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

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16   Keywords: Complement, C1q, immunosurveillance, Her2/neu, ErbB2, mammary cancer,

17   genetically engineered mice

18

19   List of abbreviations: BALB-C1KO, BALB/c mice deficient for the C1qA; BALB-C3KO, BALB/c

20   mice deficient for C3; C1KO, C1q deficient; C1qR, C1q receptor; C3, complement 3; CR3,

21   complement receptor 3; EMT, epithelial-to-mesenchymal transition; KLRK1 or NKG2D, killer

22   cell lectin-like receptor subfamily K, member 1; MDCS, myeloid derived suppressor cells;

23   neuT, rat Her2/neu transgenic; neuT-BKO mice, neuT mice deficient in antibody production;

24   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

71   differentiation, [11] coagulation, [12] angiogenesis [13] and embryonic development, including

72   neurological synapse function. [14]

73   There is mounting evidence to support the idea that C1q and its receptors are also

74   involved in the regulation of cancer. The recently postulated ability of C1q to induce apoptosis

75   in prostate cancer cells by coordinating signal transduction pathways linked to the tumor

76   suppressor WW domain containing oxidoreductase (WWOX), [15] has highlighted its important

77   role as a humoral factor which is needed to directly block cancer cell proliferation, without

78   the involvement of anti-tumor antibodies and the consequent activation of the classical

79   complement pathway. The direct anti-tumor effect played by C1q is also highlighted by the

80   fact that it is significantly down-regulated in benign prostatic hyperplasia and prostate cancer

81   despite being expressed in normal prostate tissues. [15] Furthermore, recent data demonstrate

82   that C1q receptors (C1qR) expression is up-regulated on almost all types of malignant cells. [9]

83   Interestingly, two of the best-known C1qR, [16] cC1qR and gC1qR, have opposite functions;

84   while cC1qR is detrimental to tumor growth because it is a pro-phagocytic signal, gC1qR

85   promotes tumor cell growth, angiogenesis and metastases formation. [9] Moreover, tumor cells

86   not only express gC1qR on their membrane but they can also secret it into the tumor

87   microenvironment, protecting cancer cells from C1q-induced apoptosis by impounding the C1

88   molecule. Soluble gC1qR, has also a role to play, by activating the complement system, in the

89   generation of potent vasoactive peptides which are able to enhance vascular permeability,

90   thus facilitating tumor cell escape and consequent metastatization. [9]

91   A growing number of papers in recent years have linked the expression of the

92   complement cascade components and regulators to the prognosis of breast cancer patients.

93   [17,18] CD59, a membrane complement regulatory protein, has recently been found to be

94   overexpressed in breast cancer where it facilitates tumor cell escape from complement

95   surveillance. [19] Significantly differential complement cascade pathway expression was

96   observed in luminal A, luminal B, basal-like and Her2^{+} mammary cancers. [20] The complement

97   factor C1q, which was found to be down-regulated, appears to play a noteworthy and

98   important role in this context. [18] However, the mechanisms that underlie the effects exerted

99   by C1q on breast cancer progression have not yet been sufficiently clarified, meaning that

100   deeper understanding of the molecular interactions between tumors and C1q may lead to the

101   identification of additional pathways and targets which can be exploited for combined

102   therapy.

103   The genetic predisposition of rat Her2/neu transgenic (neuT) mice to developing lethal

104   mammary carcinomas, characterized by well-defined gene expression signature, [21]

105   progression and long lasting interaction with the host microenvironment, make these mice an

106   appealing model for the evaluation of the role of immunosurveillance. [22,23] We have

107   previously demonstrated that there is a deposition of C3 on tumor cells and vessels during

108   Her2/neu-driven mammary carcinogenesis in neuT mice and that natural antibodies which

109   are directed against antigens expressed by tumor cells arise concomitantly. [24] These

110   observations suggest that complement activation through the classical pathway may play a

111   key role in tumor immunosurveillance. To verify this hypothesis, the present work sees us

112   generate neuT mice that are deficient for the C1qA molecule (neuT-C1KO mice) and then

113   evaluate the progression of carcinogenesis in their mammary glands in comparison to neuT

114   mice. Surprisingly, our data demonstrate that C1q can act as tumor-inhibiting factor,

115   regardless of complement activation.

