C1q is related to microglial phagocytosis in the hippocampus in physiological conditions

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Abstract

The complement protein C1q has lately emerged as an important molecule in the clearance of apoptotic cells. However, its role in the brain in physiological conditions is less explored. Using as a model the adult neurogenic cascade, where newborn cells undergo apoptosis and are phagocytosed by microglia, in the present study we have discovered three major findings using immunofluorescence and RTqPCR from FACS-sorted microglia in fms-EGFP mice. First, we found that C1q is mainly produced by microglia in the hippocampus in physiological conditions. Second, we observed a relationship between phagocytic microglia and C1q, as suggested by the presence of C1q within the majority of the microglial phagocytic pouches. Finally, we discovered that the functions of C1q in microglia may go beyond phagocytosis, as C1q was also found in non-phagocytic microglia.

Introduction

The complement protein C1q plays several key functions in the innate immune system as the key recognition protein of the C1 macromolecular complex, which initiates the classical complement pathway [1]. In macrophages, C1q plays a central role in autocrine and paracrine signaling, antigen presentation, danger signaling and induction of immunomodulatory molecules [2]. In addition, C1q is emerging as an important molecule in the clearance of apoptotic cells, since it has been shown to bind dead cells in vitro [3] [4] [5]. C1q directly opsonizes target cells by binding to phosphatidylserine on the apoptotic cell surface, contributing to cell clearance [6] [7] [8].

In the brain, however, the role of the complement is less explored. C1q is mainly produced by microglia [9] [2], the resident immune cells and brain professional phagocytes, although it has also been reported to be produced by neurons [10]. However, the majority of the studies on microglia are related to its overproduction upon neurotoxicity [11] or under pathological conditions such as ischemic stroke [12] or viral infection [13]. C1q participates in the elimination of synapses by tagging a subset of immature synapses that are engulfed by microglia [14] [15]. However, detrimental functions of C1q have also been suggested upon deregulation in neurodegenerative diseases such as Alzheimer’s Disease, in which C1q increases synapse targeting and promote an excessive elimination and synapse loss by microglia [16]. C1q has also been involved in the microglial phagocytosis of apoptotic cells, and its presence leads to immunosuppressive effects in vitro [17]. Here we will focus on the role of C1q in apoptotic cell clearance in vivo, using as a model the adult neurogenic cascade, where there is ongoing phagocytosis due to the continuous death of new-born cells [18].

To test the association between C1q and microglia we use immunofluorescence and confocal imaging to assess protein expression and location, and RTqPCR from FACS-sorted microglia to assess mRNA expression. To identify microglia, we use fms-EGFP mice, in which the green reporter (enhanced green fluorescent protein) is expressed under the control of the mouse Csf1r, colony stimulating factor 1 receptor, promoter [19]. In the brain of these mice, EGFP is expressed in parenchymal microglia, as well as meningeal and perivascular macrophages [20].

Objective

The objective of this paper is to study the relationship between C1q expression and microglial phagocytosis in the adult hippocampus.
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Figure Legend
Figure 1. Characterization of the presence of C1q protein in the mouse adult brain.

(A) Representative confocal Z-stack of C1q immunofluorescence in the mouse hippocampal DG in physiological conditions at 1 month. Microglia were labeled with fms EGFP+ (cyan), C1q (red) and apoptotic nuclei were detected by pyknosis/karyorrhexis (white, DAPI). C1q presented a dotted pattern in the cytoplasm of microglia cells, both in the soma and the processes. Phagocytic microglia showed C1q also in the pouches that surrounded apoptotic cells. High magnification details show each apoptotic cell individually. Apoptotic cell 1 was not phagocytosed and did not present C1q staining. Apoptotic cells 2 and 3 were engulfed by microglia and C1q was present in the phagocytic pouches. Scale bars = 20 μm (C, G), z = 16.5 μm (DG).

