individually ventilated cages at the animal care facility of the Netherlands Cancer Institute and food and water were given *ad libitum*. All animal experiments were performed in accordance with institutional guidelines and national ethical regulations.

Histology and Immunohistochemistry

Tissue samples were processed, sectioned and stained as described [25]. Details regarding antibodies, antigen retrieval methods and type of tissue sections can be found in Supplemental table 1a. All immunohistochemical experiments included negative controls for determination of background staining, which was negligible. Slides were digitally processed using the Aperio ScanScope (Aperio, Vista, CA) using ImageScope software version 10.0 (Aperio). Data shown are representative of results obtained following examination of tissues removed from a minimum of 5 patients or mice per group.

Flow cytometry

Both 4th (inguinal) mammary glands were isolated from age-matched negative littermate mice and tumours (15x15 mm) were isolated from transgenic mice. After removal of lymph nodes, glands and tumours were mechanically chopped using the McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. Ltd., Guildford, UK) and digested for 1 hr at 37˚C in a digestion mix of 3 mg/ml Collagenase Type A (Roche, Mannheim, Germany) and 1.5 mg/ml porcine pancreatic trypsin (Invitrogen, Breda, the Netherlands) in serum-free L15 medium (Invitrogen). Cells and organoids were centrifuged at 1200 rpm for 5 min, resuspended in L15 medium, 10% FCS, 100 IU/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen) and dispersed through a 70 μm cell strainer (BD Falcon). Blood was collected in heparin-containing eppendorf tubes and treated with NH_4Cl lysing buffer. Spleens were homogenized over a 70 μm filter (BD Falcon) and cells were treated with NH_4Cl lysing buffer. Cells from tissues, blood and spleen were centrifuged for 5 min at 1200 rpm and resuspended in PBS supplemented with 1% BSA (Sigma) (PBS/BSA). Samples of $0.5x10^6$ cells were incubated for 20 min at 4 ◌C in the dark with antibodies (Supplemental table 1b) Cells were washed with PBS/BSA, 7-AAD (eBioscience) was added (1:10) to exclude dead cells, and data acquisition was performed on a FACSCalibur using CellQuestPro software (BD Biosciences). Data analysis was performed using FlowJo software version 7.1.3 (Tree Star Inc., Ashland, OR).

Orthotopic tumour transplantations

Mammary tumours (15x15 mm) were isolated from two NeuT/RAG-$2^{+/-}$ and two NeuT/RAG-$2^{-/-}$ mice. Small tumour pieces (1x1 mm, mechanically minced in ice-cold PBS) were grafted into the mammary fatpad of female Balb/c mice (8–12 weeks of age). Mice were anesthetized with hypnorm/dormicum/H2O (1:1:2, 7ml/kg) and a small abdominal skin incision was made. Using watchmaker forcepts, a small pocket was generated in the 4th (inguinal) mammary gland fat pad into which a tumour piece was placed. The skin was stitched and temgesic was given for postoperative pain relief. After postoperative surveillance, tumour growth was monitored twice a week starting 1 week after transplantation.

Luminex cytokine assays

Both 4th mammary glands were isolated from age-matched negative littermate mice and tumours (15x15 mm) were isolated from transgenic mice. After removal of lymph nodes, tissue was snap frozen and stored at -80˚C. Frozen tissue was pulverized in liquid nitrogen using a mortar. The pulverized tissue was dispersed in Bio-Plex Cell Lysis Buffer (Bio-Rad, Munich, Germany), centrifuged for 15 min at 14.000 rpm at 4 °C, supernatant was collected, and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, Bonn, Germany) according to manufacturers' recommendations. Samples were stored at -80˚C. Cytokine concentrations in protein lysates were determined with Bio-Plex Pro Cytokine Kits (Bio-Rad, Munich Germany) on a Luminex system. The assay was performed according to the manufacturers' recommendations. Data acquisition and analysis was performed on a Bio-Plex 200 reader, using Bio-Plex Manager 6.0 software (Bio-Rad, Munich Germany).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA). Specific tests used were the Mann-Whitney test (unpaired, two-tailed), Log-Rank test and Fisher's exact test. P values < 0.05 were considered statistically significant.

Results

Human and mouse HER2+ breast tumours are characterized by influx of adaptive immune cells

Given the paradoxical role of the adaptive immune system during *de novo* tumourigenesis [1-3], and considering the reported association between lymphocytic infiltrates and high expression of lymphocyte-associated genes with favourable prognosis in patients with HER2+ breast cancer [17] [18], we set out to investigate whether the adaptive immune system does modulate spontaneous HER2+ breast cancer formation. We utilized a mouse model for multi-stage HER2+ breast tumourigenesis, e.g. MMTV-NeuT mice. Female MMTV-NeuT mice develop hyperplastic lesions within 2 months of age, and invasive mammary tumours and pulmonary metastases around 4 months of age, resembling human HER2+ breast tumourigenesis [21-22]. Like human HER2+ breast tumours, tumours in MMTV-NeuT mice are characterized by infiltrating $CD45^+$ immune cells (Fig. S1). We investigated the nature of the inflammatory infiltrate in human and mouse HER2+ breast tumours by immunohistochemistry, and observed a prominent influx of macrophages (Fig. S1), as well as an influx of adaptive immune cells, i.e. B cells, and $CD4^+$ and $CD8^+$ T cells (Fig. 1). In human and mouse HER2+ tumours, the infiltrating leukocytes were mainly localized in stromal areas surrounding nests of cancer cells. Thus, both human and murine HER2+ breast cancers are characterized by infiltrating innate and adaptive immune cells.

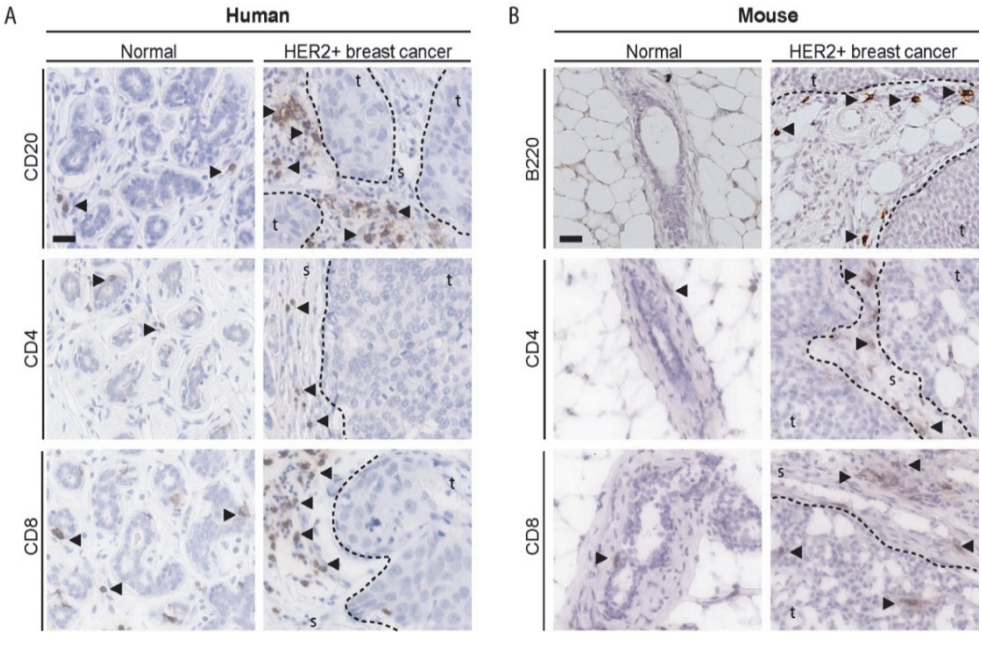

Figure 1. Increased infiltrates of adaptive immune cells in human and mouse HER2+ breast cancer. (A) Presence and location of CD20+, CD4+, and CD8+ cells (brown staining; arrowheads) in human normal mammary glands and human invasive HER2+ breast tumours. Representative images are shown (n=5/group). Scale Bar, 25 μm. Dashed line, stromal-tumour interface; tumour, t; stroma, s. B) Presence and location of $B220^{+}$, $CD4^{+}$ and $CD8^{+}$ cells (brown staining; arrowheads) in normal mammary glands of adult wild-type mice and in HER2+ breast tumours from MMTV-NeuT mice (n=5/group). Representative images are shown. Scale Bar, 25 μm. Dashed line, stromal-tumour interface; tumour, t; stroma, s.