116

117   **Results**

118   **Autochthonous Her2/neu[+] carcinoma progression is accelerated in C1q deficient**

119   **mice.**

120   Significantly accelerated tumor incidence was observed when mammary carcinogenesis

121   was evaluated in neuT-C1KO mice, with 100% of mice already bearing a palpable tumor at

122   week 19 of age, when 50% of neuT mice were still free from palpable tumors (**Fig. 1A**;

123   p<0.0001, Log-rank Mantel-Cox test). The lack of the C1q molecule in neuT mice also impacts

124   on tumor multiplicity and tumor growth. Indeed, from the 17th week of age (**Fig. 1B**; p values

125   ranging from p=0.04 to p<0.0001, Student's *t*-test), a significant increase in tumor multiplicity

126   was observed in neuT-C1KO mice, as compared to neuT mice, and tumors grew significantly

127   more quickly (**Fig. 1C**; p=0.001, Student's *t*-test). A histological analysis of mammary glands

128   from 11-, 15- and 17-week-old mice confirmed that progression from hyperplastic lesions to

129   *in situ* and diffuse carcinomas was accelerated in neuT-C1KO mice (**Fig. 1D-I**). By week 11, the

130   hyperplastic lesions appeared to be more numerous than those in neuT mice (**Fig. 1D, G**). At

131   week 15, neuT-C1KO mice displayed multifocal lesions, which were larger and evenly more

132   spread throughout the mammary gland than those observed in neuT mice (**Fig. 1E, H**). By

133   week 17, these lesions converged into multiple, large nodules similar to *in situ* carcinomas

134   that were more diffused and expanded than in neuT mice (**Fig. 1F, I**). Nevertheless,

135   carcinomas progressing in neuT-C1KO and neuT mice displayed a similar grade of

136   differentiation (**Fig. 1J, K**). However, a significantly higher number of PCNA^{+} tumor cells was

137   found in carcinomas from neuT-C1KO as compared to neuT mice suggesting an increasing

138   tumor cell proliferation in complement C1q deficient neuT mice (**Fig. 1L**; p=0.001, Student's *t*-

139   test). In addition, even if very few apoptotic cells were detectable in the mammary tumors

140   from both neuT and neuT-C1KO mice, a significant decrease in the number of Caspase-3^{+} cells

141   was observed in mice lacking C1q (**Fig. 1M-O**; p=0.002, Student's *t*-test), suggesting a

142   decreased apoptosis in the absence of C1q molecule.

143   The accelerated tumor onset observed in neuT-C1KO mice was paralleled by a

144   significantly accelerated spontaneous lung metastasis development. Indeed, at week 17, when

145   neuT mice were completely free from lung metastases, 36% of neuT-C1KO mice displayed

146   Her2/neu+ nodules in their lungs (**Fig. 2A-C**, p=0.05, Chi-square test). This accelerated

147   metastatization suggests that the epithelial-to-mesenchymal transition (EMT) occurs earlier

148   in neuT-C1KO tumors. In order to investigate this issue, we evaluated the neuT and neuT-

149   C1KO tumor expression of E-Cadherin, whose functional loss or down-regulation is

150   considered a hallmark of EMT. [25] Moreover, since a link a between C3 overexpression, C3a

151   generation and EMT has been recently reported, [26] we also evaluated the expression of E-

152   Cadherin in the tumors from neuT mice deficient for the C3 molecule (neuT-C3KO mice).

153   Western blotting showed significantly lower E-cadherin expression in neuT-C1KO and neuT-

154   C3KO as compared to neuT tumors (**Fig. 2D-E**; p<0.05, Student's *t*-test). We used fluorescence

155   microscopy (**Fig. 2F-I**) to confirm the immunoblot findings and determine the localization of

156   E-cadherin in tumor cells. While tumor cells from neuT mice displayed E-cadherin expression

157   at contact sites between cells (**Fig. 2F**), tumor cells from neuT-C1KO (**Fig. 2G**) and neuT-C3KO

158   (**Fig. 2H**) mice provided almost negative staining and fluorescent signal quantification

159   showed significantly lower E-cadherin expression in neuT-C1KO and neuT-C3KO than in neuT

160   mice (**Fig. 2I**; p<0.0001, p= 0.04, Student's *t*-test, respectively).

161

162   **Accelerated carcinogenesis in neuT-C1KO mice is independent of the absence of**

163   **the classical complement activation pathway.**

164   The accelerated pace of tumor progression in neuT-C1KO mice points towards a key role

165   for the complement system in hampering Her2/neu autochthonous mammary tumor

166   progression, as previously demonstrated in neuT-C3KO mice. [24] The contribution of C1q to

167   tumor immunosurveillance should mainly be due to the triggering of the classical pathway of

168   complement activation that occurs after binding with natural anti-tumor antibodies present

169   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

195　the tumor site in neuT-C3KO than in neuT mice (**Fig. 4E-H**; p=0.009, Student's *t*-test). The

196　confocal immunofluorescence microscopy analysis of tumors after staining with anti-C1q and

197　CD31 antibodies showed that C1q was deposited mainly in the tumor vasculature in both

198　neuT and neuT-C3KO mice (**Supplementary Fig. S2**).

