The non inflammatory role of C1q during Her2/neu driven mammary carcinogenesis

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List of abbreviations: BALB-C1KO, BALB/c mice deficient for the C1qA; BALB-C3KO, BALB/c mice deficient for C3; C1KO, C1q deficient; C1qR, C1q receptor; C3, complement 3; CR3, complement receptor 3; EMT, epithelial-to-mesenchymal transition; KLRK1 or NKG2D, killer cell lectin-like receptor subfamily K, member 1; MDCS, myeloid derived suppressor cells; neuT, rat Her2/neu transgenic; neuT-BKO mice, neuT mice deficient in antibody production; neuT-C1KO mice, neuT mice deficient for the C1qA molecule; neuT-C3KO mice, neuT mice

25 deficient for the C3 molecule; NK, natural killer; pWWOX, phospho WWOX; Treg, T regulatory;

26 WWOX, WW domain containing oxidoreductase.

Abstract

There is an ever increasing amount of evidence to support the hypothesis that complement (C)1q, the first component of the classical complement pathway, is involved in the regulation of cancer growth, in addition to its role in fighting infections. It has been demonstrated that C1q is expressed in the microenvironment of various types of human tumor, including breast adenocarcinomas. This study compares carcinogenesis progression in C1q deficient (neuT-C1KO) and C1q competent neuT mice in order to investigate the role of C1q in mammary carcinogenesis. Significantly accelerated autochthonous neu^{+} carcinoma progression was paralleled by accelerated spontaneous lung metastases occurrence in C1q deficient mice. Surprisingly, this effect was not caused by differences in the tumor infiltrating cells or in the activation of the complement classical pathway, since neuT-C1KO mice did not display a reduction in C3 fragment deposition at the tumor site. By contrast, a significant higher number of intratumor blood vessels and a decrease in the activation of the tumor suppressor WW domain containing oxidoreductase (WWOX) were observed in tumors from neuT-C1KO as compared to neuT mice. In parallel, an increase in Her2/neu expression was observed on the membrane of tumor cells. Taken together, our findings suggest that C1q plays a direct role both on halting tumor angiogenesis and on inducing apoptosis in mammary cancer cells by coordinating the signal transduction pathways linked to WWOX and, furthermore, highlight the role of C1q in mammary tumor immune surveillance regardless of complement system activation.

Introduction

It has now been clearly demonstrated that the survival and progression of a primary tumor depend on three major events: immune evasion, angiogenesis and metastasis. These complex biological processes can be promoted and halted by a number of factors, including complement cascade components. [1] There is sufficient evidence to demonstrate the presence of complement deposition on tumor tissues from patients in a variety of cancer types. [2,3] Nevertheless, the role of the complement system in tumor growth and metastatic spread has not yet been sufficiently addressed, and contradictory findings are still being reported. [4,5]

C1, the first component of the classical complement pathway, is a multimolecular complex composed of C1q, C1r and C1s molecules that associate in a calcium-dependent macromolecular complex. C1q, the ligand recognition subcomponent of the complex, is composed by six heterotrimeric structures, made of A (C1qA), B (C1qB) and C (C1qC) chains, with a collagen-like and a C-terminal globular region; the latter is the moiety responsible for ligand binding. [6] When C1q binds to IgM or clustered IgG on immunocomplexes, or to surface-bound pentraxins, other complement cascade components are sequentially activated leading to the generation of effector molecules which are able to limit infection by pathogens and play an essential homeostatic role in the clearance of damaged self-antigens, immune complexes, altered self, and apoptotic cells. [7]

Besides this role in the activation of the classical complement pathway through the recognition of immune complexes, [8] it is now recognized that C1q can carry out functions that are unrelated to complement activation. Recent studies have demonstrated its involvement in several physiological processes via binding with its specific receptors. [9] Among the processes induced by the non-classical functions of the C1q molecule, we find: the modulation of various immune cells, [10] the regulation of cell migration (chemotaxis), adhesion, survival and

differentiation, [11] coagulation, [12] angiogenesis [13] and embryonic development, including neurological synapse function. [14]

There is mounting evidence to support the idea that C1q and its receptors are also involved in the regulation of cancer. The recently postulated ability of C1q to induce apoptosis in prostate cancer cells by coordinating signal transduction pathways linked to the tumor suppressor WW domain containing oxidoreductase (WWOX), [15] has highlighted its important role as a humoral factor which is needed to directly block cancer cell proliferation, without the involvement of anti-tumor antibodies and the consequent activation of the classical complement pathway. The direct anti-tumor effect played by C1q is also highlighted by the fact that it is significantly down-regulated in benign prostatic hyperplasia and prostate cancer despite being expressed in normal prostate tissues. [15] Furthermore, recent data demonstrate that C1q receptors (C1qR) expression is up-regulated on almost all types of malignant cells. [9] Interestingly, two of the best-known C1qR, [16] cC1qR and gC1qR, have opposite functions; while cC1qR is detrimental to tumor growth because it is a pro-phagocytic signal, gC1qR promotes tumor cell growth, angiogenesis and metastases formation. [9] Moreover, tumor cells not only express gC1qR on their membrane but they can also secret it into the tumor microenvironment, protecting cancer cells from C1q-induced apoptosis by impounding the C1 molecule. Soluble gC1qR, has also a role to play, by activating the complement system, in the generation of potent vasoactive peptides which are able to enhance vascular permeability, thus facilitating tumor cell escape and consequent metastatization. [9]

A growing number of papers in recent years have linked the expression of the complement cascade components and regulators to the prognosis of breast cancer patients. [17,18] CD59, a membrane complement regulatory protein, has recently been found to be overexpressed in breast cancer where it facilitates tumor cell escape from complement surveillance. [19] Significantly differential complement cascade pathway expression was

observed in luminal A, luminal B, basal-like and Her2$^+$ mammary cancers. [20] The complement factor C1q, which was found to be down-regulated, appears to play a noteworthy and important role in this context. [18] However, the mechanisms that underlie the effects exerted by C1q on breast cancer progression have not yet been sufficiently clarified, meaning that deeper understanding of the molecular interactions between tumors and C1q may lead to the identification of additional pathways and targets which can be exploited for combined therapy.

The genetic predisposition of rat Her2/neu transgenic (neuT) mice to developing lethal mammary carcinomas, characterized by well-defined gene expression signature, [21] progression and long lasting interaction with the host microenvironment, make these mice an appealing model for the evaluation of the role of immunosurveillance. [22,23] We have previously demonstrated that there is a deposition of C3 on tumor cells and vessels during Her2/neu-driven mammary carcinogenesis in neuT mice and that natural antibodies which are directed against antigens expressed by tumor cells arise concomitantly. [24] These observations suggest that complement activation through the classical pathway may play a key role in tumor immunosurveillance. To verify this hypothesis, the present work sees us generate neuT mice that are deficient for the C1qA molecule (neuT-C1KO mice) and then evaluate the progression of carcinogenesis in their mammary glands in comparison to neuT mice. Surprisingly, our data demonstrate that C1q can act as tumor-inhibiting factor, regardless of complement activation.

Results

Autochthonous Her2/neu$^+$ carcinoma progression is accelerated in C1q deficient mice.

