C1q is an active player in cancer microenvironment promoting tumour growth and invasion independently of complement activation

Damiano Rami
C1q IS AN ACTIVE PLAYER IN CANCER MICROENVIRONMENT PROMOTING TUMOUR GROWTH AND INVASION INDEPENDENTLY OF COMPLEMENT ACTIVATION

Settore scientifico-disciplinare: MED/04 PATOLOGIA GENERALE

DOTTORANDO
DAMIANO RAMI

COORDINATORE
PROF. GUIDALBERTO MANFIOLETTI

SUPERVISORE DI TESI
PROF. ROBERTA BULLA

CO-SUPERVISORE DI TESI
DR. CHIARA AGOSTINIS

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This dissertation is dedicated to my family, my mom Daniella, my dad Gabriele, and my sister Anna, and to my love Patrizia, without whom I would have never survived this endeavour.

“You treat a disease, you win, you lose. You treat a person, I guarantee you, you'll win, no matter what the outcome... Our job is improving the quality of life, not just delaying death”

(Hunter "Patch" Adams)
# TABLE OF CONTENTS

TABLE OF CONTENTS .................................................................................................................. 1
LIST OF FIGURES AND TABLES ................................................................................................. 5
ABBREVIATIONS .......................................................................................................................... 7
SUMMARY ........................................................................................................................................ 11
INTRODUCTION ............................................................................................................................ 13

Chapter 1 ........................................................................................................................................ 13
1.1 Cancer is more than a genetic disease ..................................................................................... 13
1.2 The role of the microenvironment on the progression of cancer ............................................ 15
  1.2.1 Influence of cancer-associated fibroblasts on tumour behaviour .................................... 15
  1.2.2 Extracellular matrix remodelling in cancer ....................................................................... 16
  1.2.3 Tumour angiogenesis and hypoxia ..................................................................................... 17

Chapter 2 ........................................................................................................................................ 21
2.1 Immune system: a double-edged sword in cancer ................................................................. 21
2.2 Cancer immunoediting: from immunosurveillance to immune escape .................................. 21
  2.2.1 Elimination phase: anti-tumour immune response ............................................................. 23
  2.2.2 Equilibrium phase: tumour persistence .............................................................................. 24
  2.2.3 Evasion phase: tumour progression .................................................................................... 24
    2.2.3.1 Tumour cell intrinsic mechanisms .............................................................................. 25
    2.2.3.2 Establishment of an immunosuppressive environment ....... 25

Chapter 3 ........................................................................................................................................ 27
3.1 The Complement System ......................................................................................................... 27
  3.1.1 Pathways of complement activation ................................................................................... 27
3.2 The role of complement in tumour growth .............................................................................. 30
  3.2.1 Immune surveillance function of complement in cancer .................................................. 30
  3.2.2 Complement and immunotherapeutic monoclonal antibodies in cancer ....................... 32
  3.2.3 Cancer-associated resistance mechanism to complement activation .............................. 34
  3.2.4 Strategies for the improvement of complement-mediated immunotherapy ...................... 38
3.3 Complement activation can influence tumour growth ......................................................... 39
3.3.1 Role of the complement system in tumour angiogenesis and metastasis 40

Chapter 4 ............................................................................................................................... 41

4.1 C1q: the first component of the complement system .................................................... 41

4.1.1 Cellular sources of human C1q .................................................................................. 41

4.1.2 The structure–function relationship of the human C1q ........................................... 42

4.1.3 The main receptors of C1q ....................................................................................... 43

4.1.3.1 gC1qR: the receptor for the globular heads of C1q ........................................... 43

4.1.3.2 Calreticulin: the receptor for the collagen-like domain of C1q (cC1qR) ............... 44

4.2 The emerging functions of C1q independently on complement activation .............. 46

4.3 C1q and tumourigenesis: first evidences and beyond ................................................. 48

RATIONALE AND AIM OF THE STUDY ............................................................................. 51

MATERIALS AND METHODS ...................................................................................... 53

Antibodies and reagents .................................................................................................... 53
Human tissues .................................................................................................................... 53
Mice ...................................................................................................................................... 53
In vivo tumour models ....................................................................................................... 54
Bone marrow transplantation (BMT) and assessment of C1q levels ............................. 54
Immunohistochemical staining ......................................................................................... 55
Immunofluorescence analysis .......................................................................................... 55
Confocal analysis .............................................................................................................. 55
Evaluation of angiogenesis and metastases ..................................................................... 56
Flow cytometry .................................................................................................................. 56
Adhesion assay .................................................................................................................. 56
Assessment of Ki-67 expression ....................................................................................... 57
Migration assay .................................................................................................................. 57
Phosphorylation of ERK, Akt and GSK-3β in mouse melanoma cell line ..................... 57
Statistical analysis ............................................................................................................. 58

RESULTS .......................................................................................................................... 59

C1q is present in human tumour specimens .................................................................... 59
Prolonged survival and decreased tumour growth in C1q-deficient mice ....................... 59
Distinct features of melanoma in WT and C1qa−/− mice .................................................. 62
Effect of C1q on tumour angiogenesis and metastases.................................................. 66
Tumour growth in bone marrow transplanted mice..................................................... 67
Biologic effects of C1q on murine melanoma cells....................................................... 67

DISCUSSION.................................................................................................................. 71

CONCLUSION.............................................................................................................. 75

BIBLIOGRAPHY........................................................................................................... 77
LIST OF FIGURES AND TABLES

Introduction

Figure 1. Hallmarks of cancer.......................................................... 14
Figure 2. The tumour microenvironment.......................................... 15
Figure 3. The tumour microenvironment continually changes and evolves as the tumour grows, creating localized pockets of hypoxia, inflammation and ECM turnover that affect blood vessel growth, remodelling and maturation ................................................................................. 19
Figure 4. The “three Es” of cancer immunoediting............................. 22
Figure 5. Cascade of events during the activation of the complement system...... 29
Figure 6. Mechanisms used by cancer cells to resist complement activation........ 34
Figure 7. Potential tumour-promoting roles of complement proteins.............. 39
Figure 8. Structural organization of C1q ............................................ 42
Figure 9. The emerging and re-emerging functions of human C1q.............. 46

Table 1. Therapeutic monoclonal antibodies approved or in review in the European Union or the United States for use in cancer treatment ................. 33

Results

Figure 1A. Immunohistochemical analysis of human tumours for the presence and distribution of complement components................................. 60
Figure 1B. Analysis of tissue distribution of C1q........................................ 60
Figure 1C. Colocalization of CD34 and C1q on the vessel endothelium of colon cancer ...................................................................................... 60
Figure 1D. Deposition of C1q at the infiltration edge and in liver metastasis of colon cancer ...................................................................................... 60
Figure 2. Representative microphotographs showing differential C1q expression in the stroma of cancer-involved and non-involved mucosa ...................... 61
Figure 3A-B. Effect of C1q on tumour growth and survival of B16/F10 bearing mice 63
Figure 3C. Immunofluorescence analysis of tumour tissue for the distribution of the...
C1q, C4, C3 and endothelial vWF at day 14. ........................................... 63

Figure 3D.  Immunofluorescence staining of tumour tissue for C1q, C3 and
Macrophage CD68 at day 14. ................................................................. 63

Figure 3E.  Expression of C1q and C3 in tumour tissue collected from WT mice at
various days after B16/F10 injection. ...................................................... 64

Figure 3F-G.  Evaluation of tumour size in tumour bearing WT and C1q-deficient
mice........................................................................................................... 64

Figure 3H.  Percentage of different cell populations within the tumour mass on day
12 after the B16/F10 injection. ................................................................. 65

Figure 4A-F.  Evaluation of angiogenesis and lung metastases in tumour-bearing mice66

Figure 5A.  Tumour growth in BM transplanted mice. .................................... 68

Figure 5B.  C1q antigenic levels in reconstituted mice. ................................... 68

Figure 5C.  Localization of C1q (red) and macrophage CD68 (green) in tumour
section of BMT mice .................................................................................. 68

Figure 6A-C.  Effect of C1q on B16/F10 melanoma cell adhesion, migration and
proliferation, as well as C1q-mediated cell signalling................................. 70

Figure 6D.  Confocal analysis of B16/F10 seeded on slides coated with different
matrix ......................................................................................................... 70

Figure 6E-H.  Phosphorylation of ERK (Thr202/Tyr204), GSK-3β (Ser9) and Akt
(Thr308 and Ser473) in mouse melanoma cells. ........................................ 70
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>α4</td>
<td>Integrin, alpha 4</td>
</tr>
<tr>
<td>β1</td>
<td>Integrin, beta 1</td>
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<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AEC</td>
<td>3-Amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<tr>
<td>BMDCs</td>
<td>Bone Marrow-Derived Cells</td>
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<td>BMT</td>
<td>Bone Marrow Transplantation</td>
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<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>C</td>
<td>Complement</td>
</tr>
<tr>
<td>C1</td>
<td>C component 1</td>
</tr>
<tr>
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</tr>
<tr>
<td>C1r</td>
<td>C component 1, subunit r</td>
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<tr>
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<td>C component 3</td>
</tr>
<tr>
<td>C3a</td>
<td>C component 3, fragment a</td>
</tr>
<tr>
<td>C3b</td>
<td>C component 3, fragment b</td>
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<tr>
<td>CDC</td>
<td>C-Dependent Cytotoxicity</td>
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<td>C4BP</td>
<td>C4b-binding protein</td>
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<td>C5b-9</td>
<td>Terminal Complement Complex</td>
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<tr>
<td>CAFs</td>
<td>Cancer-Associated Fibroblasts</td>
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<tr>
<td>cC1q</td>
<td>Collagen-like domain of C1q</td>
</tr>
<tr>
<td>cC1qR</td>
<td>Receptor for the collagen-like domain of C1q</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CD4</td>
<td>T-cell co-receptor surface glycoprotein</td>
</tr>
<tr>
<td>CD8a</td>
<td>Cytotoxic T cells surface glycoprotein, alpha chain</td>
</tr>
<tr>
<td>CD11b</td>
<td>Integrin alpha M</td>
</tr>
<tr>
<td>CD14</td>
<td>Myeloid cell-specific leucine-rich glycoprotein</td>
</tr>
<tr>
<td>CD16</td>
<td>FcγRIIIa</td>
</tr>
<tr>
<td>CD31</td>
<td>Platelet endothelial cell adhesion molecule (PECAM-1)</td>
</tr>
<tr>
<td>CD32</td>
<td>Fc fragment of IgG, low affinity IIa, receptor</td>
</tr>
<tr>
<td>CD34</td>
<td>Hematopoietic progenitor cell antigen</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 molecule (Indian blood group)</td>
</tr>
<tr>
<td>CD45</td>
<td>Tyrosine phosphatase, leukocyte common antigen (LCA)</td>
</tr>
<tr>
<td>CD49b</td>
<td>Integrin, alpha 2</td>
</tr>
<tr>
<td>CD68</td>
<td>Lysosomal/endosomal-associated membrane glycoprotein (LAMP) family member</td>
</tr>
<tr>
<td>CD91</td>
<td>Low density lipoprotein receptor-related protein 1 (LRP1),</td>
</tr>
<tr>
<td>C3</td>
<td>alpha-2-macroglobulin receptor (A2MR), apolipoprotein E receptor (APOER)</td>
</tr>
<tr>
<td>CDC</td>
<td>C-Dependent Cytotoxicity</td>
</tr>
<tr>
<td>CDCC</td>
<td>C-Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>CI</td>
<td>Cancer Immunotherapy</td>
</tr>
<tr>
<td>CRPs</td>
<td>Complement Regulatory Proteins</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-Lymphocyte-Associated protein 4</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine3</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine5</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay-Accelerating Factor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific Intercellular</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>SDS/PAGE</td>
<td>Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour-Associated Antigens</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumour-Associated Macrophages</td>
</tr>
<tr>
<td>TCC</td>
<td>Terminal Complement Complex</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor α (TNFα)</td>
</tr>
<tr>
<td>Tp53</td>
<td>Tumour suppressor protein p53</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>TRIC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>USC</td>
<td>Uterine Serous Carcinoma</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>γδT</td>
<td>Gamma delta T-cells</td>
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</table>
SUMMARY

The complement (C) system is part of the local microenvironment acting as a bridge between innate and adaptive immunity and taking part in immune surveillance and homeostasis. The C activation is known to inhibit tumour development contributing to the destruction of cancer cells. This paradigm have recently been challenged by demonstrating a tumour-promoting role for C, which may support chronic inflammation, promote an immunosuppressive milieu, induce angiogenesis, and activate cancer-related signalling pathways.

C1q is the first component of the C system, which belongs to the Tumour Necrosis Factor superfamily of proteins and is mainly produced by macrophages, fibroblasts and endothelial cells. C1q plays a key role in apoptotic cell and immune complex removal. Besides activating the classical C pathway, growing evidence demonstrates that C1q has other biologic functions, including regulation of autoimmune diseases and promotion of angiogenesis and tissue remodelling.

The role of C1q in the pathogenesis of cancer is still unknown. The first evidence supporting a role of C1q in tumour progression has been proposed by Markiewski and colleagues, who interpreted the localization of C1q on tumour vessels in a mouse model of cervical carcinoma as an indication of C activation through the classical pathway.

We decided to investigate the contribution of C1q to cancer development following our previous finding that C1q is deeply involved in trophoblast invasion and placental development. The invasive behaviour of trophoblast resembles that of cancer cells, except that this is a physiological process, tightly regulated in time and space.

Our analysis of several solid human tumours revealed the presence of C1q that was distributed on the vascular endothelium and in the stroma in the absence of C4. The tumours were mildly positive for C3 and C1s. Analysis of melanoma and colon adenocarcinoma development revealed expression of C1q in a very early stage of malignant transformation. Overall, the data support a tumour-promoting role of C1q independently of classical C pathway activation.

C1qa−/− B16/F10 melanoma-bearing mice show inhibition of the tumour growth and increased survival rate compared to WT, C3−/− and C5−/− mice. Analysis of the tumour mass confirm the presence of C1q, but not C4, on vessels and in the tumour stroma. C3 was also detected mainly associated with tumour-infiltrating macrophages.
Analysis of growing murine melanoma revealed that C1q appeared earlier than C3 in the tumour mass and was only partially co-localized with CD68+ infiltrating cells, clearly suggesting that C1q was at least in part produced by non-myeloid cells.

The tumour mass in $C1qa^{-/-}$ melanoma-bearing mice showed reduced microvascular density compared to the WT supporting our recent findings that C1q induces angiogenesis by promoting wound healing. Moreover, the $C1qa^{-/-}$ mice manifested a lower number of lung metastases. No difference was observed in the percentage of tumour-infiltrating leukocytes between WT and $C1qa^{-/-}$ mice.

Bone marrow transplantation between WT and $C1qa^{-/-}$ mice revealed that the C1q-deficient background was associated with reduced tumour progression, even in the presence of C1q-producing myeloid cells in the tumour microenvironment. Analysis of the tumour mass in bone marrow transplanted mice clearly showed that tumour vessel endothelium and CD68-infiltrating cells mainly contribute to local production of C1q.

We demonstrated that solid–phase bound C1q is able to increase adhesion, proliferation and migration of B16/F10 melanoma cells in vitro, by stimulating the phosphorylation status of ERK, Akt and GSK-3β, possibly because of interaction with gC1qR and α4β1 integrin expressed on these cells. These evidences suggest C1q as an active player in the tumour microenvironment able to sustain tumour growth by enhancing several cancer-related signalling pathways.

Our data support the conclusion that C1q favours tumour growth and metastases facilitating cancer cell seeding and invasion through promotion of angiogenesis.
INTRODUCTION

Chapter 1

1.1 Cancer is more than a genetic disease

Cancer development is a multistep process where genetic changes are accumulated, thus progressively transforming cells into a cancerous phenotype. Several evidences in literature suggest that the cellular transformation process from the normal to the malignant state requires a number of steps wherein these tumourigenic features accumulate through somatic genetic alterations. These genetic aberrations are generally characterized as either gain of function in oncogenes or as loss of function of tumour suppressors. Gain of function mutations often confer new functions or inhibit normal regulatory mechanisms, leading to hyper-activation of associated pathways. Conversely, loss of function mutations in tumour suppressors inhibit the function of proteins usually involved in restraining cellular growth and replication, or response to genetic damage. Both gain or loss of function mutations can arise from a variety of mechanisms, including point mutations at critical residues within the protein, insertions or deletions that disrupt the normal gene structure, as well as variations in gene copy number that lead to aberrant expression (Hanahan and Weinberg, 2011).