199　　　　Since neuT tumors are strictly addicted to Her2/neu, [28] we evaluated its expression

200　using confocal immunofluorescence microscopy. As compared to tumors in neuT mice (**Fig.**

201　**4I, L**), Her2/neu protein expression was significantly increased in neuT-C1KO tumors (**Fig. 4J,**

202　**L**; p=0.05, Student's *t*-test). This enhanced expression was similar to what observed in neuT-

203　C3KO (**Fig. 4K, L**; p=0.02, Student's *t*-test) tumors. Her2/neu appeared to be expressed mainly

204　at the cell membrane of neuT tumor cells and its expression appeared to be higher and

205　broader in both complement-deficient strains.

206

207　　　　**C1q deficiency affects tumor vessel density but not the frequency of tumor-**

208　**infiltrating leukocytes.**

209　　　　Our data indicate that the more aggressive phenotype of neuT-C1KO, as compared to

210　neuT tumors (**Fig. 1**), may be a result of the reduced activation of tumor suppressor WWOX

211　and the consequent survival of tumor cell clones that present stronger Her2/neu oncoprotein

212　expression. To evaluate whether C1q deficiency also impacts on the tumor microenvironment,

213　we evaluated the vessel density (**Fig. 5A-H**) and the frequency of tumor-infiltrating immune

214　cells (**Fig. 5I-M**) in neuT, neuT-C1KO and neuT-C3KO tumors of equivalent size.

215　　　　Immunohistochemical staining for endothelial cell markers (**Fig. 5A-C**) displays a

216　statistically higher number of intratumor vessels in neuT-C1KO as compared to neuT and

217　neuT-C3KO mice (**Fig. 5D**; p=0.04, Student's *t*-test). On the other hand, the assessment of

218　vessel diameter indicates that, although higher in number, tumor-associated blood vessels in

219　neuT-C1KO tumors are similar in dimension when compared to those of neuT mice (**Fig. 5E-F,**

220 **H**). By contrast, as previously reported [24], significantly larger intratumor vessels are evident

221 in carcinomas developing in the absence of C3 (**Fig. 5G-H**; p<0.0001 Student's *t*-test).

222   The frequency of tumor-infiltrating immune cells was evaluated using flow-cytometry.

223 Tumors of neuT and neuT-C1KO mice displayed no statistically significant differences in the

224 frequency of T regulatory (Treg) cells, CD4$^+$, CD8$^+$ and γδ T cells, natural killer (NK) cells,

225 macrophages and myeloid-derived suppressor cells (MDSC) (**Fig. 5I-M**). These data suggest

226 that the C1q molecule is not involved in regulating leukocyte recruitment at the tumor site.

227 This is in contrast to observations in C3 deficient mice which instead show a significant

228 increase in infiltrating Treg cells (**Fig. 5I**; p=0.005, Student's *t*-test), as previously shown, [24]

229 and a significant reduction in NK cells (**Fig. 5L**; p=0.005, Student's *t*-test). These differences,

230 together with those in vessel dimension and complement activation, justify the fact that neuT-

231 C1KO tumors are less aggressive than those of neuT-C3KO mice (**Fig. 6A, B**; p=0.05 and

232 p=0.02, Log-rank Mantel-Cox test) despite showing comparable levels of pWWOX and

233 Her2/neu expression (**Fig. 4D, L**).

234

235 **Discussion**

236   Several studies have suggested that the complement system plays a critical role in

237 cancer growth and spread. It has been demonstrated that cancer cells establish a balance

238 between complement activation and inhibition. [29] However, controversial and conflicting data

239 on the complement system's tumor-promoting, [30,31] and inhibiting activities, [24] have been

240 published, [2] and the mechanisms of complement-specific activities in the tumor

241 microenvironment are still unclear and demands further study.

242   The use of genetically engineered mice, which are predestined to develop fatal tumors

243 and are knocked out for one of the key complement cascade molecules, enabled us to test the

244   role of complement activation in cancer growth and development and will possibly lead to the

245   design of complement-related anticancer strategies.

