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Original Citation:
Early onset and enhanced growth of autochthonous mammary carcinomas in C3-deficient Her2/neu transgenic mice / Bandini S; Curcio C; Macagno M; Quaglino E; Arigoni M; Lanzardo S; Hysi A; Barutello G; Consolino L; Longo DL; Musiani P; Forni G; Iezzi M; Cavallo F. - In: ONCOIMMUNOLOGY. - ISSN 2162-4011. - 2:9(2013), pp. 1-14.

Availability:
This version is available http://hdl.handle.net/2318/138317 since

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(Article begins on next page)
This is an author version of the contribution published on:

Questa è la versione dell’autore dell’opera:

[Oncoimmunology. 2013 Sep 1;2(9):e26137. Epub 2013 Sep 12.]

ovvero [Bandini S et al., Oncoimmunology, 2, Landes Bioscience, 2013]

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Early onset and enhanced growth of autochthonous mammary carcinomas in C3 deficient Her2/neu transgenic mice

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Keywords: Complement, Immunosurveillance, Her2/neu, mammary cancer, genetically engineered mice
Abstract

Beside its role against infections, complement is an important component of the tumor microenvironment. However, its role as a regulator of tumor growth is still debated. To assess the role of complement in the progression of autochthonous mammary carcinogenesis we have crossed C3 deficient BALB/c mice (C3KO) with BALB/c females transgenic for the activated rat Her2/neu oncogene (neuT), which develop autochthonous mammary cancer with 100% penetrance. A significantly earlier onset of the first palpable tumor, higher tumor multiplicity and a dramatic increase in the tumor growth rate were found in neuT-C3KO as compared to neuT females. The accelerated tumor onset observed in neuT-C3KO mice was paralleled by the earlier onset of spontaneous lung metastases and by an increase in Her2/neu expression on tumor cells. The percentage of tumor infiltrating immune cells in neuT-C3KO mice was similar to that found in neuT females, except for a significant increase in the frequency of T regulatory cells in neuT-C3KO tumors. Interestingly, the enhanced immunosuppression determined by C3 deficiency influenced the immunogenic phenotype of autochthonous mammary tumors, since tumor cells from neuT-C3KO displayed significantly delayed and reduced incidence as compared to those from neuT mice, when transplanted into syngeneic immunocompetent hosts. Finally, increased vessel permeability was evident in neuT-C3KO tumors, even if a similar number of tumor vessels was found in neuT and neuT-C3KO tumors. All together, these data point to the existence of a crucial role for the complement in the immunosurveillance and editing of Her2/neu autochthonous mammary tumors.
Introduction

There is a growing body of evidence to suggest that the host immune system plays an important role in modulating the development and spread of cancer, particularly in its early stages. Many of the cellular and molecular immune mechanism enhancing\textsuperscript{1, 2} and hampering\textsuperscript{3-5} the early stages of tumor growth have been worked out in detail.

Complement is one of the innate immune defense systems whose activation products and membrane-bound regulators interact with cells of the adaptive immune response. Its effector functions in host defense and inflammation can also have a role during tumorigenesis. They can be triggered at the tumor development site by danger associated molecular pattern-elicited activation or via the infiltration of inflammatory cells, which express complement components.\textsuperscript{6-8} Spontaneous anti-tumor antibodies also play an important role in complement activation at the tumor site.\textsuperscript{9-11} Nevertheless, the study of the role of the complement in tumor growth and metastases has not been explored a great deal and has given contradictory results.\textsuperscript{12, 13} Most studies have evaluated the growth of transplantable tumors in complement deficient mice, while very few data\textsuperscript{14} about the influence of the complement on the development of autochthonous tumors are available.

Genetically engineered mice (GEM) that naturally develop tumors as a consequence of a defined gene alteration, which is artificially inserted in their genome, allow scientists to study the progressive stages of carcinogenesis and the natural occurrence of cancer.\textsuperscript{15, 16} These mice are of special interest when they develop tumors that recapitulate both the molecular and genetic changes found in human cancers. The slow tumor progression, the natural occurrence of invasion and metastasis, and the presence of a long-lasting interaction between the tumor, its environment and the host immune system allow a direct assessment of the weight played by immune mechanisms in affecting both the pace of tumor progression and the intensity of the metastatic spread to be carried out.\textsuperscript{17} The progressive stages of the mammary lesions displayed inbred BALB/c female mice
expressing the activated rat Her2/neu oncogene under the transcriptional control of the mouse mammary tumor virus promoter (neuT mice) mimic several features of the devastating progression and metastasizing ability of Her2/neu+ breast cancer in women. From the 4th week of life, cells in the not yet completely formed mammary ducts of neuT mice start to express the Her2/neu protein, proliferate and form side buds. These initial lesions appear as foci of atypical hyperplasia in carcinomatous progression, that gradually coalesce to give rise to large in situ carcinoma, and then invasive microscopic carcinoma. The shift from preneoplastic to invasive lesions is associated with the vigorous proliferation of microvessels that express αvβ3 integrin. The microscopic lesions become palpable through the skin, and gradually occupy the bulk of the subcutaneous fibro adipose tissue of the mammary pad. Moreover, waves of tumor cells disseminate from the primary tumor to the bone marrow during the 4th to 9th weeks. Neoplastic cells also settle in lung capillaries and vessels to form roundish intravascular nodules that eventually invade the parenchyma.

Although the increased expression of the activated rat Her2/neu is sufficient to drive mammary lesions in neuT mice, the pace of their progression is modulated by the type of extracellular matrix, by perforin dependent immunosurveillance, and by the tumor-induced negative regulations of the immune response. Cancer progression is also modulated by the cytokines in the tumor microenvironment. Four transcriptional networks whose hub genes (IL-1β, TNF-α, IFN-γ, and MCP-1/CCL2) are directly linked to inflammation become increasingly expressed with neuT carcinogenesis progression. The presence of MCP-1/CCL2 in the reactive stroma around tumoral lesions enhances the rate of cancer progression. This is markedly slower in neuT MCP-1/CCL2 KO mice. On the other hand, the tumor-elicited expression of IFN-γ plays a major role in tumor inhibition. In neuT IFN-γ KO mice, more intense tumor angiogenesis goes along with faster cancer progression.