Significantly accelerated tumor incidence was observed when mammary carcinogenesis was evaluated in neuT-C1KO mice, with 100% of mice already bearing a palpable tumor at week 19 of age, when 50% of neuT mice were still free from palpable tumors (**Fig. 1A**; $p<0.0001$, Log-rank Mantel-Cox test). The lack of the C1q molecule in neuT mice also impacts on tumor multiplicity and tumor growth. Indeed, from the 17th week of age (**Fig. 1B**; p values ranging from $p=0.04$ to $p<0.0001$, Student's *t*-test), a significant increase in tumor multiplicity was observed in neuT-C1KO mice, as compared to neuT mice, and tumors grew significantly more quickly (**Fig. 1C**; $p=0.001$, Student's *t*-test). A histological analysis of mammary glands from 11-, 15- and 17-week-old mice confirmed that progression from hyperplastic lesions to *in situ* and diffuse carcinomas was accelerated in neuT-C1KO mice (**Fig. 1D-I**). By week 11, the hyperplastic lesions appeared to be more numerous than those in neuT mice (**Fig. 1D, G**). At week 15, neuT-C1KO mice displayed multifocal lesions, which were larger and evenly more spread throughout the mammary gland than those observed in neuT mice (**Fig. 1E, H**). By week 17, these lesions converged into multiple, large nodules similar to *in situ* carcinomas that were more diffused and expanded than in neuT mice (**Fig. 1F, I**). Nevertheless, carcinomas progressing in neuT-C1KO and neuT mice displayed a similar grade of differentiation (**Fig. 1J, K**). However, a significantly higher number of PCNA$^+$ tumor cells was found in carcinomas from neuT-C1KO as compared to neuT mice suggesting an increasing tumor cell proliferation in complement C1q deficient neuT mice (**Fig. 1L**; $p=0.001$, Student's *t*-test). In addition, even if very few apoptotic cells were detectable in the mammary tumors from both neuT and neuT-C1KO mice, a significant decrease in the number of Caspase-3$^+$ cells was observed in mice lacking C1q (**Fig. 1M-O**; $p=0.002$, Student's *t*-test), suggesting a decreased apoptosis in the absence of C1q molecule.

The accelerated tumor onset observed in neuT-C1KO mice was paralleled by a significantly accelerated spontaneous lung metastasis development. Indeed, at week 17, when

neuT mice were completely free from lung metastases, 36% of neuT-C1KO mice displayed Her2/neu$^+$ nodules in their lungs (**Fig. 2A-C**, p=0.05, Chi-square test). This accelerated metastatization suggests that the epithelial-to-mesenchymal transition (EMT) occurs earlier in neuT-C1KO tumors. In order to investigate this issue, we evaluated the neuT and neuT-C1KO tumor expression of E-Cadherin, whose functional loss or down-regulation is considered a hallmark of EMT. [25] Moreover, since a link a between C3 overexpression, C3a generation and EMT has been recently reported, [26] we also evaluated the expression of E-Cadherin in the tumors from neuT mice deficient for the C3 molecule (neuT-C3KO mice). Western blotting showed significantly lower E-cadherin expression in neuT-C1KO and neuT-C3KO as compared to neuT tumors (**Fig. 2D-E**; $p<0.05$, Student's *t*-test). We used fluorescence microscopy (**Fig. 2F-I**) to confirm the immunoblot findings and determine the localization of E-cadherin in tumor cells. While tumor cells from neuT mice displayed E-cadherin expression at contact sites between cells (**Fig. 2F**), tumor cells from neuT-C1KO (**Fig. 2G**) and neuT-C3KO (**Fig. 2H**) mice provided almost negative staining and fluorescent signal quantification showed significantly lower E-cadherin expression in neuT-C1KO and neuT-C3KO than in neuT mice (**Fig. 2I**; $p<0.0001$, $p= 0.04$, Student's *t*-test, respectively).

Accelerated carcinogenesis in neuT-C1KO mice is independent of the absence of the classical complement activation pathway.

The accelerated pace of tumor progression in neuT-C1KO mice points towards a key role for the complement system in hampering Her2/neu autochthonous mammary tumor progression, as previously demonstrated in neuT-C3KO mice. [24] The contribution of C1q to tumor immunosurveillance should mainly be due to the triggering of the classical pathway of complement activation that occurs after binding with natural anti-tumor antibodies present in the sera of tumor-bearing mice. [24] If this were the case, C3 fragments deposition in the

mammary glands would be drastically lower in neuT-C1KO mice than in neuT. Nevertheless, the confocal immunofluorescence microscopy analysis of tumors after staining with anti-C3b/iC3b/C3c antibodies showed that comparable levels of C3 cleavage products are present in neuT and neuT-C1KO tumors (**Fig. 3A, B, D**). Moreover, as previously observed in neuT mice, [24] C3 cleavage products were clearly evident both in the tumor vasculature and stroma of mammary tumors developing in neuT-C1KO mice (**Supplementary Fig. S1**). A further investigation was therefore performed on neuT mice that were deficient in antibody production (neuT-BKO mice). [27] C3 deposition in neuT-BKO tumors was similar to that observed in neuT and neuT-C1KO mice (**Fig. 3C, D**), confirming that complement activation is independent of the classical pathway in neuT mice.

C1q deficiency is associated with decreased activation of the oncosuppressor WWOX and increased Her2/neu expression

The influence of C1q on tumor progression does not appear to be due to complement cascade activation and so we decided to determine whether it acts on the tumor cell phenotype directly. Recent studies have reported that C1q protein may induce the apoptosis of cancer cells during the initial hyperplasia and cancerous stages of cancer progression by activating the tumor suppressor WWOX pathway. [15] In order to study whether a correlation between C1q deficiency and WWOX activation exists in neuT tumors, immunofluorescence staining of tumors from neuT and neuT-C1KO mice was performed. While clear positivity for the phospho (p) WWOX protein was observed in neuT tumor cells (**Fig. 4A, D**), it was found to be significantly reduced in neuT-C1KO mice (**Fig. 4B, D**; p=0.01, Student's *t*-test). Interestingly, a similar reduction in pWWOX was also observed in tumors from neuT-C3KO mice (**Fig. 4C, D**; p=0.02, Student's *t*-test), despite the C1qA gene not being knocked out in these mice. However, significantly lower levels of C1q fragment deposition were observed at

the tumor site in neuT-C3KO than in neuT mice (**Fig. 4E-H**; p=0.009, Student's *t*-test). The confocal immunofluorescence microscopy analysis of tumors after staining with anti-C1q and CD31 antibodies showed that C1q was deposited mainly in the tumor vasculature in both neuT and neuT-C3KO mice (**Supplementary Fig. S2**).

Since neuT tumors are strictly addicted to Her2/neu, [28] we evaluated its expression using confocal immunofluorescence microscopy. As compared to tumors in neuT mice (**Fig. 4I, L**), Her2/neu protein expression was significantly increased in neuT-C1KO tumors (**Fig. 4J, L**; p=0.05, Student's *t*-test). This enhanced expression was similar to what observed in neuT-C3KO (**Fig. 4K, L**; p=0.02, Student's *t*-test) tumors. Her2/neu appeared to be expressed mainly at the cell membrane of neuT tumor cells and its expression appeared to be higher and broader in both complement-deficient strains.

C1q deficiency affects tumor vessel density but not the frequency of tumor-infiltrating leukocytes.

Our data indicate that the more aggressive phenotype of neuT-C1KO, as compared to neuT tumors (**Fig. 1**), may be a result of the reduced activation of tumor suppressor WWOX and the consequent survival of tumor cell clones that present stronger Her2/neu oncoprotein expression. To evaluate whether C1q deficiency also impacts on the tumor microenvironment, we evaluated the vessel density (**Fig. 5A-H**) and the frequency of tumor-infiltrating immune cells (**Fig. 5I-M**) in neuT, neuT-C1KO and neuT-C3KO tumors of equivalent size.