246   We have recently exploited the genetic predestination of neuT female mice to mammary

247   carcinogenesis to assess the weight of C3 complement-mediated [24] immune surveillance in

248   the development of autochthonous carcinomas. The data obtained demonstrated that the loss

249   of C3 activation, whose fragments were found to specifically accumulate in vessels and stroma

250   in and around the incipient cancers, was responsible for the dramatic increase in the

251   aggressiveness of neu+ mammary tumors. Concomitantly, as had already been demonstrated

252   in cancer patients, [32,33,34] spontaneous antitumor antibodies increased in neuT mice during

253   the course of tumor progression. [24] Taken together, these observations suggest that the

254   complement system can take on a tumor-inhibiting role that is played out through the

255   activation of the classical pathway.

256   The involvement of classical complement pathway activation in tumor cell killing was

257   further investigated by generating neuT-C1KO mice. An accelerated carcinoma progression

258   was evident in these mice. The comparable level of C3 fragment deposition observed in the

259   mammary glands of neuT-C1KO mice and neuT-BKO mice, which are unable to produce

260   antibodies, highlight the fact that tumor cell killing is not related to complement classical

261   pathway activation in neuT mice. By contrast, the decreased activation of the oncosuppressor

262   WWOX and the over-expression of the neuT protein on the mammary tumor cells found in

263   neuT-C1KO mice point to the key and direct role that C1q plays in tumor cell elimination.

264   Indeed, recent data have highlighted the existence of non-canonical functions that are exerted

265   by C1q on several types of cells, including cancer cells. Irrespectively of the beneficial or

266   harmful impact that the complement system has on tumor growth, the C1q molecule's

267   contribution to tumor progression and metastasization has been demonstrated regardless of

268   complement activation, both in prostatic cancer cells [15, 35] and very recently in melanoma. [31]

12

269   These results provide a new perspective on our previously published data which

270   demonstrated that the neuT up-regulation on tumor cells observed in neuT-C3KO tumors was

271   not only directly dependent on lack of C3 activation. In particular, the extremely aggressive

272   phenotype displayed by neuT-C3KO tumors can also be caused by the impaired activation of

273   WWOX, which is a result of the unexpected lack of C1q deposition on the tumor site of neuT-

274   C3KO mice. A relationship between C1q-induced expression and hypoxia has been

275   demonstrated in neurons. [36] Although the mechanisms by which C1q mRNA expression is up-

276   regulated by hypoxia remain to be investigated, it has been demonstrated that cultured cells,

277   which did not express C1q before hypoxia, did express C1q mRNA and protein during and

278   after hypoxia, respectively. [36] It has recently been demonstrated, by both

279   immunohistochemistry and western blotting experiments, that neuT tumor cells express a

280   low but detectable level of HIF1 protein. [37] Similar amounts of HIF1 protein were also

281   observed in neuT-C1KO tumors, while it was almost absent in neuT-C3KO tumors

282   (**Supplementary Fig. S3 A-C**). These data, together with the previously reported increased

283   blood vessel permeability and reduced necrotic areas within neuT-C3KO neoplasms, as

284   compared with neuT lesions, suggest that the hypoxic phenotype of neuT-C3KO is lower than

285   that of both neuT and neuT-C1KO tumors. [24] Altogether, these observations lead to speculate

286   that the unexpected lack of the C1q protein observed in neuT-C3KO tumors may be due to the

287   fact that C1q mRNA transcription is not up-regulated by hypoxia in these tumors, as it is in

288   neuT and neuT-C1KO tumors. Indeed, using the LASAGNA-Search tool [38]

289   (http://biogrid.engr.uconn.edu/lasagna_search/) to perform binding sites searches on mouse

290   C1q promoter, we confirmed the presence of binding sites specific for the transcriptional

291   factors RelA, [39] Meis-1a, [40] AhR [41] and Arnt, [42] known to be present on human C1q promoter

292   and linked to hypoxia (GeneCards®, Human Gene Data base; http://www.genecards.org/cgi-

293   bin/carddisp.pl?gene=C1QA&keywords=c1qa).

294   Contradictory effects of complement in modulating vascularization and angiogenesis are

295   described in the literature. [30,31,43] Our immunohistochemistry data showed a marked

296   deposition of C1q on vascular endothelium and stroma of neuT tumors. On the other hand, the

297   increased tumor vascularization observed in neuT-C1KO mice suggests that C1q could be

298   considered an inhibitor of tumor angiogenesis, at least in our model of autochthonous Her2-

299   driven mammary cancer that display a process of angiogenesis [44] not superimposable to that

300   observed in transplantable tumors. [31] Further investigations are warranted to define the

301   underlying mechanisms of this C1q-mediated inhibition of tumor vasculature.