In the current study we evaluated the consequences of complement system deficiency during mammary cancer progression by generating neuT mice that were deficient for C3 (neuT-C3KO
mice). The early onset and faster progression of mammary cancer observed in these neuT-C3KO mice show that the complement hampers the growth of Her2/neu+ autochthonous mammary lesions. This protective effect of the complement rests on multiple factors, such as a less immunosuppressive microenvironment, a limitation of Her2/neu expression and proliferative ability of tumor cells, and an alteration in tumor vascularization. In addition, tumors that arise in neuT-C3KO mice are more immunogenic than those in neuT mice, showing that complement should be considered one of the many mechanisms involved in the immunosurveillance of Her2/neu+ tumors.
Results

Spontaneous anti-tumor antibodies increase during cancer progression in neuT mice. As cancer progresses, antibodies able to bind tumor cells are frequently found in cancer patients.\textsuperscript{9-11} These are one of the main mechanisms of complement activation at the tumor site.\textsuperscript{9} We thus evaluated whether antibodies that are able to bind neuT tumor cells (TUBO cells) are present in the sera of neuT mice (Fig. 1A). Sera were collected at 8, 19 and 34 weeks of age, when the mammary glands of neuT mice display diffuse atypical hyperplasia lesions, small multifocal carcinomas and large coalescing invasive carcinomas, respectively. The amount of serum antibodies that was able to bind TUBO cells was comparable in neuT and wild type BALB/c mice at 8 weeks of age. At 19 weeks it increased in both BALB/c and neuT mice, but it was significantly higher in the latter. At 34 weeks, the amount of serum antibodies binding TUBO cells was further increased in neuT sera, while it remained unchanged in BALB/c mice (Fig. 1A). A similar pattern was found in neuT-C3KO mice (Supplementary Fig. S1A).

Deposition of C3 fragments occurs in neuT carcinomas. Spontaneous antibodies directed against tumor-associated antigens can trigger complement activation at the tumor site. To evaluate whether complement activation occurs in the mammary glands of neuT mice during tumor development, we monitored the presence of C3 fragments in the mammary tumor lesions using immunofluorescence staining (Fig. 1B). C3 cleavage products, detected with an anti-C3b/iC3b/C3c antibody, were clearly evident in the tumor vasculature and stroma of neoplastic mammary lesions in neuT females (Fig. 1B, left panel), whereas they were absent, as expected, in neuT-C3KO tumors (Fig. 1B, right panel). While C3 cleavage suggests that the activation of complement proteins had occurred at the tumor site and had likely led to the generation of complement effectors, it remains uncertain whether this results in the assembly of the membrane attack complex on neuT tumor cells, as they markedly express the decay-accelerating factor (CD55; Supplementary Fig. S1B).
The onset of autochthonous Her2/neu carcinomas is accelerated in neuT-C3KO mice. To assess whether the marked complement activation occurring at the tumor site affects the pace of mammary lesions, we compared mammary tumor onset and growth in neuT and neuT-C3KO mice (Fig. 2A-C). In neuT-C3KO mice, early onset of the first palpable lesion (Fig. 2A) and higher tumor multiplicity (Fig. 2B) were found with respect to what observed in neuT mice. Early tumor onset was also evident in C3 heterozygous neuT mice (neuT-C3Het, Supplementary Fig. S2). Moreover, tumors arising in neuT-C3KO mice grew faster than those arising in neuT mice (Fig. 2C). A comparative whole mount microscopic analysis of the mammary glands showed that the progression from hyperplastic lesions to in situ carcinoma and invasive solid carcinomas is accelerated in neuT-C3KO mice. While large microscopic lesions, equivalent to in situ carcinomas, become evident from the 15th and 17th week of age in neuT mice (Fig. 2D-F), they are already evident at 11 weeks of age in neuT-C3KO mice (Fig. 2G). By week 15, these lesions are notably enlarged (Fig. 2H) while at week 17 their further expansion and clumping occupy the whole mammary gland (Fig. 2I). Despite their different growth kinetics, carcinomas of neuT and neuT-C3KO mice displayed a similar grade of differentiation (Fig. 2L and N).

The accelerated tumor onset observed in neuT-C3KO mice was paralleled by the early onset of spontaneous lung metastases (Table 1; Figure 2M and O) that are evident at week 15, when all neuT mice were still free of lung metastases. All together, these data point to the crucial role C3 activation plays in hampering Her2/neu autochthonous mammary cancer.

Accelerated carcinoma progression in neuT-C3KO mice is associated with an increase in Her2/neu expression. As compared to mammary lesions of neuT mice (Fig. 3A), those of neuT-C3KO display a higher expression of the proliferation marker proliferating cell nuclear antigen (PCNA; Fig. 3B and C). This matches the faster growth rate of clinically evident carcinomas (Fig. 2C). Since Her2/neu is the driving force behind neuT tumors, we compared Her2/neu protein
expression in tumors from neuT and neuT-C3KO mice. Western blot quantification showed that Her2/neu is expressed 6-8 times more in neuT-C3KO than in neuT tumors (Fig. 3D). Her2/neu appeared to be expressed mainly at the cell membrane of neuT tumor cells, with various intensities, under confocal microscopy (Fig. 3E-G; Supplementary Fig. S1A). Higher and more broader expression was found in neuT-C3KO tumor cells (Fig. 3H-L; Supplementary Fig. S1A). These data suggest that C3 cleavage at the tumor site limits Her2/neu expression and consequently the proliferative ability of neuT tumor cells.