Immunohistochemical staining for endothelial cell markers (**Fig. 5A-C**) displays a statistically higher number of intratumor vessels in neuT-C1KO as compared to neuT and neuT-C3KO mice (**Fig. 5D**; p=0.04, Student's *t*-test). On the other hand, the assessment of vessel diameter indicates that, although higher in number, tumor-associated blood vessels in neuT-C1KO tumors are similar in dimension when compared to those of neuT mice (**Fig. 5E-F,**

H). By contrast, as previously reported [24], significantly larger intratumor vessels are evident in carcinomas developing in the absence of C3 (**Fig. 5G-H**; p<0.0001 Student's *t*-test).

The frequency of tumor-infiltrating immune cells was evaluated using flow-cytometry. Tumors of neuT and neuT-C1KO mice displayed no statistically significant differences in the frequency of T regulatory (Treg) cells, $CD4^+$, $CD8^+$ and γδ T cells, natural killer (NK) cells, macrophages and myeloid-derived suppressor cells (MDSC) (**Fig. 5I-M**). These data suggest that the C1q molecule is not involved in regulating leukocyte recruitment at the tumor site. This is in contrast to observations in C3 deficient mice which instead show a significant increase in infiltrating Treg cells (**Fig. 5I**; p=0.005, Student's *t*-test), as previously shown, [24] and a significant reduction in NK cells (**Fig. 5L**; p=0.005, Student's *t*-test). These differences, together with those in vessel dimension and complement activation, justify the fact that neuT-C1KO tumors are less aggressive than those of neuT-C3KO mice (**Fig. 6A, B**; p=0.05 and p=0.02, Log-rank Mantel-Cox test) despite showing comparable levels of pWWOX and Her2/neu expression (**Fig. 4D, L**).

Discussion

Several studies have suggested that the complement system plays a critical role in cancer growth and spread. It has been demonstrated that cancer cells establish a balance between complement activation and inhibition. [29] However, controversial and conflicting data on the complement system's tumor-promoting, [30,31] and inhibiting activities, [24] have been published, [2] and the mechanisms of complement-specific activities in the tumor microenvironment are still unclear and demands further study.

The use of genetically engineered mice, which are predestined to develop fatal tumors and are knocked out for one of the key complement cascade molecules, enabled us to test the

role of complement activation in cancer growth and development and will possibly lead to the design of complement-related anticancer strategies.

We have recently exploited the genetic predestination of neuT female mice to mammary carcinogenesis to assess the weight of C3 complement-mediated [24] immune surveillance in the development of autochthonous carcinomas. The data obtained demonstrated that the loss of C3 activation, whose fragments were found to specifically accumulate in vessels and stroma in and around the incipient cancers, was responsible for the dramatic increase in the aggressiveness of neu$^+$ mammary tumors. Concomitantly, as had already been demonstrated in cancer patients, [32,33,34] spontaneous antitumor antibodies increased in neuT mice during the course of tumor progression. [24] Taken together, these observations suggest that the complement system can take on a tumor-inhibiting role that is played out through the activation of the classical pathway.

The involvement of classical complement pathway activation in tumor cell killing was further investigated by generating neuT-C1KO mice. An accelerated carcinoma progression was evident in these mice. The comparable level of C3 fragment deposition observed in the mammary glands of neuT-C1KO mice and neuT-BKO mice, which are unable to produce antibodies, highlight the fact that tumor cell killing is not related to complement classical pathway activation in neuT mice. By contrast, the decreased activation of the oncosuppressor WWOX and the over-expression of the neuT protein on the mammary tumor cells found in neuT-C1KO mice point to the key and direct role that C1q plays in tumor cell elimination. Indeed, recent data have highlighted the existence of non-canonical functions that are exerted by C1q on several types of cells, including cancer cells. Irrespectively of the beneficial or harmful impact that the complement system has on tumor growth, the C1q molecule's contribution to tumor progression and metastasization has been demonstrated regardless of complement activation, both in prostatic cancer cells [15, 35] and very recently in melanoma. [31]

These results provide a new perspective on our previously published data which demonstrated that the neuT up-regulation on tumor cells observed in neuT-C3KO tumors was not only directly dependent on lack of C3 activation. In particular, the extremely aggressive phenotype displayed by neuT-C3KO tumors can also be caused by the impaired activation of WWOX, which is a result of the unexpected lack of C1q deposition on the tumor site of neuT-C3KO mice. A relationship between C1q-induced expression and hypoxia has been demonstrated in neurons. [36] Although the mechanisms by which C1q mRNA expression is up-regulated by hypoxia remain to be investigated, it has been demonstrated that cultured cells, which did not express C1q before hypoxia, did express C1q mRNA and protein during and after hypoxia, respectively. [36] It has recently been demonstrated, by both immunohistochemistry and western blotting experiments, that neuT tumor cells express a low but detectable level of HIF1 protein. [37] Similar amounts of HIF1 protein were also observed in neuT-C1KO tumors, while it was almost absent in neuT-C3KO tumors (**Supplementary Fig. S3 A-C**). These data, together with the previously reported increased blood vessel permeability and reduced necrotic areas within neuT-C3KO neoplasms, as compared with neuT lesions, suggest that the hypoxic phenotype of neuT-C3KO is lower than that of both neuT and neuT-C1KO tumors. [24] Altogether, these observations lead to speculate that the unexpected lack of the C1q protein observed in neuT-C3KO tumors may be due to the fact that C1q mRNA transcription is not up-regulated by hypoxia in these tumors, as it is in neuT and neuT-C1KO tumors. Indeed, using the LASAGNA-Search tool [38] (http://biogrid.engr.uconn.edu/lasagna_search/) to perform binding sites searches on mouse C1q promoter, we confirmed the presence of binding sites specific for the transcriptional factors RelA, [39] Meis-1a, [40] AhR [41] and Arnt, [42] known to be present on human C1q promoter and linked to hypoxia (GeneCards®, Human Gene Data base; http://www.genecards.org/cgi-bin/carddisp.pl?gene=C1QA&keywords=c1qa).

Contradictory effects of complement in modulating vascularization and angiogenesis are described in the literature. [30,31,43] Our immunohistochemistry data showed a marked deposition of C1q on vascular endothelium and stroma of neuT tumors. On the other hand, the increased tumor vascularization observed in neuT-C1KO mice suggests that C1q could be considered an inhibitor of tumor angiogenesis, at least in our model of autochthonous Her2-driven mammary cancer that display a process of angiogenesis [44] not superimposable to that observed in transplantable tumors. [31] Further investigations are warranted to define the underlying mechanisms of this C1q-mediated inhibition of tumor vasculature.