302   The hypothesis that C1q may have an effect in the induction of an immunosuppressive

303   tumor microenvironment, was excluded by an analysis of tumor infiltrating lymphocytes.

304   Contrary to results in tumors from neuT-C3KO mice, [24] no differences in the percentages of

305   myeloid derived suppressor cells and Treg cells were found. The significant reduction in NK

306   cells in tumors from neuT-C3KO mice, as compared to both neuT and neuT-C1KO tumors, is

307   certainly worthy of note. The lack of a C1q-induced immunosuppressive tumor phenotype,

308   together with the presence of a significant amount of NK cells, can help explain why neuT-

309   C1KO tumors are less aggressive than their neuT-C3KO analogues. Indeed, NK cells can lyse

310   tumor cells after the recruitment of complement receptor 3 (CR3) by iC3b, especially when

311   MHC class I molecules are poorly expressed. [45] NeuT+ tumor cells are susceptible to the

312   activity of NK cells, as an inverse correlation exists between the expression levels of neuT

313   protein and those of MHC class I molecules as well as other components of the antigen-

314   processing machinery. [46] Moreover, signaling through the Her2/Her3 pathway in breast

315   tumor cell lines has been shown to enhance their recognition by NK and T cells thanks to the

316   killer cell lectin-like receptor subfamily K, member 1 (KLRK1, or NKG2D). [47]

317   In conclusion, our data demonstrate that the C1q protein protects against the

318   development of Her2/neu+ mammary carcinomas, at least in our preclinical BALB-neuT

319   female mouse model. The tumor-inhibiting C1q-mediated effects do not appear to be

320   associated with the classical pathway of complement, but with its direct role on endothelial

321   and tumor cells. Based on our results and on data from the literature we propose the

322   following mechanism of C1q direct antitumoral role in neuT mammary carcinogenesis. The

323   C1q binding with its receptor leads to the phosphorylation and consequent activation of

324   WWOX, which is a known oncosuppressor required to block cancer cell proliferation. [15]

325   Indeed, activated WWOX may induce the apoptosis of mammary tumor cancer cells, probably

326   by interacting with p53, [48] and inhibit the EMT processes through the expression of E-

327   Cadherin. Conversely, the lack of C1q prevents WWOX activation leading to tumor cell growth

328   and metastases formation. As far as the mechanisms linking the absence of C1q and the

329   increased levels of neuT protein expression on mammary tumor cells are concerned, our

330   hypothesis is that WWOX activation may be involved in neuT post-translational negative

331   regulation (**Fig. 7**). By contrast, WWOX-dependent transcriptional regulation of neuT gene is

332   unlikely since this transgene is under the control of the MMTV promoter and not of the

333   endogenous one.

334   However, as expected for a system with various distinct activities, this protective role in

335   mammary cancer does not appear to be applicable to every tumor type. Indeed, recent

336   findings have highlighted C1q's role as a cancer-promoting factor in a transplantable model of

337   melanoma. [31] However, numerous variables, including the genetic background of mice used,

338   the type of tumors and especially the use of transplantable instead of autochthonous tumors,

339   may all influence how the complement system affects tumor progression, culminating in very

340   different biological outcomes. Moreover, considering the opposite function exerted by the two

341   known C1qR, we can speculate that the tumor inhibiting- or promoting- effect mediated by

342   the C1q protein may results from different degrees of cC1qR or gC1qR expression in the

343   tumor.

344   Nevertheless, it is important to note that C1q can exert its functions directly on tumor

345   cells, independently of complement activation pathways, both in breast cancer and in

346   melanoma. Defining the role of C1q in different tumor types may lead to new drugs in the

347   clinic, thanks to its role as a major contributor to the immunosurveillance and control of

348   cancer progression as well as tumor growth and dissemination.

349

350   **Materials and methods**

351   **Mice.** BALB/c mice that were deficient for the C1qA (BALB-C1KO) and C3 (BALB-C3KO)

352   complement component were provided by Prof. Marina Botto (Imperial College, London, UK).

353   [49] NeuT male mice [28] from Biogem (Ariano Irpino, Italy) were crossed with BALB-C1KO and

354   with BALB-C3KO females in order to obtain neuT$^+$ C1$^{+/-}$ and neuT$^+$ C3$^{+/-}$ heterozygous male

355   mice, respectively. These heterozygous male mice were then crossed with BALB-C1KO and

356   BALB-C3KO females; the progeny was genotyped in order to identify neuT$^+$ C1$^{-/-}$ (neuT-C1KO)

357   and neuT$^+$ C3$^{-/-}$ (neuT-C3KO) female mice that were then used in the experiments. NeuT-BKO

358   mice were generated by crossing neuT mice with BALB/c mice KO for the immunoglobulin μ

359   chain gene, as previously described. [27] The mammary glands of all neuT mice were inspected

360   and palpated twice a week for tumor appearance. Individual neoplastic masses were

361   measured with calipers in two perpendicular diameters and the average value was recorded.