Genetic elimination of the adaptive immune system does not alter latency, multiplicity, outgrowth and phenotype of mammary tumours

To functionally address the modulating role of the adaptive immune system during HER2+ mammary tumourigenesis, we intercrossed MMTV-NeuT mice with Recombination-Activating Gene-2 homozygous null (Rag-$2^{-/-}$) mice deficient for mature T and B lymphocytes, and generated cohorts of immuno-proficient NeuT/Rag-$2^{+/-}$ and immuno-deficient NeuT/Rag-$2^{-/-}$ mice (Fig. S2). We first examined whether absence of the adaptive immune system altered mammary tumour development. Strikingly, complete lymphocyte deficiency did not alter tumour latency (Fig. 2A) or tumour multiplicity (Fig. 2B). In addition, no differences in speed of tumour outgrowth between NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice (Fig. 2C) were observed. These data were further confirmed by comparable *in vivo* BrdU incorporation into breast cancer cells of both cohorts (Fig. S3). In order to address whether absence of the adaptive immune system resulted in mammary tumours with increased immunogenicity, 1x1 mm pieces of mammary tumours isolated from two independent NeuT/Rag-$2^{+/-}$ and two independent NeuT/Rag-$2^{-/-}$ mice were orthotopically transplanted in mammary glands of syngeneic Balb/c mice. Tumour take and latency were independent of the immunological status of the donor mice (Fig. 2D), suggesting that the adaptive immune system does not shape the immunogenicity of mammary tumours in MMTV-NeuT mice. We next evaluated morphological features of mammary glands of wild type RAG-$2^{+/-}$ and RAG-$2^{-/-}$ mice, as well as neoplastic mammary glands (2-months of age) and tumours of NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice. Absence of lymphocytes did not alter the phenotype of normal mammary glands, nor the progression towards early hyperplastic mammary lesions or their phenotype (Fig. 3). In addition, the histological phenotype of primary mammary tumours was comparable between T and B cell proficient and deficient MMTV-NeuT mice (Fig. 3). Taken together, these data indicate that the adaptive immune system has neither a protective nor a promoting role during mammary tumourigenesis in MMTV-NeuT mice.

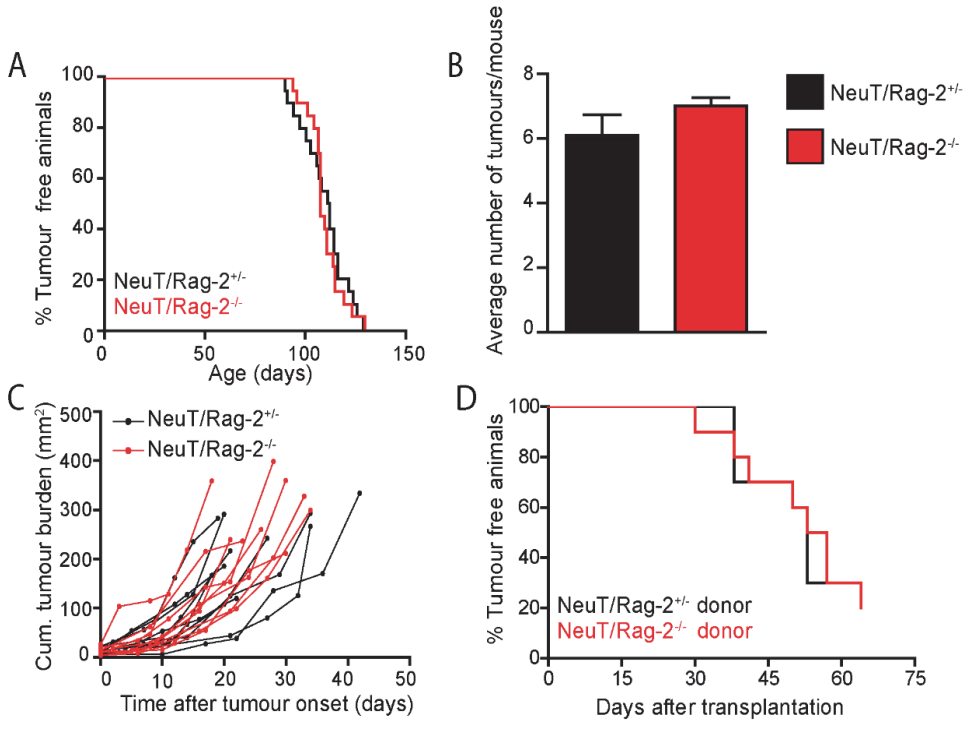

Figure 2. The adaptive immune system does not regulate primary breast cancer development in MMTV-NeuT mice. (A) Kaplan-Meier tumour-free survival curve of NeuT/Rag-2$^{+/-}$ mice and NeuT/Rag-2$^{-/-}$ mice (n=20/group). Mice were considered tumour free until a palpable tumour mass of 2x2 mm was detected. No statistically significant difference was observed as evaluated by Log-Rank test (p=0.84). (B) Average number of primary breast tumours per NeuT/Rag-2$^{+/-}$ mouse and NeuT/Rag-2$^{-/-}$ mouse (n=20/group) evaluated at the day the largest tumour reached 15x15 mm. No statistically significant differences were observed between both cohorts as evaluated by Mann-Whitney test (p=0.83). (C) Cumulative tumour burden (mm^2) followed over time in individual NeuT/Rag-2$^{+/-}$ mice and NeuT/Rag-2$^{-/-}$ mice (n=10/group). (D) Kaplan-Meier tumour-free survival curve of Balb/c mice orthotopically transplanted with 1x1 mm tumour pieces isolated from NeuT/Rag-2$^{+/-}$ mice and NeuT/Rag-2$^{-/-}$ mice (n=10/group). Mice were considered tumour free until a palpable tumour mass of 3x2 mm was detected. No statistically significant difference was observed as evaluated by Log-Rank test (p=0.92).