The tumor microenvironment in neuT-C3KO mice is more immunosuppressive. We then evaluated whether C3 deficiency in neuT mice also has an impact on the composition of tumor infiltrating leukocytes. C3 deficiency did not cause any difference in the percentage of either circulating, as shown by the white blood cell count (Supplementary Fig. S3), or tumor infiltrating, as shown by immunohistochemistry, leucocytes (Supplementary Fig. S4). No significant differences in the frequency of tumor-infiltrating macrophage, CD4+ and CD8+ T cells, B cells and myeloid-derived suppressor cells (MDSC) were found in tumors of neuT and neuT-C3KO mice on the flow cytometric analysis (Fig. 4A). The amount of MDSC present in the spleen was also similar (Supplementary Fig. S5A). By contrast, when the frequency of T regulatory (T reg) cells was evaluated, a significant increase (p = 0.02) was found in neuT-C3KO tumors as compared to neuT ones (Fig. 4B), and a similar trend was also found in the spleens (Supplementary Fig. S5B). The increase in T reg cells was confirmed by immunohistochemistry, showing that FoxP3+ cells are more abundant in neuT-C3KO tumors (Fig. 4C). This finding is in line with the notion that reduced signaling through the receptors for the complement fragments C3a and C5a supports the development of FoxP3+ induced T reg cells.28 The same receptors also have a role in limiting the functions of naturally occurring T reg cells.29 C3a cannot be generated in neuT-C3KO mice, while C5a is present, even if strongly reduced as compared to neuT mice (Fig. 4D, p = 0.002).
Carcinomas arising in neuT-C3KO mice are more immunogenic than those of neuT mice. To assess whether the enhanced immunosuppression caused by C3 deficiency can influence the immunogenic phenotype of autochthonous tumors arising in neuT mice, the tumorigenicity of mammary carcinomas from neuT and neuT-C3KO mice was compared using transplantation approaches. When transplanted into naïve syngeneic immunocompetent hosts, tumor cells from neuT-C3KO mice displayed reduced take and delayed incidence (Fig. 5A) and growth kinetics (Fig. 5B) as compared to those from neuT mice. Similar results were found when the same tumor cells were injected into C3KO BALB/c mice (Fig. 5C and D). Altogether, these data support the hypothesis that tumors from C3 deficient mice undergo decreased levels of immunoediting. Moreover, since tumors from neuT mice displayed significantly earlier incidence when injected into C3KO (Fig. 5C) as compared to immunocompetent (Fig. 5A) recipients, C3 also has a role to play in counteracting the growth of immunoedited tumors.

Tumor vessels in C3 deficient mice are enlarged and display an increased permeability. The increased proliferative ability of tumors and their suppressive microenvironment accounts for the earlier incidence and faster growth of carcinomas in neuT-C3KO mice. However, a fast growing cancer needs a high blood supply. Because complement is known to have an important role in modulating vascularization,14, 30 we evaluated tumor microvascular density by endothelial cell staining in neuT (Fig. 6A) and neuT-C3KO (Fig. 6B) tumors of the same size.

While a similar number of intra-tumor vessels was found in neuT and neuT-C3KO tumors (Fig. 6C), tumor-associated vessels in neuT carcinomas (Fig. 6A) appeared to be thinner than those seen in neuT-C3KO carcinomas (Fig. 6B). A statistical assessment of vessel diameter confirmed that tumor-associated vessels are significantly larger in carcinomas growing in the absence of C3 (Fig. 6D). The larger diameter of neuT-C3KO tumor-associated vessels went along with an increased number of NG2+ pericytes associated with endothelial cells, as compared to neuT tumors (Fig. 6E and F). The small increase in NG2+ pericytes became much more evident when only
mature pericytes were visualized by αSMA staining (Fig. 6G and H). The high number of by αSMA+ mature pericytes in neuT-C3KO carcinomas shows that the lack of C3 affects pericyte investment of endothelial cells and, in a more marked way, their maturation.

Because tumor-induced angiogenesis is largely dependent on vascular endothelial growth factor (VEGF), and neuT carcinomas produce VEGF26, 31, an analysis of serum VEGF levels in neuT and neuT-C3KO mice was performed. A significant increase in VEGF levels went along with the expansion of mammary lesions both in neuT and neuT-C3KO mice. However, no differences were found in the amount of VEGF in neuT and neuT-C3KO mice with the same tumor burden (Fig. 6I). This finding was in accordance with the observation that neuT and neuT-C3KO mice have the same number of tumor vessels. The thinner diameter and the minor tumor vessel area found in neuT carcinomas can be ascribed to the direct effect of complement deposition on endothelial cells.32

We next used the dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) technique to assess whether the wider area of tumor-associated vessels found in neuT-C3KO carcinomas and their prominent mature and stable phenotype result in major functional differences. The quantitative analysis of the DCE-MRI images inside the tumor regions showed that the vessel permeability of neuT-C3KO carcinomas is enhanced in comparison to neuT ones (Fig. 7A, Ktrans = 8.2 x 10⁻⁵ and 1.6 x 10⁻⁴ for neuT and neuT-C3KO, respectively). Similarly, carcinomas from neuT-C3KO mice displayed a markedly higher plasmatic volume percentage (Vp), which reflect the percentage of tumor area occupied by vessels (Fig. 7B, Vp = 0.012 and 0.035 for neuT and neuT-C3KO, respectively, p = 0.05). Considering the amount of injected contrast agent extravasation, we found wider regions showing low - if any - contrast enhancement in neuT carcinomas, corresponding to a higher measured necrosis extent (Fig. 7C, 53.3% and 30.6% for neuT and neuT-C3KO, respectively). Moreover, the mean apparent diffusion coefficient (ADC) value measured in neuT-C3KO tumors was higher than in neuT tumors (Fig. 7D, 8.6 x 10⁻⁴ and 1.1 x 10⁻³ mm²/sec, respectively). This difference is likely to be the result of different stromal or extracellular
extravascular space content, or related to the different cellularity of the tumor microenvironment following the unequal kinetics of carcinoma growth in neuT and neuT-C3KO mice.
Discussion