362   Progressively growing masses > 1 mm in mean diameter were regarded as tumors. Neoplastic

363   growth was monitored until the first tumor that exceeded a mean diameter of 10 mm was

364   found, at which point mice were euthanized for ethical reasons. Tumor multiplicity was

365   calculated as the cumulative number of incident individual tumors/total number of mice and

366   is reported as mean ± SEM. All mice were maintained at the Molecular Biotechnology Center,

367   University of Torino, in specific pathogen free conditions (Allentown Caging Equipment,

368   Allentown Inc., Allentown, NJ) and treated in conformity with current European guidelines

369   and policies. The Bioethical Committee of the University of Torino approved the experimental

370   plan.

371

372   **Morphological analyses.** The whole mount preparation of mammary glands was

373   carried out by removing the mouse skin and fixing it overnight in 10% buffered formalin.

374   Mammary fat pads were scored into quarters and gently scraped from the skin. These were

375   immersed in acetone overnight and then rehydrated and stained in ferric hematoxylin

376   (Sigma-Aldrich), dehydrated in increasing concentrations of alcohol, cleared with histo-

377   lemon, and stored in methyl salicylate (Sigma-Aldrich). Digital pictures were taken using a

378   Nikon Coolpix 995 (Nital, Medley, FL) mounted on a stereoscopic microscope (MZ6; Leica

379   Microsystems, Milano, Italy) and analyzed as previously described in detail. [50]

380

381   **Histology, immunohistochemistry and immunofluorescence.** Lung samples from 14

382   neuT and 19 neuT-C1KO 17 week-old mice were fixed in formalin and embedded in paraffin.

383   To optimize the detection of microscopic metastases and ensure systematic uniform and

384   random sampling, lungs were processed as previously described. [24] For the evaluation of

385   spontaneous metastases of all the experimental groups, sections were stained with

386   hematoxylin/eosin and with anti-Her2 immunohistochemistry. Metastases were counted

387   independently by two pathologists in a blind fashion.

388   Mammary glands were fixed in formalin and embedded in paraffin or fixed in

389   paraformaldehyde 1% and frozen in a cryo-embedding medium (OCT, Bioptica) for

390   histological and immunohistochemical analyses. Six to eight mm mammary tumors were fixed

391   in paraformaldehyde 1% and frozen in a cryo-embedding medium (OCT, Bioptica).

392   Sections were incubated with the mouse monoclonal anti-PCNA antibody (Dako

393   Corporation, M0879), rabbit anti-active caspase-3 (R&D systems, AF835), rabbit anti-human

394 Her-2 (Dako Corporation, A0485), rabbit anti-mouse E-Cadherin (Cell Signaling, 24E10),

395 rabbit anti-phospho-WWOX (pTyr[33]) (Sigma-Aldrich, SAB4504681), rat anti-mouse C1q

396 (Abcam, ab11861), rat anti-mouse C3b/iC3b/C3c mAb (HyCult biotech, 2/11), rat anti-CD105

397 (BD Pharmingen, 550546) and rat anti-CD31 (BD Pharmingen, 550274). For

398 immunohistochemical staining, sections were then incubated with the appropriate

399 biotinylated secondary antibody (Jackson Immunoresearch Laboratories). Immunocomplexes

400 were detected using NeutrAvidin™ Alkaline Phosphatase Conjugated (Thermo Scientific-

401 Pierce Biotechnology) and Vulcan Fast Red (Biocare Medical) or Streptavidin Peroxidase

402 (Thermo Scientific) and DAB Chromogen System (DakoCorporation). For

403 immunofluorescence analysis, sections were then incubated with the appropriate Alexa 488

404 and 546 labeled secondary antibodies (all from Molecular Probes); nuclei were stained with

405 DAPI (Sigma-Aldrich) or TO-PRO®-3 iodide (Thermo-Fisher Scientific). Images were acquired

406 on a Zeiss ApoTome fluorescence microscope (Axiovert 200M, Zeiss, Jena, Germany) and

407 captured using a CCD cool digital camera (Zeiss) or on a Zeiss LSM 510 META confocal

408 microscopy.