The hypothesis that C1q may have an effect in the induction of an immunosuppressive tumor microenvironment, was excluded by an analysis of tumor infiltrating lymphocytes. Contrary to results in tumors from neuT-C3KO mice, [24] no differences in the percentages of myeloid derived suppressor cells and Treg cells were found. The significant reduction in NK cells in tumors from neuT-C3KO mice, as compared to both neuT and neuT-C1KO tumors, is certainly worthy of note. The lack of a C1q-induced immunosuppressive tumor phenotype, together with the presence of a significant amount of NK cells, can help explain why neuT-C1KO tumors are less aggressive than their neuT-C3KO analogues. Indeed, NK cells can lyse tumor cells after the recruitment of complement receptor 3 (CR3) by iC3b, especially when MHC class I molecules are poorly expressed. [45] NeuT$^+$ tumor cells are susceptible to the activity of NK cells, as an inverse correlation exists between the expression levels of neuT protein and those of MHC class I molecules as well as other components of the antigen-processing machinery. [46] Moreover, signaling through the Her2/Her3 pathway in breast tumor cell lines has been shown to enhance their recognition by NK and T cells thanks to the killer cell lectin-like receptor subfamily K, member 1 (KLRK1, or NKG2D). [47]

In conclusion, our data demonstrate that the C1q protein protects against the development of Her2/neu$^+$ mammary carcinomas, at least in our preclinical BALB-neuT

female mouse model. The tumor-inhibiting C1q-mediated effects do not appear to be associated with the classical pathway of complement, but with its direct role on endothelial and tumor cells. Based on our results and on data from the literature we propose the following mechanism of C1q direct antitumoral role in neuT mammary carcinogenesis. The C1q binding with its receptor leads to the phosphorylation and consequent activation of WWOX, which is a known oncosuppressor required to block cancer cell proliferation. [15] Indeed, activated WWOX may induce the apoptosis of mammary tumor cancer cells, probably by interacting with p53, [48] and inhibit the EMT processes through the expression of E-Cadherin. Conversely, the lack of C1q prevents WWOX activation leading to tumor cell growth and metastases formation. As far as the mechanisms linking the absence of C1q and the increased levels of neuT protein expression on mammary tumor cells are concerned, our hypothesis is that WWOX activation may be involved in neuT post-translational negative regulation (**Fig. 7**). By contrast, WWOX-dependent transcriptional regulation of neuT gene is unlikely since this transgene is under the control of the MMTV promoter and not of the endogenous one.

However, as expected for a system with various distinct activities, this protective role in mammary cancer does not appear to be applicable to every tumor type. Indeed, recent findings have highlighted C1q's role as a cancer-promoting factor in a transplantable model of melanoma. [31] However, numerous variables, including the genetic background of mice used, the type of tumors and especially the use of transplantable instead of autochthonous tumors, may all influence how the complement system affects tumor progression, culminating in very different biological outcomes. Moreover, considering the opposite function exerted by the two known C1qR, we can speculate that the tumor inhibiting- or promoting- effect mediated by the C1q protein may results from different degrees of cC1qR or gC1qR expression in the tumor.

Nevertheless, it is important to note that C1q can exert its functions directly on tumor cells, independently of complement activation pathways, both in breast cancer and in melanoma. Defining the role of C1q in different tumor types may lead to new drugs in the clinic, thanks to its role as a major contributor to the immunosurveillance and control of cancer progression as well as tumor growth and dissemination.

Materials and methods

Mice. BALB/c mice that were deficient for the C1qA (BALB-C1KO) and C3 (BALB-C3KO) complement component were provided by Prof. Marina Botto (Imperial College, London, UK). [49] NeuT male mice [28] from Biogem (Ariano Irpino, Italy) were crossed with BALB-C1KO and with BALB-C3KO females in order to obtain neuT^{+} C1$^{+/-}$ and neuT^{+} C3$^{+/-}$ heterozygous male mice, respectively. These heterozygous male mice were then crossed with BALB-C1KO and BALB-C3KO females; the progeny was genotyped in order to identify neuT^{+} C1$^{-/-}$ (neuT-C1KO) and neuT^{+} C3$^{-/-}$ (neuT-C3KO) female mice that were then used in the experiments. NeuT-BKO mice were generated by crossing neuT mice with BALB/c mice KO for the immunoglobulin μ chain gene, as previously described. [27] 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. 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. 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 Torino approved the experimental plan.

Morphological analyses. The whole mount preparation of mammary glands was carried out by removing the mouse skin and fixing it overnight in 10% buffered formalin. Mammary fat pads were scored into quarters and gently scraped from the skin. These were immersed in acetone overnight and then rehydrated and stained in ferric hematoxylin (Sigma-Aldrich), dehydrated in increasing concentrations of alcohol, cleared with histo-lemon, and stored in methyl salicylate (Sigma-Aldrich). Digital pictures were taken using a Nikon Coolpix 995 (Nital, Medley, FL) mounted on a stereoscopic microscope (MZ6; Leica Microsystems, Milano, Italy) and analyzed as previously described in detail. [50]

Histology, immunohistochemistry and immunofluorescence. Lung samples from 14 neuT and 19 neuT-C1KO 17 week-old mice were fixed in formalin and embedded in paraffin. To optimize the detection of microscopic metastases and ensure systematic uniform and random sampling, lungs were processed as previously described. [24] For the evaluation of spontaneous metastases of all the experimental groups, sections were stained with hematoxylin/eosin and with anti-Her2 immunohistochemistry. Metastases were counted independently by two pathologists in a blind fashion.

Mammary glands were fixed in formalin and embedded in paraffin or fixed in paraformaldehyde 1% and frozen in a cryo-embedding medium (OCT, Bioptica) for histological and immunohistochemical analyses. Six to eight mm mammary tumors were fixed in paraformaldehyde 1% and frozen in a cryo-embedding medium (OCT, Bioptica).

Sections were incubated with the mouse monoclonal anti-PCNA antibody (Dako Corporation, M0879), rabbit anti-active caspase-3 (R&D systems, AF835), rabbit anti-human

Her-2 (Dako Corporation, A0485), rabbit anti-mouse E-Cadherin (Cell Signaling, 24E10), rabbit anti-phospho-WWOX ($pTyr^{33}$) (Sigma-Aldrich, SAB4504681), rat anti-mouse C1q (Abcam, ab11861), rat anti-mouse C3b/iC3b/C3c mAb (HyCult biotech, 2/11), rat anti-CD105 (BD Pharmingen, 550546) and rat anti-CD31 (BD Pharmingen, 550274). For immunohistochemical staining, sections were then incubated with the appropriate biotinylated secondary antibody (Jackson Immunoresearch Laboratories). Immunocomplexes were detected using NeutrAvidin™ Alkaline Phosphatase Conjugated (Thermo Scientific-Pierce Biotechnology) and Vulcan Fast Red (Biocare Medical) or Streptavidin Peroxidase (Thermo Scientific) and DAB Chromogen System (DakoCorporation). For immunofluorescence analysis, sections were then incubated with the appropriate Alexa 488 and 546 labeled secondary antibodies (all from Molecular Probes); nuclei were stained with DAPI (Sigma-Aldrich) or TO-PRO®-3 iodide (Thermo-Fisher Scientific). Images were acquired on a Zeiss ApoTome fluorescence microscope (Axiovert 200M, Zeiss, Jena, Germany) and captured using a CCD cool digital camera (Zeiss) or on a Zeiss LSM 510 META confocal microscopy.

The percentage of $PCNA^+$ tumor cells was evaluated counting positive and negative cells on the digital images of 10 neuT and 10 neuT-C1KO tumors (3 ×400 microscopic fields per tumor) by 2 pathologists, independently and in a blind fashion.

The number of activated Caspase 3^+ tumor cells was evaluated counting positive cells on the digital images of 10 neuT and 10 neuT-C1KO tumors (3 ×200 microscopic fields per tumor) by 2 pathologists, independently and in a blind fashion.

The intensity of E-Cadherin expression was evaluated and recorded using the Image J software and by analyzing ROI with similar cell numbers (3 ×630 microscopic fields per tumor, 3 tumors per group each from different mice).

