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A NEW ROLE FOR COMPLEMENT C3: REGULATION OF ANTIGEN PROCESSING THROUGH AN INHIBITORY ACTIVITY

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Abstract

Increasing evidence underlines the involvement of complement component C3 in the establishment of acquired immunity which appears to play a complex role and to act at different levels. As antigen proteolysis by antigen presenting cells is a key event in the control of antigen presentation efficiency, and consequently in the quality of the immune response, we investigated whether C3 could modulate this step. Our results demonstrate for the first time that C3 can interfere with antigen proteolysis: i) proteolysis of tetanus toxin (TT) by the lysosomal fraction from a human monocytic cell line (U937) is impaired in the presence of C3, ii) this effect is C3-specific and involves the C3c fragment of the protein, iii) C3c is effective even after disulfide disruption, but none of its three constitutive peptides is individually accountable for this inhibitory effect, iv) the target protease(s) exhibit(s) a serine-protease activity. The physiological relevance of our results is demonstrated by experiments showing a subcellular colocalisation of TT and C3 after their uptake by U937 and the reduction of TT proteolysis once internalised together with C3.
These results highlight a novel role for C3 that broadens its capacity to modulate acquired immune response.

Keywords: Complement; C3; Antigen presentation; Macrophages; Proteolysis

1. Introduction

The involvement of the complement system, a key component of innate immunity, in the establishment of the adaptative immune response is now well-documented (reviewed in references (Carroll, 2004; Walport, 2001)). Among the components of the complement cascade, C3 plays a unique role, due to its different characteristics: not only is it the most abundant complement protein in serum, but it is also the meeting point of the three activation pathways of the complement cascade. Moreover, C3 belongs to the α2-macroglobulin (α2-M) family that contains an intrachain thioester bond (Dodds and Law, 1988). The breakdown of this bond upon activation of C3 to C3b results in a transient capacity for the molecule to form a covalent bond with nucleophilic acceptors such as amino

Abbreviations : α2-M, alpha2-macroglobulin; Ag, antigen; DFP, di-isopropyl fluorophosphate; LF, lysosomal fraction; SBTI, soybean trypsin inhibitor; TT, tetanus toxin.

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or hydroxyl groups (Law et al., 1979; Sim et al., 1981). Finally, C3b can undergo successive rounds of cleavage leading to different fragments (iC3b, C3dg), each of them being able to interact specifically with receptors expressed by most immune cells (Villiers and Villiers, 2003; Villiers et al., 2004). These characteristics confer a variety of different properties to C3 and its fragments, including acting as bifunctional ligands between antigens (Ag) and cells (Villiers et al., 2003; Villiers et al., 1996; Weiss et al., 1989), as signalling effectors (Cherukuri et al., 2001; Fearon and Carter, 1995; Wagner et al., 2006), or as anaphylatoxins (Hawlisch et al., 2004; Schulman et al., 1988; Takafuji et al., 1994). The role of C3 in acquired immune response modulation has first been demonstrated "in vivo", using complement-depleted or -deficient animals (Bottger et al., 1986; Ochs et al., 1983; Pepys, 1974). Increasing evidence from "in vivo" as well as "in vitro" experiments has raised since, revealing the complexity of this modulatory effect which operates at different levels, including cytokine release, T cell proliferation, regulatory T cell development and B cell activation/differentiation (reviewed in references (Carroll, 2004; Morgan et al., 2005; Wagner and Hansch, 2006)). The mechanisms involved are not yet fully understood and are still under investigation.