Despite the notion that tumours arise through clonal expansion of cells that have acquired genetic alterations advantageous for proliferation, survival and metastasis, it is now accepted that epigenetic modifications play a key role in generating dynamic tumour heterogeneity (You and Jones, 2012). Epigenetic changes affect gene transcription by modulating the packaging of chromatin, including DNA methylation, histone variants and modifications, nucleosome remodelling as well as small non-coding regulatory RNAs, thereby regulating the accessibility of DNA to sequence-specific transcription factors (Sharma et al., 2009; Rodríguez-Paredes and Esteller, 2011). During tumour initiation and progression, the epigenome goes through multiple alterations, including a genome-wide loss of DNA methylation, frequent increases in promoter methylation of CpG islands, changes in nucleosome occupancy and modification profiles. More recently, intriguing evidence has emerged that genetic and epigenetic mechanisms are not separate events in cancer; they intertwine and take advantage of each other during tumourigenesis. Alterations in epigenetic mechanisms can lead to genetic mutations and genetic mutations
in epigenetic regulators leads to an altered epigenome (You and Jones, 2012). The readout of the epigenome variation dictates the diversity in cellular phenotypes of cells harbouring the same genome. Furthermore, a specific combination of genetic and epigenetic marks has been shown to be able to produce drug-resistant phenotypes in a heterogeneous tumour cell population (Wilting and Dannenberg, 2012). The fact that the epigenome acts at the pinnacle of the hierarchy of gene control mechanisms, by affecting multiple pathways relevant to the cancer phenotype, has reached mainstream oncology in the development of new biomarkers of the disease and new pharmacological strategies (Rodríguez-Paredes and Esteller, 2011).

Over the last decades, numerous investigators have studied the underlying molecular mechanisms for malignant transformation. This has resulted in many models that explain the development of a malignant phenotype of human cells, for instance the “Hallmarks of Cancer” by Hanahan and Weinberg (Hanahan and Weinberg, 2011). Cells undergoing malignant transformation acquire the ability to sustain proliferative signalling, evade growth suppressors, and enable replicative immortality by resisting cell death, promote inflammation, induce angiogenesis and deregulate cellular metabolism, avoid immune distraction, invade the surrounding tissue and spread to distant organs via the circulatory or lymphatic systems (Figure 1).

**Figure 1. Hallmarks of cancer.** Malignant tumours have overcome cell intrinsic suppressors of carcinogenesis such as apoptosis, scarcity of growth stimuli, anti-proliferative signalling, DNA-repair mechanisms and telomere shortening. Extrinsic hallmarks of cancer involve the induction of angiogenesis, tissue-remodelling leading to metastasis, evasion of anti-tumour immune responses, as well as the induction of tumour promoting inflammation. (Figures adapted from Hanahan and Weinberg, Cell, 2011; **144**:646-74).
1.2 The role of the microenvironment on the progression of cancer

There is strong evidence that tumour growth not only depends on the accumulation of genetic abnormalities in the originating cancer cells, but also on the local microenvironment that can provide a permissive niche for the survival, growth, and migration of the cancer cells (Barcellos-Hoff et al., 2013) (Figure 2). The host microenvironment undergoes extensive change during the evolution and progression of cancer, so that it is becoming evident that its characterization can provide important prognostic and predictive information about tumours, independent of the tumour cell phenotype (McAllister and Weinberg, 2014).

![Figure 2. The tumour microenvironment.](image)

**Figure 2. The tumour microenvironment.** The tumour microenvironment comprises the cancer cells, and either with invasive or staminal phenotype. In addition, endothelial cells, pericytes, fibroblasts, stromal cells as well as various immune inflammatory cells contribute to the malignant outgrowth (Figures adapted from Joyce and Pollard, Nat Rev Cancer, 2009; 9:239–252).

1.2.1 Influence of cancer-associated fibroblasts on tumour behaviour

*Cancer-Associated Fibroblasts (CAFs)* are among the most abundant cell type in the microenvironment of solid tumours. CAFs form a heterogeneous population, probably related to their diverse origin. Whilst activation of local stromal fibroblasts has traditionally been considered the major source of CAFs (Kalluri and Zeisberg, 2006), experimental models using genetically marked *Bone Marrow-Derived Cells* (BMDCs) have demonstrated that these cells can contribute to the tumour-associated stroma and develop markers and functions of CAFs. It has also been suggested that CAFs may be derived from normal or tumour cells that undergo *Epithelial–Mesenchymal Transition* (EMT), a theory that may explain the identification of identical genetic alterations in tumour epithelium and its associated stroma (Allen and Jones, 2011).
There are abundant evidences reporting that CAFs are able to release classical growth factors, such as Epidermal Growth Factor (EGF), Transforming Growth Factor Beta (TGFβ) and Hepatocyte Growth Factor (HGF), as well as a range of chemokines shown to influence different aspects of tumour cell behaviour. Moreover, CAFs directly promote tumour growth and mediate the recruitment of endothelial progenitor cells into the tumour mass, leading to enhanced angiogenesis (Kalluri and Zeisberg, 2006). Orimo et al. found that several unique characteristics of CAFs are maintained even in the absence of contact with tumour cells, implying that CAF function is not merely a response to tumour-derived signals (Orimo et al., 2005). In addition to promoting growth of established tumours, there is strong evidence that altered fibroblast signalling may be critical in the initiation of carcinogenesis, in regulating its phenotype and in mediating metastatic spread of tumours (Allen and Jones, 2011). Cunha et al. reported that the pro-tumour function of CAFs does not simply relate to enhanced mitogenesis. They showed that CAFs does not stimulate tumour development in a non-immortalized benign epithelium, suggesting that some epithelial abnormality is necessary to respond to the pro-tumourigenic effects of CAFs (Olumi et al., 1999). Erez et al. also demonstrated that CAFs support tumourigenesis by mediating tumour-enhancing inflammation through a NF-kB–dependent activation of a pro-inflammatory gene expression signature (Erez et al., 2010). A recent study by Vermeulen et al. has highlighted the role for CAFs also in the regulation of tumour behaviour, that of modulating the Cancer Stem Cell (CSC) phenotype (Vermeulen et al., 2010). This study indicates that the stem cell phenotype is plastic and is dependent on the tumour microenvironment, suggesting that targeting the CSC-microenvironment interface may be an effective approach to overcome stem cell resistance to current therapies.

### 1.2.2 Extracellular matrix remodelling in cancer

Besides releasing a range of chemokines and growth factors, CAFs also generate an altered extracellular matrix (ECM) environment. Most solid tumours exhibit a very different profile of ECM proteins in the stroma compared to their normal counterparts, and many of these proteins interact directly with tumour cells, via integrins and other cell surface receptors, to influence functions such as proliferation, apoptosis, migration and differentiation (Lu et al., 2012) (Figure 3). Among the number of proteins consistently up-regulated in solid tumours, fibronectin (FN) has attracted interest in cancer research, owing to its role in tumour progression. FN, a
class of high-molecular-weight adhesive glycoproteins, plays a prominent role in mediating ECM function, because of its high abundance and its interaction with cellular components (Kaspar et al., 2006). In tumours, alternative splicing of FN pre-mRNA, as well as post-translational modification, give rise to several isoforms of this complex protein. The oncofoetal form of FN that contain the extra-domain (ED)A is known to be required for the transduction of TGFβ signals, and the conversion of fibroblasts to myofibroblasts, a key event in the tumour microenvironment, whereas the variant that contain the extra-domain (ED)B is particularly associated with neovascular structures in many different tumour types (Allen and Jones, 2011).

As well as the composition of the ECM, the mechanical properties of the stroma also have a profound impact on function. The tensile strength or stiffness of the ECM can regulate epithelial cell growth, differentiation and migration, and reduction of ECM stiffness can repress the malignant behaviour the epithelial cells. These stromal changes are mainly associated with increased focal adhesion formation and increased Focal Adhesion Kinase (FAK) activity, and inhibition of β1-integrin in this context has been shown to prevent tumour invasion, suggesting that changes in ECM stiffness regulate epithelial cells through integrin signalling (Levental et al., 2009).

The ECM undergoes significant remodelling during tumour progressions, and this is mediated largely by the extracellular proteinases, particularly the matrix metalloproteinases (MMPs), and the major source of these are stromal cells (Egeblad and Werb, 2002). MMPs are implicated in the promotion of tumour invasion and metastasis. By contrast, several recent evidences in literature have shown that some MMPs act as tumour suppressors rather than tumour promoters and, as well as inducing angiogenesis, may in some instances lead to the activation of anti-angiogenic mechanisms (Deryugina and Quigley, 2010).

1.2.3 Tumour angiogenesis and hypoxia

Angiogenesis, the creation of new vessels from pre-existing ones, is a key mechanism of carcinogenesis that is directly related to the aggressiveness of cancer (Carmeliet, 2005) (Figure 3). Angiogenesis is required if a tumour is to progress past a certain size, and the microenvironment plays an important role in dictating when the “angiogenic switch” will occur. This event separates cancer development from small lesions, which are dormant, to an exponential growth phase. Tumours, as well as normal tissues, require a supply of nutrients and the removal of waste products to survive and
proliferate. As tumours grow, areas of nutrient deprivation and hypoxia arise because of an insufficient blood supply.

Although a limiting factor for tumour growth, oxygen deprivation also represents a stimulus for invasion and metastasis. A number of studies have shown that hypoxia is a negative prognostic and predictive factor in cancer, owing to its multiple contributions to chemoresistance, radioresistance, angiogenesis, vasculogenesis, resistance to cell death, altered metabolism and genomic instability (Wilson and Hay, 2011). Hypoxia stimulates Hypoxia-Inducible Family (HIF) of proteins, which regulate diverse cellular processes, including metabolism, angiogenesis, cell proliferation, apoptosis and tissue remodelling (Carmeliet, 2005). In addition, hypoxia can down-regulate epithelial E-cadherin, thus promoting EMT (Esteban et al., 2006) and activate Wnt/β-catenin signalling pathway, promoting a more motile and invasive phenotype (Sahlgren et al., 2008); moreover, low oxygen tension causes cancer cells to switch to anaerobic metabolism, which greatly increases the genetic instability of the cells (Kim et al., 2006). One of the key roles of HIF-1α in hypoxia is the induction of pro-angiogenic factors, including Vascular Endothelial Growth Factor (VEGF), angiopoietin-2, Platelet-Derived Growth Factor (PDGF) and Fibroblast Growth Factor (FGF), as well as downregulation of anti-angiogenic factors, such as thrombospondin. Whereas the major focus on hypoxia has been its role in enhancing angiogenesis, a number of angiogenesis-independent mechanisms for hypoxia-induced tumour progression have already been described (Fraisl et al., 2009). In one key study, Pennacchietti et al. showed that HIF-1α binds to the Hepatocyte Growth Factor Receptor (HGFR) promoter, also called c-Met, leading to an “invasive switch” in the tumour cells, increasing degradation of the ECM and allowing tumour cells to move freely towards more oxygen-rich areas (Pennacchietti et al., 2003). This has therapeutic implications, since targeting angiogenesis alone may not be sufficient, and indeed may even aggravate this invasive response to hypoxia (Allen and Jones, 2011).
Figure 3. The tumour microenvironment continually changes and evolves as the tumour grows, creating localized pockets of hypoxia, inflammation and ECM turnover that affect blood vessel growth, remodelling and maturation. Environmental or genetic events transform normal epithelial cells into tumour cells, which grow and divide with little effect on their surroundings until their size exceeds 1–2 mm. At that point, hypoxia and nutrient deprivation trigger the requirement for angiogenesis. Tumour cells release soluble growth factors, chemokines and cytokines, which create a concentration gradient that initiates the sprouting and proliferation of formerly quiescent endothelial cells on nearby blood vessels and lymphatics. These signals also recruit fibroblasts that deposit a repertoire of ECM proteins and enzymes in an attempt to remodel and repair the site. Most tumours elicit an inflammatory response that attracts myeloid cells into the tumour microenvironment, and these cell types release their stores of soluble factors to escalate the angiogenic response, thus favouring tumour progression (Figure from Weis and Cheresh, Nat Med, 2011; 17:1359–1370).
Chapter 2

2.1 Immune system: a double-edged sword in cancer

Inflammation is a complex physiological process that normally functions to maintain tissue homeostasis in response to tissue stressors such as infection or tissue damage, and it has recently been proposed to be the seventh hallmark of cancer (Colotta et al., 2009). The inflammatory response consists of an innate system of cellular and humoral responses following injury, in which the body attempts to restore the tissue to its pre-injury state. In the acute inflammatory response, there is a complex orchestration of events, involving activation of many different types of cells, such as endothelial and stromal cells, as well as activation of platelets and complement system, and recruitment of leukocytes, all of which may assist in coping with the state of injury. Acute inflammation frequently precedes the development of protective adaptive immune responses to pathogens and cancer. On the other hand, long-term inflammatory processes, directed at a particular endogenous or exogenous antigen, define the chronic inflammatory response. Chronic inflammation contributes to tumourigenesis at all stages. It takes part to cancer initiation by generating genotoxic stress, to cancer promotion by inducing cellular proliferation, and to cancer progression by enhancing angiogenesis and tissue invasion (Grivennikov et al., 2010). On the basis of these observations, it has been proposed that tumour-promoting inflammation and protective tumour immunity are dynamically interconnected processes that vie for dominance as tumour cells develop and transit through cancer immunoediting (Schreiber et al., 2011).

2.2 Cancer immunoediting: from immunosurveillance to immune escape

The immune system plays a dual role in cancer (Hagerling et al., 2014). It can not only suppress tumour growth, by destroying cancer cells or inhibiting their outgrowth, but also promote tumour progression, either by selecting for tumour cells that are more fit to survive in an immunocompetent host or by establishing conditions within the tumour microenvironment that facilitate tumour outgrowth (Schreiber et al., 2011). There is a growing consensus that the relationship between the immune system and cancer has more dimensions than just immunosurveillance (Dunn et al., 2002; Schreiber et al., 2011). Apart from eliminating cancer cells, immunity also sculpts the immunogenic phenotype of tumours to evade immune control. Analogous to Darwinian evolution, the immune system exerts selection pressure on the rapidly mutating, heterogeneous and genetically unstable
tumour mass to develop variants with reduced immunogenicity, to escape immunologic detection and destruction. These dual effects of the immune system on developing tumours prompted a refinement of the cancer immnosurveillance concept into the one termed cancer immunoediting (CI). This is a dynamic process composed of three phases: elimination, also known as immunosurveillance or protection, equilibrium or persistence, and escape or progression, which are collectively denoted the “three Es” of CI (Figure 4).

**Figure 4. The “three Es” of cancer immunoediting.** Cancer immunoediting (CI) is an extrinsic tumour suppressor mechanism that engages only after cellular transformation has occurred and intrinsic tumour suppressor mechanisms have failed. CI consists of three sequential phases: elimination, equilibrium and evasion. In the elimination phase, transformed cells are detected by the innate and adaptive immune system sensing stress signals, Natural Killer-Receptor (NKR) ligands and the presence of Tumour-Associated Antigens (TAAs). This leads to elimination of the nascent tumour, before it reaches clinically apparent stages. In the equilibrium phase, tumour cells that were not deleted are merely held in check by the immune system. The genetically unstable tumour mass is being selected to develop less immunogenic variants and induce an immunosuppressive tumour promoting microenvironment. Just like the elimination phase, the equilibrium phase is clinically not apparent and can last many years. In the evasion phase, the tumour has evolved into a non-immunogenic, highly immunosuppressive malignancy that attracts various innate and adaptive immune cells fostering invasive growth and maintaining a permissive environment while suppressing effector T-cell and CTL function (Figure from Schreiber et al., Science, 2011; 331:1565–1570).
2.2.1 Elimination phase: anti-tumour immune response

The elimination phase of CI is an updated version of the original concept of cancer immunosurveillance, in which the innate and adaptive immune systems work together to detect the presence of a developing tumour and destroy it before it becomes clinically apparent. In the case of partial tumour elimination, the theory of immunoediting is that a temporary state of dynamic equilibrium can then develop between the immune system and the developing tumour (Schreiber et al., 2011). The elimination phase has only been observed indirectly in vivo, but its existence has been inferred from the earlier onset or greater penetrance of neoplasia in immunodeficient mice (Vesely et al., 2011).