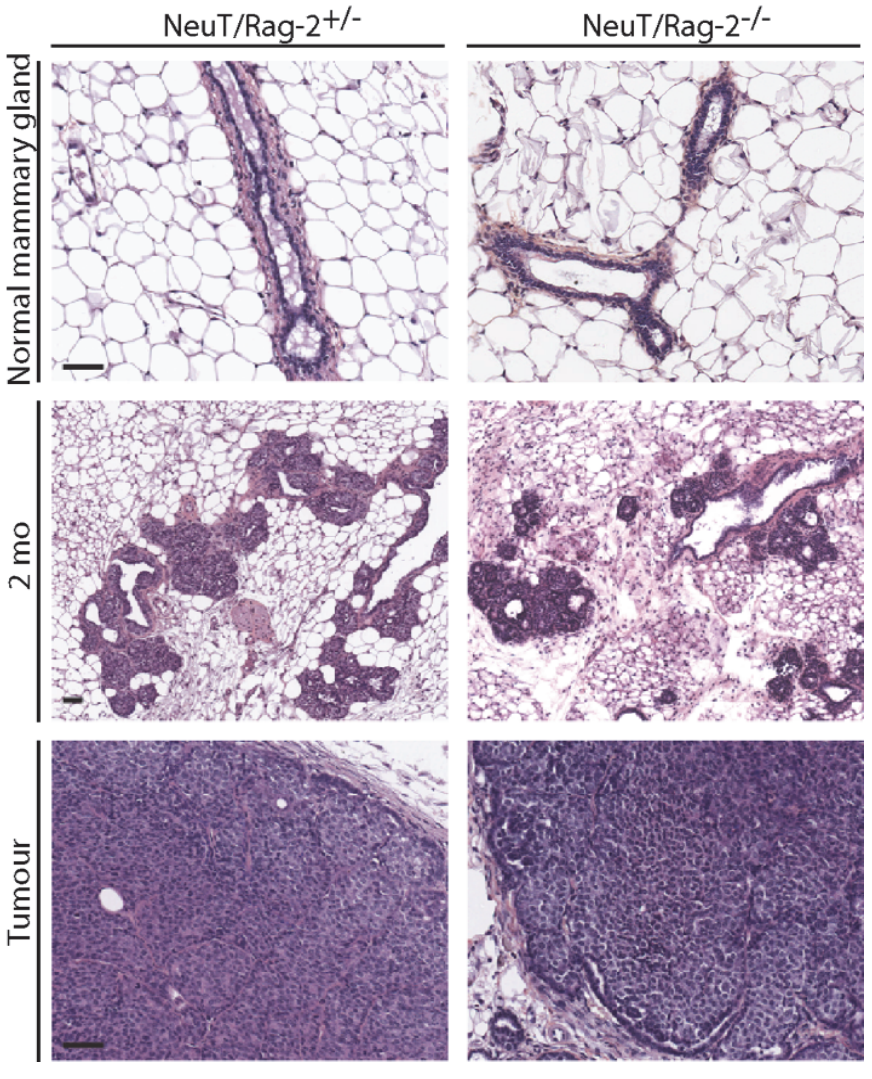

Figure 3. Absence of the adaptive immune system does not alter tumour phenotype. H&E stainings of normal mammary glands from age-matched wild type Rag-$2^{+/-}$ and Rag-$2^{-/-}$ mice, hyperplastic mammary lesions (2-mo of age) and mammary tumours of age-matched (4.5 mo of age) NeuT/Rag-$2^{+/-}$ mice and NeuT/Rag-$2^{-/-}$ mice. Representative images are shown (n=10/group). Scale Bar, 50 μm.

The adaptive immune system does not regulate the inflammatory tumour microenvironment in MMTV-NeuT mice

Adaptive immune cells have been shown to exert pro-tumour functions through activation of tumour promoting innate immune responses [8,10-11,26-27]. Mammary tumourigenesis in MMTV-NeuT mice is accompanied by increased influx of innate immune cells (Fig. 1B, Fig. S1B). We therefore examined whether presence of innate immune cells in HER2+ tumours was regulated by the adaptive immune system. Flow cytometric analysis of $CD45^+$ leukocytes in tumours from both NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice revealed a two-fold increase in $CD45^+$ cells as compared to normal mammary glands (Fig. 4A). However, no significant difference in $CD45^+$ leukocyte infiltrate in tumours of NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice was observed (Fig. 4A). Immunohistochemical analysis of hyperplasias and primary mammary tumours of NeuT/Rag-$2^{-/-}$ and NeuT/Rag-$2^{+/-}$ mice confirmed these results and showed no differences in the degree and location of infiltrating $CD45^+$ leukocytes (Figure 4B). To investigate whether the composition of the inflammatory infiltrates in tumours of the two cohorts was different, we profiled the two major leukocyte populations, *i.e.* $F4/80^+CD11b^+$ macrophages and $Gr1^+CD11b^+$ granulocytes. Both immune cell populations were increased in tumours of NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice as compared to normal mammary tissue (Fig. 4C, D and S4A). However, no significant changes were observed in the magnitude and location of both immune subsets between tumours of NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice (Fig. 4C, D, S4A). In addition, spleens of both tumour-bearing NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice were characterized by accumulation of $Gr1^+CD11b^+$ leukocytes (Fig. S4B).

Cytokines, either secreted by cancer cells or by tumour-associated immune cells, are part of the tumour microenvironment and can influence cancer progression and prognosis [28-29]. We performed cytokine profiling on protein lysates generated from tumours of NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice to dissect whether the adaptive immune system regulates the local cytokine milieu in HER2+ tumours. None of the twenty-five cytokines and growth factors tested (Fig. 5 and data not shown for IL1a, IL1b, IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL12p49, IL12p70, IL13, IL17, Eotaxin, G-CSF, GM-CSF, MIP1b, RANTES and CSF-1) displayed significantly altered levels in tumours samples from NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice. Together, these data indicate that the adaptive immune system does not sculpt the composition or cytokine profile of the inflammatory tumour microenvironment in HER2+ mammary tumours.