While several of the mechanisms by which the immune system enhances or impairs tumor growth have been worked out in detail, the complement’s role in this respect has been relatively under-explored. The few data so far available are contradictory. A few studies point towards a protective role played by complement against malignant cells,\textsuperscript{6, 28, 33} whereas others highlight tumor promoting roles of complement.\textsuperscript{14, 34-37} This disagreement is not so surprising, considering that complement takes part in a plethora of distinct immunological and inflammatory processes,\textsuperscript{13} each of them having different types of effect in different tumor models. With the present study, we are adding fresh information on the role of C3, the central component of the complement cascade, in the autochthonous Her2/neu driven mammary carcinogenesis of neuT mice. These mice provide a well-defined study model of Her2/neu mammary carcinogenesis.\textsuperscript{18}

We herein show the accumulation of activated C3 fragments in tumor vessels and stroma of the areas of the mammary gland undergoing neoplastic transformation, but not in the surrounding tissues. This finding suggests that complement activation was specific for tumors, rather than systemic. The presence of spontaneous antibodies that react with antigens expressed on the tumor cell membrane is one of the possible reasons of this local activation.\textsuperscript{38} Indeed, as frequently happens in cancer patients,\textsuperscript{9-11} spontaneous anti-tumor antibodies increased during tumor progression in neuT mice.

To investigate the consequences of complement activation in the tumor microenvironment, we generated neuT-C3KO mice and evaluated their mammary tumor progression. We found that complement deficiency in neuT mice causes the early onset of palpable mammary tumors and spontaneous lung metastases, higher tumor multiplicity, and a dramatic increase in the tumor growth rate. This dramatically aggressive behavior discloses the role played by complement-dependent mechanisms in halting Her2/neu carcinogenesis in neuT mice.
The more aggressive behavior acquired by mammary carcinogenesis in neuT-C3KO mice appears not to be due to the lack of a single activity of complement, but rather of multiple distinct activities requiring the C3 activation. First, the drastic increase in carcinoma growth rate in neuT-C3KO mice was paralleled by an increase in Her2/neu expression. This suggests that one of the consequences of C3 activation at the tumor site is the down-regulation of Her2/neu. Her2/neu down-regulation is one of the direct mechanisms by which anti-Her2/neu antibodies inhibit carcinogenesis progression in neuT mice.39, 40 Spontaneous anti-tumor antibodies, part of which probably directed against Her2/neu, were found to increase during tumor progression in both neuT and neuT-C3KO mice. Complement activation may markedly enhance their ability to down-modulate Her2/neu expression. Alternatively, tumor cells expressing the higher amount of Her2/neu could be eliminated following C3 deposition activated by anti-tumor antibodies. The killing of tumor cells is probably not due to the formation of the membrane attack complex, as neuT tumor cells express CD55 that is responsible for the accelerated dissolution of C3 and C5 convertases and eliminates the release of anaphylotoxins.41 Instead, C3 coated Her2/neu overexpressing tumor cells might be killed by natural killer (NK) cells that express the complement receptor 3 (CR3). CR3, upon stimulation via iC3b, can induce cytotoxic responses in NK cells, especially when class I molecules of the major histocompatibility complex (MHC I) are poorly expressed by the target cell.42 Indeed, an inverse correlation exists between Her2/neu expression and the expression of MHC I and the components of the antigen-processing machinery.43 In this way, Her2-overexpressing tumor cells escape TCR guided cytotoxic T lymphocyte-mediated lysis, while are more susceptible to natural killer (NK) cell-mediated lysis. Moreover, signaling via the Her2/Her3 pathway in breast carcinoma cell lines may lead to the enhanced ligand recognition by the activating receptor NK group 2, member D (NKG2D) expressed by NK cells and T cells.44

Carcinomas expanding in neuT-C3KO mice display a marked increase in the frequency of Treg cells in the tumor microenvironment. This may contribute to their not hindered expansion. Complement is known to have an integral role in modulating the induction and function of T reg
cells and thus immunological tolerance.\textsuperscript{28, 29} During the early events of T cell activation, signaling through C3a and C5a receptors onto CD4\textsuperscript{+} T cells and dendritic cells (DC) up-regulates the expression of costimulatory molecules and increases T cell survival.\textsuperscript{45} Moreover, C3a and C5a receptor signaling into circulating T reg cells modulates their function by inducing FoxP3 down-regulation.\textsuperscript{29} On the other hand, the absence of this signaling deflects naïve T cells into induced T reg cells\textsuperscript{28} and frees natural occurring T reg function.\textsuperscript{29} In neuT-C3KO mice, C3a should not be generated, while the C5 convertase-like activity of thrombin\textsuperscript{46, 47} allows the generation of C5a, that was actually present in neuT-C3KO mice, but in lower amounts than in neuT mice. Consequently, the complete lack of C3a receptor signaling and reduced signaling through C5a receptor in neuT-C3KO mice may explain the observed T reg increase.

The highly suppressive tumor microenvironment of neuT-C3KO carcinomas may be instrumental for their escape form immunoediting and selection.\textsuperscript{48} Actually, neuT-C3KO carcinomas display an impaired growth when transplanted into syngeneic immunocompetent BALB/c mice.

Altogether these data suggest that the complement system is a key factor in the interrelationship between tumor and the host immune system, participating in the immunosurveillance and editing of the tumor, and may in itself be sufficient to explain the increased malignancy of tumors developing in neuT-C3KO mice. Nevertheless, beyond its role in immunity, complement also has a role in modulating tissue-remodeling processes, including neo-angiogenesis.\textsuperscript{30} Whether the complement system is pro- or anti-angiogenic is still controversial. Few studies – and none on breast cancer - have evaluated the role of the complement system in \textit{in vivo} tumor angiogenesis, and in doing so have given to contradictory results.\textsuperscript{14, 36, 49} The only study that evaluated the consequences of a genetic C3 blockade\textsuperscript{14} showed reduced tumor angiogenesis, with reduced microvascular density in tumors from mice that were heterozygous for C3, suggesting the existence of a pro-angiogenic role for C3. In the present study we show that C3 activation on the tumor vasculature results in impaired angiogenic function. In neuT-C3KO mice, no differences in
tumor vascular density were observed as compared to neuT mice. However, vessels in neuT-C3KO mice were wider and with a mature and functional phenotype, as indicated by the presence of increased number of both young and mature pericytes and by the increased Vp as measured by MRI. Due to their mature structure and ameliorated permeability and water diffusion within the tumor, these vessels result more capable to provide an adequate oxygen and nutrient supply required by the fast growing tumor, leading to a decrease in tumor necrotic areas in tumors from neuT-C3KO as compared to those of neuT mice.