(B) Percentage of phagocytosed and non-phagocytosed apoptotic cells with and without C1q labeling. The majority of the phagocytosed apoptotic cells had C1q in their surrounding microglia pouch. A small percentage of the phagocytic pouches did not present C1q in the pouch per se, but the majority of the microglia forming those pouches did have C1q in their soma and/or branches. Few phagocytosed apoptotic cells did not express C1q, neither in their pouch nor in the microglia engulfing them. A total of 159 apoptotic cells were quantified along the DG of 3 different mice (2 sections per mouse) by confocal microscopy.

(C) The orthogonal projection of a confocal Z-stack of C1q immunofluorescence in the mouse hippocampal DG in physiological conditions at 1 month. Microglia were labeled with fms EGFP+ (cyan), C1q (red) and apoptotic nuclei were detected by pyknosis/karyorrhexis (white, DAPI).

(D) Percentage of microglia with C1q labeling in the soma, branches or pouch. A total of 289 microglia cells were quantified along the DG of 3 different mice (2 sections per mouse) by confocal microscopy.

(E) Representative confocal Z-stack of the presence of C1q in the CA1 and CA3 of 1mo (1 month old) fms-EGFP mice in physiological conditions. Microglia were labeled with fms EGFP+ (cyan), C1q (red), and cell nuclei were labeled with DNA marker DAPI (White). C1q presented a dotted pattern inside microglia cells, both in the soma and the processes. High magnification details show each microglia cell individually. C1q staining colocalized with the soma and/or branches of microglia. Scale bars = 20 μm, z = 7.5 μm (CA1), 9 μm (CA3).

(F) Experimental design used to isolate microglia (GFP+) vs non-microglial cells (GFP-) from 1mo fms-EGFP mice. Flow cytometry analysis of the expression of C1q subunits in hippocampal cells. First, debris was excluded using the P1 gate in FSC versus SSC (left panel). Next, gates for GFP+ microglia cells (P3) and GFP- non-microglial cells (P2) were defined based on the distribution of the fms-EGFP+ cells in EGFP vs FSC (right panel).

(G) Expression of C1q subunits in microglia (GFP+) vs non-microglial cells (GFP-) by RTqPCR in FACS-sorted cells from fms-EGFP mice hippocampi (n = 3, each from 8 pooled hippocampi). OAZ1 was selected as a reference gene. Bars represent mean ± SEM. ***indicates \( p < 0.001 \) by Student’s t test.

Results & Discussion

Phagocytosed apoptotic cells are related to C1q

To test whether C1q played a role in phagocytosis in vivo, we analyzed by immunofluorescence and confocal imaging the presence of C1q in the neurogenic niche of the hippocampus, the subgranular zone (SGZ) of the dentate gyrus (DG) (Fig. 1A). We used fms-EGFP+ mice, in which microglia express the green fluorescent protein at 1mo of age to increase the chances to observe phagocytosed apoptotic cells, because at this age the process of hippocampal neurogenesis reaches its peak [21], and consequently, apoptosis...
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and microglial phagocytosis are more robust [18]. We studied a total of 159 DG apoptotic cells pooled from 3 different mice. SGZ apoptotic cells, likely to be newborn cells were phagocytosed by ramified, unchallenged microglia (93.0% of the apoptotic cells), as shown by the formation of a three-dimensional phagocytic pouch surrounding the apoptotic cell, as already described [18].

C1q was expressed in the SGZ and showed a dotted pattern. To assess the relationship between C1q labeling and apoptosis, we quantified the expression of C1q in apoptotic cells (Fig. 1B) and found that C1q labeling was associated with 84.9% of the phagocytosed apoptotic cells. To distinguish whether the C1q signal belonged to the cytoplasm of the apoptotic cell or the microglial pouch, we analyzed orthogonal projections of confocal Z-stacks. Orthogonal projections showed C1q labeling within the microglial cytoplasm (Fig. 1C), strongly suggesting that C1q was part of the microglial pouch, rather than the apoptotic cell cytoplasm. Moreover, we found that out of 8.1% of apoptotic cells without C1q staining in the phagocytic pouch, up to 69.3% belonged to C1q+ microglia, as C1q staining was found either in the microglial soma and/or branches (Fig. 1B). The remaining 30.7% of the C1q+ pouches were formed by microglia that we cannot exclude as C1q+ because part of the microglial cell was located outside the Z-stack. On the other hand, none of the non-phagocytosed apoptotic cells presented any C1q staining. Altogether, this data suggested a clear relationship between C1q and phagocytosis.