Previous work using C3b-TT complexes demonstrated the influence of C3b on Ag presentation at the cellular level. Ag recognition by T lymphocytes requires Ag processing by antigen presenting cell (APC), which involves limited proteolysis, loading of the antigenic peptides onto MHC-II molecules and presentation of the MHC-II – peptide complexes to the cell surface. We demonstrated previously that C3b fixation on TT results in increased Ag uptake by APC (Rey-Millet et al., 1994; Villiers et al., 1996), improved antigenic peptide binding on MHC class II molecules (Serra et al., 1997), modified intracellular trafficking (Perrin-Cocon et al., 2004; Rey-Millet et al., 1994) and delayed Ag proteolysis in subcellular compartments (Jacquier-Sarlin et al., 1995). To gain new insight into this last point, we assessed here whether C3b *per se*, affects the activity of lysosomal proteases involved in Ag processing, using TT as model Ag. Experiments were performed with monocytic cells (U937) as i) they have higher protease content than B cells or dendritic cells (Delamarre et al., 2005) and ii) they are known to secrete larger amount of C3 at sites of inflammation than other APC (Einstein et al., 1977). Our results constitute the first evidence that C3b can indeed inhibit lysosomal proteases. This inhibitory activity is specific of C3b and is supported by the C3c fragment. The presence of the three C3c polypeptides is required, although their disulfide linkage is not imperative. The use of known protease inhibitors suggests that the target-protease(s) of C3b belong(s) to the serine-protease family. Finally, experiments using living cells demonstrate the physiological relevance of these results.

2. Materials and methods

2.1. Proteins

Purified TT was generously supplied by Dr J.R. Cartier (Institut Mérieux, France) and was further purified as described previously (Villiers et al., 1991). Trypsin (L-tosylphenylalanyl chloromethane-treated), soybean trypsin inhibitor (SBTI), ovalbumin, BSA, and hemoglobin were from Sigma Aldrich Co., St Louis, Mo., USA. Elastase was from Roche Diagnostic, Meylan, France. α2-M was a kind gift from Dr J.-J. Feige (IRTSV/APV, Grenoble, France). Immunoglobulin G2a was produced by a hybridoma derived from BALB/c.

2.2. Purification of C3 and its fragments

C3 was purified from outdated human citrated plasma obtained from the Etablissement Français du Sang (Grenoble, France) according to (Al Salihi et al., 1982). C3b was prepared by incubating purified C3 with 0.1% (w/w) trypsin at 23°C for 1 h and the reaction was stopped by adding 0.2% (w/w) SBTI and incubating at 37°C for 15 min. C3c and C3de were obtained by incubating C3 with elastase (100/5, w/w) in PBS during 18 h at

37°C. The two fragments were further purified using a Sephadex G-100 superfine (2.6 x 90) column equilibrated in 150 mM NaCl, 20 mM Tris-HCl, pH 7.2, at a flow rate of 8 ml/h. The three polypeptides constituting C3c (α1, α2 and β) were obtained after reduction of the molecule (1h at 37°C in the presence of 50 mM dithiothreitol) followed by alkylation (30min at 37°C in the presence of 137 mM iodoacetamide), and purification by reverse phase-HPLC using a Vydac C4 column (Interchim, Montluçon, France). The polypeptides were acidified using trifluoroacetic acid 0.1% (v/v), loaded onto the column at 0.25ml/min and eluted using a linear acetonitril gradient (0-80%, v/v). Absorbance was monitored at 215 nm. The concentration of purified proteins was estimated from absorbance at 280 nm, using E1%, 1 cm = 11.5 for TT (Villiers et al., 1993), 9.7 for C3 and C3b (Tack and Prahl, 1976), 10.0 for C3c, 13.34 for C3de and for the fragments (α1: 4.57, α2: 11.23, and β: 7.97), values were calculated using the ExPASy facilities.

2.3. Protein labelling

2.3.1 Radioactivity: Proteins were labelled with Na ^{125}I using the iodogen method (Salacinski et al., 1981). ^{125}I (3.7GBq/ml) was from GE-Healthcare (France) and the Iodogen iodination reagent was from Pierce Chemical Co. (Interchim, Montluçon, France).
Free iodine was eliminated by filtration through a Sephadex G50-fine column (GE-Healthcare, France). Specific radioactivity was 170 Bq/µg.

2.3.2. Fluorescence: Proteins were labelled with Cy2 (TT) and Cy3 (C3) (GE-Healthcare, France) as recommended by the manufacturer.

2.4. Cells and culture condition

U937 cells (Sundström and Nilsson, 1976) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in culture at 0.5 x 10^6 cells/ml in RPMI-1640 (Invitrogen, France), supplemented with 2 mM L-glutamine and 10% heat-inactivated (56°C, 30min) foetal calf serum. Cultures were routinely passaged every 3 days and cells were used 24 h after the last passage.