In conclusion, our data support the notion that complement has a protective role against the development of autochthonous Her2/driven mammary cancer in neuT mice. However, as expected when dealing with a system with numberless distinct activities, this protective role does not appear to rest on a single function of crucial importance, but rather on the concomitance of multiple distinct activities concurring in hampering the onset and expansion of mammary carcinomas in neuT mice. While these data should be seriously taken into account in a clinical perspective, the fact that the protection observed stems from the concurrence of several distinct mechanisms may explain why in a different cancer-prone GEM model the lack of C3 may result in an attenuated tumor phenotype and even abrogate tumor establishment and progression. It is conceivable that the genetic background, the transgene and its promoter, the fact that the transgene protein product may or may not be a self tolerated protein, the kind of tumor induced by the transgene, and the tumor penetrance of the model may all contribute to determining how complement influences tumor progression and leads to different outcomes. However, the molecular definition of a few peculiarities of the tumor may allow exploiting the complement as a major contribute to the immunosurveillance and control of cancer progression in clinics.
Materials and methods

Mice. BALB/c C3KO (C3KO) mice,\textsuperscript{50} kindly provided by Prof. Marina Botto (Imperial College, UK), were crossed with Her2/neu transgenic BALB/c (neuT) male mice.\textsuperscript{18} Heterozygous C3 and neu\textsuperscript{+} (neuT-C3Het) male mice were then crossed with C3KO females and the progeny was genotyped in order to identify the C3KO neu\textsuperscript{+} males (neuT-C3KO) that were to be crossed with C3KO females. The neuT-C3KO progeny was used for the experiments. The mammary glands of all neuT mice were inspected and palpated twice a week for tumor appearance. Individual neoplastic masses were measured with calipers in two perpendicular diameters and the average value was recorded. Progressively growing masses > 1 mm in mean diameter were regarded as tumors and measured with caliper in two perpendicular diameters. Neoplastic growth was monitored until the first tumor that exceeded a mean diameter of 10 mm was found, at which point mice were euthanized for ethical reasons. Tumor multiplicity was calculated as the cumulative number of incident individual tumors/total number of mice and is reported as mean ± SEM. Tumor volume was calculated as \((X^2 Y) / 2\), where \(X\) and \(Y\) represent the short and long tumor diameters, respectively. Tumor burden was calculated as the sum of individual tumor volumes of each mouse and is reported as mean ± SEM. All mice were maintained at the Molecular Biotechnology Center, University of Torino, in specific pathogen free conditions (Allentown Caging Equipment, Allentown Inc., Allentown, NJ) and treated in conformity with current European guidelines and policies. The Bioethical Committee of the University of Turin approved the experimental plan.

Cells. TUBO carcinoma cells that expressed H-2K\textsuperscript{d} and neu molecules are from a mammary carcinoma that arose in a neuT mouse.\textsuperscript{51} Cells were cultured in DMEM with Glutamax 1 (DMEM, Life Technologies) supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen) at 37°C in a humidified 5% CO\textsubscript{2} atmosphere.
Flow Cytometry Assay for serum antibody detection. Sera collected from BALB/c and neuT mice at different time points were diluted 1:20 in PBS with 0.1% sodium azide and 2% bovine serum albumin (BSA, Sigma-Aldrich) (PBS-azide-BSA) and incubated for 30 minutes at 4°C with $2 \times 10^5$ TUBO cells pretreated with Fc receptor blocker (anti CD16/CD32; BD Biosciences, 01245B) for 15 min at 4°C. After washing with PBS-azide-BSA, the cells were incubated for 30 minutes with polyclonal rabbit anti-mouse immunoglobulins/FITC (reacting with all mouse IgG subclasses, mouse IgM and mouse IgA; from DakoCytomation, F 0313), washed two times with PBS-azide-BSA and then resuspended in PBS-azide-BSA that contained 1 mg/ml of propidium iodide for the acquisition. Flow cytometry was performed on a CyAn ADP (DakoCytomation, Beckman Coulter, Milan, Italy). Results were expressed as mean fluorescence intensity (MFI) and analyzed using Summit 4.2 (DakoCytomation) software. Differences in MFI were analyzed using the Student's t test.

Morphological analyses. The whole mount and immunohistochemical preparation of mammary glands were carried out as previously described in detail.23 Digital pictures were taken with a Nikon Coolpix 995 (Nital, Turin, Italy) mounted on a stereoscopic microscope (MZ6; Leica Microsystems, Milan, Italy). Mammary glands were frozen in a cryo-embedding medium (OCT, Bioptica) for histological and immunohistochemical analyses. Sections were incubated with the following primary antibodies: mouse monoclonal antibodies (MoAb) to Proliferating Cell Nuclear Antigen (PCNA) (Dako Corporation, M0879); anti-CD105 (550546), anti-CD31 (550274) (both from BD Biosciences); anti-FoxP3 (eBioscience, 14-5773); followed by the appropriate secondary antibodies. Immunocomplexes were detected using Streptavidin Peroxidase (Thermo Scientific-Lab Vision Corporation, Bioptica) and the DAB Chromogen System (Dako Corporation) or the Bajoran Purple Chromogen System (Biocare Medical), or NeutrAvidin™ Alkaline Phosphatase Conjugated (Thermo Scientific-Pierce Biotechnology, EuroClone) and Vulcan Fast Red (Biocare Medical) or
the DAB Chromogen System (Dako Corporation). The percentage of PCNA\(^+\) tumor cells and the number and area of CD31\(^+\)/CD105\(^+\) vessels were evaluated on the digital images of 10 neuT and 10 neuT-C3KO tumors (5 x400 microscopic fields per tumor) by two pathologists, independently and in a blind fashion. Vessels area (in pixels) was evaluated with Adobe Photoshop by selecting vessels with the lasso tool and reporting the number of pixels indicated in the histogram window. Lung samples were fixed in formalin and embedded in paraffin. To optimize the detection of microscopic metastases and ensure systematic uniform and random sampling, lungs were cut transversely, to the trachea, into 2.0-mm-thick parallel slabs with the first cut being in a random position of the first cut in the first 2.0 mm of the lung, resulting in 5-8 slabs per lung. The slabs were then embedded cut surface down. Sections were stained with hematoxylin/eosin and immunohistochemistry with the anti-Her2/neu antibody for the evaluation of spontaneous metastasis of all the experimental groups. Metastases were counted independently by two pathologists in a blind fashion.