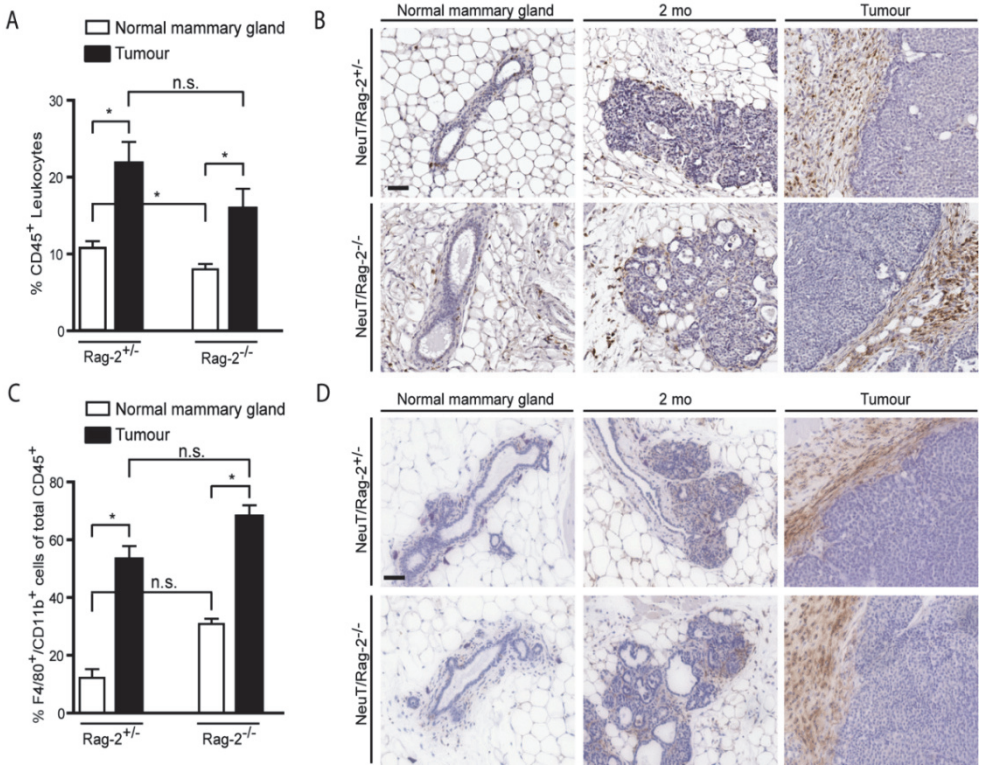

Figure 4. The adaptive immune system does not regulate the inflammatory tumour microenvironment in MMTV-NeuT mice. (A and C) Flow cytometric analysis of $CD45^+$ leukocytes (A) and $F4/80^+CD11b^+$ macrophages (C) in normal mammary glands from age-matched wild type Rag-$2^{+/-}$ and Rag-$2^{-/-}$ mice and tumours of NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice. Data on $CD45^+$ leukocytes are depicted as the mean percentage gated on live cells ± SEM (A) and data on $F4/80^+CD11b^+$ macrophages are depicted as mean percentage gated on live $CD45^+$ leukocytes ± SEM (C). (n=4/wild type cohort; n=8/tumour cohort). *$p<0.05$ by Mann-Whitney test. n.s., not significant by Mann-Whitney test. (B and D) Immunodetection of $CD45^+$ leukocytes (B) and $F4/80^+$ macrophages (D) in normal mammary glands from age-matched wild type Rag-$2^{+/-}$ and Rag-$2^{-/-}$ mice, and in hyperplastic mammary lesions (2-mo of age) and mammary tumours of NeuT/Rag-$2^{+/-}$ mice and NeuT/Rag-$2^{-/-}$ mice. Representative images are shown. Scale Bar, 50 µm.

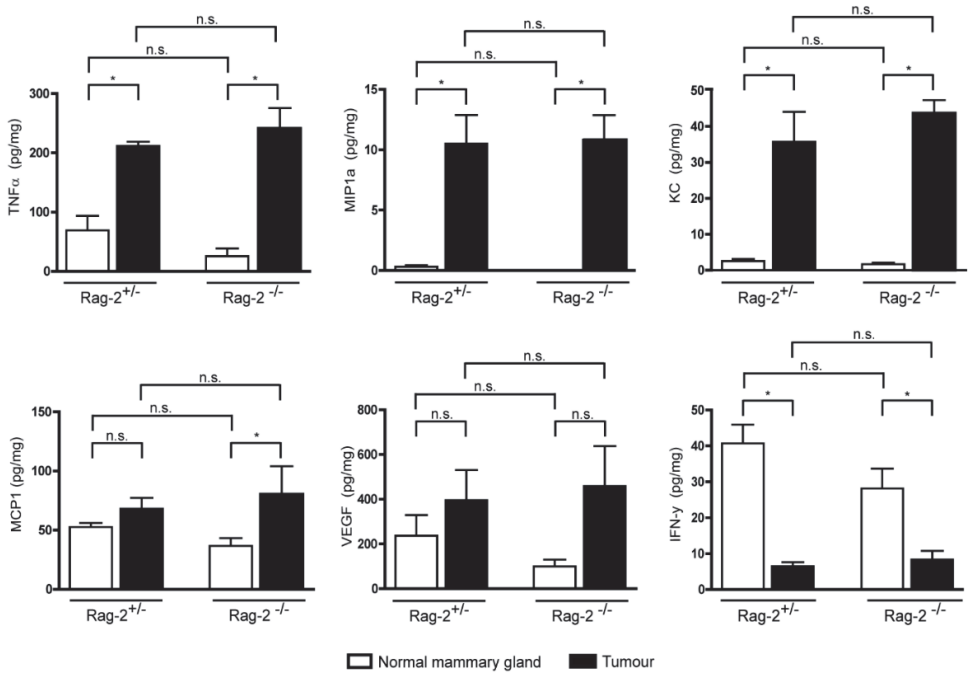

Figure 5. The adaptive immune system does not sculpt the cytokine profile in breast tumours. The cytokine profile in protein lysates of normal mammary glands from age-matched wild type Rag-$2^{+/-}$ and Rag-$2^{-/-}$ mice or mammary tumours (15x15 mm) of NeuT/Rag-$2^{+/-}$ mice and NeuT/Rag-$2^{-/-}$ mice was assessed using the Bio-Plex Pro Cytokine assay. Concentrations of TNFα, MIP1a, KC, MCP-1, VEGF and IFN-γ are shown as pg/mg and are depicted as mean ± SEM (n=5 mice/group). * $p < 0.05$ by Mann-Whitney test. n.s., not significant.

Pulmonary metastasis formation in MMTV-NeuT mice is not regulated by the adaptive immune system

Deficiency of the adaptive immune system did not alter any characteristics of primary HER2+ mammary cancer formation. Yet, it has been reported that lymphocytes can promote mammary cancer metastasis formation [10]. Therefore we set out to investigate spontaneous pulmonary metastasis formation in NeuT/Rag-2$^{+/-}$ and NeuT/Rag-2$^{-/-}$ mice. Serial sections of lungs isolated from NeuT/Rag-2$^{+/-}$ and NeuT/Rag-2$^{-/-}$ mice bearing end-stage mammary tumours were microscopically screened for the presence of metastases. This analysis did not reveal a significant change in metastasis incidence between both tumour cohorts (Fig. 6A). In addition, the size of metastases, the average number of metastases per mouse and the metastasis phenotype were unaffected in lymphocyte deficient MMTV-NeuT mice (Fig. 6B, C, D and S5). In conclusion, spontaneous pulmonary metastasis formation in MMTV-NeuT mice is not influenced by the endogenous adaptive immune system.

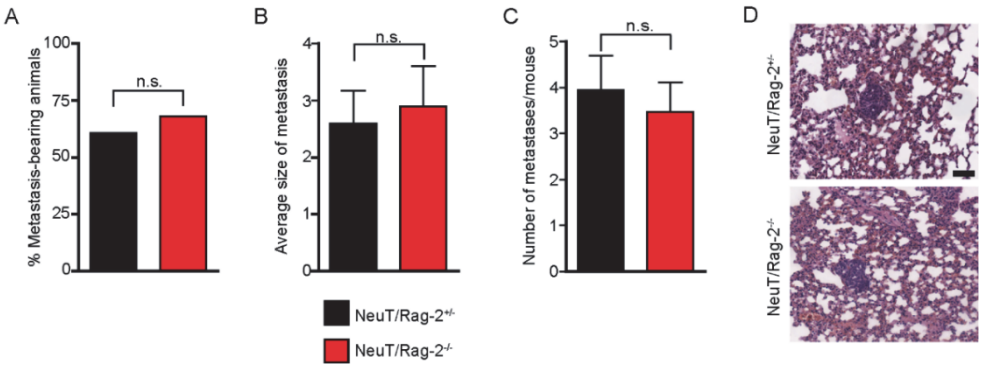

Figure 6. Pulmonary metastasis formation in MMTV-NeuT mice is not regulated by the adaptive immune system. Lungs of mice bearing end-stage mammary tumours were serially sectioned and ~11 sections 135 µm apart were H&E stained and microscopically screened for the presence of metastases (n=15 mice/cohort). (A) Percentage of tumour-bearing NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice with one or more pulmonary metastases. n.s., not significant by Fisher's exact test (p=0.77). (B) The average size of metastasis per mouse was determined by categorizing individual lung metastases based on their average diameter (Fig. S5). n.s., not significant by Mann-Whitney test (p=0.52). Error bars represent SEM. (C) The average number of metastases per metastasis-bearing mouse. n.s., not significant by Mann-Whitney test (p=0.83). Error bars represent SEM. (D) H&E stainings of lung tissue sections with a metastasis from NeuT/Rag-$2^{+/-}$ and NeuT/Rag-$2^{-/-}$ mice. Representative images are shown. Scale Bar, 50 µm.