418 The quantification of C3 fragment deposition, pWWOX, C1q and Her2/neu protein was
419 performed by image analysis with Adobe Photoshop. Positive red fluorescent pixels were
420 selected using the magic wand tool and quantified in the histogram window in images from 10
421 tumors per group (3 ×400 microscopic fields per sample) for C3 and C1q quantification and 7
422 tumors per group (3 ×400 microscopic fields per sample) for pWWOX and Her2/neu protein
423 quantification. Results are represented as means ± SEM.
424 The number and the lumen area of $CD31^+/CD105^+$ vessels were evaluated on the digital
425 images of 3-5 tumors per group (5 x200 microscopic fields per tumor) by 2 pathologists,
426 independently and in a blind fashion. Vessels area (in pixels) was evaluated with Adobe
427 Photoshop by selecting vessels with the lasso tool and reporting the number of pixels
428 indicated in the histogram window.
429
430 **Protein preparation and immunoblotting.** Total protein extracts were obtained from
431 neuT, neuT-C1KO and neuT-C3KO mammary tumors. Briefly flash frozen specimens were
432 dissociated using an IKA-Ultra-Turrax® T8 homogenizer (IKA-Werke, Staufen Germany) in a
433 buffer containing 10mM Tris, 5 mM EDTA, 50 mM NaCl, 30 mM Sodium pyrophosphate
434 decahydrate ($Na_4O_7P_2$-$10H_2O$), 50 mM Sodium Fluoride (NaF), 1 mM Sodium orthovanadate
435 (Na3VO4), 1% Triton X (Adjust the pH 7.6), 1 mM Phenylmethanesulfonyl fluoride100 (all
436 from Sigma-Aldrich) and a cocktail of protein inhibitors (Sigma-Aldrich, P8340). Samples
437 were then centrifuged twice at 12000 rpm at 4°C for 5 minutes. 40 μg of total proteins, as
438 determined by BCA Protein Assay (Pierce, Thermo Fisher), were separated by SDS-PAGE and
439 electroblotted onto polyvinylidene fluoride membranes (BioRad). Membranes were blocked
440 in 5% BSA (Sigma Aldrich) Tris-buffered saline (TBS)-Tween buffer (137 mM NaCl, 20 mM
441 Tris/HCl, pH 7.6, 0.05% Tween-20) for 1 h at RT and then incubated with appropriate
442 primary (anti e-Cadherin, Cell Signaling, 3195; anti β-actin, Santa Cruz Biotechnology, sc-

69879) and appropriate secondary antibodies (goat anti-rabbit, Sigma_Aldrich, A0545 and goat anti mouse Sigma_Aldrich, A4416, respectively) in 3% BSA TBS-Tween buffer overnight at 4° C and for 1 h at room temperature, respectively, and visualized using enhanced chemiluminescence (ECL Plus, Thermo Scientific Pierce). Protein modulations were normalized on the actin loading control and expressed as Adjusted Volume Intensity/mm^2 (background subtraction) using Quantity ONE software (Biorad, Milano, Italy).

Cytometric identification of tumor infiltrating leukocytes. For the infiltrating-cell phenotypic analyses, fresh primary tumor specimens of 6-8 mm mean diameter from 5 neuT, 7 neuT-C1KO and 6 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 h in an orbital shaker. After washing in PBS supplemented with 2% fetal bovine 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 min and re-suspended in an erylise buffer. Tumor infiltrating leukocytes were collected, washed, re-suspended in PBS, treated with Fc receptor blocker (anti CD16/CD32; 01245B; BD Bioscences), and stained with the following antibodies: anti-mouse CD45 VioGreen (Miltenyi Biotec, 130097), anti-mouse CD3 FITC (Miltenyi Biotec, 130-092962), anti-mouse CD4 APC-Vio770 (Miltenyi Biotec, 130-102-179), anti-mouse CD8 VioBlue (Miltenyi Biotec, 130-094-360), anti-mouse γδ PE/Cy7 (BioLegend, 118124), anti-mouse CD49b PE (Miltenyi Biotec, 130-091-816), anti-mouse F4/80 APC (Miltenyi Biotec, 130-102-379), anti-mouse CD11b FITC (Miltenyi Biotec, 130-081-201), and anti-mouse GR-1 PE (Miltenyi Biotec, 130-102-426). To detect $FoxP3^+$ T regulatory cells, samples were permeabilized with the FoxP3 anti-mouse staining kit (eBioscience) and stained with the anti-

mouse/rat-Foxp3-FITC antibody (eBioscience, FJK-16s;). Samples were acquired and analyzed on a CyAn ADP flow cytometer using Summit 4.3 software (Beckman Coulter, Milano, Italy).

Captions to figures

Figure 1. C1q deficiency is responsible for accelerated tumor growth in neuT mice. Tumor incidence (**A**) and multiplicity (**B**) of mammary carcinomas in neuT (n = 30, gray line) and neuT-C1KO (n = 18, black line) mice. Earlier incidence (*** p<0.0001, Log-rank Mantel-Cox Test) and higher tumor multiplicity (starting from the 17th week of age, p values ranging from p=0.04 to p<0.0001, Student's *t*-test) were found in neuT-C1KO as compared to neuT mice. **C**: Time required for a 2 mm mean diameter tumor to reach an 8 mm threshold. Tumors that arose in neuT-C1KO (black bar) mice grew significantly faster than those growing in neuT (grey bar) mice (** p=0.001, two-tailed Student's *t*-test). (**D-I**) Representative whole mount images of the fourth (inguinal) mammary glands of 11- (**D, G**), 15- (**E-H**), 17- (**F, I**) week-old neuT (**D-F**) and neuT-C1KO (**G-I**) mice. The central oval black shadows are the intra-mammary lymph nodes. Magnification x6.3. (**J, K**) Histological and immunohistochemical staining for the PCNA of mammary tumor lesions in neuT (**J**) and neuT-C1KO (**K**) mice. Magnification x400. PCNA^{+} tumor cell quantification (**L**) in neuT (grey bar) and in neuT-C1KO (black bar) mice (** p=0.001, two-tailed Student's *t*-test). (**M, N**) Histological and immunohistochemical staining for the active caspase-3 in mammary tumor lesions of neuT (**M**) and neuT-C1KO (**N**) mice. Black arrows indicate apoptotic tumor cells. Magnification x400. Active caspase-3^{+} tumor cell quantification (**O**) in neuT (grey bar) and in neuT-C1KO (black bar) mice (** p=0.002, two-tailed Student's *t*-test).

Figure 2. C1q deficiency is associated with anticipated metastatic spread and epithelial-to-mesenchymal transition in neuT tumors. Histological and immunohistochemical staining for Her2/neu of lungs from 17-week-old neuT (**A**) and neuT-C1KO (**B**) mice reveal earlier metastatic infiltration in neuT-C1KO mice. Magnification x400. (**C**): Percentage of neuT (n = 19, grey bar) and neuT-C1KO (n = 14, black bar) mice (*p=0.05 Chi-square test) bearing lung metastatic lesions at 17 weeks of age. (**D-I**) Decreased expression of E-Cadherin in neuT-C1KO and neuT-C3KO vs. neuT tumors. (**D**) E-Cadherin (upper panel) and actin (lower panel) protein levels as measured using the immunoblotting of whole cell lysates from 6-8 mm mean diameter carcinomas. A representative blot from three independent experiments is shown. (**E**) Quantification of E-Cadherin protein expression in neuT (grey bar), in neuT-C1KO (black bar) and neuT-C3KO (white bar) tumors (*p<0.05, two-tailed Student's *t*-test). (**F-H**) Representative microscopy images of tumor sections from neuT (**F**), neuT-C1KO (**G**) and neuT-C3KO (**H**) mice (n = 3 per group) labeled with anti-E-Cadherin antibody (red) and DAPI (blue, labeling nuclei). Magnification x400. (**I**) E-Cadherin protein was quantified in neuT (grey bar), neuT-C1KO (black bar) (***p<0.0001, two-tailed Student's *t*-test) and neuT-C3KO (white bar) tumors (*p=0.04, two-tailed Student's *t*-test). Results are represented as means ± SEM**.**