The initiation of the anti-tumour immune response occurs when cells of the innate immune system become alerted of the presence of the growing tumour, although the mechanisms of their activation are not yet completely understood. Local tissue disruption, as a result of increased angiogenesis, hypoxia, genome instability or tissue-invasive growth, and release of antigens during tumour cell necrosis, as well as oncogene-induced expression of stress-ligands, induces proinflammatory molecules, such as Interleukin 1 and 15 (IL-1; IL-15), Tumour Necrosis Factor α (TNFα), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), leading to the recruitment of innate immune cells. Dendritic cells may act as sentinel cells sensing tissue stress, damage and transformation. Their ability to uptake heat shock proteins and products of ECM breakdown promotes differentiation and subsequent crosstalk between Dendritic Cells (DCs) and Natural Killer cells (NK-cells). These cells, as well as Macrophages (MΦ), gamma delta T-cells (γδ T-cells) and Natural Killer T-cells (NKT-cells) are among the first cells attracted by cancerous lesions and comprise the first line of defence against tumours. They are recruited to the site of “danger” and recognize molecules that can be induced on tumour cells upon cellular transformation, such as stress induced ligands for the activating Natural-Killer Group 2, member D receptor (NKG2D) (Smyth et al., 2005). In addition, either Major Histocompatibility Complex (MHC)-tumour-associated peptide or glycolipid-CD1 complexes expressed on developing tumour cells can be recognized by T-cells and NKT-cells, respectively (Smyth et al., 2002). These immune cells exert several effector mechanisms to eliminate tumour cells including the release of Interferon-γ (IFN-γ) and perforin, or the expression of death receptor ligands, such as the TNF-Related Apoptosis-Inducing Ligand (TRAIL). Secretion of IFN-γ controls tumour growth and amplifies immune responses through the production of chemokines. In return, chemokines attract more immune cells to the tumour site generating a positive feedback loop for tumour elimination. In the next phase, tumour antigens released, during tumour
cell killing, drive the development of tumour-specific adaptive immune responses. Recruited immature DCs become activated through the tumour cytokine milieu and uptake of tumour antigens and migrate to the lymph nodes, where they induce the activation of naïve tumour-specific CD4+ and CD8+ T-cells (Griffith et al., 2014). Once activated, tumour-specific T-cells home to the tumour site to eliminate antigen-expressing tumour cells.

2.2.2 Equilibrium phase: tumour persistence

In the equilibrium phase immune cells and tumour cells, which survived the elimination phase, enter a dynamic process where new tumour variants with increased resistance to the immune attack arise (Aguirre-Ghiso, 2007). Schreiber and colleagues hypothesized that this immune-mediated latency phase is characterized by tumour dormancy and may be the longest one, occurring over a period of many years in humans (Smyth et al., 2006). Moreover, Koebel et al. showed that, unlike in the elimination phase, only the adaptive immune system plays a crucial role in the equilibrium phase (Koebel et al., 2007). During this period, heterogeneity and genetic instability of remaining cancer cells may favour the generation of poorly recognized tumours by the immune system, that have acquired mechanisms that suppress immune effector functions.

2.2.3 Evasion phase: tumour progression

The escape phase of CI is always clinically apparent, since it leads to a rapid progression of tumours. It is characterized by the presence of tumour variants that evade immune detection, are resistant to immune-mediated killing and that are able to induce an immunosuppressive microenvironment tolerating or even fostering tumour growth (Schreiber et al., 2011). Overcoming immunosurveillance by escape is one of the hallmarks of cancer (Colotta et al., 2009; Hanahan and Weinberg, 2011). Tumour cell evasion can occur through the development of intrinsic mechanisms at the cancer cell level; alternatively, escape may result from the establishment of an immunosuppressive state within the tumour microenvironment (Schreiber et al., 2011).
2.2.3.1 Tumour cell intrinsic mechanisms

A number of alterations in the tumour cell itself can reduce the chance of detection and destruction by the immune system. Selected tumour cell variants may avoid immune recognition by interfering with antigen presentation via down-regulation or loss of MHC class I protein expression, blocking the antigen processing and loading machinery (Khong and Restifo, 2002) or by becoming insensitive to IFN-γ (Dunn et al., 2005). Moreover, since the immune system negatively selects against specific tumour antigens, variants not expressing these antigens will prevail (Schreiber et al., 2011). In a similar fashion, cells may lose the expression of ligands for activating NK-cell receptors, such as NKG2D to escape detection of the innate immune system (Spear et al., 2013). Another important cell intrinsic alteration is the resistance to apoptosis, a classical hallmark of cancer (Hanahan and Weinberg, 2011). Anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family, the expression of mutated inactive death receptors, such as Fas, and the persistent activation of pro-oncogenic transcription factors, such as Signal Transducer and Activator of Transcription 3 (STAT3) (Schreiber et al., 2011) are frequently found in progressively growing tumours. In addition to being resistant to extrinsic induction of apoptosis, expression of inhibitory ligands such as Programmed Death Ligand-1 (PD-L1) may directly dampen cytotoxicity of T-cells and even induce apoptosis of cancer cells. Further evidences also reported that a direct “counterattack” by expression of Fas-ligand on tumour cells induces apoptosis in Fas-expressing T-cells (Vesely et al., 2011).

2.2.3.2 Establishment of an immunosuppressive environment

Beyond cell intrinsic mechanisms and alterations involving direct juxtacrine signalling, tumours establish a local immunosuppressive environment that prevents a protective anti-tumour response despite systemic immunocompetence (Schreiber et al., 2011). Tumour-derived immunosuppression is achieved by secretion of immunosuppressive soluble mediators. These molecules include VEGF, that leads to increased angiogenesis to overcome shortage of oxygen and nutrient supply, TGFβ, which suppresses effector NK-cells, DCs and T-cells, whereas it takes part into the differentiation of regulatory T-cells (Treg) cells and Th-17 cells, as well as indoleamine 2,3-dioxygenase (IDO) and interleukin (IL)-10, able to inhibit T-cells (Vesely et al., 2011). Furthermore, soluble factors that inhibit the activation of the innate immune system include decoy
ligands for NKG2D, that are shed from the cell membrane and thus interfere with innate recognition of transformed cells (Spear et al., 2013). Apart from blocking effector function of infiltrating immune cells, progressing tumours often attract regulatory and suppressive immune cells, that in turn suppress anti-tumour responses (Vesely et al., 2011). Treg cells and Myeloid-Derived Suppressor Cells (MDSCs) are two major types of immunosuppressive leukocyte populations that play key roles in inhibiting host-protective anti-tumour responses. Treg cells are CD4+ T-cells, which constitutively express Interleukin-2 Receptor Alpha chain (IL2RA; CD25) and the Forkhead box P3 transcription factor (FoxP3). Treg cells are able to inhibit the function of cytotoxic T lymphocytes (CTL; CD8+ T-cells). This occurs either by producing the immunosuppressive cytokines IL-10 and TGF-β or by expressing the negative co-stimulatory molecules Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4; CD152), Programmed cell Death protein 1 (PD-1; CD279), and PD-L1 and by consuming IL-2, a cytokine that is critical for maintaining CTL function (Schreiber et al., 2011).

Various tumour-derived soluble mediators, including VEGF, IL-1β and Prostaglandin-E2 (PGE2) also attract a heterogeneous group of immature myeloid cells and myeloid progenitor cells collectively termed MDSCs (Khaled et al., 2013). MDSCs directly inhibit lymphocyte function, by inducing Treg cells, secrete a number of immunosuppressive molecules, including IL-10 and TGF-β, as well as sequester the amino acids arginine, tryptophan, and cysteine required for T-cell function, or nitrating T-cell receptors or chemokine receptors on tumour-specific T-cells (Schreiber et al., 2011). Tumour-associated macrophages (TAMs) are another myeloid cell type that contribute to the immunosuppressive microenvironment of the tumour. Attracted by IL-4 and IL-13, TAMs are usually M2-polarized macrophages and mediate suppression by secretion of TGF-β and IL-10 (Noy and Pollard, 2014).

Taken together, the escape phase of cancer immunoediting involves various cell types of both adaptive and innate immunity, a fundamental change in the prevalent signalling molecules, and is not only dependent on various types of cells attracted to the tumour, but also on repolarization of inflammatory cells that already present surrounding the tumour.
Chapter 3

3.1 The Complement System

Complement (C) is a well-known arm of innate immunity, providing an early warning signal and fast response upon encountering a foreign antigen, although recent findings have revealed that C orchestrates many more immunological and inflammatory processes that contribute to maintain the homeostasis of different organs and tissues (Ricklin et al., 2010). The C system is populated by more than 50 proteins found in the fluid phase and on cell surfaces, which function as a proteolytic cascade amplifying an initial signal (Walport, 2001; Pio et al., 2014). These proteins can be zymogens, which become active enzymes upon activation of C, effectors, receptors, or control proteins, which help maintain well-balanced activation and inhibition of the system. The role that C plays in immunity is multifaceted and complex. It functions in infection control through cytolysis of invading pathogens, opsonisation for phagocytosis and release of anaphylatoxins, which recruit and activate immune cells. It is also important in immune complex clearance. C can act as a bridge between innate and adaptive immunity helping to regulate B- and T-cell responses. Apart from these key immune functions, it is implicated in processes including regulation during development of neuronal networks, tissue regeneration and angiogenesis (Pio et al., 2014).

3.1.1 Pathways of complement activation

The C system has three well-established activation pathways, classical, lectin and alternative (Walport, 2001) (Figure 5). The first component of the classical pathway is C1 (C1qr2s2) complex, which is formed by C1q together with C1r and C1s, two serine protease proenzymes (Kojouharova et al., 2010). Activation of this pathway is primarily initiated by the binding of C1q to either IgG or IgM bound to antigen in immune complexes (Degn and Thiel, 2013). This pathway can also be activated in the absence of antibodies, following the recognition by C1q of several endogenous ligands, including dying cells (Nauta et al., 2002), ECM proteins (Sjöberg et al., 2009) and the fluid phase pattern recognition molecule pentraxin 3 (PTX3) (Doni et al., 2012), as well as the acute-phase protein C-reactive (PCR) protein (McGrath et al., 2006). Binding of C1q induce a conformational change leading to auto-activation of the associated C1r, which then cleaves and activates C1s (Venkatraman Girija et al., 2013). Thus, C1s can cleave both the C proteins C4, to release the anaphylatoxin C4a, and the
C4b fragment, and C2, to produce C2a and C2b fragments, which forms part of classical C3-convertase (C4b2a), in conjunction with C2a. The resulting C3 convertase is responsible for the initiation of the common terminal pathway (Degn and Thiel, 2013).

The lectin pathway is initiated upon binding of mannose-binding lectin (MBL) or one of the ficolins (M, L and H) to patterns of N-acetyl residues and those of carbohydrate surfaces. MBL and ficolins are soluble pattern-recognition molecules, that share with C1q collagen-like regions (Degn and Thiel, 2013). These proteins form a C1q-like complex with MBL-Associated Serine Proteases (MASPs), cleaving the C components C4 and C2 to form the C3-convertase, which is common to the classical pathway activation route (Fujita, 2002).

The alternative pathway could be activated by spontaneous low-level hydrolysis of the central component of the C system, C3, to form C3(H2O). This mechanism of activation is referred to be as the “tick-over” of C3. C3(H2O) can bind to factor B, which is cleaved by factor D to form the initial alternative pathway C3-convertase, C3(H2O)Bb. Kinin-generating proteases kallikrein and thrombin are also capable of activating C (Kolev et al., 2014) by cleaving C3.

All C pathways converge on the C3 molecule, which upon cleavage by the C3-convertase (C4b2a) forms C3a, a potent anaphylatoxin, and C3b, an opsonin (Walport, 2001; Kolev et al., 2014). C activation is tightly regulated in vivo, so that various regulators rapidly cleave C3b to its inactive form, iC3b. However, some C3b can bind to C-activating surfaces and associate with factor B, and this protein complex again can be cleaved by factor D, forming the predominant alternative pathway C3-convertase (C3bBb). The binding of properdin enhance the stability of this convertase. C3bBb can also generate large amounts of C3b through the alternative pathway amplification loop. Binding of another C3b-fragment to the C3-convertase creates a C5 convertase, which consists of C3b3bBb in the alternative pathway, instead of C4b2a3b to the classical or lectin pathway. C5 convertase can cleave the C protein C5 to release C5a, another potent anaphylatoxin of the C system, and C5b, that associates with C6 and C7 to form C5b67 complex, which inserts into the cell membrane and then binds to C8 and C9 to form the membrane attack complex (MAC), also known as C5b-9 complex. The latter, also known as Terminal Complement Complex (TCC) promotes lysis of cells and microorganisms when in sufficient quantities (Walport, 2001; Kolev et al., 2011).
Figure 5. Cascade of events during the activation of the complement system. C activation represents a well-preserved innate immune response, which is able to act fast, within seconds to minutes, as well as sustainable (over days). The activation of the C system can occur through at least three well-known pathways: the classical, the lectin and the alternative pathway.

Activated C1q, in complex with C1s and C1r, initiates the classical pathway upon binding of antigen-antibody complexes. The lectin pathway starts upon recognition of certain oligosaccharide moieties by Mannose/Mannan Binding Lectin (MBL) or ficolins, in complex with Mannan-binding lectin-Associated Serine Protease (MASPs). The alternative pathway can be activated either by the presence of foreign surfaces, such as lipopolysaccharides (LPS), or through C3(H2O), generated by spontaneous hydrolyses, the so-called “tick-over” mechanism.

All three pathways merge at the level of C3. Through the formation of C3 convertases (C4b2a for the classical and lectin pathways, and C3bBb or C3(H2O)Bb for the alternative pathway), these pathways culminate in the generation of the opsonin C3b, and the anaphylatoxin C3a. Subsequent C5 convertase formation (C4b2aC3b for the classical and lectin pathways, and C3bBbC3b for the alternative pathway) leads to C5b and anaphylatoxin C5a generation, C5b is then able to bind C6 and be released from the surface. C5b6 once bound to C7 and C8 begins to deposit and allow C9 polymerisation, to form the Membrane Attack Complex (MAC) and its insertion into target membranes.

Self-tissue is protected from C deposition through fluid-phase and cell-bound regulators. C1-inhibitor (C1-INH) is an esterase that irreversibly bind to and inactivate C1r, C1s, MASP-1 and MASP-2 proteases, thus preventing the proteolytic cleavage of later C components C4 and C2 by C1 and MBL. Convertases are regulated through disassembly by several regulators, such as the serine protease Factor I (FI) as well as one of several cofactor proteins, such as surface-bound CD46 and the decay-accelerating factors CD55 and CD35, also known as C receptor type 1 (CR1), or fluid-phase Factor H (FH) and C4b-binding protein (C4BP). The formation of the MAC is controlled by CD59, which can prevent the polymerization of C9, and vitronectin, a multifunctional glycoprotein able to block the binding of C5b-7 on the cell membrane and to prevent the C9 polymerization (Adapted from Kolev, Friec and Kemper, Nat Rev Immunol, 2014; 14, 811–820. Figure modified from Pio, Ajona and Lambris, Semin Immunol, 2013; 25:54-64).
3.2 The role of complement in tumour growth

3.2.1 Immune surveillance function of complement in cancer

The C system is one of the immune actors present in the tumour microenvironment and plays an important role in the control of tumour growth (Macor and Tedesco, 2007; Pio et al., 2014). The numerous genetic and epigenetic alterations associated with carcinogenesis dramatically change the morphology and composition of the cell membrane. Altered glycosylation is considered a hallmark of cancer cells (Hanahan and Weinberg, 2011; Christiansen et al., 2014), and progression of epithelial cells from a normal to malignant phenotype is associated with an aberrant increase in the metabolism of membrane phospholipids (Glunde et al., 2011). Cancer-related membrane modifications can be recognized by innate and adaptive immune mechanisms that protect the host against the development of cancer (Pardoll, 2003). Several studies of many different types of solid and hematopoietic cancer have suggested that the C system is activated in response to the expression of tumour-associated antigens, with the subsequent deposition of C components on tumour tissue (Markiewski and Lambris, 2009a; Pio et al., 2014). The exact trigger for C activation upon transformation is still not fully understood, although activation through immune complexes, antibody binding or direct C1q binding to necrotic and apoptotic cellular components are all possibilities. The relative contribution of each activation pathway has not yet been clarified, but increasingly data suggest an influence of all three pathways (Pio et al., 2013, 2014).