Discussion

Human HER2+ breast cancers are characterized by influx of adaptive immune cells (Fig. 1A)[17]. Presence of tumour infiltrating lymphocytes and expression of lymphocyte-associated genes in human HER2+ breast cancers have been reported to correlate with a good prognosis [17-18]. Whether this favourable prognosis is actually caused by the increased lymphocyte infiltration cannot be concluded from these clinical observations. In this study, we have investigated whether there is a causal relationship between the adaptive immune system and HER2 driven mammary tumourigenesis and metastasis formation. Using MMTV-NeuT mice, we found that absence of the adaptive immune system did not delay nor accelerate premalignant progression. Likewise, latency, growth, multiplicity, immunogenicity, histology and the inflammatory microenvironment of primary breast tumours arising in T and B cell deficient MMTV-NeuT mice were identical to those of immune proficient MMTV-NeuT mice. In addition, we demonstrate that pulmonary metastasis formation in MMTV-NeuT mice is not dependent on the adaptive immune system. Thus, our findings reveal that spontaneous HER2 driven mammary tumourigenesis and metastasis formation are not suppressed by immunosurveillance mechanisms, nor promoted by the adaptive immune system.

If we put these findings into context with previous studies, it becomes clear that the outcome of the dynamic interplay between adaptive immune system and nascent malignancies can be divided into three scenarios: protection, inertia and promotion [30]. Whereas spontaneous adaptive immune responses protect against chemical-induced sarcoma formation [5-6], viral oncogene driven skin tumourigenesis and lymphotoxin driven hepatocellular carcinoma development are promoted by the adaptive immune system [8-9,11] and large T antigen driven pancreatic cancer is not affected by the adaptive immune system [7]. Both a recent study by DeNardo et al. [10] and our study reveal in two independent transgenic mouse models for *de novo* breast cancer formation that primary mammary tumourigenesis is not influenced by the adaptive immune system. Thus, the tissue of origin likely plays an important role in determining the nature of the interplay between cancer cell and the adaptive immune system.

The absence of immunosurveillance in MMTV-NeuT mice might seem rather surprising, given the reported observation of adaptive immune responses directed against HER2/neu in the MMTV-NeuT mouse model during the pre-

malignant phase [31-32]. In addition, anti-HER2 $CD4^+$ and $CD8^+$ T cell responses have been described in patients with HER2+ breast cancer [33-34]. These observations suggest that the endogenous adaptive immune cell repertoire is not completely devoid of tumour-specific immune cells, and could thus -in theory- be involved in immunosurveillance mechanisms. Tumour transplantation studies have however shown that such spontaneous immune responses failed to reject transplanted $Her2^+$ tumour cells [32] and antibody-mediated depletion of T cells in MMTV-NeuT mice resulted only in a temporary marginal increase in tumour multiplicity [35]. Myeloid derived suppressor cells, regulatory T cells and regulatory dendritic cells have been reported to be involved in suppression of anti-tumour T cell responses in MMTV-NeuT mice [36-38]. Our study extents these observations by showing that -despite reported incomplete tolerance at early stages of tumourigenesis in MMTV-NeuT mice- spontaneous pre-malignant progression, tumour formation and development of metastases are not delayed or phenotypically altered by the unmanipulated adaptive immune system. That said, interventions aimed at increasing immunity towards HER2+ tumours, such as vaccination strategies or antibody therapies, have been reported to overcome the unresponsiveness of the adaptive immune system and result in successful tumour inhibition [39-41]. In addition, the therapeutic effect of anti-Her2 antibody therapy has been reported to depend on the adaptive immune system [42].

Strikingly, pulmonary metastasis formation in the MMTV-NeuT mouse model is not dependent on the adaptive immune system, whereas pulmonary metastasis formation in the MMTV-PyMT mouse model is promoted by the adaptive immune system [10]. These findings indicate that the outcome of the interplay between adaptive immune system and cancers is not solely dependent on the tissue context, but also on the genetic pathways underlying tumour initiation and tumour maintenance. This notion is underscored by recent publications reporting direct instruction of the inflammatory phenotype by particular oncogenes, such as Myc and Ras [43-45]. Thus, the genetic make-up of a particular tumour is critical for determining the nature of its crosstalk with the immune system. Given the heterogeneous nature of breast cancer, it is likely that also distinct subtypes of breast cancer are differently dependent on cancer cell extrinsic processes.

Similarly, functional differences between NeuT and PyMT might explain why the adaptive immune system affects pulmonary metastasis formation in the

MMTV-PyMT mouse model [10] but not in MMTV-NeuT mice. In the MMTV-PyMT mouse model, IL-4 producing $CD4^+$ T cells promoted pulmonary metastasis formation through enhancing epidermal growth factor (EGF) production by macrophages [10]. Macrophage-derived EGF subsequently stimulated epidermal growth factor receptor (EGFR)-dependent metastasis formation. In contrast, mammary tumourigenesis in the MMTV-NeuT mouse model is initiated by overexpression of an activated form of the EGFR family member HER2/ErbB2 [46], which does not require ligand for receptor activation. Hence, the metastatic capacity of NeuT-overexpressing tumours might be a cell-autonomous trait which is independent of EGFR activation by (macrophage-derived) EGF. However, the exact explanation for the observed differences awaits further investigation.

How could we translate these findings to the human situation? Lymphocyte infiltration and expression of a T cell gene expression signature have been shown to correlate with lower recurrence rates of HER2+ breast cancers [17-18]. In view of our data, it is plausible that the presence of lymphocytes in HER2+ breast cancers is not causal to improved metastasis-free survival rates, but rather a consequence of a distinct natural history of the good prognosis HER2+ tumours compared to the poor prognosis tumours. Importantly, the majority of patients analysed in these clinical studies were treated with radiotherapy [17] or chemotherapy [18]. The influx of lymphocytes in tumours of these patients might therefore be induced by cancer cells dying in response to anti-cancer therapy [47-48]. Thus, presence of lymphocytes or expression of a T cell metagene in treated HER2+ breast cancers might be a predictive rather than a prognostic indicator. Our data revealing absence of immunosurveillance in spontaneous HER2+ breast tumourigenesis are supported by epidemiological studies of cancer incidence in patients with a suppressed adaptive immune system. These patients suffer from increased incidence of viral-associated malignancies; however, the relative risk for breast cancer is not increased in these patients, and might actually be lower than in the general population [3,19-20].

Our data suggest that without a careful dissection of the dependence of different (breast) cancer subtypes on the adaptive immune system, therapies aimed at suppressing the adaptive immune system in unselected patient groups might actually have no effect, or even exacerbate disease. Mouse tumour models recapitulating many different subtypes of human (breast) cancer are available [49-51] and will be extremely useful for

predicting which patients would benefit from therapeutic targeting of the adaptive immune system. A deeper understanding of tumour- intrinsic and -extrinsic characteristics that influence the nature of the crosstalk with the immune system might open opportunities to suppress tumour-promoting immune responses and tip the balance over in favour of anti-tumour immune responses.

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Statement of author contributions

MC, JJ and KEdV conceived the study and participated in its design and coordination. MC, KV, C-SH, ENS, CWD and KEdV performed the intercrossings, established the tumour cohort, performed mouse colony maintenance and monitoring, performed weekly tumour palpations and sections. KV, TP, C-SH and ENS participated in handling of mouse tissues and performed immunohistochemical stainings. MC, CWD and KEdV performed microscopy. Flow cytometry and cytokine arrays were performed by MC, KV, KW and C-SH. MC, JJ and KEdV analysed data. MC, JJ and KEdV drafted the manuscript. All authors read and approved the final manuscript.