For immunofluorescence analysis cryo-sections were tested with rabbit polyclonal anti-human Her2/neu (Dako Corporation), rat monoclonal anti-CD105 and anti-CD31, all from BD Biosciences, mouse monoclonal anti-\(\alpha\) smooth muscle actin (Sigma Aldrich), rabbit polyclonal anti NG-2 (Millipore), hamster anti-mouse CD31 mAb (Chemicon), and rat anti-mouse C3b/iC3b/C3c (HyCult biotech) antibodies. Appropriate Alexa 488 and 546 labeled secondary antibodies were then utilized. Nuclei were stained with TO-PRO®-3 Iodide (all from Molecular Probes).

Protein preparation and immunoblotting. Total protein extracts were obtained from mammary glands from 17-week-old neuT and neuT-C3KO mice by using a boiling buffer containing 0.125 M Tris/HCl, pH 6.8 and 2.5 % sodium dodecyl sulphate (SDS). Protein level evaluation of Her2 was performed on total lysates. 30 \(\mu\)g of proteins were separated by SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (BioRad). Membranes were blocked in 5 % Blotto non-fat milk (Santa Cruz) Tris buffered saline (TBS)-Tween buffer (137 mM NaCl, 20 mM Tris/HCl, pH 7.6,
0.1 % Tween-20) for 1 h at 37° C then incubated with appropriate primary and secondary antibodies in 1 % milk TBS-Tween buffer overnight at 4° C and for 1 h at room temperature, respectively and visualized by enhanced chemiluminescence (ECL®, Amersham Biosciences). The following antibodies were used: anti-Her2/neu (Ab-3) monoclonal antibody (Calbiochem, OP15); anti-actin mouse monoclonal antibody (Santa Cruz, sc-69879), and goat anti-mouse IgG HRP-conjugated (Sigma, A4416). Her2/neu protein modulation in the mammary glands of neuT-C3KO mice was calculated relative to Her2/neu mean expression in the mammary glands of neuT mice, normalized on the actin loading control and expressed as percentages using Quantity One software.

Blood collection and C5a and VEGF determination. Blood samples were collected from the retroorbital sinus at the indicated time points from neuT and neuT-C3KO mice, and were allowed to clot for 2 hours at room temperature before centrifugation for 20 minutes at 1000 x g. Sera were then isolated from the clot and stored at -20°C or -80°C until used. The presence of C5a and VEGF in the sera was determined by the commercial Elisa kit for C5a, (USCN) and Quantikinine VEGF, (R&D) immunoassay kit, respectively, according to the manufacturer’s directions. For VEGF detection, supplied standards were used to generate a standard curve.

Cytometric identification of tumor infiltrating leukocytes. For infiltrating-cell phenotypic analyses, fresh primary tumor specimens of 8-10 mm mean diameter from neuT and neuT-C3KO mice were finely minced with scissors and then digested by incubation with 1 mg/ml collagenase IV (Sigma Aldrich) in RPMI-1640 (Life Technologies) at 37° C for 1 hour in an orbital shaker. After washing in PBS supplemented with 2% fetal calf serum (GIBCO), the cell suspension was incubated in an erylise buffer (155mM NH₄Cl, 15.8mM Na₂CO₃, 1mM EDTA, pH 7.3) for 10 minutes at RT. After washing in RPMI-1640 supplemented with 10% FBS, the cell suspension was passed through a 70-µm pore cell strainer, centrifuged at 1400 rpm for 10 minutes and resuspended in an erylise buffer. Cells were collected, washed, re-suspended in PBS, treated with Fc receptor
blocker (anti CD16/CD32, BD Bioscences, 01245B), and stained with the following antibodies: anti-mouse CD3e PerCP, (553067), anti-mouse CD11b APC-Cy7 (557657), anti-mouse Ly-6G and Ly-6C FITC (551460), anti-mouse B220 Pacific blue (558108) all from BD Biosciences; anti-mouse CD4 PE/Cy7 (100528) and anti-mouse F/480 PE (122615) from BioLegend; anti-mouse CD45 APC (130-091-811) (Miltenyi Biotech); anti-mouse CD8 FITC (11-0081-86) and anti-mouse CD25 APC (102012) (eBioscience). To detect FoxP3+ T regulatory cells, samples were permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences) and stained with anti-mouse/rat Foxp3 FITC (FJK-16s) (eBioscience). Samples were acquired and analyzed on the CyAn ADP (DakoCytomation, Beckman Coulter) using Summit 4.3 software (DakoCytomation).