C1q protein is mainly expressed in microglia in the hippocampus

In addition to its presence in phagocytic pouches (Fig. 1A, C) C1q labeling was also located in the microglial soma—surrounding the nucleus—and throughout their processes (Fig. 1D) in 100% of the DG microglia. We only considered whole cells for the quantification of microglia, and therefore, microglial branches that had their soma outside the Z-stack or microglial somas with branches outside the Z-stack were not included in the analysis. In addition, very few scattered C1q dots were found in non-microglial cells. To test whether microglia expression of C1q solely occurred in the presence of apoptotic cells, we also analyzed its expression in the Cornu Ammonis (CA), a nearby region of the hippocampus where there is no neurogenesis and therefore no apoptosis nor phagocytosis [18]. We analyzed both CA1 and CA3 and found that here microglia also expressed C1q protein either in their soma (perinuclearly) and/or within the microglial processes, distributed as a dotted pattern along the branches (Fig. 1E). Similarly to DG, almost no presence of C1q was found in non-microglial cells. Therefore, in the adult brain, microglia were the main cell type expressing the complement protein C1q.

Thus, although we cannot exclude that the C1q plays other roles in non-phagocytic microglia (from CA), the association of C1q to the phagocytic pouches strongly suggests that C1q is involved in the process of phagocytosis.

Microglia is the major source of C1q in the hippocampus

C1q was almost exclusively found inside microglia but, nonetheless, the immunofluorescence data could not disregard that C1q was originated in apoptotic cells and incorporated in microglia during their engulfment and degradation. To further confirm the microglial origin of C1q, we compared the C1q mRNA expression in microglia versus non-microglial cells. The two cell populations were acutely purified from the hippocampus of fms-EGFP mice by fluorescent-activated cell sorting (FACS) (Fig. 1F). Microglia were selected as GFP+ cells, whereas other non-microglial cell types were characterized as GFP−. Next, the mRNA expression of the three subunits conforming C1q protein (C1qA, C1qB, and C1qC) was assessed by RT-qPCR (Fig. 1G). We found that the mRNA of each subunit was almost exclusively restricted to microglia, with enrichment of 98.1 ± 0.5% for C1qA, 97.5 ± 1.4% for C1qB, and 84.1 ± 7.5% for C1qC. Therefore, in agreement with previous reports [9] the translation of this mRNA into protein likely renders microglia as the major source of C1q in the hippocampus.

Conclusions
In the present paper, we have discovered that microglia is the main source of C1q mRNA and protein in the hippocampus in physiological conditions. In addition, we have found that most of the phagocytic pouches contained C1q and that the majority of microglial cells presented C1q within the microglial cytoplasm conforming the phagocytic pouch, suggesting a strong interaction between C1q and phagocytosis. Finally, we have also described C1q within non-phagocytic microglia, which suggests a close interaction between C1q and microglia and further functions of the complement protein beyond phagocytosis.

Limitations

One of the evidence we provide in the present work is the presence of C1q in phagocytic microglia in physiological conditions. However, although the orthogonal projection clearly shows the presence of C1q within the phagocytic pouches of microglia rather than in the surface of the apoptotic cell, confocal microscopy has a spatial resolution of 400–500 nm [22] [23], and therefore complementary techniques such as electron microscopy could provide information to support our results.

In addition, we used fms-EGFP mice in order to FACS-sort microglial cells. Fms-EGFP mice express the fluorescent protein in the mononuclear phagocytic system, which includes microglia, macrophages, and monocytes [19] [20]. Our dissection technique of the hippocampus includes careful peel off of the meninges and the plexus choroideus in order to remove macrophages and circulating monocytes, and therefore it is unlikely that residual cDNA of these cell types might be present in our RT-qPCR samples. However, this possibility should always be taken into the consideration.

Finally, this work is focused on the microglial phagocytosis of apoptotic cells specifically on the hippocampus. A similar analysis of the presence of C1q in different regions of the brain such as the subventricular zone where there is also ongoing apoptosis and therefore phagocytosis would be of great interest in order to provide a better understanding of the relationship between C1q and microglial phagocytosis.