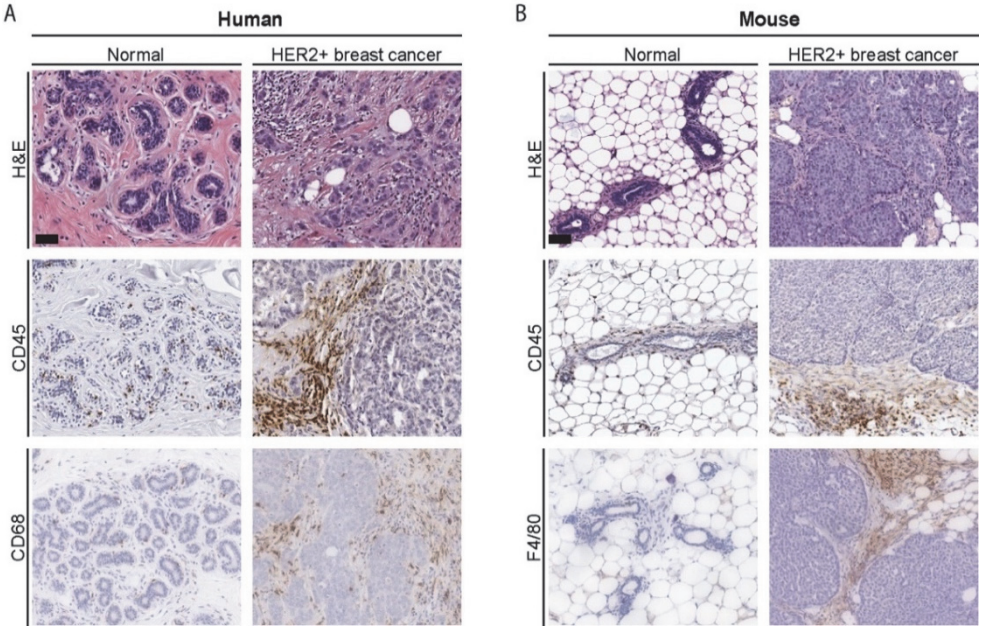

Figure S1. Increased infiltration of immune cells in human and mouse HER2+ breast cancer. Hematoxylin and eosin (H&E)-stained sections of human normal mammary glands, human invasive HER2+ breast tumors (A) and normal mammary glands of adult wild-type mice and HER2+ breast tumors of MMTV-NeuT mice (B). Immunodetection of $CD45^+$ leukocytes (brown staining) and macrophages ($CD68^+$ cells in human tissues and $F4/80^+$ cells in mouse tissues; brown staining). Representative images are shown. (n = 5 for human tissues, n = 8 for mouse tissues). Scale Bar, 50 µm.

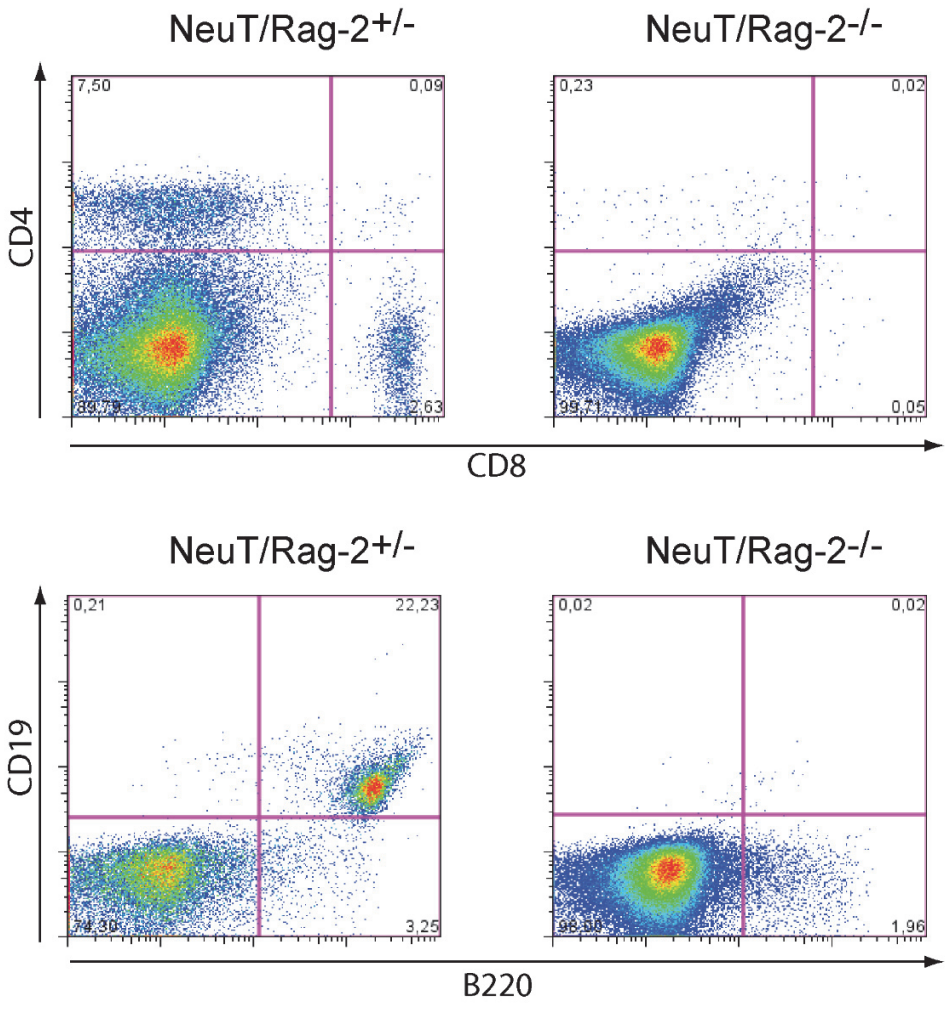

Figure S2. Absence of CD4⁺ and CD8⁺ T cells and CD20⁺/CD19⁺ B cells in NeuT/Rag-2$^{-/-}$ mice. Flow cytometric analysis of CD4⁺ and CD8⁺ T cells and CD19⁺/B220⁺ B cells in blood from NeuT/Rag-2$^{+/-}$ mice and NeuT/Rag-2$^{-/-}$ mice. Representative dot plots including percentages of CD4⁺ and CD8⁺ T cells (upper panel) and CD19⁺/B220⁺ B cells (lower panel) gated on live CD45⁺ leukocytes are shown.

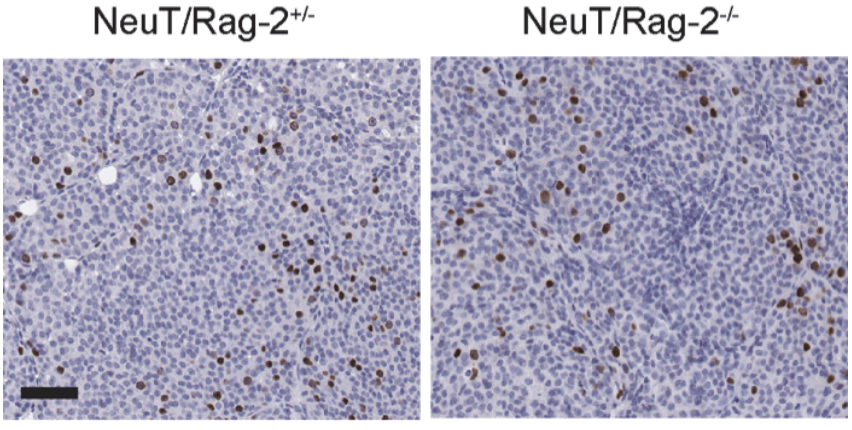

Figure S3. The adaptive immune system does not modulate proliferation of breast cancer cells in MMTV-NeuT mice. Immunodetection of proliferating cells positive for BrdU (brown staining). Ninety minutes before sacrifice, tumour-bearing mice were injected with BrdU (n= 7/group). BrdU incorporation was assessed by immunohistochemistry. Scale Bar, 50 μm.