Figure 3. The dispensable role of the classical complement activation pathway in neuT tumor immunosurveillance. C3 fragment deposition at the tumor site is not altered in the absence of C1q or antibodies. (**A-C**) Confocal microscopy images representative of frozen tumor sections from mammary glands of 17-week-old neuT (**A**), neuT-C1KO (**B**) and neuT-BKO (**C**) mice labeled with anti-C3b/iC3b/C3c antibody (red) and TO-PRO®-3 iodide (blue). Magnification x400. C3 fragment deposition was quantified (**D**) in neuT (grey bar), neuT-C1KO (black bar) and neuT-BKO (white bar) mice (n = 10 each group). No differences in C3

518 fragments deposition were found (p=ns, two-tailed Student's *t*-test). Results are represented
519 as means ± SEM from 3 x400 microscopic fields per sample.
520
521 **Figure 4. Decrease of pWWOX and increase of Her2/neu expression in neuT-C1KO**
522 **tumors.** Confocal microscopy images of frozen tumor sections from neuT (**A, E, I**), neuT-C1KO
523 (**B, F, J**) and neuT-C3KO (**C, G, K**) mice (n = 7 per group) labeled with anti-pWWOX (red, **A-C**),
524 anti-C1q (red, **E-G**) and anti-Her2/neu (red, **I-K**) antibodies. Nuclei were stained with TO-
525 PRO®-3 iodide (blue). Magnification x100. pWWOX (**D**), C1q (**H**) and Her2/neu (**L**) protein
526 quantification was performed in neuT (grey bar), neuT-C1KO (black bar) and neuT-C3KO
527 (white bar) mice (*p=0.02 for pWWOX; *p=0.04 and **p=0.009 for C1q; *p≤0.04 for
528 Her2/neu; two-tailed Student's *t*-test). Results are represented as means ± SEM from 3 x400
529 microscopic fields per sample.
530
531 **Figure 5. C1q deficiency affects intratumoral vessel density but does not modify tumor**
532 **infiltrating leukocyte recruitment.** (**A–C**) Representative images of immunohistochemical
533 staining for endothelial cell markers (CD31 and CD105, red) to visualize blood vessels in
534 mouse tumors of equal volume developed in neuT (**A**) neuT-C1KO (**B**) and neuT-C3KO (**C**)
535 mice. Magnification x200. Quantification of the number (**D**) of vessels in neuT (gray bar; n =
536 3), neuT-C1KO (black bar; n = 5) and neuT-C3KO (white bar; n = 5) carcinoma (*p=0.04; two-
537 tailed Student's *t*-test). Results are represented as means ± SEM from 5 x200 microscopic
538 fields per sample. (**E–H**) Representative confocal microscopy images of tumors from neuT (**E**)
539 neuT-C1KO (**F**) and neuT-C3KO (**G**) mice stained with anti-CD31 antibodies (green).
540 Magnification x400. Quantification of the vessel area (**H**) in neuT (gray bar; n = 3), neuT-C1KO
541 (black bar; n = 5) and neuT-C3KO (white bar; n = 5) carcinoma. (***p<0.0001; two-tailed
542 Student's *t*-test)Results are represented as means ± SEM from 5 ×200 microscopic fields. (**I-**

M) Flow cytometry analysis of infiltrating leukocytes in 6-8 mm mean diameter tumors from neuT (n = 5; gray bars), neuT-C1KO (n = 7; black bars) and neuT-C3KO (n = 6; white bars) mice. (**I**) $CD3^+$ leukocytes were gated and $CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ were identified as Tregs (**p=0.005; two-tailed Student's t-test). (**L**) $CD45^+$ leukocytes were gated and $CD3^+$ $CD4^+$ cells were identified as CD4 T, $CD3^+$ $CD8^+$ as CD8 T, $CD3^+$ $\gamma\delta^+$ as γδ T and $CD3^-$ $CD49b^+$ as NK (**p=0.005; two-tailed Student's t-test). (**M**) $CD45^+$ $CD11b^+$ leukocytes were gated and $F4/80^+$ cells were identified as macrophages (MΦ) while $GR\text{-}1^+$ cells were identified as myeloid derived suppressor cells (MDSC). Bars represent the percentage of positive cells ± SEM.

Figure 6. NeuT-C1KO tumors are less aggressive than those of neuT-C3KO mice. Tumor incidence of mammary carcinomas (**A**) and overall mice survival (**B**) in neuT (n = 20, continuous gray line), neuT-C1KO (n = 14, continuous black line) and neuT-C3KO (n = 15, dotted black line) mice. Neu-T-C3KO mice displayed earlier tumor incidence (*p=0.05, Log-rank Mantel-Cox Test) and lower overall survival (*p=0.02, Log-rank Mantel-Cox Test) than neuT-C1KO mice.

Figure 7. Proposed mechanism of C1q influence on neuT tumor progression. C1q component of C1 complex (C1q, C1s and C1r) appears to act directly both on tumor vasculature (on the right) and on tumor cells (on the left). Deposition of C1q on vascular endothelium inhibits tumor angiogenesis through a still undefined mechanism. C1q binding with its receptor(s) (C1qR) on tumor cells leads to the phosphorylation of tyrosine 33 (Y33) on WWOX. Activated WWOX in turn inhibits the EMT processes, through the expression of E-Cadherin, and induces Caspase-3-mediated apoptosis, probably by engaging p53. We hypothesize activated WWOX may be also involved in neuT post-translational negative

regulation further contributing to tumor inhibition. Green lines: anti-tumor activities; red lines: pro-tumor activities.