409 The percentage of PCNA$^+$ tumor cells was evaluated counting positive and negative

410 cells on the digital images of 10 neuT and 10 neuT-C1KO tumors (3 ×400 microscopic fields

411 per tumor) by 2 pathologists, independently and in a blind fashion.

412 The number of activated Caspase 3$^+$ tumor cells was evaluated counting positive cells

413 on the digital images of 10 neuT and 10 neuT-C1KO tumors (3 ×200 microscopic fields per

414 tumor) by 2 pathologists, independently and in a blind fashion.

415 The intensity of E-Cadherin expression was evaluated and recorded using the Image J

416 software and by analyzing ROI with similar cell numbers (3 ×630 microscopic fields per

417 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-

443 69879) and appropriate secondary antibodies (goat anti-rabbit, Sigma_Aldrich, A0545 and

444 goat anti mouse Sigma_Aldrich, A4416, respectively) in 3% BSA TBS-Tween buffer overnight

445 at 4° C and for 1 h at room temperature, respectively, and visualized using enhanced

446 chemiluminescence (ECL Plus, Thermo Scientific Pierce). Protein modulations were

447 normalized on the actin loading control and expressed as Adjusted Volume Intensity/mm^2

448 (background subtraction) using Quantity ONE software (Biorad, Milano, Italy).

449

450 **Cytometric identification of tumor infiltrating leukocytes.** For the infiltrating-cell

451 phenotypic analyses, fresh primary tumor specimens of 6-8 mm mean diameter from 5 neuT,

452 7 neuT-C1KO and 6 neuT-C3KO mice were finely minced with scissors and then digested by

453 incubation with 1 mg/ml collagenase IV (Sigma Aldrich) in RPMI-1640 (Life Technologies) at

454 37° C for 1 h in an orbital shaker. After washing in PBS supplemented with 2% fetal bovine

455 serum (GIBCO), the cell suspension was incubated in an erylise buffer (155mM NH4Cl,

456 15.8mM Na2CO3, 1mM EDTA, pH 7.3) for 10 minutes at RT. After washing in RPMI-1640

457 supplemented with 10% FBS, the cell suspension was passed through a 70-µm pore cell

458 strainer, centrifuged at 1400 rpm for 10 min and re-suspended in an erylise buffer. Tumor

459 infiltrating leukocytes were collected, washed, re-suspended in PBS, treated with Fc receptor

460 blocker (anti CD16/CD32; 01245B; BD Bioscences), and stained with the following antibodies:

461 anti-mouse CD45 VioGreen (Miltenyi Biotec, 130097), anti-mouse CD3 FITC (Miltenyi Biotec,

462 130-092962), anti-mouse CD4 APC-Vio770 (Miltenyi Biotec, 130-102-179), anti-mouse CD8

463 VioBlue (Miltenyi Biotec, 130-094-360), anti-mouse γδ PE/Cy7 (BioLegend, 118124), anti-

464 mouse CD49b PE (Miltenyi Biotec, 130-091-816), anti-mouse F4/80 APC (Miltenyi Biotec,

465 130-102-379), anti-mouse CD11b FITC (Miltenyi Biotec, 130-081-201), and anti-mouse GR-1

466 PE (Miltenyi Biotec, 130-102-426). To detect FoxP3$^+$ T regulatory cells, samples were

467 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

469 analyzed on a CyAn ADP flow cytometer using Summit 4.3 software (Beckman Coulter,

470 Milano, Italy).

471

472 **Captions to figures**

473

474 **Figure 1. C1q deficiency is responsible for accelerated tumor growth in neuT mice.**

475 Tumor incidence (**A**) and multiplicity (**B**) of mammary carcinomas in neuT (n = 30, gray line)

476 and neuT-C1KO (n = 18, black line) mice. Earlier incidence (*** p<0.0001, Log-rank Mantel-

477 Cox Test) and higher tumor multiplicity (starting from the 17th week of age, p values ranging

478 from p=0.04 to p<0.0001, Student's *t*-test) were found in neuT-C1KO as compared to neuT

479 mice. **C**: Time required for a 2 mm mean diameter tumor to reach an 8 mm threshold. Tumors

480 that arose in neuT-C1KO (black bar) mice grew significantly faster than those growing in neuT

481 (grey bar) mice (** p=0.001, two-tailed Student's *t*-test). (**D-I**) Representative whole mount

482 images of the fourth (inguinal) mammary glands of 11- (**D, G**), 15- (**E-H**), 17- (**F, I**) week-old

483 neuT (**D-F**) and neuT-C1KO (**G-I**) mice. The central oval black shadows are the intra-

484 mammary lymph nodes. Magnification x6.3. (**J, K**) Histological and immunohistochemical

485 staining for the PCNA of mammary tumor lesions in neuT (**J**) and neuT-C1KO (**K**) mice.