Alternative Explanations

As the majority of microglia express C1q in the hippocampus (both phagocytic and non-phagocytic), it is possible that the location of C1q to the phagocytic pouches is the result of some sort of biophysical process that leads to its grouping or a condensation in the pouch membrane, but not its functional implication in the process of phagocytosis.

Conjectures

In the present work, we have shown that microglia is responsible for the majority of the intrinsic presence of C1q in the hippocampus, which opens a new insight in the understanding of the relevance of C1q in the physiological functions of microglia and more specifically, in phagocytosis. Such a close relationship between C1q and microglia in physiological conditions should be further studied in order to fully unravel the different functions that C1q might exert in the healthy brain. In addition, it also opens the question of whether microglial C1q production and signaling are balanced upon different neurological and neurodegenerative diseases, in which apoptosis is abundant. In addition, microglia and C1q production might be affected in such diseases and therefore contribute to the detrimental consequences such as neuroinflammation and neurodegeneration.

In addition, this paper reopens the debate on how microglia should be cultured in vitro. We found a relationship between C1q and microglial phagocytosis in physiological conditions in the CNS, and the presence or absence of the protein would have dramatic differences in the downstream experiments, which would determine the immunomodulatory nature of the process [17], suggesting that in order to mimic in vivo physiological...
conditions, C1q should be present in the in vitro phagocytosis assays. However, traditionally microglia cultures have been performed in the presence of heat-inactivated serum in order to inhibit complement proteins [24]. While the absence of serum in the culture leads to higher microglial survival and a more ramified, in vivo-like morphology, it also prevents microglia from engulfing latex microbeads or myelin [25]. Therefore, taking this data into consideration, this paper advocate for including C1q in the culture conditions by using a non-inactivated serum when performing phagocytosis assays.

**Additional Information**

**Methods**

**0.0.1 Animals**

Fms-EGFP (MacGreen) mice were used in every experimental procedure. These transgenic mice had a C57BL/6 background and expressed the enhanced green fluorescent protein (EGFP) under the CSF1R promoter specific of the mononuclear phagocytic system [19] [20]. The mice were 4 weeks old and were always maintained 12:12 h light cycle housing conditions and free access to food and water. All procedures were approved by the European Directive 2010/63/EU and NIH guidelines, and local Ethics Committees.

**Immunofluorescence**

Mice were perfused transcardially with 4% paraformaldehyde (PFA), and brains post-fixed for 3 h at RT. Then, the brains were rinsed in PBS and stored at 4°C until processing. The mouse brains were coronally sliced in a vibratome (Leica vibrating blade microtome) in order to obtain 6 series of 50 μm thickness sections. Standard immunofluorescence procedures were followed for tissue immunostaining [26] [18]. The staining was performed in free-floating tissue slices which were first blocked and permeabilized for 3 h at RT in 0.3% Triton-X100, 0.5% BSA in PBS solution and second, sections were treated for 2 overnight with a combination of the primary antibodies rat-anti-C1q and chicken-anti-GFP diluted in 0.3% Triton-X100, 0.5% BSA in PBS at 4°C. After this period of time, the tissue sections were rinsed with 0.3% Triton-X100 in PBS and incubated with the secondary antibodies (RRX and Alexa-488) and DAPI in 0.3% Triton-X100, 0.5% BSA in PBS at RT. Afterward, brain sections were thoroughly rinsed with PBS and mounted on glass slides with mounting medium (DakoCytomation).

**Confocal microscopy/Image Analysis**

Images of the DG of the immunostained brain sections were obtained using confocal microscopy (Leica SP8) under a 40X objective and scanning at a Z-step of 0.7 μm. A total of 3–4 sections were fully analyzed under the microscope with a zoom of 2.5 in order to find apoptotic cells.

The presence of pyknotic and/or karyorrhectic nuclei observed by DAPI staining was used to define apoptotic cells. On the other hand, characteristic of phagocytosed apoptotic cells were only attributed to those dead cells completely surrounded by an enclosed three-dimensional pouch of a microglial branch.