Some of the first evidence for the classical pathway of C activation by malignant transformed cells was revealed by immunohistochemical analysis of breast cancer tissue, which showed deposition of the MAC and the C components C4 and C3, in contrast to the lack of such components in adjacent benign breast tissue (Niculescu et al., 1992). In vitro studies have shown spontaneous activation of the classical C pathway by two neuroblastoma cell lines (Gasque et al., 1996). Evidence for activation of this pathway has been found also in patients with papillary thyroid carcinoma (Yamakawa et al., 1994; Lucas et al., 1996), on which deposits of MAC, C3d, C4d, C5, IgG1 and IgG4 were detected, as well as in patients with follicular lymphoma, and mucosa-associated lymphoid tissue lymphoma (Bu et al., 2007). In vivo alterations in the activation of the classical pathway have been described in patients with chronic lymphatic leukaemia (Schlesinger et al., 1996), with a strong positive correlation between survival and the initial activity this pathway of complement (Varga et al., 1995). Recently, Markiewski et al. demonstrated...
that the classical pathway is the main contributor to C activation in a syngeneic mouse model of cervical cancer (Markiewski et al., 2008).

The lectin pathway of C activation has been found to be significantly increased in patients with colorectal cancer (Ytting et al., 2004) and pancreatic cancer (Rong et al., 2010) when compared to healthy subjects, suggesting MBL may be activated in response to malignant transformation. Swierzko et al. recently reviewed that MBL may play various roles in malignancy, depending on the type of cancer, age or ethnicity of patients, as well as mode of treatment (Swierzko et al., 2013). The concentration of MASP-2 in serum has also been reported to be an independent prognostic marker for poor survival (Ytting et al., 2005).

The alternative C pathway has been found to be activated in lymphoblastoid cell lines (Theofilopoulos and Perrin, 1976; McConnell et al., 1978) and in patients with multiple myeloma (Kraut and Sagone, 1981). In childhood acute lymphoblastic leukaemia, amplification of the alternative pathway after activation of the classical pathway has also been suggested (Kalwinsky et al., 1976). The presence of elevated levels of C3a and soluble MAC in the intraperitoneal ascitic fluid of patients with ovarian cancer also indicates activation of the alternative and classical pathways in this tumour type, supported by the deposition of some activation products, such as C3d and C1q, on the surface of tumour cells (Bjørge et al., 2005).

Recently, C activation has also been observed in in vitro studies of cancer cell lines, in the absence of an exogenous source of C components (Pio et al., 2014). Lung tumour cells deposit C5 and generate the active product C5a more efficiently than non-malignant bronchial epithelial cells (Corrales et al., 2012), although the antigens responsible for this activation and the pathways involved are not known yet. Moreover, a significant increase in C5a was found in the plasma samples of patients with NSCLC, suggesting that the local generation of C5a within tumours may be followed by its systemic diffusion (Corrales et al., 2012). Recently, Cho et al have also identified a prominent role for tumour-derived C production and activation in ovarian cancer growth and progression (Cho et al., 2014). Ovarian cancer cells have been shown to secrete C3 in the tumour mass, and the production of C3a has confirmed that C proteins, involved in the C activation inside the tumour, are originated locally (Cho et al., 2014). These evidences suggest that, apart from the traditional pathways of C activation, cancer cells may have the capacity to activate C by extrinsic activation mechanisms (Corrales et al., 2012). The production of anaphylatoxins by cancer cells has been recently reported to be mediated, at least in part, by soluble and membrane-bound proteases, such as serine proteases of the coagulation and
fibrinolysis systems or cell-bound proteases (Huber-Lang et al., 2002, 2006; Amara et al., 2008).

3.2.2 Complement and immunotherapeutic monoclonal antibodies in cancer

The involvement of C in cancer immunosurveillance has long been neglected until monoclonal antibodies (mAbs) were introduced in cancer therapy (Scott et al., 2012). mAbs target tumour-specific and Tumour-Associated Antigens (TAA) and block important cancer activities. In addition, many of them are able to activate the immune system and mediate Fc domain–based reactions by Antibody-Dependent Cellular Cytotoxicity (ADCC) (Kolev et al., 2011). Besides mediating ADCC, some mAbs trigger C activation by C-Dependent Cytotoxicity (CDC) and C-Dependent Cellular Cytotoxicity (CDCC) (Markiewski and Lambris, 2009b; Meyer et al., 2014), that helps to control tumour growth by a direct cytotoxic effect on cancer cells and by promoting inflammation (Taylor and Lindorfer, 2014a). The advantage of the C system over ADCC is that it is made of soluble components readily available at tissue site, where they are secreted by various cell types recruited from the circulation as a result of the inflammatory process and sometime also by the tumour cells (Macor and Tedesco, 2007). Successful C activation by these therapeutic antibodies can also have other effects on the immune response against tumours, such as the formation of the MAC, opsonization, and release of proinflammatory anaphylatoxins (Pio et al., 2014).

The number of mAbs approved for cancer treatment has rapidly increased since the anti-CD20 rituximab was first used for the treatment of malignant lymphomas (Table 1). Rituximab exerts its effects through a variety of mechanisms, including CDC, although its efficacy has been reported to be limited by the expression of CRPs in B-cell lymphoma cell lines (Taylor and Lindorfer, 2014b). Several studies are underway to enhance anticancer efficacy of mAbs by overcoming the resistance to C-activation exerted by CRPs.
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<td>Perjeta</td>
<td>HER2; Hum IgG1</td>
<td>Breast Cancer</td>
<td>2013</td>
<td>2012</td>
</tr>
<tr>
<td>Brentuximab vedotin</td>
<td>Adcetris</td>
<td>CD30; Chi IgG1</td>
<td>Hodgkin lymphoma, systemic ALCL</td>
<td>2012</td>
<td>2011</td>
</tr>
<tr>
<td>Iplimumab</td>
<td>Yervoy</td>
<td>CTLA-4; Hu IgG1</td>
<td>Metastatic melanoma</td>
<td>2011</td>
<td>2011</td>
</tr>
<tr>
<td>Ofatumumab</td>
<td>Arzerra</td>
<td>CD20; Hu IgG1</td>
<td>CLL</td>
<td>2010</td>
<td>2009</td>
</tr>
<tr>
<td>Catumaxomab</td>
<td>Removab</td>
<td>EPCAM/CD3; Rat Bis IgG2b/mouse IgG2a</td>
<td>Malignant ascites</td>
<td>2009</td>
<td>NA</td>
</tr>
<tr>
<td>Panitumumab</td>
<td>Vectibix</td>
<td>EGFR; Hu IgG2</td>
<td>Colorectal cancer</td>
<td>2007</td>
<td>2006</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Avastin</td>
<td>VEGF; Hum IgG1</td>
<td>Colorectal cancer</td>
<td>2005</td>
<td>2004</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Erbitux</td>
<td>EGFR; Chi IgG1</td>
<td>Colorectal cancer</td>
<td>2004</td>
<td>2004</td>
</tr>
<tr>
<td>Tositumomab-I131</td>
<td>Bexxar</td>
<td>CD20; Mu IgG2a</td>
<td>Non-Hodgkin lymphoma</td>
<td>NA</td>
<td>2003#</td>
</tr>
<tr>
<td>Ibritumomab tiuxetan</td>
<td>Zevalin</td>
<td>CD20; Mu IgG1</td>
<td>Non-Hodgkin's lymphoma</td>
<td>2004</td>
<td>2002</td>
</tr>
<tr>
<td>Alectuzumab</td>
<td>Lemitrada, MabCampath, Campath-1H</td>
<td>CD52; Hum IgG1</td>
<td>CML#</td>
<td>2001#; 2013</td>
<td>2001#; Rev</td>
</tr>
<tr>
<td>Gemtuzumab ozogamicin</td>
<td>Mylotarg</td>
<td>CD33; Hum IgG4</td>
<td>AML</td>
<td>NA</td>
<td>2000#</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Herceptin</td>
<td>HER2; Hum IgG1</td>
<td>Breast cancer</td>
<td>2000</td>
<td>1998</td>
</tr>
<tr>
<td>Rituximab</td>
<td>MabThera, Rituxan</td>
<td>CD20; Chi IgG1</td>
<td>Non-Hodgkin's lymphoma</td>
<td>1998</td>
<td>1997</td>
</tr>
</tbody>
</table>

Table 1. Therapeutic monoclonal antibodies approved or in review in the European Union or the United States for use in cancer treatment. (#, Withdrawn; NA, Not Approved; Rev, In Review; P, pending; EC, European Community; Mu, Murine; Hu, Human; Hum, Humanized; Chi, Chimeric; Bis, Bispecific. Tumour types: NSCLC, Non-small cell lung cancer; ALL, Acute lymphoblastic leukaemia; CLL, Chronic lymphocytic leukaemia; ALCL, anaplastic large cell lymphoma; CML, Chronic myeloid leukaemia; AML, Acute myeloid leukaemia. Table adapted from Janice M. Reichert, Reichert Biotechnology Consulting LLC; updated November 5th, 2014. http://www.antibodysociety.org/news/approved_mabs.php).
3.2.3 Cancer-associated resistance mechanism to complement activation

The contribution of C to the antibody-mediated cancer cell killing remains controversial (Pio et al., 2014). Cancer cells exhibit a number of strategies to resist C attack (Jurianz et al., 1999). Many of these resistance mechanisms are used also by normal cells to avoid accidental activation or bystander effects from a local activation of C. However, cancer cells develop additional mechanisms to inhibit C activation, thus promoting tumour growth (Figure 6).

Figure 6. Mechanisms used by cancer cells to resist complement activation. The efficacy of C-dependent cytotoxicity is strongly restricted due to the expression and acquisition of regulators of C activation. Cancer-resistance associated mechanisms can be divided into two categories: extracellular, which are spontaneously expressed in cells without a need for prior activation, whereas the induced intracellular mechanisms develop in cells subjected to stimulation with cytokines, hormones, drugs or with sublytic doses of C and other pore-formers. Membrane-associated C regulatory proteins, such as CD55, CD46, CD35 and CD59, serve as an important mechanism of self-protection and render autologous cells insensitive to the action of C. These C regulators appear to be overexpressed on several tumours. Furthermore, tumour cells secrete several soluble C inhibitors. Tumour cells may also release proteases that degrade C proteins, such as C3; moreover, cancer cells may express ecto-protein kinases (ecto-PK), which can phosphorylate C9 protein in the C9a fragment, responsible for the interaction of the unfolded C9 with the preformed C5b-8 complex in the formation of the MAC. Besides this basal resistance, nucleated cells resist, to some extent, C damage by removing the MAC from their surface. Several biochemical pathways, including protein phosphorylation, activation of G-proteins and turnover of phosphoinositides have been implicated in resistance to C. Calcium ion influx and activation of PKC and of MAPK have also been demonstrated to be associated with the C-induced enhanced resistance to lysis. Red lines represent inhibitory activity (Adapted from Jurianz et al., Mol Immunol, 1999; 36:929–939. The figure is from Pio, Corrales and Lambris, Adv Exp Med Biol, 2014; 772:229-62).
One of the best characterized cancer-associated resistance mechanism is the expression of membrane-bound C Regulatory Proteins (mCRPs), CD46, CD55 and CD59, that are overexpressed on tumour cells (Jurianz et al., 1999; Fishelson et al., 2003; Kolev et al., 2011).

CD46, also known as Membrane Cofactor Protein (MCP), is a type I membrane regulatory protein of the C. It has cofactor activity for inactivation of C components C3b and C4b by serum factor I, which protects the host cell from damage by C (Kolev et al., 2011). CD46 is perhaps the mCRP with the lowest level of variation between tumours and normal tissue. Nevertheless, CD46 levels are correlated with tumour grade and recurrence in breast tumours (Rushmere et al., 2004; Madjd et al., 2005).

CD55, also known as C Decay-Accelerating Factor (DAF), is a glycophsphatidylin (GPI)-anchored protein that interferes with the ability of C4b and C3b to catalyse the conversion of C2 and factor B to active C2a and Bb, and thereby prevents the formation of C4b2a and C3bBb, thus blocking the formation of the Membrane Attack Complex (MAC) (Kolev et al., 2011). Prostatic tumours and medullary thyroid carcinomas were reported to overexpress CD55 and its receptor CD97 (Loberg et al., 2006). The correlation between the level of CD55 expression in human breast cancers and disease prognosis is still unclear: high CD55 expression has been correlated to a higher tumour size and poor outcome (Ikeda et al., 2008), while others demonstrated that the loss of CD55 is related to poor prognosis (Madjd et al., 2004, 2005). In colorectal carcinoma, the expression of CD55 has also been shown to be associated with poor prognosis (Durrant et al., 2003). Overexpression of CD55 in human cervical cancer tissue was shown to correlate with reduced deposition of C3b compared to surrounding tissue, suggesting C inhibition is relevant in vivo (Gao et al., 2009).

CD59, also known as MAC-inhibitory protein (MAC-IP), is a GPI-anchored protein able to bind the C proteins C8 and/or C9, thereby inhibiting the incorporation of multiple copies of C9 during the assembling of the MAC (Kolev et al., 2011). In several cancer types, increased levels of CD59 have been found to be associated with resistance to CDC (Brasoveanu et al., 1996; Chen et al., 2000; Coral et al., 2000; Jarvis et al., 1997), increased metastatic potential (Loberg et al., 2005), or poor prognosis (Xu et al., 2005; Watson et al., 2006). A recent study shown also that the expression of CD59 determines huge xenograft tumours in a mouse model of ovarian cancer (Shi et al., 2009). More recently, Li et al. reported that the suppression of CD59 expression enhanced C-mediated apoptosis of lung cancer cells through the induction of Fas (Li et al., 2013).
The expression of both these mCRPs was found to correlate to prognosis in Non-Small Cell Lung Cancer (NSCLC) and contribute to disease resistance to herceptin, an anti-Her2/neu mAb, treatment, whose efficacy relies upon C activation (Zhao et al., 2009). The inhibition of CD55 and CD59 has also been shown to sensitize primary Uterine Serous Carcinoma (USC) to both CDC and ADCC in vitro and, if specifically targeted to tumor cells, to significantly increase trastuzumab-mediated therapeutic effect in vivo (Bellone et al., 2012). A more recent study has demonstrated that also Head and Neck Squamous Cell Carcinoma (HNSCC) massive upregulate all mCRPs on cancer cells and in the surrounding tissue, leading to the enhancement of C resistance in the tumor microenvironment (Kesselring et al., 2014).

Overall, the correlation between expression and prognosis of the mCRPs in several human carcinomas suggests a key role for C during tumourigenesis, and provide evidences for the use of mCRPs as candidate targeting gene in cancer therapy (Geis et al., 2010; Carter and Lieber, 2014).

Several types of cancer cell lines release soluble forms of the mCRPs, and many of these factors also have been detected in patients with cancer (Pio et al., 2014). These forms of mCRPs, including C Factor H (FH) and its truncated form Factor H-Like protein 1 (FHL-1), have been extensively reported to bind to tumor cells, contributing to their resistance to C activation and CDC (Ajona et al., 2004).

FH is a soluble glycoprotein that regulates the C activation by possessing both cofactor activity for the Factor I (FI) mediated C3b cleavage, and decay accelerating activity against the alternative pathway C3-convertase. FH has been shown to regulate the activation of C in the SK-MEL-93-2 melanoma cell line (Ollert et al., 1995), while Cheng et al. reported that FH is a tumour diagnostic marker for bladder cancer (Cheng et al., 2005). Downregulation of FH has also been reported to reduce the growth of lung cancer cells in vivo (Ajona et al., 2007), and its expression in lung adenocarcinomas may be associated with worse prognosis (Cui et al., 2011). Lung cancer cell lines downregulate the expression of FH, FI, CD46, and CD55 under hypoxic conditions and during reoxygenation of tissues, implying that, under these conditions, cancer cells reduce their reliance on mechanisms to control C activation while keeping free from CDC (Okroj et al., 2009). FH and FHL-1 are highly expressed by ovarian carcinomas, both proteins are abundantly present in ascites from these tumors (Junnikkala et al., 2002) and are able to promote the inactivation of C3b in the H2 glioblastoma cells (Junnikkala et al., 2000). In vitro studies have shown that also lung cancer cell lines are more resistant to CDC than human nasal epithelium primary
cell cultures thank to the expression and secretion of these two factors to the extracellular milieu (Ajona et al., 2004).

Factor I (FI) is a serine protease capable of degrading the activated C components C3b and C4b, whilst C4b-binding protein (C4BP) acts as its cofactor. One of the first evidence of the involvement of C4BP in cancer showed that this protein is able to bind to ovarian tumour cells and retain its cofactor activity for FI-mediated inactivation of C4b, thus increasing the control of classical C pathway activation on the surfaces of cancer cells (Holmberg et al., 2001). Recently, Okroj et al. showed that NSCLC cell lines express both FI and C4BP, efficiently supporting C inhibition and contributing to the aggressive tumour phenotype in vitro (Okroj et al., 2008). This study provides further protection from C beyond the level ensured by mCRPs.