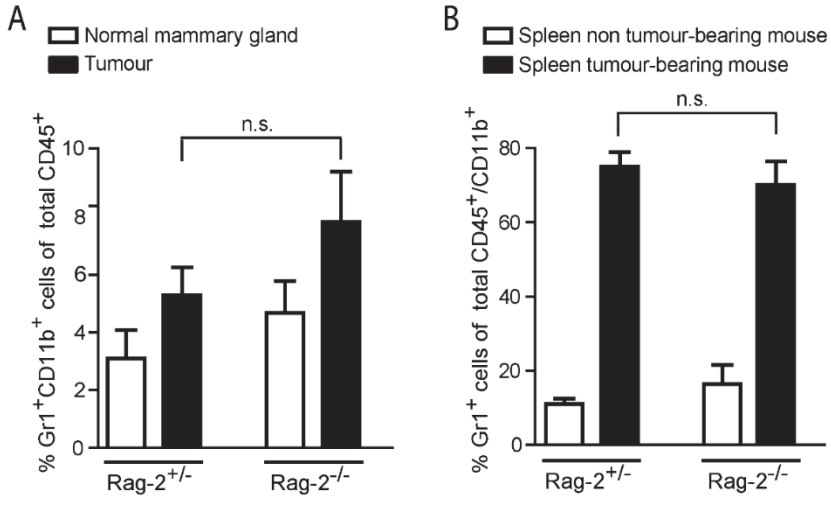

Figure S4. Accumulation of Gr1+CD11b+ granulocytes in the tumormicroenvironment and spleen is not altered by the absence of the adaptive immune system. (A) Flow cytometric analysis of $Gr1^+CD11b^+$ granulocytes in normal mammary glands from age-matched wild type Rag-$2^{+/-}$ and Rag-$2^{-/-}$ mice and in tumors of NeuT/Rag-$2^{+/-}$ mice and NeuT/Rag-$2^{-/-}$ mice. Data on $Gr1^+CD11b^+$ leukocytes are depicted as mean percentage gated on live $CD45^+$ leukocytes ± SEM (n = 4 per wild type cohort; n = 8 per tumor cohort). n.s., not significant by Mann-Whitney test. (B) Flow cytometric analysis of $Gr1^+$ leukocytes in spleens from age-matched wild type Rag-$2^{+/-}$ and Rag-$2^{-/-}$ mice (non tumour-bearing) and in spleens of tumour-bearing NeuT/Rag-$2^{+/-}$ mice and NeuT/Rag-$2^{-/-}$ mice. Data on $Gr1^+$ leukocytes are depicted as mean percentage gated on live $CD45^+/CD11b^+$ leukocytes ± SEM (n = 4 per wild type cohort; n = 8 per tumor cohort). n.s., not significant by Mann-Whitney test.

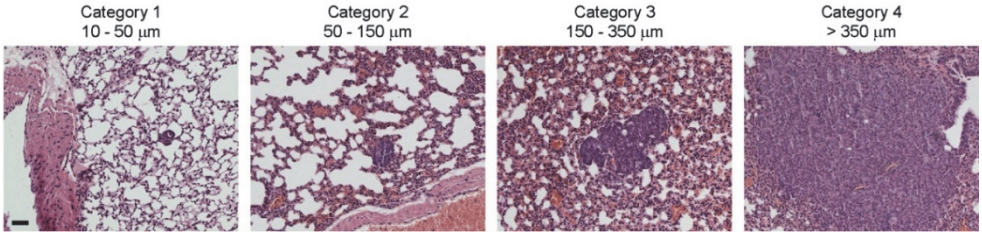

Figure S5. Size-categories of pulmonary metastases. H&E stainings of lung metastases from tumor-bearing NeuT/Rag-2$^{+/-}$ mice representing the different size categories. Size of individual metastases was determined by measurement of the average diameter (n = 15 mice/cohort). Category 1: 10 – 50 μm diameter; Category 2: 50 – 150 μm diameter; Category 3: 150 - 350 μm diameter; Category 4: > 350 μm diameter. Scale Bar, 50 μm.

Supplemental Table 1a

Detailed information about antibodies used for immunohistochemistry

Antibody specificity	Clone (company)	Dilution	Antigen retrieval	Tissue
Mouse anti-human CD45	HI30 (eBioscience[1])	1:100	Citra buffer (Biogenex[2])	Paraffin[9]
Mouse anti-human CD4	4B12 (Monosan[3])	1:100	Citra buffer (Biogenex)	Paraffin
Mouse anti-human CD8	C8/144B (DAKO[4])	1:200	Citra buffer (Biogenex)	Paraffin
Mouse anti-human CD20	L26 (DAKO)	1:1000	Citra buffer (Biogenex)	Paraffin
Mouse anti-human CD68	PG-M1 (DAKO)	1:150	3 min Proteinase K (DAKO)	Paraffin
Rat anti-mouse CD45	30-F11 (BD biosciences[5])	1:200	Citra buffer (Biogenex)	Paraffin
Rat anti-mouse CD4	GK1.5 (hybridoma supernatant, NKI[6])	1:1000	none	Cryo[10]
Rat anti-mouse CD8	2.43 (hybridoma supernatant, NKI)	1:1000	none	Cryo
Rat anti-mouse B220	RA3-6B2 (BD biosciences)	1:500	Citra buffer (Biogenex)	Paraffin
Rat anti-mouse F4/80	CI:A3-1 (Serotec[7])	1:300	none	Paraffin
Mouse anti-BrdU	Bu20a (DAKO)	1:100	Citra buffer (Biogenex)	Paraffin
Biotinylated goat anti-rat	(Southern Biotech[8])	1:300	NA	
Biotinylated goat anti-mouse	(DAKO)	1:300	NA	

[1]San Diego, CA; [2]San Ramon, CA; [3]Uden, the Netherlands; [4]Denmark; [5]San Diego, CA; [6]purified hybridoma supernatant produced by protein core facility of NKI; [7]Dusseldorf, Germany; [8]Birmingham, AL

[9] Collected tissues and tumors were fixed for 24 hours in 10% neutral buffered formalin. Tissues were embedded in paraffin. For immunohistochemical analysis, 5μm thick paraffin sections were cut, deparaffinized, and stained.