**FACS Sorting**

FACS Sorting was carried out following the previously well-described protocols of Abiega et al. [27] and Sierra et al. [20]. Briefly, both enzymatic (papain, 20 U/ml and DNase I, 150 U/ml) and mechanic homogenization were used in order to digest the hippocampal tissue of the mice. Afterward, the homogenate was filtered through a 40 μm nylon strainer and the enzymatic activity was quenched with 5 ml of 20% PBS in HBSS. Then, cells were centrifuged at 200 g for 5 min and resuspended in 20% SIP (Solution of Isotonic Percoll, in HBSS). Afterward, fire-polished pipettes were used to slowly add HBSS on top of the 20% SIP in order to create a Percoll gradient. Next, samples were
centrifuged at 200 g for 20 min with the lowest acceleration or deceleration in order to avoid any disruption of the gradient. Then myelin (the interphase) was removed and cells were rinsed with HBSS in a 200 g centrifugation for 5 min. Finally, the resulting pellet was resuspended in sorting buffer and cells were sorted in a FACS Jazz (BD) in order to collect the green fluorescent cell population. This population was directly collected in Lysis Buffer (Qiagen) with RNAse inhibitors (0.7% β-mercaptoethanol) and stored at -80°C.

RNA Isolation and RTqPCR

For the RNA isolation of microglia, the RNeasy Plus micro kit (Qiagen) manufacturer’s instructions were followed. iScript kit (iScript Advanced cDNA Synthesis Kit, Biorad) was used in order to retrotranscribed the resulting RNA. For the RT-qPCR, cDNA was amplified with SsoFast EvaGreen Supermix (Biorad) and detected in a CFX96 System (Biorad). All the RT-qPCR experiments were performed according to the MIQE guidelines (Minimal Information for Publication of Quantitative Real-Time Experiments [28].

In order to avoid unspecific detection of genomic DNA contamination, primers were specifically designed (PrimerBlast, NIH) against exon-exon junctions of the genes of interest and the resulting amplicon was confirmed by melting curves in the RT-qPCR and by running in a 2% agarose gel electrophoresis. For each set of primers, the amplification efficiency was calculated using the software LinRegPCR [29] [30] and the relative quantification was measured with the following formula:

$$\Delta \Delta Ct = (1 + eff. target \ gene) \exp(Ct \ sample - Ct \ control)/(1 + eff. reference \ gene) \exp(Ct \ sample - Ct \ control)$$

C1q genes RT-qPCR was always performed in parallel with two independent reference genes, OAZ-1 and HPRT. OAZ-1 encodes an ornithine decarboxylase antienzyme [31] and HPRT encodes hypoxanthine guanine phosphoribosyl transferase [32]. Both reference genes expression was constant and therefore, OAZ-1 was selected as a reference in the statistical analysis due to the lower intragroup variability. Primer sequences are listed below:

- OAZ1 (NM_008753; 51bp): Fwd AGCGAGAGTTCTAGGGTTGCC, Rev CCCCGGACCCAGTTACTAC
- HPRT (NM_013556.2; 150bp): Fwd ACAGGCCAGACTTTGTTGGA, Rev ACTTGCGCTCATCTTAGGCT
- C1qA (NM_007572.2; 96bp): Fwd CACGGAGGCAGGGACAC, Rev GGCAGCATCTTCAGCCACT.
- C1qB (NM_009777.2; 77bp): Fwd ATTCCATAACAGGAAGCCCC, Rev GCAGTAAACAGGTGTGTCAGA.
- C1qC (NM_007574.2; 147bp): Fwd GCTGCTGCTGTTTCTTCTGG, Rev GGGATGCCCTGGCTCTCCCT

Statistics

Gene expression was compared between microglia and non-microglia cells using Student’s t-test after assessing for normality and homoscedasticity with Sigmaplot.

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Ethics Statement

All procedures followed the European Directive 2010/63/EU and NIH guidelines and were approved by the Ethics Committees of the University of the Basque Country EHU/UPV (Leioa, Spain; CEBA/205/2011, CEBA/206/2011, CEIAB/82/2011, CEIAB/105/2012).