2.5. Subcellular fractionation

Cells (70 x 10^6 cells/ml in 250 mM sucrose, 1 mM EDTA, 1 mM hepes, pH 7.2) were disrupted and fractionated on a Percoll gradient as previously described (Villiers et al., 1996). LF was characterized as previously described (Perrin-Cocon et al., 2004) and stored at -80°C until use.

2.6. Analysis of TT proteolysis

2.6.1. by lysosomal fraction: ^{125}I-TT (5 µg) was incubated in 50 mM Na acetate (50 µl) for 18 h at 37°C, in the presence of LF (equivalent to 2.5 x 10^6 cells) and of different proteins (5 µg). The mixture was analyzed on SDS-PAGE (10-15% acrylamide) in non reducing conditions and the radioactivity associated with the protein bands was quantified using a Phosphorimager (GE-Healthcare, France). TT proteolysis was assumed to be the reduction in the 150 kDa band (= intact TT) radioactivity compared to the total radioactivity present on the gel.

2.6.2. by U937 cells: 10 x 10^6 cells were incubated at 37°C for different times in the presence of ^{125}I-TT (0.1mg/ml) alone or with C3b (0.1mg/ml). Then, cells were washed three times in PBS and disrupted using a “cell-disrupter” (1 shot-0.75 kW model, Constant systems

Ltd., Warwick, U.K.) with a pressure of 350 bar. Two successive centrifugations were performed first to remove intact cells and membranes (3min at 1,500g) and then to concentrate the subcellular compartments (10min at 10,000g). The pellet was resuspended in 50µl of denaturing buffer (8M urea, 2% SDS, 0.2M tris, pH 8.0) and analysed on SDS-PAGE (7.5% acrylamide).

2.7. Confocal microscopy

Cells in 100 µl culture medium (10^7/ml) were incubated in the presence of fluorescent TT and C3b (0.1 mg/ml each) at 37°C for 2h. Cells were washed three times in PBS and allowed to adhere on poly-L-lysine-coated lamellas for 40 min at room temperature. Lamellas were washed three times in PBS and cells were fixed with paraformaldehyde 3% (w/v) in PBS, 10min at room temperature. Samples were mounted in Dako fluorescent mounting medium (Beckman Coulter, Roissy CDG, France), sealed, and stored at 4 °C in the dark. Fixed cells were imaged at room temperature using the inverted confocal and two-photon laser-scanning microscope (LSM 510 NLO, Carl Zeiss) equipped with a 40×/1.3 Plan-Neofluar oil immersion objective. The pinhole adjustment to 1 Airy unit resulted in less than 0.7 µm optical slice at 488 nm excitation wavelength. The image plane was chosen to be near the equator of the cell body and nucleus. No significant photobleaching was induced during image acquisition in either detection channel.

3. Results

3.1. Inhibitory effect of C3 upon tetanus toxin (TT) proteolysis by lysosomal fraction (LF)

In order to assess whether C3 could affect some proteolytic processes, ^{125}I-TT proteolysis by the LF of U937 cells was analyzed in the presence or absence of C3, as described in the Materials and Methods section. As shown in Fig.1, TT is present as two close bands (M_r = 150,000 and 140,000) on SDS-PAGE under non-reducing conditions (lane 1) and is partially proteolyzed by LF (lane 2), as indicated by the appearance of three new major bands (M_r = 100,000, 48,000 and 46,000) together with small fragments (M_r < 15,000) migrating with the dye front, and the parallel decrease in band intensity corresponding to intact TT. An additional, fainter, band featuring an apparent M_r = 120,000 can also be detected. When C3 is added to the mixture prior to incubation of TT with LF, a similar electrophoretical pattern is observed, although the amount of non-proteolyzed TT remains higher than in the absence of C3 (lane 3): C3 does therefore not modify the proteolytic fragments generated by LF, but rather at least partially inhibits its proteolytic activity.
The inhibitory effect of C3 on TT proteolysis by LF was confirmed by a kinetic analysis: As shown in Fig.2, in these conditions, only 15% of input TT is proteolyzed after a 24 h incubation in the presence of C3 against 45% in its absence.