In addition to the expression of mCRP and soluble regulators, there are several alternative mechanisms used by cancer cells to control C activation (Pio et al., 2014). Tumour cells can either release proteases that cleave C components, or express them in their cell membrane. The overexpression of procathepsin-L in DX-3 melanoma cells was reported to increase the tumourigenicity of the cells and switch their phenotype from non-metastatic to highly metastatic (Frade et al., 1998). Recently, the overexpression of MMP-1 in the B16/F1 murine melanoma cells has been shown to protect these cells from the damaging effects of C and promote the formation of lung metastasis in vivo (Rozanov et al., 2006). More recent evidences showed that the matrix secreted and cell-surface associated proteoglycan serglycin protects the survival of myeloma (Skliris et al., 2011) and breast (Korpetinou et al., 2013) cancer cells from the attack specifically of the classical and the lectin pathways of the C system.

Tumour cells are able to eliminate the MAC by endocytosis or vesiculation (Moskovich and Fishelson, 2007). Interestingly, sublytic doses of the MAC have been shown to provide intracellular protection against C attack. Insertion of the MAC into the cell membrane causes a variety of biological effects, including entrance into the cell cycle, resistance to apoptosis, expression of adhesion molecules, or augmentation of C resistance (Liu et al., 2012), although the mechanisms responsible for this protection are still under investigation. Kraus et al. reported that cell desensitization in the chronic myelogenous leukemia K562 cancer cell line by sublytic doses of the C5b-9 protein complex involves a signalling cascade that includes Protein kinase C (PKC)-mediated extracellular signal-regulated kinase (ERK) activation (Kraus et al., 2001). Vlaicu and colleagues showed that sublytic MACs play a role in tumour cell activation, proliferation and differentiation.
These effects have been shown to result in the regulation of cell cycle genes, activated by the Phosphatidylinositol 3-Kinase (PI3K)/Akt and ERK1 pathways (Vlaicu et al., 2013). Sublytic MACs have also shown to inhibit apoptosis, by inducing the phosphorylation of the pro-apoptotic Bcl-2-associated death promoter (Bad) and blocking the activation of caspase-8, and the pro-apoptotic protein BH3 interacting-domain death agonist (Bid) (Tegla et al., 2011).

### 3.2.4 Strategies for the improvement of complement-mediated immunotherapy

The possible role CRPs have in protecting tumours has been of great interest to those studying resistance to C fixing antibody in immunotherapy (Meyer et al., 2014). The abrogation of inhibitory activity of CRPs has been demonstrated in several in vitro and in vivo studies (Kolev et al., 2011), in which CRPs protection is removed, leading to a significant increase in tumour cell sensitivity to mAb-based C lysis. Strategies include blockade of the activity of the regulators, for example with anti-CD55 and anti-CD59 in human mammary (Ellison et al., 2007), ovarian and prostate carcinoma cells (Donin et al., 2003), as well as B-cell tumours (Kuraya et al., 1992), erythroleukaemic (Jurianz et al., 2001) and renal cancer cell lines (Gorter et al., 1996). Other approaches include the downregulation of the CRPs expression (Bellone et al., 2012) or their removal from the cell surface (Geis et al., 2010). Various studies have also reported the use of mixtures of several antibodies to bypass the action of CRPs (Spiridon et al., 2002; Kennedy et al., 2003; Macor et al., 2006). However, targeting inhibitory molecules to C regulators in vivo is technically challenging, because of possible unwelcome consequences for normal cells. An alternative approach would be to improve the C-mediated effector mechanisms of mAbs through genetic engineering or conjugation. Several strategies have been devised for turning non-C-fixing antibodies in those able to activate C, in order to be employed in immunotherapy. These include the selection of the IgG subclasses 1 (IgG1) and 3 (IgG3), that are most efficient in activating C, and the production of IgG1-containing recombinant variants of Fc, that exhibit increased capacity to induce CDC or ADCC (Ziller et al., 2005; Macor and Tedesco, 2007; Moore et al., 2010; Elvington et al., 2012). In addition, to overcome the inhibitory effect on the tumour microenvironment, some researchers have proposed the use of bispecific mAbs that target a tumour antigen and simultaneously block a major C regulatory protein (Gelderman et al., 2005; Macor et al., 2015). The potential of these antibodies is to enhance the clinical efficacy of therapeutic mAbs if delivered
selectively to their targets on cancer cells (Macor et al., 2015). To now, the molecular architecture of the antigens selected for immunotherapy, and the antibody concentration, still seem to be essential for the proper induction of CDC (Meyer et al., 2014).

### 3.3 Complement activation can influence tumour growth

Researchers have long thought that C activation in the cancer setting is extremely useful for proper elimination of tumour cells. However, recent data have also revealed a tumour-promoting role for the C system (Markiewski and Lambris, 2009b) (Figure 7).

![Figure 7. Potential tumour-promoting roles of complement proteins.](image)

**Figure 7. Potential tumour-promoting roles of complement proteins.** Complement proteins contribute to each major hallmark of cancer, including mitogenic signalling cascades and growth factor production, angiogenesis, protection from C-mediated lysis, cellular invasion and migration through the extracellular matrix, and suppression of antitumor immunity (Adapted from Rutkowski et al, Mol Cancer Res, 2010; 8:1453–1465. Figure modified from Pio, Corrales and Lambris, Adv Exp Med Biol, 2014; 772:229–262).

Markiewski and colleagues (Markiewski et al., 2008) first reported that C5a, generated in the tumour microenvironment as a result of C activation, favours cervical cancer growth by recruiting and activating MDSCs, which worked to dampen a CD8+ T-cell-mediated anti-tumour response. This observation and similar findings in mouse models of lung
(Corrales et al., 2012) and ovarian cancers (Nunez-Cruz et al., 2012) challenge the concept that C activation at tumour site is always beneficial for cancer patients. However, the relevance of these in vitro and animal data remains to be established, and the clinical and therapeutic implications of these findings warrant further investigation as C5a has been found to exert opposite effects depending on the amount available in the tumour microenvironment (Gunn et al., 2012). Supporting this idea, Kim and colleagues has recently demonstrated a slower tumour growth in C5a expressing mammary sarcoma cells, compared to those lacking C5a in an in vivo mouse model (Kim et al., 2005). Irrespective of the beneficial or harmful effect of C on tumour growth, both effects have been found to be associated with C fragments, such as C3a and C5a, indicating complement activation (Sayegh et al., 2014).

### 3.3.1 Role of the complement system in tumour angiogenesis and metastasis

Recent evidences in literature demonstrate the ability of C to assist the escape of tumour cells by promoting angiogenesis and participating in tumour cell invasion and migration (Rutkowski et al., 2010). C-activated factors are related, either directly or indirectly, to neovascularization in cancer. Because of different types of tumour examined, there is still some controversy about the pro- or anti-angiogenic role of the C system in neovascularization (Pio et al., 2014). In a mouse model of epithelial ovarian cancer, a genetic C3 deficiency has been shown to impair tumour vascularization by altering the function of endothelial cells (Nunez-Cruz et al., 2012). However, in end point tumour specimens in a murine model of cervical cancer, C5a receptor (C5aR) blockade has been reported not to compromise tumour angiogenesis (Markiewski et al., 2008). Based on these results, Pio et al. suggested that C activation may be important in the promotion of angiogenesis only during the early steps of tumour formation (Pio et al., 2014). In vitro studies, using endothelial cells, support this conclusion. C5a has been recently demonstrated to stimulate chemotaxis and the formation of tube-like structures in both human umbilical endothelial cells (Corrales et al., 2012) and human microvascular endothelial cells (Nunez-Cruz et al., 2012). Rutkowski et al. reported that various C proteins might also contribute, either directly or indirectly, to release the metalloproteinases MMP-2/9 in the tumour microenvironment (Rutkowski et al., 2010), although their direct contribution in tumour invasion is still far from being clearly elucidated.
Chapter 4

4.1 C1q: the first component of the complement system

Extensive research conducted over the past 20 years has allowed us to identify in detailed the structural and functional role of human C1q in the activation of the classical pathway of the C system, as a well-recognized molecular sensor of damage-modified self or non-self antigens. In the last few years, increasingly data are emerging about its participation in a rapidly expanding list of pathological disorders, such as in placental development (Agostinis et al., 2010), preeclampsia (Singh et al., 2011), wound healing (Bossi et al., 2014) and cancer (Hong et al., 2009). In these diseases, either the soluble form, or the membrane-associated form, of C1q appear to be intimately involved. The chimeric structure of C1q, containing two functional domains, together with the fact that it is synthesized by a wide range of proinflammatory cell types, makes it likely that it is a potent orchestrator of molecular pathways involved not only in innate and adaptive immunity, but also in a wide range of inflammatory diseases, including cancer. (Ghebrehiwet et al., 2012).

4.1.1 Cellular sources of human C1q

C1q is the first component of the classical C activation pathway, belonging to the TNF superfamily of proteins (Kishore et al., 2004). This protein has been demonstrated to be highly conserved among more than a dozen of vertebrate species (Petry et al., 1996). Unlike most of the C proteins, which are produced in the liver, C1q is synthesized extrahepatically by a wide range of cell types, mainly macrophages (Petry et al., 2001), dendritic cells and fibroblasts, as well as epithelial, mesenchymal (Ghebrehiwet et al., 2012), endothelial (Bulla et al., 2008; Bossi et al., 2014) and trophoblast (Agostinis et al., 2010) cells. Recent papers showed the expression of C1q also in the central nervous system, by microglial cells (Lynch et al., 2004) and in the brain, by postnatal neurons (Stevens et al., 2007), as well as in the eye, by Retinal Ganglion Cells (RGCs) (Bialas and Stevens, 2013). The synthesized molecule in turn, is either transiently expressed as a cell membrane associated molecular ligand (mC1q), or secreted into the pericellular milieu, where it can modulate cell-specific biological responses in a manner that involves an autocrine and/or paracrine signalling through cell surface receptors and their signalling partners (Ghebrehiwet et al., 2012).
4.1.2 The structure–function relationship of the human C1q

The diversity of immunological functions mediated by C1q seem to be explained by having a unique structural feature. C1q consists of three similar, but distinct, polypeptide chains, A (28 kDa), B (25 kDa), C (24 kDa), which are the product of three distinct genes clustering in the same orientation, and in the order A–C–B, on a 24 kb stretch of DNA on chromosome 1p. Each of the three polypeptide chains consists of a C-terminal globular “head” domain, also called gC1q domain, linked to a N-terminal “stalk”, known as collagen-like domain or cC1q domain. Of these, the gC1q domain, is considered to be key to the versatility of C1q function (Gaboriaud et al., 2003). These proteins are thus assembled to form disulfide-linked A–B and C–C dimers, while the A–B dimer is non-covalently (--) linked to the C-chain. Three A–B–C–C–B–A doublets are linked by non-covalent forces, to get a collagen-like hexameric glycoprotein of about 460 kDa, resembling a “bouquet of tulips” (Ghebrehiwet et al., 2012) (Figure 8). Once formed, C1q associates with the Ca\(^{2+}\)-dependent C1r2–C1s2 tetramer, of about 360 kDa, to form the soluble pentameric C1 complex.

![Figure 8. Structural organization of C1q](image)

The figure represents schematically the subunit structure and assembly of intact of C1q. Three similar chains, such as A, B and C, are assembled to form disulphide-linked A-B and C-C dimers. One strand of the molecule consists of an A-B dimer non-covalently (--) linked to a C chain forming (A-B--C). The C-chain of one strand is then disulfide linked to the C chain of a neighbouring strand to give an A-B--C-C--B-A doublet and three such doublets are linked by non-covalent forces giving C1q its signature “bouquet-like” structure (The figure adapted from Peerschkeia and Ghebrehiwet, Mol Immunol, 2014; 61:100–109).
4.1.3 The main receptors of C1q

C1q receptors comprise diverse cell surface and intracellular proteins, which recognize and bind to C1q, either via its gC1q or cC1q domain (Ghebrehiwet et al., 2012). These interactions mediate a plethora of immunologic functions (Nayak et al., 2012), including those associated with innate and adaptive immunity, and cancer (Peerschke and Ghebrehiwet, 2014).

The receptor for the globular heads of C1q, gC1qR, also known as Hyaluronan-Binding Protein 1 (HABP1), p32 or p33, and the receptor for the collagen-like domain of C1q, cC1qR or calreticulin (CRT) are the two well-studied and ubiquitously distributed C1q receptors (Ghebrehiwet and Peerschke, 2004; Nayak et al., 2012). Although each of these molecules lacks a consensus sequence motif for a traditional transmembrane segment, the lack of direct conduit to intracellular signalling elements is bypassed by the formation of a docking/signalling complex with associated adapter proteins, such as CD91, CD44, DC-SIGN and β1 integrin (Ghebrehiwet and Peerschke, 2004; Ghebrehiwet et al., 2012). Growing evidences show that both CRT and gC1qR are also implicated in carcinogenesis, albeit their roles seem to be rather divergent. Whereas cC1qR is reported to have tumour suppressive effect, gC1qR appears to be requisite for tumour cell progression and metastasis (Peerschke and Ghebrehiwet, 2014).

4.1.3.1 gC1qR: the receptor for the globular heads of C1q

The 33-kDa protein gC1qR is a multiligand binding protein, localized in every compartment of the cell, predominantly to the mitochondrial matrix and on the cell surface, and can be secreted by highly proliferating or activated cells. It has been shown to recognise and bind to the gC1q domain with high affinity interactions, under physiologic ionic strength (Peerschke and Ghebrehiwet, 2014). The binding site for gC1qR on C1q has been reported to be primarily on the C1qA chain, which is the same or overlapping site to a putative IgG site (Ghebrehiwet et al., 2012). Although the biologic significance of the wide distribution of gC1qR is yet to be elucidated, the relevance of gC1qR as an important modulator of ligands both inside and outside the cell is recognized increasingly (Dembitzer et al., 2012). The C1q/gC1qR interaction has been reported to lead to classical C pathway activation, with generation of inflammatory cytokines, as well as the binding of gC1qR to high-molecular-weight kininogen activates the kinin system, to produce bradykinin, ensuing vascular permeability (Peerschke and Ghebrehiwet, 2014).
To date, neither a mutation in the gC1qR gene, nor a genetic deficiency has been identified, which suggests that gC1qR is essential for survival (Peerschke and Ghebrehiwet, 2014). The human gene C1QBP, encoding the gC1qR protein, is located on the short arm of chromosome 17, in cytogenetic band 13.3 (17p13.3). Interestingly, bioinformatics analysis reveal that this region contains some of the major tumour suppressor genes, such as Tp53, BRCA1, as well as OVCA1 and OVCA2, which are frequently mutated in most cancer types, suggesting a potential role of gC1qR in carcinogenesis (Peerschke and Ghebrehiwet, 2014).

Recent experiments (Dembitzer et al., 2012) compared the presence of gC1qR either in normal and malignant tissue of epithelial and mesenchymal origin. These histochemical analyses showed that the large majority, but not all, of the analysed human solid tumour tissues revealed marked differential expression of gC1qR compared to the non-malignant histologic counterparts (Peerschke and Ghebrehiwet, 2014). Recently, Fogal et al. showed that cell surface expressed gC1qR binds the tumour homing peptide LyP-1 (Fogal et al., 2008). Moreover, knocking down gC1qR in cancer cells has been demonstrated to make them less tumorigenic (Fogal et al., 2010). The involvement of gC1qR in tumour promotion has been further substantiated by more recent studies, which reported that gC1qR in the membrane of human lung carcinoma A549 cells is a key regulator for lamellipodia formation and cancer metastasis via receptor tyrosine kinase activation (Kim et al., 2011). By contrast, two very recent papers demonstrated that increased gC1qR levels induce human cervical cancer cell apoptosis through mitochondrial dysfunction, by activating the p38 MAPK/JNK signalling pathway (Gao et al., 2013; Chen et al., 2014).

Overall, the role of gC1qR in carcinogenesis is still contradictory, although Ghebrehiwet et al. have recently suggested its use as target for therapeutic intervention (Peerschke and Ghebrehiwet, 2014). Moreover, it remains to be established whether C1q acts in concert with gC1qR to sustain malignant transformation, tumour growth and invasion.

4.1.3.2 Calreticulin: the receptor for the collagen-like domain of C1q (cC1qR)

Calreticulin (CRT) is a 42-kDa molecule, belonging to the family of heat shock proteins, which is localized primarily in the lumen of the endoplasmic reticulum. CRT is expressed in all types of cells, with the exception of erythrocytes (Nayak et al., 2012). CRT is one of the crucial proteins responsible for calcium homeostasis, and together with its
homolog calnexin, regulates glycoprotein folding and quality control of nascent proteins as a molecular chaperon (Michalak et al., 2009).