[10] Tissue samples were frozen directly in glycerol-based freezing medium (OCT). 10-μm thick OCT-embedded tissue sections were cut using a Leica CM3050 S cryostat. Sections were air-dried, fixed in ice-cold acetone for 5-min, incubated for 10 min with avidin solution (DAKO), PBS washed, incubated for 10 min with biotin solution (DAKO), PBS washed and blocked for 30-min in blocking buffer (5% goat serum/2.5% bovine serum albumin/PBS). Antibodies were diluted in 0.5X blocking buffer and incubated with tissue sections for 1.5-hr at room temperature. Sections were washed with PBS and incubated with secondary antibody for 45-min at RT

Supplemental Table 1b

Detailed information about antibodies used for flow cytometry

Antibody specificity	Clone (company)	Dilution
FITC anti-mouseCD4	GK1.5 (eBioscience)	1:200
PE anti-mouse CD8	53-6.7 (eBioscience)	1:200
FITC anti-mouse CD19	MB19-1 (eBioscience)	1:200
PE anti-mouse B220	CD45R RAS3-6B2 (eBioscience)	1:200
APC anti-mouse CD45	30-F11 (eBioscience)	1:200
PE anti-mouse CD11b	M 1/70 (eBioscience)	1:200
FITC anti-mouse GR1	RB6-8C5 (eBioscience)	1:200
FITC anti-mouse F4/80	BM8 (eBioscience)	1:200

References

1. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004; **21**: 137-148.
2. Willimsky G, Blankenstein T. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature* 2005; **437**: 141-146.
3. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* 2006; **6**: 24-37.
4. Pardoll DM. Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 2003; **21**: 807-839.
5. Shankaran V, Ikeda H, Bruce AT*, et al.* IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 2001; **410**: 1107-1111.
6. Dunn GP, Bruce AT, Ikeda H*, et al.* Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002; **3**: 991-998.
7. Casanovas O, Hicklin DJ, Bergers G*, et al.* Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 2005; **8**: 299-309.
8. de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 2005; **7**: 411-423.
9. Haybaeck J, Zeller N, Wolf MJ*, et al.* A lymphotoxin-driven pathway to hepatocellular carcinoma. *Cancer Cell* 2009; **16**: 295-308.
10. DeNardo DG, Barreto JB, Andreu P*, et al.* CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 2009; **16**: 91-102.
11. Andreu P, Johansson M, Affara NI*, et al.* FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* 2010; **17**: 121-134.
12. Perou CM, Sorlie T, Eisen MB*, et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747-752.
13. Linn SC, Van 't Veer LJ. Clinical relevance of the triple-negative breast cancer concept: genetic basis and clinical utility of the concept. *Eur J Cancer* 2009; **45 Suppl 1**: 11-26.
14. Burstein HJ. The distinctive nature of HER2-positive breast cancers. *N Engl J Med* 2005; **353**: 1652-1654.
15. Desmedt C, Haibe-Kains B, Wirapati P*, et al.* Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. *Clin Cancer Res* 2008; **14**: 5158-5165.
16. Slamon DJ, Godolphin W, Jones LA*, et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; **244**: 707-712.
17. Alexe G, Dalgin GS, Scanfeld D*, et al.* High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. *Cancer Res* 2007; **67**: 10669-10676.

18. Rody A, Holtrich U, Pusztai L*, et al.* T-cell metagene predicts a favorable prognosis in estrogen receptor-negative and HER2-positive breast cancers. *Breast Cancer Res* 2009; **11**: R15.
19. Stewart T, Tsai SC, Grayson H*, et al.* Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation. *Lancet* 1995; **346**: 796-798.
20. Engels EA, Goedert JJ. Human immunodeficiency virus/acquired immunodeficiency syndrome and cancer: past, present, and future. *J Natl Cancer Inst* 2005; **97**: 407-409.
21. Muller WJ, Sinn E, Pattengale PK*, et al.* Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 1988; **54**: 105-115.
22. Boggio K, Nicoletti G, Di Carlo E*, et al.* Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. *J Exp Med* 1998; **188**: 589-596.
23. Shinkai Y, Rathbun G, Lam KP*, et al.* RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 1992; **68**: 855-867.
24. Weijer K, Uittenbogaart CH, Voordouw A*, et al.* Intrathymic and extrathymic development of human plasmacytoid dendritic cell precursors in vivo. *Blood* 2002; **99**: 2752-2759.
25. Evers B, Speksnijder EN, Schut E*, et al.* A tissue reconstitution model to study cancer cell-intrinsic and -extrinsic factors in mammary tumourigenesis. *J Pathol* 2010; **220**: 34-44.
26. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. *Nat Immunol* 2004; **5**: 971-974.
27. Shanker A. Adaptive control of innate immunity. *Immunol Lett* 2010.
28. Seruga B, Zhang H, Bernstein LJ*, et al.* Cytokines and their relationship to the symptoms and outcome of cancer. *Nat Rev Cancer* 2008; **8**: 887-899.
29. Balkwill F. Tumour necrosis factor and cancer. *Nat Rev Cancer* 2009; **9**: 361-371.
30. de Visser KE. Spontaneous immune responses to sporadic tumors: tumor-promoting, tumor-protective or both? *Cancer Immunol Immunother* 2008; **57**: 1531-1539.
31. Takeuchi N, Hiraoka S, Zhou XY*, et al.* Anti-HER-2/neu immune responses are induced before the development of clinical tumors but declined following tumorigenesis in HER-2/neu transgenic mice. *Cancer Res* 2004; **64**: 7588-7595.
32. Kmieciak M, Morales JK, Morales J*, et al.* Danger signals and nonself entity of tumor antigen are both required for eliciting effective immune responses against HER-2/neu positive mammary carcinoma: implications for vaccine design. *Cancer Immunol Immunother* 2008; **57**: 1391-1398.
33. Disis ML, Calenoff E, McLaughlin G*, et al.* Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res* 1994; **54**: 16-20.

34. Peoples GE, Goedegebuure PS, Smith R*, et al.* Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci U S A* 1995; **92**: 432-436.

35. Park JM, Terabe M, Donaldson DD*, et al.* Natural immunosurveillance against spontaneous, autochthonous breast cancers revealed and enhanced by blockade of IL-13-mediated negative regulation. *Cancer Immunol Immunother* 2008; **57**: 907-912.

36. Melani C, Chiodoni C, Forni G*, et al.* Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity. *Blood* 2003; **102**: 2138-2145.

37. Ambrosino E, Spadaro M, Iezzi M*, et al.* Immunosurveillance of Erbb2 carcinogenesis in transgenic mice is concealed by a dominant regulatory T-cell self-tolerance. *Cancer Res* 2006; **66**: 7734-7740.

38. Norian LA, Rodriguez PC, O'Mara LA*, et al.* Tumor-infiltrating regulatory dendritic cells inhibit CD8+ T cell function via L-arginine metabolism. *Cancer Res* 2009; **69**: 3086-3094.

39. Rovero S, Amici A, Carlo ED*, et al.* DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J Immunol* 2000; **165**: 5133-5142.

40. Spadaro M, Lanzardo S, Curcio C*, et al.* Immunological inhibition of carcinogenesis. *Cancer Immunol Immunother* 2004; **53**: 204-216.

41. Rolla S, Ria F, Occhipinti S*, et al.* Erbb2 DNA vaccine combined with regulatory T cell deletion enhances antibody response and reveals latent low-avidity T cells: potential and limits of its therapeutic efficacy. *J Immunol* 2010; **184**: 6124-6132.

42. Park S, Jiang Z, Mortenson ED*, et al.* The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* 2010; **18**: 160-170.

43. Shchors K, Shchors E, Rostker F*, et al.* The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. *Genes Dev* 2006; **20**: 2527-2538.

44. Soucek L, Lawlor ER, Soto D*, et al.* Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med* 2007; **13**: 1211-1218.

45. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 2004; **6**: 447-458.

46. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001; **2**: 127-137.

47. Casares N, Pequignot MO, Tesniere A*, et al.* Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med* 2005; **202**: 1691-1701.

48. Apetoh L, Ghiringhelli F, Tesniere A*, et al.* Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007; **13**: 1050-1059.

49. Herschkowitz JI, Simin K, Weigman VJ*, et al.* Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol* 2007; **8**: R76.

50. Derksen PW, Liu X, Saridin F*, et al.* Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* 2006; **10**: 437-449.

51. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer* 2007; **7**: 645-658.