486 Magnification x400. PCNA+ tumor cell quantification (**L**) in neuT (grey bar) and in neuT-C1KO

487 (black bar) mice (** p=0.001, two-tailed Student's *t*-test). (**M, N**) Histological and

488 immunohistochemical staining for the active caspase-3 in mammary tumor lesions of neuT

489 (**M**) and neuT-C1KO (**N**) mice. Black arrows indicate apoptotic tumor cells. Magnification

490 x400. Active caspase-3+ tumor cell quantification (**O**) in neuT (grey bar) and in neuT-C1KO

491 (black bar) mice (** p=0.002, two-tailed Student's *t*-test).

492

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-**

23

543   **M**) Flow cytometry analysis of infiltrating leukocytes in 6-8 mm mean diameter tumors from

544   neuT (n = 5; gray bars), neuT-C1KO (n = 7; black bars) and neuT-C3KO (n = 6; white bars)

545   mice. (**I**) CD3$^+$ leukocytes were gated and CD3$^+$ CD4$^+$ CD25$^+$ FoxP3$^+$ were identified as Tregs

546   (**p=0.005; two-tailed Student's t-test). (**L**) CD45$^+$ leukocytes were gated and CD3$^+$ CD4$^+$ cells

547   were identified as CD4 T, CD3$^+$ CD8$^+$ as CD8 T, CD3$^+$ γδ$^+$ as γδ T and CD3$^-$ CD49b$^+$ as NK

548   (**p=0.005;   two-tailed Student's t-test). (**M**) CD45$^+$ CD11b$^+$ leukocytes were gated and

549   F4/80$^+$ cells were identified as macrophages (MΦ) while GR-1$^+$ cells were identified as

550   myeloid derived suppressor cells (MDSC). Bars represent the percentage of positive cells ±

551   SEM.

552

553   **Figure 6. NeuT-C1KO tumors are less aggressive than those of neuT-C3KO mice.** Tumor

554   incidence of mammary carcinomas (**A**) and overall mice survival (**B**) in neuT (n = 20,

555   continuous gray line), neuT-C1KO (n = 14, continuous black line) and neuT-C3KO (n = 15,

556   dotted black line) mice. Neu-T-C3KO mice displayed earlier tumor incidence (*p=0.05, Log-

557   rank Mantel-Cox Test) and lower overall survival (*p=0.02, Log-rank Mantel-Cox Test) than

558   neuT-C1KO mice.

559

560   **Figure 7. Proposed mechanism of C1q influence on neuT tumor progression.** C1q

561   component of C1 complex (C1q, C1s and C1r) appears to act directly both on tumor

562   vasculature (on the right) and on tumor cells (on the left). Deposition of C1q on vascular

563   endothelium inhibits tumor angiogenesis through a still undefined mechanism. C1q binding

564   with its receptor(s) (C1qR) on tumor cells leads to the phosphorylation of tyrosine 33 (Y33)

565   on WWOX. Activated WWOX in turn inhibits the EMT processes, through the expression of E-

566   Cadherin, and induces Caspase-3-mediated apoptosis, probably by engaging p53. We

567   hypothesize activated WWOX may be also involved in neuT post-translational negative

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

569   lines: pro-tumor activities.

570

578

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Bandini et al., Figure 1

neuT   neuT-C1KO

A   B   C

lung metastases (%)

D

E-Cadherin

actin

neuT   neuT-C1KO   neuT-C3KO

E

area (pixel) x 10⁻³

F   G   H   I

area (pixel) x 10⁻³

Bandini et al., Figure 2

neuT

neuT-C1KO

neuT-BKO

area (pixel) x 10^{-3}

pWWOX

A B C **D**

area (pixel)

300
200
100
0

*
*
ns

C1q

E F G **H**

area (pixel)

20
15
10
5
0

**
**
*

Her2/neu

I J K **L**

area (pixel)

300
200
100
0

*
*
ns

neuT   **neuT-C1KO**   **neuT-C3KO**

Bandini et al., Figure 4

Bandini et al., Figure 5

Bandini et al., Figure 6

neuT

C1r **C1q** C1s

Tumor Progression

C1qR

WWOX P **Y33**

Cytoplasm

Tumor cell

WWOX P
p53

Apoptosis

Nucleus

Caspase-3

Promoter
↑ E-Cadherin
↓ EMT

Metastatization

Tumor mass

Blood vessel

Angiogenesis

?

Bandini et al., Figure 7