Surface expressed CRT, however, has been shown to play a major role in cellular adhesion, and in clearance of cell debris. The interactions between CRT and the collagenous region of C1q are crucial in apoptotic cell clearance via macrophages, with C1q opsonizing on the surface of apoptotic cells, and CRT linked to CD91 over the phagocyte cell surface (Nayak et al., 2012). However, CRT has optimal binding to C1q at sub-physiologic ionic strength, thus neutral pH has the ability to block most of the activities of C1q (Peerschke and Ghebrehiwet, 2014). CRT is also referred as the collectin receptor, due to its ability to bind to the collagen-like regions of various collectin, such as mannose-binding lectin (MBL), surfactant protein (SP)-A and SP-D (Nayak et al., 2012).

Calreticulin has been found to be upregulated in most malignant cells (Zamanian et al., 2013), where it functions as a pro-phagocytic signal, promoting macrophages to engulf hazardous cancerous cells. However, this “eat-me” signal is counterbalanced by the presence on tumour cells of the receptor for thrombospondin-1, CD47, which is able to block the functions of cC1qR (Chao et al., 2010). Blockade of the “don't-eat-me signal” CD47 on tumour cells may allow already expressed “eat-me” signals to induce macrophages to eliminate tumour cells. A recent study revealed a key molecular mechanisms adopted by macrophages to recognize and phagocytise tumour cells. This study demonstrated that the activation of Toll-like receptor pathways in macrophages induces the phosphorylation of Bruton's tyrosine kinase (Btk), which catalyses cell-surface exposure of CRT. Calreticulin on or secreted by macrophages plays a critical role in mediating adjacent tumour cell recognition and phagocytosis, playing an essential role in tumour surveillance and elimination (Feng et al., 2015). The group of Tosato and colleagues also found that the N-terminal domain of CRT is able to prevent tumour development, as well as to inhibit the growth of established ones, by interfering with endothelial cell growth and neovascularization. Moreover, this study demonstrated that the inhibition of endothelial cell proliferation was due to direct binding of the cC1qR fragment to the ECM protein, laminin, thereby interfering with endothelial cell attachment (Yao et al., 2002). Since adhesion and proliferation are key steps leading to neovascularization, and C1q has been shown to be a strong angiogenic factor (Bossi et al., 2014), it is possible to speculate that interfering with the C1q-cC1qR axis may provide a suitable therapeutic approach to fight against cancer.
On the other hand, evidence from numerous studies suggests that CRT overexpression is also a pro-tumourigenic event in various cancers. However, the exact roles of CRT is yet be clarified, due to its diverse interactions into various cellular processes and signalling pathways (Zamanian et al., 2013).

4.2 The emerging functions of C1q independently on complement activation

Besides the role of C1q as a C initiating molecule, it is also engaged in an array of processes that is completely independent of the C activation (Nayak et al., 2012). Most inflammatory cells, or those that undergo phenotypic differentiation or proliferation, are known to express and/or secrete C1q (Bulla et al., 2008; Ghebrehiwet et al., 2012). Recent evidences have revealed that locally secreted C1q modulates many cellular functions by an autocrine and/or paracrine signalling mechanism (Ghebrehiwet et al., 2012).

The locally synthesized C1q has recently been suggested to control microglial activation and recruitment (Färber et al., 2009). Fraser et al. showed that C1q has a neuroprotective role in the form of activating microglial cells, in order to ingest apoptotic neurons and by suppressing the production of pro-inflammatory cytokines (Fraser et al., 2010).
C1q mediates apoptotic cell clearance both in a C-dependent and C-independent manner. The non-C activating way of clearing apoptotic neurons and neuronal blebs is through C1q binding to CRT on macrophages and microglial cells which, in turn, leads to apoptotic cell phagocytosis through CD91-induced micropinocytosis (Ogden et al., 2001; Fraser et al., 2009, 2010).

C1q has been demonstrated to be associated in the maturation of dendritic cells (DCs) (Csomor et al., 2007; Nayak et al., 2010) and involved in modulating T-cell and B-cell activity (Nayak et al., 2012). The pro-proliferative function of C1q is not yet fully clarified. One of the first study reported that C1q induces an anti-proliferative signal in peripheral T cells (Chen et al., 1994). In contrast, a recent study showed that C1q positively regulates CD8+ T-cell proliferation and also influences the cytokine profile of antigen-specific T-cells (van Montfoort et al., 2007). Moreover, C1q stimulates the production of IgG by B-lymphocytes and also plays a role in the negative selection of autoreactive B cells (Young et al., 1991).

C1q is also involved in the migration of eosinophils, mast cells, neutrophils and fibroblasts, thus acting as a potent chemotactic molecule. This ability of C1q depends on the expression of gC1qR and CLR on the surface of these immune cells (Nayak et al., 2010). Several groups showed that C1q is able to bind to various ECM proteins with different affinity (Nayak et al., 2012). C1q binds strongly to decorin and biglycan, which are leucine-rich proteoglycans involved in matrix assembly and bone mineralisation, respectively (Groeneveld et al., 2005). In contrast, C1q binds to laminin (Bohsack et al., 1985) and fibronectin (Sorvillo et al., 1985) with a lower affinity. This binding has been implied in deposition and retention of immune complexes in the basement membrane (Sjöberg et al., 2009). Sjöberg et al. reported that the interaction between ECM proteins and C1q occurs without activating C, probably involving the CLR domain of C1q. Wherever the interaction involves the gC1q domain, C activation mostly results (Sjöberg et al., 2009).

The nature of C1q involvement into non-C associated functions has been recently extended to pregnancy and development (Ghebrehiwet et al., 2012; Nayak et al., 2012). Our group showed that foetal trophoblast cells invading the decidua in the early stages of pregnancy not only actively synthesize and express C1q on their surface, but this C1q is actively involved in promoting trophoblast invasion of the decidua (Agostinis et al., 2010). This, in turn, is mediated by two cell surface receptors, gC1qR and α4β1 integrin, which promote trophoblast adhesion and migration through the activation of ERK1/2 MAPKs. In support
of these findings, Agostinis et al. also demonstrated that $C1qa^{-/-}$ mice manifested increased frequency of foetal resorption, reduced foetal weight, and smaller litter size when compared to their wild-type counterparts, suggesting that defective local production of C1q may be involved in pregnancy disorders, such as preeclampsia (Agostinis et al., 2010). The significance of C1q in pregnancy disorders has been further supported by an independent study, which showed that pregnant $C1qa^{-/-}$ mice express the key features of human preeclampsia that correlate with increased foetal death (Singh et al., 2011).

More recently, Naito et al. reported that C1q activates canonical Wnt signalling and promotes aging-associated decline in tissue regeneration (Naito et al., 2012); moreover, our group showed that C1q is a strong angiogenic factor and promotes wound healing (Bossi et al., 2014). These studies suggest novel and unexpected roles of this C component, either in the modulation of mammalian aging, or in cancer neovascularization, respectively.

4.3 C1q and tumourigenesis: first evidences and beyond

The role of C1q in carcinogenesis is still largely elusive. To date, only few papers in literature indicate a possible direct involvement of this protein in neoplastic proliferation (Peerschke and Ghebrehiwet, 2014). As already previously described, the autocrine or paracrine signalling effects of C1q seem to occur through its cell surface receptors and signalling partners (Ghebrehiwet et al., 2012).

The first indication that C1q may regulate cancer cell proliferation showed that the addition of soluble C1q to diverse types of cultured haematological tumour cell lines, expressing C1q receptors, such as the B-cells Raji, Daudi and Wil2-WT, the T-cells Molt4, and the monocytes U937, temporary inhibits tumour cell growth (Ghebrehiwet et al., 1990). This study also reported that polyclonal F(ab')$_2$ and anti-C1q antibodies, as well as monoclonal antibodies against C1q receptors abrogate the antiproliferative effect of C1q. Based on these findings, Ghebrehiwet et al. suggested that C1q receptors alone, or in conjunction with other cellular factors, support cell growth upon ligand binding. The latter event has been thought to suppress post-receptor events, which are necessary for cell proliferation. More important, these early studies were performed in vitro using only haematological cancer cells, and lacked the necessary reagents to identify which of the two C1q receptors was responsible for the C1q-mediated tumor-suppressive or antiproliferative effect (Peerschke and Ghebrehiwet, 2014).

A more recent study reported the involvement of C1q in the regulation of cancer cell survival and progression by inactivating the apoptotic cascade (Hong et al., 2009). C1q has
been shown to be expressed on basal and epithelial cells of prostate tissues, sustaining the activation of the tumour suppressor *WW-domain containing oxydoreductase* (WOX1), which is needed for blocking cancer cell proliferation. This data suggests that C1q may induce apoptosis of prostate cancer cells by activating WOX1 and destabilizing cell adhesion. The same authors also reported that the downregulation of C1q was found to enhance prostate hyperplasia and cancerous formation, due to failure of WOX1 activation. It is important to take into account that Hong et al. focused only on the prostate cancer, using the human DU-145 tumour cell line to perform all the experiments *in vitro*, without any evidence of tumour growth inhibition *in vivo* (Hong et al., 2009). In addition, this study lacked to unveil which of the two C1q domain was responsible for the C1q-mediated apoptosis. This study also revealed that C1q is significantly reduced in benign prostatic hyperplasia and prostate cancer tissues compared to the normal prostate (Hong et al., 2009), although these findings are not corroborated by those obtained in other tumour types (Bulla, Tripodo, Rami et al., in submission).

Ghebrehiwet et al. has recently suggested that these evidences highlight the “cytokine-like” activity of C1q (Ghebrehiwet et al., 2012), while the functions exert by locally expressed C1q in the tumour microenvironment are still unknown.
RATIONALE AND AIMS OF THE STUDY

The complement (C) system is part of the local microenvironment acting as a bridge between innate and adaptive immunity and taking part in immune surveillance and homeostasis. The activation of the C system has been demonstrated to exert an active and beneficial role in the fight against malignant cells, based on the ability to promote inflammation and to cause direct cell killing. The advantage of this defence system is to be made up of several components readily available at the tissue site, where they are secreted by various stromal and immune cells surrounding the tumour mass, and also by tumour cells or recruited from the circulation as result of an inflammatory process.

Circumstantial evidences obtained from the analysis of different tumour tissue types showing deposition of C activation products on cancer cells have suggested a role for C in the immune surveillance of cancer. Although C has proven to be an efficient weapon to control tumour growth, provided that the membrane-bound C regulatory proteins (CRPs) on cancer cells are neutralized, a recently published Nature Immunology paper and others have challenged the concept that increased C activation at tumour site is merely beneficial for cancer patients.

Here we present novel data showing that C can adopt an alternative strategy that does not require activation of the system to promote cancer progression and invasion.

Guided by our recent findings that the first component, C1q, promotes trophoblast migration through the decidua during pregnancy and exerts a pro-angiogenic effect in wound healing, we aimed to investigate the role of the locally produced C1q in the tumour microenvironment.
MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were used: rabbit antiserum to human C1q (Dako); goat antiserum to human C3, C4, C1s (Quidel); rat monoclonal anti-mouse C1q (clone 7H8; HyCult) and rabbit polyclonal anti-mouse C1q (kindly provided by Prof M. Daha, Leiden, The Netherlands); FITC-conjugated F(ab’)2 fragment goat anti-mouse C3 (Cappel) and rat monoclonal anti-mouse C4 (clone 16D2; HyCult); rabbit polyclonal pan-specific Von Willebrand Factor (code number A0082; Dako); rat monoclonal anti-mouse CD68 (clone FA-11; AbD Serotec); rabbit polyclonal anti-mouse Ki-67 (code number 15580; Abcam); FITC-labeled swine anti-rabbit IgG (code number F0205), TRITC-labeled swine anti-rabbit IgG (code number R0156) and FITC-labeled rabbit anti-rat IgG (code number F0234) (Dako); polyclonal Cy3-conjugated F(ab’)2 fragment goat anti-rabbit IgG (H+L) and polyclonal Alexa Fluor 488 goat anti-rat IgG (H+L) (Jackson ImmunoResearch); alkaline phosphatase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG (Sigma–Aldrich). The following reagents were used: fibronectin (FN) (Roche, Milan, Italy), Polylysine (PolyLys) and bovine serum albumin (BSA) (Sigma–Aldrich). Purified C1q, was purchased from Sigma–Aldrich and its purity was checked by SDS/PAGE analysis.

Human tissues

Tumour tissue samples were obtained from the archives of the Department of Human Pathology, University of Palermo, Italy. Thirty invasive malignant neoplasm specimens were selected including six cases of each of the following tumour type, such as colon adenocarcinoma, melanoma, lung adenocarcinoma, breast adenocarcinoma, and pancreatic adenocarcinoma. The study was approved by the Institutional review board of the University of Palermo.

Mice

C57BL/6 WT mice were purchased from Harlan Laboratories. Mice deficient in complement components C1q, C3 or C5 (respectively C1qa−/−, C3−/− and C5−/−) were generated as described previously (Botto et al., 1998; Wessels et al., 1995; Wetsel et al., 1990). All strains were backcrossed onto the C57BL/6 (B6) genetic background for more than 10 generations. The mice were sex- and age-matched. All animals were handled in accordance with the institutional guidelines and in compliance with the European
In vivo tumour models

B16/F10 murine melanoma cell line was purchased from the American Tissue Culture Collection (Manassas, VA) and maintained in Minimum Essential Medium (Euroclone) supplemented with 10% (v/v) Fetal Bovine Serum, 1% (v/v) Penicillin–Streptomycin solution, 2 mM L-Glutamine, 100 μM Non-Essential Amino Acids, 1 mM Sodium Pyruvate and 10 mM HEPES (all from Life Technologies) in a humidified 5% CO₂ atmosphere at 37°C. C57BL/6 WT, C57BL/6.C1qa⁻/⁻, C57BL/6.C3⁻/⁻ or C57BL/6.C5⁻/⁻ were injected intramuscularly into the left flank of the mice (2×10⁶) with viable B16/F10 cells and tumour development was monitored over time. Tumour volume was measured with calliper by determining two orthogonal axis, and calculated using the following formula: \((\pi/6)\cdot a^2\cdot b\), with \(a\) the shorter and \(b\) the longer axis (Gava et al., 2006). At day 14 the animals were sacrificed and tumours, spleens and lungs were collected for analyses.

Bone marrow transplantation (BMT) and assessment of C1q levels

Mice were irradiated at 8 Gy using a \(^{137}\text{Cs}\) γ-ray source and reconstituted with 10⁷ bone marrow cells from sex-matched WT or \(C1qa^{-/-}\) donors. Mice were kept under sterile conditions for the first two months after BMT. The successful reconstitution of the haematopoietic lineages after BMT was monitored by flow cytometry and by measuring C1q levels in serum. Two months after the BMT mice were injected intramuscularly with 2×10⁶ viable B16/F10 cells and tumour growth monitored up to day 14. Serum C1q levels were measured by ELISA as previously described (Petry et al., 2001; Cortes-Hernandez et al., 2004). Briefly, microtiter plates were coated with 1 μg/ml of anti-mouse C1q Ab (clone Rm C7H8; Connex) and blocked with 5% milk in PBS. Serum samples were diluted appropriately in PBS-2% BSA-0.05%Tween 20-0.02% NaN₃. Bound C1q was detected with a biotinylated goat anti-mouse C1q antibody (kindly provided by Prof F. Petry, Mainz, Germany) followed by a streptavidin-alkaline phosphatase conjugate (BD Bioscience, San Diego, Ca), and revealed with p-Nitrophenyl Phosphate (pNPP) alkaline phosphates substrate (Sigma). Optical density (OD) was measured at 405 nm. The results were expressed in μg/ml, referring to a standard curve derived from a known concentration of purified human C1q. C1q-deficient mouse serum was included as negative control.
**Immunohistochemical staining**

Four-micrometer-thick paraffin tissue sections were stained for immunohistochemical analysis as previously reported (Bulla et al., 2008; Bossi et al., 2014). After microwave antigen retrieval, the sections were incubated overnight with C1q (Dako, Denmark), C1s (Quidel, USA), C3 (The Binding Site, UK), C4 (The Binding Site, UK) and CD31 (Novocastra, UK) primary antibodies and staining was revealed by streptavidin–biotin–peroxidase complex method. 3-Amino-9-ethylcarbazole (AEC; red signal, DAKO, Denmark) was used as a chromogenic substrate, and sections were counterstained with Hematoxylin. Negative control stainings were performed by using mouse, rabbit, or goat immune sera instead of the primary antibodies. For double-marker immunohistochemistry, sections underwent two sequential rounds of single-marker immunostaining. Briefly, the samples were incubated with mouse anti-human CD34 (Novocastra, UK), and after Fc blocking, with rabbit anti-human C1q (Dako, Denmark). The binding was revealed using specific secondary antibodies phosphatase alkaline or peroxidase conjugated (Sigma, USA) and BCIP/NBT and AEC as substrates respectively.