Tumor cell isolation from primary carcinoma specimens and in vivo transplantation. Primary tumor specimens obtained from 8-10 mm mean diameter spontaneous mammary carcinomas developed in neuT and neuT-C3KO mice (each time 2-4 spontaneous tumors from the same mouse; n = 5) were finely minced with scissors and then digested by incubation with 1 mg/ml collagenase IV (Sigma Aldrich) in RPMI-1640 (Life Technologies) at 37°C for 1 hour in an orbital shaker. After washing in PBS supplemented with 2% fetal calf serum (GIBCO), the cell suspension was incubated in an erylise buffer for 10 minutes at RT and then washed twice with cold PBS. Cell pellets were re-suspended in Trypsin + 0.05% EDTA (GIBCO) for 15 minutes at 37°C and disaggregated with serological pipette. Tumor cells were washed once in PBS +2% FBS, passed through a 70 μm pore cell strainer (Becton Dickinson, San Jose, CA) to separate the cell components from stroma and aggregates, and re-suspended in endotoxin-free PBS. One million viable cells were injected subcutaneously into the flank of wild type BALB/c or C3KO BALB/c mice (Charles River Laboratories, Calco, Italy). Tumor growth was monitored weekly as described above.
Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI). Magnetic resonance images were acquired on anesthetized mice using an Aspect M2 MRI System (Aspect Magnet Technologies Ltd., Netanya, Israel) working at 1 Tesla. Mice were placed supine inside a solenoid Tx/Tr coil with an inner diameter of 3.5 cm. A phantom filled with diluted ProHance (Bracco Imaging SpA) was included in the field of view (FOV), close to each animal, to allow correction for potential spectrometer variation. After the scout image was acquired, a T2-weighted (w) anatomical image was obtained using a Fast Spin Echo sequence (TR 2500 sec; TE 41 msec; number of slices 8; slice thickness 1.5 mm; FOV 40 mm; matrix 128 x 128; four averages; acquisition time 2 min 40 sec). The baseline tumor T1 map was acquired by using a variable flip-angle Gradient-Echo (VFA-GRE) sequence (7 flip angles in the range 15°-160°). The DCE-MRI dynamic protocol was carried out using an axial T1w 3D spoiled Gradient Echo sequence with three initial pre-contrast images and 47 dynamic post-contrast images with the following parameters: TR/TE = 40/1.8 msec, flip angle = 60°, number of slices = 10, slice thickness = 1.5 mm, FOV = 40 mm, matrix = 128 x 128. The Gd-containing serum albumin binding contrast agent (Phenoquant, Cage Chemicals) was injected into the tail vein of neuT (n = 2) and neuT-C3KO (n = 4) mice bearing 6 mm mean diameter autochthonous mammary tumors in the IV mammary glands, at a dose of 0.05 mmol/kg.

The acquired raw DCE-MRI data were analyzed using a quantitative method and by implementing a two-compartment extended Tofts model on in-house developed C++ software, which yielded the relevant parametric maps (Ktrans and Vp). A two-tailed unpaired Student’s t-test was used to compare mean tumor parametric maps in neuT and neuT-C3KO mice. Diffusion Weighted Images (DWI) were acquired by using a Spin-Echo sequence with seven b-values between 0 and 600 sec/mm². The Apparent Diffusion Coefficient (ADC, mm²/sec) was calculated by fitting the obtained images as a function of b-values on a pixel-by-pixel basis.
Acknowledgements

This work was supported by grants from the Italian Association for Cancer Research (IG 5377 and IG 11675), Fondazione Ricerca Molinette Onlus, the University of Turin, and the Compagnia di San Paolo (Progetti di Ricerca Ateneo/CSP). We thank Dr Dale Lawson for his revision and editing of the manuscript.
Captions to figures

Figure 1. Increase in circulating spontaneous anti-tumor antibodies and C3 fragment deposition on mammary tumor lesions in neuT mice.
A: anti-TUBO antibody titer in the sera of 8, 19 and 34 week-old neuT (n = 6; black bars) and BALB/c (n = 5; grey bars) mice. Results are expressed as mean ± SEM of the mean fluorescence intensity. Differences in antibody titers were evaluated using a two-tailed Student’s t test (* p = 0.03; ** p = 0.008). B: Confocal microscopy of frozen tumor sections labeled with anti-C3b/iC3b/C3c antibody (red), anti-CD31 (green) and TO-PRO®-3 Iodide for nuclei (blue). Original magnification, ×400. Images are representative of ten neuT and neuT-C3KO tumors analyzed.

Figure 2. Accelerated pace of ErbB-2 mammary carcinogenesis and metastatization in neuT-C3KO mice.
A, B: Incidence (A), and multiplicity (B) of mammary carcinomas in female neuT (n = 26, continuous line) and neuT-C3KO mice (n = 10, dashed line). Earlier incidence (*** p = 0.0001, Log-rank Mantel-Cox Test) and higher tumor multiplicity (* p = 0.02; ** p = 0.004; *** p < 0.0001, two-tailed Student’s t test) were found in neuT-C3KO as compared to neuT mice. C: Time required for a 2 mm mean diameter tumor to reach a 8 mm threshold. neuT-C3KO (white bar) vs. neuT (black bar) *** p < 0.0001, two-tailed Student’s t test. D-I: Whole mounts of the fourth (inguinal) mammary gland. The central oval black shadows (N) are the intra-mammary lymph nodes. Arrows indicate tumor multifocal hyperplastic lesions appearing as black spots with a roundish shape along the path and within the ducts. Normal tumor progression in neuT mice (D-F) is accelerated in neuT-C3KO mice with more extended hyperplastic foci and small in situ tumors evident at 11 weeks of age (G), and small tumors already palpable at 15 weeks (H). Larger lesions and tumor masses filled most of the mammary fat pad at week 17th (I). Magnification x6.3. L-O:
Histological and immunohistochemical staining for Her2/neu of mammary tumor lesions (L, N) and lungs (M, O) from 17 week-old neuT and neuT-C3KO mice. Magnification x400.

Figure 3. Increased proliferation and expression of Her2/neu in mammary tumors of neuT-C3KO mice.