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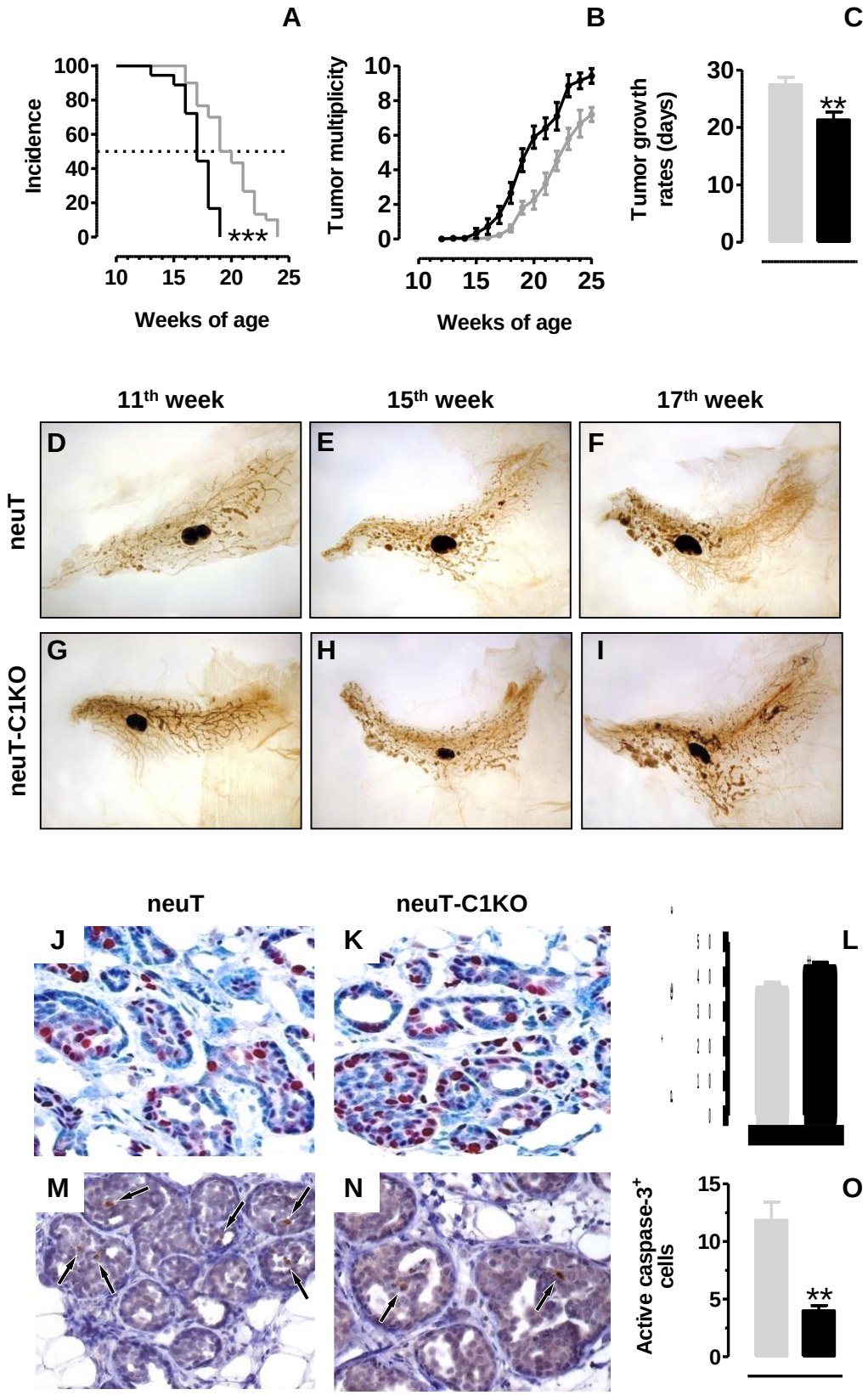
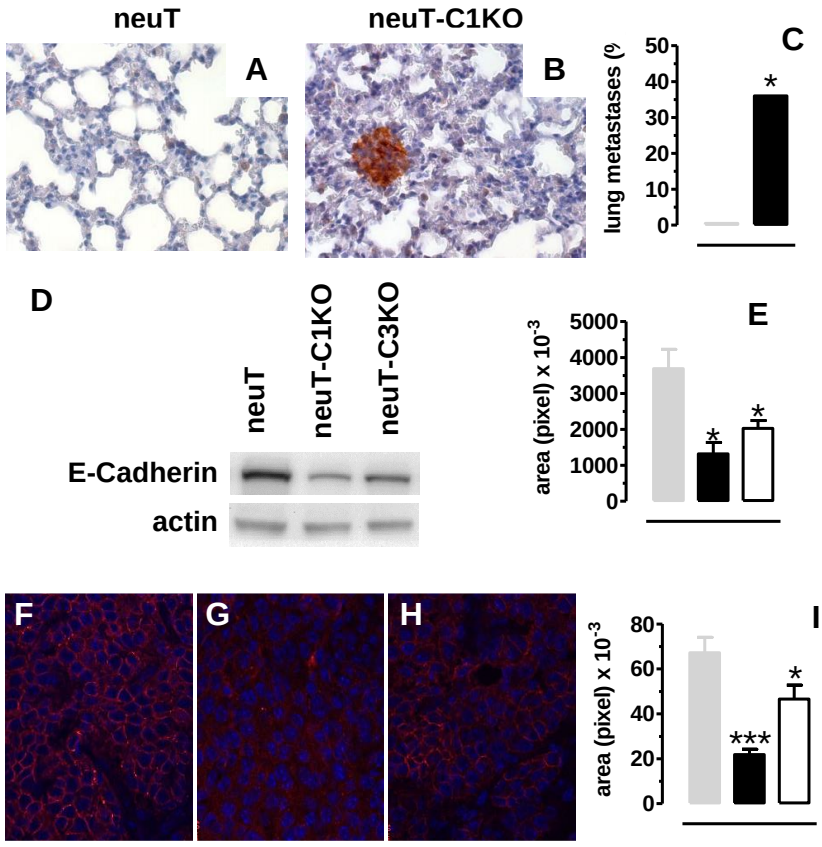
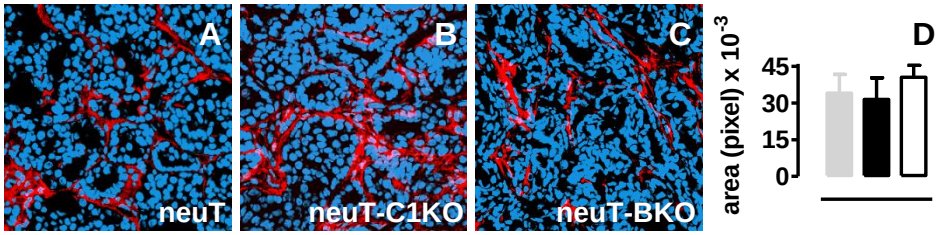