**Immunofluorescence analysis**

Seven-micrometer-thick tissue sections of snap-frozen tumour masses from WT and C1qa<sup>-/-</sup> mice at the indicated time-points, embedded in OCT medium (Diagnostic Division; Miles Inc), were stained for immunofluorescence analysis with the appropriate antibodies and incubated with DAPI (Sigma-Aldrich) to counterstain cell nuclei. The slides were mounted with the Mowiol based antifading medium (Sigma-Aldrich). Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Wetzlar, Germany) equipped with Leica DFC420 camera.

**Confocal analysis**

B16/F10 cells (3×10<sup>5</sup>) were plated at 37°C on eight-chamber culture slides (BD Biosciences) coated with C1q (20 μg/ml), FN (20 μg/ml), FN+C1q (20 μg/ml each) or PolyLys (70 μg/ml) and left to adhere for 30 min. The cells were fixed and permeabilized with the FIX & PERM Cell Permeabilization Kit (Società Italiana Chimici, Roma, Italy), stained with N-(7-nitrobenz-2-oxa-1,3-diazol-4-y)-conjugated phallacidin (NBD-phallacidin) (Molecular Probes, Invitrogen) and mAb anti-paxillin followed by Cy3-conjugated F(ab’)<sub>2</sub> goat anti-mouse IgG as previously described (Spessotto et al., 2006; Agostinis et al., 2010). Images were acquired with the Leica TCS SP2 confocal system
(Leica Microsystems) using the Leica Confocal Software and a 633 fluorescence objective on a Leica DM IRE2 microscope (Leica Microsystems) or with a Nikon C1Si confocal system, using the Nikon EZ-C1 Confocal Software and a 633 fluorescence objective on a Nikon TE2000-U inverted microscope (Nikon, Melville, NY).

**Evaluation of angiogenesis and metastases**

The microvascular density was evaluated on CD31-immunostained sections from mouse tumour samples by counting the number of vessels out of 10 microscopic fields at ×200 magnification and averaging the counts. The number of lung parenchymal metastases was evaluated on H&E-stained sections and the overall metastatic burden was assessed as the sum of the areas of the metastatic foci in the evaluated sections. All the measurements were performed using a Leica DMD108 digital microscope.

**Flow cytometry**

Cells were stained using standard protocols in the presence of a saturating concentration of anti-CD16/CD32 (2.4G2) mAb. The following antibodies were used: anti-CD4 (clone RM4-5), anti-CD8a (clone53-6.7), anti-CD11b (clone M1/70), anti-CD14 (clone Sa2-8), anti-CD45 (clone 30-F11), anti-CD49b (clone Dx5) and Gr-1 (clone RB6-8C5). Antibodies were all purchased from eBioscience (Life Technologies, Paisley, UK). Data were acquired using a FACSVerse (Becton-Dickinson, Mountain View, CA) and analysed using FlowJo software, version 7.6 (TreeStar, Ashland, OR).

**Adhesion assay**

The adhesion assay was performed as previously reported (Agostinis et al., 2010). Briefly, {10^5} B16/F10 cells/100 μl [RPMI 1640 (Life technologies) containing 0.1% (v/v) BSA], labelled with the fluorescent dye FAST DiI (Molecular Probes, Invitrogen), were added to a 96-well plate (Iwaki, Bibby Scientific Italia, Milan, Italy) for 35 min at 37°C in an air/CO₂ incubator. The wells were coated with BSA, C1q and FN used at the concentration of 20 μg/ml in pH>9 sodium bicarbonate-buffered medium (100 mM). Double-coated wells were prepared by adding first FN (20 μg/ml) and then incubated with C1q (25 μg/ml) in PBS-BSA 0.1% (v/v) and 0.7 mM Ca²⁺ and Mg²⁺ for 90 min at 37°C, to allow the interaction of C1q with bound FN. The number of adherent cells were counted with Infinite200 (absorbance 544 nm, emission 590 nm) (TECAN Italia,
Milan, Italy) and referred to a calibration curve established with an increasing number of labelled cells.

**Assessment of Ki-67 expression**

The proliferation assay was performed by modifying the protocol previously reported (Bossi et al., 2014). Briefly, serum-starved B16/F10 (8×10^4 cells) for 24 hours were seeded for 6 hours on eight-chamber culture slides (BD Biosciences Discovery Labware, Milan, Italy) coated with PolyLys (70 μg/ml), C1q (20 μg/ml), FN (20 μg/ml) or double-coated wells as described above in serum-free culture medium with 0.5% (v/v) BSA and 0.7 mM Ca^{2+} and Mg^{2+}. The cells were fixed and permeabilized with the FIX & PERM Cell Permeabilization Kit (Società Italiana Chimici), and stained with the anti-mouse Ki-67, followed by the FITC-labelled secondary antibody. The percentage of Ki-67-positive tumour cells was then determined by relating the total number of DAPI-positive nuclear staining to the total number of Ki-67-positive tumour cells in ten fields with an high-density number of cells (×200). The mean value was calculated for each section.

**Migration assay**

The migration assay was performed as previously reported (Agostinis et al., 2010). The migration assay was performed in transwell chambers using FAST DiI-labeled (Molecular Probes, Invitrogen) B16/F10 cells (2×10^5 cells), resuspended in RPMI 1640 with 0.1% (v/v) BSA and added to the upper chamber. The cells were allowed to migrate through HTS FluoroBlok systems with polycarbonate membranes of 8 μm pore size (Becton Dickinson, Falcon, Milan, Italy) coated on the upper side with BSA, C1q and FN used at the concentration of 20 μg/ml in pH>9 sodium bicarbonate-buffered medium (100 mM). Double-coated wells were prepared as described above. The cells were left to migrate for 18 hours. The number of cells transmigrated to the lower side of the insert was evaluated as outlined above for the adherent cells.

**Phosphorylation of ERK, Akt and GSK-3β in mouse melanoma cell line**

Pathway analysis was performed according to the manufacturer’s instructions of the PathScan® Intracellular Signaling Array Kit (Fluorescent Readout) (code number 7744; Cell Signaling Technology, EuroClone, Milan, Italy). Briefly, 24 hours serum-starved B16/F10 cells (1.8×10^6 cells) were left to adhere to PolyLys-, C1q-, FN- or double-coated wells as described above for the indicated periods of time at 37°C. At the end of the
incubation period, the cells were washed with ice-cold 1X PBS and lysed in 1X ice-cold Cell Lysis buffer, containing a cocktail of protease inhibitors (1X) (Roche Diagnostics). The Array Blocking Buffer was added to each well and incubated for 15 min at RT. Subsequently, an equal amount of total lysate (0.8 mg/ml) was added to each well and incubated for 2 hours at RT. After washing, the biotinylated detection antibody cocktail was added to each well and incubated for 1 hour at RT. Streptavidin-conjugated DyLight 680 was added to each well and incubated for 30 min at RT. Fluorescence readout was acquired using the LI-COR Biosciences Infrared Odyssey imaging system (Millennium science) and pixel intensity was quantified using Array Vision software.

**Statistical analysis**

Data from *in vivo* mouse models were analysed using Two-way ANOVA, Tukey-Kramer test, survival data by Kaplan-Meier test and *in vitro* experiments with unpaired two-tailed Student *t* test. Results were expressed as mean ± SEM. *Ex vivo* analysis were assessed by Mann–Whitney U tests. Results were expressed as median and interquartile range. P values of less than 0.05 were considered significant. All statistical analyses were performed using Prism 6 software (GraphPad Software Inc, La Jolla, CA).
RESULTS

C1q is present in human tumour specimens

We have recently reported that C1q favours trophoblast invasion of maternal decidua (Agostinis et al., 2010) and promotes angiogenesis in wound healing (Bossi et al., 2014). As these features are shared to some degree by growing tumours, the possibility that C1q may contribute to this process was raised. To support this notion we initially searched for the presence of C1q, C1s, C4, and C3, in a panel of 30 invasive malignant neoplasm specimens including 6 cases of each of the following tumour type, such as colon adenocarcinoma, melanoma, lung adenocarcinoma, breast adenocarcinoma, and pancreatic adenocarcinoma. As shown in Figure 1, a strong signal for C1q was detected in all tumour specimens examined, while other complement components were either absent (C4) or mildly expressed (C1s and C3) suggesting that C1q deposition did not result in the activation of the classical pathway. Within the tumour microenvironment, C1q was mainly expressed by vascular endothelia (Figure 1B, black arrows), mesenchymal cells and histiocytes (Figure 1B, black arrowheads). C1q expression within newly formed tumour-associated vascular endothelia was further confirmed by double-staining with CD34, an endothelial marker, which revealed co-localization (Figure 1C). Notably, C1q expression in the mesenchymal elements appeared to be associated with cancer invasion since C1q was clearly detected in stromal elements at the tumour invasion edge, whereas it was absent in the distant stroma (Figure 1D, left panel, black arrows). Consistent with this, C1q was not detected in non-transformed and non-infiltrated peri-tumoural tissues (Figure 2). Moreover, the association between C1q expression and tumour stroma was not confined to primary lesions as it was also detected in the stroma of metastatic tumour foci (Figure 1D, right panel, black arrows).

Prolonged survival and decreased tumour growth in C1q-deficient mice

The presence in tumour tissues of C1q in the absence of other components of the classical complement pathway led us to evaluate the contribution of this molecule to tumour growth in a syngeneic model of melanoma developed in C57BL/6 (WT) mice. After pilot experiments to define the optimal experimental conditions for inducing tumour growth and lung metastasis in a high proportion of mice, we selected the intramuscular injection of 2×10^6 B16/F10 melanoma cells. The tumour mass grew progressively in WT
Figure 1. (A) Immunohistochemical analysis of human tumours for the presence and distribution of complement components. Representative microphotographs of immunohistochemical analyses for the complement components C1q, C1s, C3, and C4 performed on different malignant cancer histotypes showing the consistent expression of C1q in the tumour-associated stroma in spite of a scant or absent expression of other C components involved in the classical pathway. Streptavidin-biotin-peroxidase complex system with AEC (red) chromogen. Original magnification ×200.

(B) Analysis of tissue distribution of C1q. C1q expression in the tumour-associated stroma of the different cancer histotypes highlights monocytoid cells suggestive of tumour-infiltrating myeloid elements (arrowheads) and mesenchymal elements including vascular endothelial cells and spindle-shaped fibroblasts
(arrows). Streptavidin-biotin-peroxidase complex system with AEC (red) chromogen. Original magnification ×630.

(C) **Colocalization of CD34 and C1q on the vessel endothelium of colon cancer.** Actual expression of C1q on tumour stroma vascular endothelia is demonstrated by double-marker immunohistochemical analysis showing co-localization of C1q (blue signal) and CD34 (red signal) in vessels. Streptavidin-biotin-peroxidase complex system with AEC (red) and alkaline-phosphatase-anti-alkaline-phosphatase method with NBT/BCIP (blue) chromogen. Original magnification ×400.

(D) **Deposition of C1q at the infiltration edge and in liver metastasis of colon cancer.** Left panel: stromal expression of C1q characterizes the stromal tissue neighbouring neoplastic glandular foci at the edge of tumour infiltration in colon adenocarcinoma infiltrating the muscularis propria (arrows), while the distant stroma has no significant C1q expression. Right panel: C1q expression also characterizes the tumor-associated stroma (particularly the fibrovascular meshwork) at sites of metastatic colonization as shown in a parenchymal liver metastasis by colon adenocarcinoma. Streptavidin-biotin-peroxidase complex system with AEC (red) chromogen. Original magnification ×400.

Figure 2. Representative microphotographs showing differential C1q expression in the stroma of cancer-involved (left edge of the upper panel; red contoured panel) and non-involved mucosa (right edge of the upper panel; green contoured panel). C1q-expressing tumour-infiltrating myeloid elements (arrowheads) and mesenchymal elements including vascular endothelial cells and spindle-shaped fibroblasts (arrows) are differently enriched in the two conditions. Streptavidin-biotin-peroxidase complex system with AEC (red) chromogen. Original magnifications: upper panel ×200; lower panels ×630.
animals reaching a mean of 6 cm$^3$ 20 days after the cell injection (Figure 3A). Thereafter, the mice started to die and were all deceased within 32 days (Figure 3B). Conversely, $C1qa^{-/-}$ mice exhibited a significantly slower tumour growth throughout the period of observation associated with a prolonged survival, while the tumour growth and the survival rates of both C3- and C5-deficient animals did not differ from those of WT animals (Figure 3A-B).

Immunofluorescence analysis of the tumour masses showed marked deposition of C1q on vascular endothelium and stroma in the absence of C4 and a scattered distribution of C3 that was undetectable on endothelial cells and was mainly associated with tumour infiltrating cells (Figure 3C). To identify the cell source of C1q and C3, double staining of tumour sections was performed with antibodies to CD68 and each of the two complement components. As shown in Figure 3D, C3 was expressed almost exclusively in CD68+ macrophages while C1q staining was seen both in CD68+ macrophages and in CD68- mesenchymal stromal cells. Kinetic analysis of the growing tumour revealed the presence of C1q as early as 2 days after tumour cell injection preceding the expression of C3 that started to be detected two days later and was clearly seen on day 6 (Figure 3E).

**Distinct features of melanoma in WT and $C1qa^{-/-}$ mice**

To validate the results of the different tumour progression observed in $C1qa^{-/-}$ and WT mice, the *in vivo* experiment was independently repeated in another institution’s animal facility using B16/F10 cells. The data shown in Figure 3F-G essentially confirm the slower growth rate and the markedly reduced size of tumour in $C1qa^{-/-}$ mice as compared with WT animals. The possibility that C1q expressed in the tumour microenvironment may promote tumour growth by modulating the local immune response was explored by flow cytometry analysis of tumour-infiltrating immune cells. No difference was found in the percentage of cells of both the innate (NK, monocytes and macrophages) and adaptive (CD4+ and CD8+ T cells) immune system (Figure 3H). In addition, we failed to detect a higher percentage of myeloid derived suppressor cells in WT mice that may explain the enhanced tumour growth in these animals as compared with $C1qa^{-/-}$ mice (Figure 3H).
Figure 3A-D. Effect of C1q on tumour growth and survival of B16/F10 bearing mice. WT (n=14), C1qa−/− (n=7), C3−/− (n=11) and C5−/− (n=10) mice received an intramuscular injection of 2×10⁶ B16/F10 melanoma cells and the tumour size (A) and survival (B) was analysed at various days after cell injection. Data of tumour size are presented as mean ± SEM. ** p<0.01 (two-way ANOVA, Tukey-Kramer test). Survival data have been analysed by Kaplan-Meier test (C1qa−/− vs WT p<0.01; C1qa−/− vs C3−/− p<0.01; C1qa−/− vs C5−/− p<0.01; C3−/− or C5−/− vs WT not significant). (C) Immunofluorescence analysis of tumour tissue for the distribution of the C1q, C4, C3 and endothelial vWF at day 14. (D) Immunofluorescence staining of tumour tissue for C1q, C3 and macrophage CD68 at day 14. Nuclei were stained with DAPI. Original magnification ×200.
Figure 3E. Expression of C1q and C3 in tumour tissue collected from WT mice at various days after B16/F10 injection. Immunofluorescence analysis of tumour tissue for the presence of the C1q and C3 at day 2, 4 and 6 after the intramuscular injection of $2 \times 10^6$ B16/F10 melanoma cells. Nuclei were stained with DAPI. Original magnification ×200.