A, B: Immunohistochemical staining with anti-PCNA, showing a lower number of positive cells in neuT mice (A) compared to neuT-C3KO mice (B). Magnification x400. Results are representative of 10 neuT and 10 neuT-C3KO analyzed tumors. C: Percentage of PCNA\(^+\) cells. neuT (black bar) vs. neuT-C3KO (white bar) *** \(p < 0.0001\), two-tailed Student’s \(t\) test. D: Her2/neu (upper panel) and actin (lower panel) protein levels measured by Western blot in mammary glands from neuT (lane 1 and 2) and neuT-C3KO (lane 3 and 4) mice. The numbers under each line indicate the ratio between Her2 and actin protein level values as measured by Quantity One software. Three experiments were performed and a single representative example is shown. E-L: Confocal microscopy of frozen tumor sections from neuT and neuT-C3KO mice labeled with anti-Her2/neu antibody (green) and TO-PRO®-3 Iodide for nuclei (blue). Original magnification, \(\times 400\). Results are representative of 8 neuT and 8 neuT-C3KO analyzed tumors.

Figure 4. Autochthonous mammary tumors from neuT-C3KO mice have a more suppressive microenvironment.

A, B: Infiltrates in neuT and neuT-C3KO tumors were analyzed by flow cytometry. Mammary tumors from neuT (\(n = 6\); black bars) and neuT-C3KO (\(n = 7\); white bars) mice were dissociated and stained for CD45, CD11b, F4/80, CD3, CD4, CD8, Gr-1 and B220 (A) or CD3, CD25, CD4 and FoxP3 (B). CD45\(^+\) leukocytes were gated and macrophages were identified as CD11b\(^+\) F4/80\(^-\), CD4 T cells as CD3\(^+\) CD4\(^+\), CD8 T cells as CD3\(^+\) CD8\(^+\), B cells as B220\(^+\)CD11b\(^-\) (A). CD45\(^+\) CD11b\(^+\) leukocytes were gated and MDSC cells were identified as GR-1\(^+\) cells (A). CD3\(^+\) leukocytes were gated and T reg cells were identified as CD4\(^+\) CD25\(^+\) FoxP3\(^+\) (B). Bars represent
the percentage of positive cells ± SEM. Four independent experiments were performed and a single representative example is shown. C: Immunohistochemical staining of neuT and neuT-C3KO mammary tumors for FoxP3 showing a prominent increase in FoxP3+ cells in tumor stroma. Results are representative of 8 neuT and 8 neuT-C3KO tumors analyzed. D: Sera from 15-17 weeks-old neuT (n = 13; black bar) and neuT-C3KO (n = 12; white bar) mice were tested for C5a protein presence by ELISA. Three independent experiments were performed and a single representative example is shown. Results are expressed as mean ± SEM of the OD. Differences between the two experimental groups were evaluated using a two-tailed Student’s t test (* p = 0.02; ** p = 0.002).

Figure 5. Increased immunogenicity of tumors from neuT-C3KO mice.

A, B: Incidence (A), and tumor growth (B) of mammary carcinomas from neuT (n = 8; grey lines) and neuT-C3KO mice (n = 8; black lines) injected subcutaneously in BALB/c females. A: reduced (50 %) and later incidence (*** p < 0.0001, Log-rank Mantel-Cox Test) was found in tumors from neuT-C3KO as compared to those from neuT mice. B: Tumor growth plotted as mean tumor diameter. Each line refers to an individual tumor. Five independent experiments were performed and a single representative example is shown. C, D: Incidence (C), and tumor growth (D) of mammary carcinomas from neuT (n = 3; grey lines) and neuT-C3KO mice (n = 8; black lines) injected subcutaneously in C3KO females. C: reduced (62.5 %) and later incidence (*** p=0.0008, Log-rank Mantel-Cox Test) was found in tumors from neuT-C3KO as compared to those from neuT mice. D: Tumor growth plotted as mean tumor diameter. Each line refers to an individual tumor. Three independent experiments were performed and a single representative example is shown.

Figure 6. C3 deficiency does not modify absolute vessel number but changes vessel architecture.

A, B: Immunohistochemical staining for endothelial cells (CD31 and CD105, red) showing that tumor vessels in neuT mice (A) are as numerous as in neuT-C3KO (B), but in neuT-C3KO mice
they are wider. C, D: Quantification of the number (C) and lumen area (D) of vessels in neuT (black bar) and neuT-C3KO (white bar) mice. Results are represented as the mean ± SEM in 7 x100 microscopic fields of 10 tumors. E-H: Confocal microscopy analysis of samples stained with anti-CD31 (red) and anti NG2 (E and G, green) or anti SMA (F and H, green) showing that, as compared to vessels in neuT tumors (E, F), those in neuT-C3KO (G, H) are well formed and display a higher number of both young (NG2⁺) and mature (SMA⁺) pericytes surrounding endothelial cells. I: VEGF amounts in the sera of neuT (n = 5, continuous grey line) and neuT-C3KO (n = 6, dotted grey line) mice was evaluated by ELISA and plotted together with mice tumor burden (continuous black line and dotted black line for neuT and neuT-C3KO mice respectively). A significant (***) p = 0.0002 between week 22 and 25 for neuT mice and *** p = 0.0003 between week 19 and 21 for neuT-C3KO mice) increase in serum VEGF was observed in both neuT and neuT-C3KO mice in accordance with the tumor burden increase.

Figure 7. C3 deficiency increases permeability/perfusion and water diffusion in neuT tumors.

MRI evaluation of tumor permeability/perfusion, % of necrosis and water diffusion in mammary tumors from neuT (n = 4; black bars) and neuT-C3KO (n = 4; white bars) mice. Tumor permeability (Ktrans) (A) and plasmatic volume (Vp) (B) were estimated from DCE-MRI data. C: Percentage of necrotic areas was calculated as tumor regions showing contrast enhancement lower than 1.5 fold increase in comparison to pre-injection images. D: Molecular water diffusion (ADC) values were calculated from DWI images in tumor regions. Data were expressed as mean values ± SD.
Table 1. Spontaneous lung metastatization in neuT and neuT-C3KO mice

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<sup>a</sup> percentage of mice with lung metastases.

<sup>b</sup> number of mice with lung metastases/total mice.
References

Bandini et al., Figure 1
Bandini et al., Figure 2
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