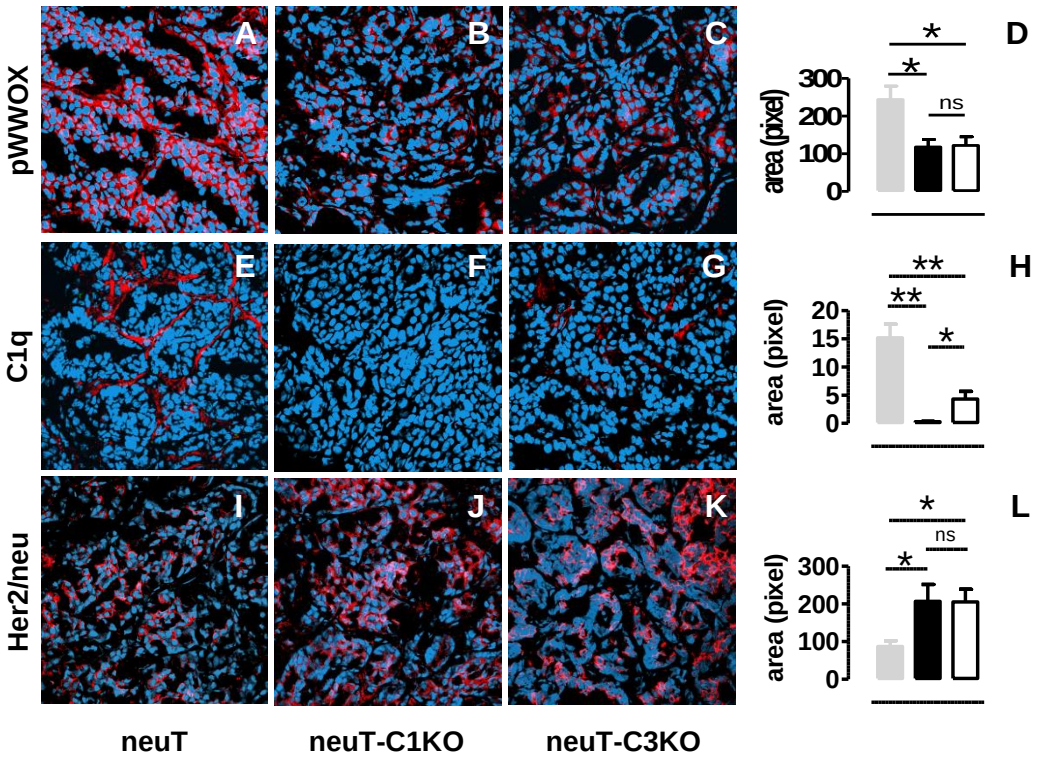
Bandini et al., Figure 1



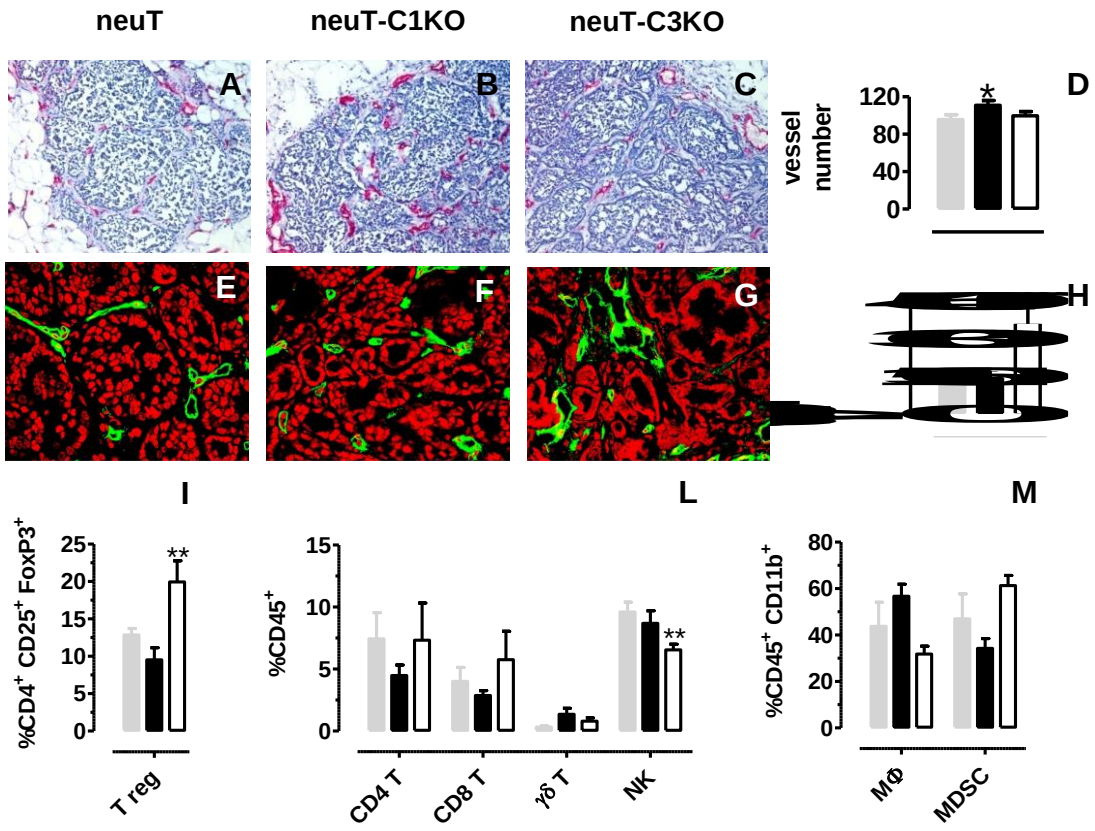
Bandini et al., Figure 2



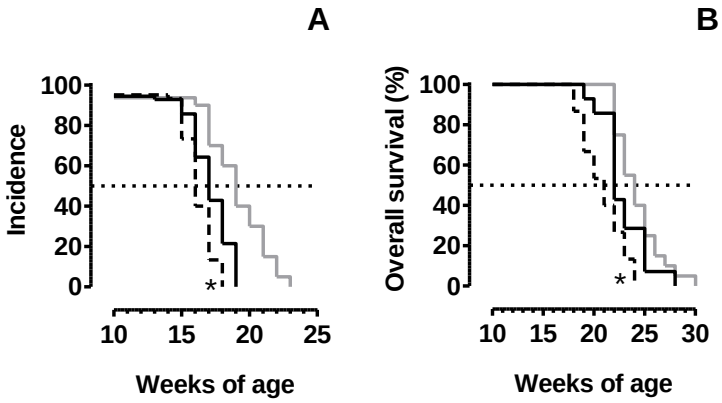
Bandini et al., Figure 3



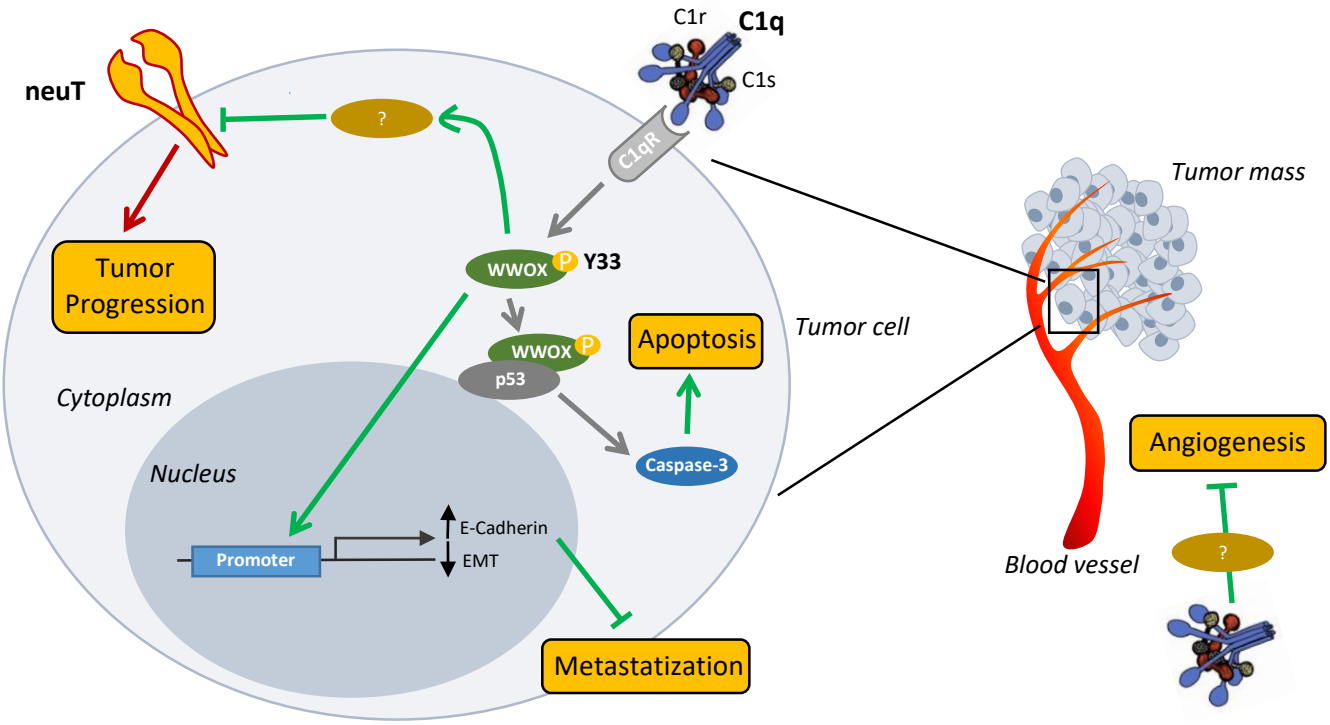
Bandini et al., Figure 4



Bandini et al., Figure 5



Bandini et al., Figure 6



Bandini et al., Figure 7