Figure 3F-G. Evaluation of tumour size in tumour bearing WT and C1q-deficient mice. (F) Tumour mass of C1q-deficient mice (n=7; triangle) and WT mice (n=7; circle) measured on various days after B16/F10 melanoma cell injection. Data are shown as mean ± SEM. ** p<0.01, Student t-test. (G) Imagine of the tumours and spleens of one representative experiment on sacrificing (day 12). Scale bar, 1 cm.
Figure 3H. Percentage of different cell populations within the tumour mass on day 12 after the B16/F10 injection. Upper panel - Tumour tissues were mechanically disaggregated and the infiltrating leukocytes were separated by gradient centrifugation on Histopaque. Representative data from 2 independent experiments are shown. Statistical analysis by two-tailed t-test, p values are indicated. Each symbol represents one mouse and the lines designate means ± SEM. Left panel - representative flow cytometric plot showing the gate strategy used to identify the mononuclear MDSCs (MO-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) among CD45+ tumour infiltrating cells. PMN-MDSCs were characterized by higher expression of CD11b and Gr-1 compared to MO-MDSCs. Right panel - ratio of PMN-MDSCs to MO-MDSCs in total tumour MDSC populations of WT and C1q-deficient mice. P value is indicated, t-test. Each symbol represents one mouse and the line designates mean ± SEM.
Effect of C1q on tumour angiogenesis and metastases

As cancer development largely depends on angiogenesis (Carmeliet, 2005), we examined the tumour tissue for the degree of new vessel formation and discovered that the vascular density in tumour bearing WT mice was significantly higher than that in C1qa<sup>-/-</sup> animals, particularly in the peri-tumoural area (Figure 4A-D). We also searched for metastatic melanoma cells in the lungs, the target organ preferentially colonized by the cancer cells that escape from the primary tumour site (Braeuer et al., 2014), and found metastases in 6 out of 11 WT mice and only in 1 out of 12 C1qa<sup>-/-</sup> mice (Figure 4E-F).

Figure 4A-F. Evaluation of angiogenesis and lung metastases in tumour-bearing mice. Quantification of intra-tumoural (A) and peri-tumoural (C) microvascular density (MVD) on five different ×200 microscopic fields of tumour sections from WT and C1q-deficient mice immunostained for CD31. Representative pictures of CD31 immunostained sections of tumour tissue from WT and C1q-deficient mice showing intra-tumoural (B) and peri-tumoural (D). Measurement of metastatic areas in the lungs of tumour bearing WT (n=11) and C1q-deficient mice (n=12) (E) and representative images showing metastases in both group of mice (F). Data are expressed in μm<sup>2</sup> as median and interquartile range. * p<0.05, ** p<0.01, *** p<0.0002 (Mann–Whitney U test).
Tumour growth in bone marrow transplanted mice

To investigate whether the manipulation of C1q levels by BMT could influence the development of the tumour, we produced reciprocal radiation BM chimeras between C1qa-/- and WT mice. In agreement with our previous findings (Petry et al., 2001; Cortes-Hernandez et al., 2004), two months after the BMT, C1q levels in the C1q-deficient host were almost comparable to those in control WT chimeras (Figure 5B). In contrast, the C1q levels in WT mice reconstituted with C1qa-/- BM cells were hardly detectable. The development of the tumour in the bone marrow chimeras was then evaluated by measuring the tumour size at different time points (Figure 5A). A progressive increase in tumour was observed in all experimental groups during the period of observation. However, at each time point this increase was markedly less pronounced in the C1qa-/- irradiated recipient mice regardless of the circulating C1q levels. Syngeneic reconstituted C1qa-/- mice (△; 718.9±364.1 mg) and the chimeric mice becoming C1q-sufficient (▲; 240.8±104.8 mg) after BMT developed smaller tumours. Conversely WT mice reconstituted with C1qa-/- BM cells (○; 2587±1391 mg) and syngeneic (●; 6750±1715 mg) reconstituted WT mice showed accelerated tumour growth (Figure 5A). Taken together these data indicate that the non-bone marrow derived C1q was a critical factor for the tumour growth. The cell source of C1q was analysed by immunofluorescence using antibodies to C1q (red) and to CD68 (green) to identify bone marrow derived macrophages (Figure 5C). As expected, C1q was undetectable in C1qa-/- mice reconstituted with C1qa-/- BM while it was widely distributed on endothelial cells and in CD68+ cells in WT mice receiving WT BM. Vascular endothelium and CD68- non-BM derived stromal cells expressed C1q in WT mice reconstituted with C1qa-/- BM. Conversely, endothelial cells did not stain for C1q in C1qa-/- recipient of WT BM that colonize the tumour mass with macrophages expressing C1q.

Biologic effects of C1q on murine melanoma cells

Having established that C1q is present in the tumour mass, we sought to investigate the mechanism by which C1q contributed to tumour growth analysing its ability to promote adhesion, proliferation and migration of cancer cells. To evaluate cell binding to C1q, melanoma cells were labelled with the fluorescent probe FAST Dil and allowed to adhere to solid phase-bound C1q or a mixture of FN and C1q, in 96-well plates for 30 min at 37°C, using BSA and FN as negative and positive controls, respectively. C1q was able to promote the adhesion of 29% of cells as opposed to nearly 13% of cells adhering to
Figure 5A-C. Tumour growth in BM transplanted mice. (A) Tumour mass measured on various days after tumour cell injection (left graph) and on end-point (day 16) when mice were killed and tumours were removed (right graph). Irradiated recipient mice are represented by a circle (WT) or a triangle (C1qa−/−). Mice reconstituted with C1q-sufficient BM cells are represented with a closed symbol while those reconstituted with C1q-deficient BM cells with an open symbol. Data are shown as means ± SEM. Each symbol represents an individual mouse; p values are indicated (two-way ANOVA). (B) C1q antigenic levels in reconstituted mice. Levels were measured by ELISA as described in the Materials and Methods section after two months after BMT. Results are expressed in μg/ml, referring to a standard curve derived from a known concentration of purified C1q. (C) Localization of C1q (red) and macrophage CD68 (green) in tumour section of BMT mice. Nuclei were stained with DAPI. Original magnification ×200.
BSA and enhanced the pro-adhesive activity of FN to approximately 80% following its interaction with this ECM protein (Figure 6A). A high percentage of cells bound to FN appeared spread out and displayed actin-containing stress fibres, in contrast with the round morphology exhibited by the great majority of those attached to C1q and by a proportion of cells adhering to C1q bound to FN (Figure 6D).

The ability of C1q to induce cell migration was examined by adding the tumour cells to the upper chamber of a transwell and allowing them to migrate through an insert coated with C1q or FN or both. C1q was slightly more effective than FN causing migration of 87% of cells that increased to 96% using the mixture of C1q and FN (Figure 6B), and the difference was statistically compared to FN.

Given the in vivo finding that C1q present in the tumour microenvironment is associated with cancer progression, we asked the question whether C1q may contribute to tumour growth by stimulating the proliferation of cancer cell. To this purpose, the melanoma cells were incubated with solid-phase bound C1q and either FN or the mixture of FN and C1q for 6 hours and the number of proliferating cells was evaluated using anti-Ki-67 antibody. As shown in Figure 6C, C1q induced significantly higher cell proliferation than FN (40% vs 27%) and the percentage of proliferating cells slightly increased after stimulation with the mixture of C1q and FN.

To further elucidate the mechanism of cell activation, we analysed the signalling pathways potentially involved in tumour cell adhesion, migration and proliferation examining more specifically the activation of ERK1 and the regulation of Akt and GSK-3β signalling molecules.

To this purpose, 24 hours serum-starved B16/F10 cells were allowed to adhere to wells coated with C1q, FN or the mixture of C1q and FN for various lengths of time and the phosphorylation status of ERK1 (Thr202/Tyr204), GSK-3β (Ser9) and Akt (Thr308 and Ser473) was evaluated by immunofluorescence using specific antibodies. As shown in Figure 6E-H, binding of B16/F10 cells to C1q resulted in the activation of all the signalling molecules, which was already seen at 20 min and substantially decreased or became undetectable at 40 min post-stimulation. Interestingly, the phosphorylation level of all the signalling molecules in cells seeded onto double matrix (FN+C1q) was greater than the combined values observed in FN- and C1q-stimulated cells. This was particularly evident when analysing the phosphorylation level of GSK-3β and Akt (Ser473) in double matrix-activated cells that by far exceeded those induced by FN and C1q tested individually.
Figure 6. Effect of C1q on B16/F10 melanoma cell adhesion, migration and proliferation, as well as C1q-mediated cell signalling. (A-C) The experiments of cell adhesion, migration and proliferation were performed as detailed in Materials and Methods. Data from at least five independent experiments are presented as mean ± SEM. * p<0.05, ** p<0.01 (Student t test). (D) Confocal analysis of B16/F10 seeded on slides coated with different matrix. Tumour cells fixed after incubation on C1q, FN and FN+C1q for 30 min and stained with NBD-phallacidin (green) and mouse monoclonal anti-paxillin Ab followed by Cy3-conjugated goat anti-mouse IgG (red) were analysed by confocal microscopy. Original magnification: ×630. (E-H) Phosphorylation of ERK (Thr202/Tyr204), GSK-3β (Ser9) and Akt (Thr308 and Ser473) in mouse melanoma cells. B16/F10 were allowed to adhere to C1q, FN and FN+C1q and the phosphorylation status of ERK1, GSK-3β and Akt and was evaluated on total cell lysates as described in Materials and Methods.
DISCUSSION

The tumour microenvironment comprising infiltrating immune and non-immune cells as well as the extracellular matrix undergoes substantial changes during cancer development that may largely influence tumour progression (Swartz et al., 2012; Junttila and de Sauvage, 2013). A recently discovered function of complement activation products released at tumour site is to recruit myelomonocytic cells that suppress the adaptive immune response to tumour antigens and promotes tumour growth (Markiewski et al., 2008). This novel aspect of the complement activity in tumour-host interaction have disclosed a dual and opposing role of this system (Loveland and Cebon, 2008; Stover, 2010). Besides being effective in cancer immunosurveillance, using complement fixing mAbs to tumour associated antigens combined with CRPs neutralizing Abs (Macor et al., 2015), favours tumour growth in situation of slow and chronic C activation (Rutkowski et al., 2010). The data presented in this study indicate that C1q contributes to changes of tumour microenvironment acting as an external component of the extracellular matrix and favouring tumour growth and invasion.

Deposits of C components have been reported in different human tumours by various groups and interpreted as the results of complement activation induced by several triggering factors including antibodies to tumour associated antigens, immune complexes, and cell damaged by necrosis and apoptosis (Stover, 2010; Pio et al., 2014). The extent of complement activation, that in some cases proceeds to the assembly of the terminal complex (Vlaicu et al., 2013), is probably dependent on the tumour type, and the degree of inflammation associated with tumour invasion. C1q was the predominant complement component deposited in all tumour examined in this study and its localization on endothelial cells and stroma is reminiscent of a similar distribution in human decidua where is locally synthesized and secreted by several cells including endothelial cells and trophoblast (Bulla et al., 2008; Agostinis et al., 2010).

Although C1q deposition is usually regarded as an indication of classical pathway activation, our failure to detect C4 in all tumours and C1s in most of them makes this an unlikely possibility and rather suggests an alternative mechanism of C1q involvement at tumour site not necessarily related to complement activation. Data accumulated in recent years have revealed non canonical functions exerted by C1q on cells of both innate and adaptive immunity (Nayak et al., 2010, 2012; Ghebrehiwet et al., 2012), as well as on cells selectively localized in some tissues, such as microglial cells in the central nervous system.
and trophoblasts in placental decidua (Veerhuis et al., 2011; Agostinis et al., 2010). The level of this recognition molecule of the complement system has been reported to increase substantially in aging brain and to contribute to age-related cognitive decline (Stephan et al., 2013). In pregnancy C1q has been implicated in tissue remodelling in maternal decidua required for successful embryo implantation (Bulla et al., 2012).

The critical role of C1q in tumour development is supported by the finding of prolonged survival, and slower progression, of tumour growth in C57BL/6.C1qa−/− bearing a syngeneic B16/F10 melanoma, as compared to WT mice. Failure to detect significant difference in tumour growth and survival between C3−/− and WT mice excludes the possibility that the cancer promoting effect of C1q was dependent on complement activation. This conclusion is further supported by the immunofluorescence analysis of the tumour mass developed in WT mice, which revealed marked deposition of C1q on vascular endothelium and stroma in complete absence of C4, while staining for C3 was almost exclusively restricted to CD68+ cells. C5−/− mice was included in this study as a control group because previous reports have shown that C5a generated through local complement activation promotes tumour growth by recruiting myeloid-derived suppressor cells (MDSCs) that inhibit T cell-mediated anti-tumour response (Markiewski et al., 2008; Rutkowski et al., 2010). The finding that melanoma grew in C5−/− mice in a manner similar to that observed in C3−/− and WT mice further rules out the contribution of complement activation products and more specifically of C5a to tumour progression. The potential contribution of C1q to modulate the anti-tumour immune response was excluded by the analysis of peri-tumoural cell infiltrates that revealed no difference in the percentage of effector T cells, NK cells, macrophages and myeloid derived suppressor cells between C1qa−/− and WT mice.

The ex vivo and in vivo data pointed to a direct contribution of C1q to tumour growth independent of complement activation and raised the question of whether locally produced C1q was responsible for its cancer promoting effect. Although several cell types may synthesise C1q, it was unclear which cells were implicated in this process. The answer was obtained from the analysis of tumour development in bone marrow transplanted mice. Surprisingly, bone marrow from WT mice, despite the ability to reconstitute normal C1q serum level in C1qa−/− animals, had only a negligible effect on tumour growth in C1q-deficient mice, suggesting that non bone marrow-derived cells were the relevant source of C1q needed for its tumour promoting activity. Included in this group of cells, besides stroma cells, are endothelial cells that exhibited strong C1q staining in tumour developed
in WT mice. Serum is an unlikely source of C1q bound to endothelial cells because tumour ECs from WT mice reconstituted with C1qa-/- bone marrow cells still express C1q despite its nearly undetectable level in the circulation. Moreover, our failure to detect C4 on ECs both in human tumours and in the animal model of melanoma tend to exclude that C1q deposition is the result of complement activation. An alternative possibility to explain C1q expression on the surface of ECs is that these cells start synthesizing and secreting C1q in response to developing tumour. Although this issue was not specifically addressed, we have collected evidence from our previous studies indicating ECs may acquire the ability to produce C1q at some tissue sites and in certain pathophysiologic conditions like decidual endothelial cells in pregnancy (Bulla et al., 2008) and dermal microvascular endothelial cells in wound healing (Bossi et al., 2014).

The finding that C1q was localized on ECs of newly formed vessels identified by CD34 expression in the tumour tissue led us to hypothesize a possible contribution of C1q to the induction of angiogenesis. We were justified in pursuing this issue by our published work showing that C1q exhibits angiogenic activity promoting skin wound healing (Bossi et al., 2014). Consistent with these observations is the increased number of vessels observed both within the tumour mass and in the peritumoral area. As melanoma is considered a highly angiogenic tumour that exploits newly formed vessels to receive oxygen and nutrients and to establish metastasis (Emmett et al., 2011; Braeuer et al., 2014), it is not surprising to see larger metastatic areas in tumour bearing WT mice compared to C1qa-/- mice.

By interacting with proteins of the extracellular matrix like fibronectin, C1q offers an anchor to tumour cells for their initial seeding. The pro-adhesive effect of C1q does not seem to be restricted to the primary tumour, but can also be seen at metastatic sites as suggested by its presence at lung metastases of colon carcinoma. As already seen with trophoblasts (Agostinis et al., 2010), melanoma cells tend to bind loosely to C1q making it easier for cells to move toward surrounding stromal areas expressing this complement component. However, the tumour cells would not have much chance of expanding if they were not stimulated by C1q to proliferate as clearly documented by our data.

C1q signals B16/F10 cells stimulating the phosphorylation status of ERK, Akt and GSK-3β possibly as a result of interaction with gC1qR and α4β1 integrin expressed on these cells (Park et al., 2012; Prakash et al., 2011; Ratheesh et al., 2007; Zhao et al., 2008). These receptors have been shown to be engaged by C1q on the surface of trophoblasts leading to activation of ERK1/2 MAPKs and enhanced adhesion and migration of these cells (Agostinis et al., 2010). Evidence has been collected indicating that MAPK/ERK,
PI3K/Akt/GSK-3β and Wnt/GSK-3β signalling pathways are often activated in cancer and cooperate to promote cancer progression and metastasis (Anastas and Moon, 2013; McCubrey et al., 2012, 2014).
CONCLUSION

In conclusion, evidence collected from human tumours revealed a constant presence of C1q, which was mainly localized in the stroma and on vascular endothelium in the absence of C4. A reduced tumour size and a prolonged animal survival were documented in a syngeneic melanoma model developed in C1q-deficient mice compared to tumour established in WT, as well as in C3 and C5 deficient mice. Our data support the conclusion that C1q favours tumour growth and metastases, facilitating cancer cell seeding and invasion, through promotion of angiogenesis and sustain the activation of cancer-related signalling pathways.
BIBLIOGRAPHY