3.2. Specificity of the C3 inhibitory effect

To assess the specificity of the observed C3 inhibitory effect, ^{125}I-TT was incubated with LF in the presence of various unlabelled proteins (TT=150kDa, C3=185kDa, ovalbumine (Ova)=43kDa, BSA=67kDa, hemoglobine (Hem)=68kDa, IgG2a=150kDa, α2-M=4x180kDa) and its proteolysis was quantified after SDS-PAGE. An assay including only ^{125}I-TT and LF was used as reference (100% proteolysis). As shown in Fig.3A, proteolysis is substantially inhibited only in the presence of C3; the other proteins tested, by contrast, only slightly decrease TT proteolysis by LF. Surprisingly, this is also the case of α2-M, which belongs to the same family as C3 (thioester-containing proteins). The observed inhibitory effect is therefore specific to C3. The mild proteolysis inhibitory effect featured by all the tested proteins with the exception of C3 is probably due to the fact that these molecules are all potential substrates for proteases, and therefore potential ^{125}I-TT competitors for LF proteolysis.

Therefore, in the following experiments, the reference assays will always include unlabelled TT (5µg) in order to eliminate the slight inhibition due to this phenomenon and to take in account only the effect due to C3.

3.3. *Identification of the C3 fragment involved in proteolysis inhibition*

Under physiological conditions, C3 undergoes successive proteolytic cleavages leading to various fragments, namely C3b, which is subsequently cleaved into iC3b (M_r = 175,000 and 173 000 respectively), then into C3c (M_r = 135,000) and C3de (M_r = 40,000) (Villiers and Villiers, 2003). In order to identify the C3 fragment involved in proteolysis inhibition, ^{125}I-TT was incubated with LF in the presence of each of the different C3 fragments and analyzed using SDS-PAGE. As shown in Fig.3B, TT degradation by lysosomal proteases is impaired in the presence of C3, C3b and C3c fragments (40-50% inhibition), but this effect is less significant in the presence of C3de (80%). Therefore, the observed proteolysis inhibitory effect of C3 is related to the C3c fragment. As evidenced using SDS-PAGE (Fig.4A), the C3c fragment (apparent M_r = 135,000 in non reducing conditions) is made of three disulfide-linked polypeptides: the C3 β chain (M_r = 75,000) and the two α chain-derived polypeptides (α_1, M_r = 40,000 and α_2, M_r = 27,000). We wondered whether the C3c inhibitory effect required the integrity of the molecule. To that aim, C3c was further incubated with trypsin (trypsin/C3c = 1/100, w/w, 2h at 37°C): further cleavage of β, α_1 and α_2 was obtained, leading to three other polypeptides (β*, M_r = 58,000, α_1*, M_r = 34,000 and α_2*, M_r = 23,000). ^{125}I-TT proteolysis by LF in the presence or absence of trypsin-treated C3c was no longer inhibited by C3c after trypsin digestion (Fig.4B), testifying to the need for the integrity of this fragment. As i) proteolytic activities are present in LF and ii) proteolyzed C3c does not retain any inhibitory effect, we investigated whether the C3 fragment is degraded upon incubation in the presence of LF. C3c is fairly resistant to proteolysis by lysosomal proteases (12.5% degradation upon a 18h incubation, data not shown), which is consistent with its inhibiting activity upon TT degradation by LF.

3.4. *Identification of the C3c domains involved in proteolysis inhibition*

To localize the proteolysis inhibitory activity within C3c, the protein was reduced and we purified its three polypeptide constituents (β, α_1 and α_2) using HPLC (Fig.5A). Each polypeptide was then tested individually for its capacity to inhibit the proteolytic activity of LF. As shown in Fig.5B, whereas reduction of C3c by itself does not abolish its inhibitory effect, neither fragment is alone sufficient to impair TT proteolysis; in other words, the inhibitory effect of C3c, although independent of disulfide bridge integrity, cannot be attributable to only one of its three constitutive peptides.

3.5. Characterisation of the protease family inhibited by C3

The protease(s) involved in TT proteolysis by LF was (were) characterized by incubating TT and LF in the presence of different inhibitors: DFP, EDTA, pepstatin A, iodoacetic acid . As illustrated in Fig.6, proteolysis of TT is mainly due to DFP-sensitive protease(s) rather than metallo-, acid- or thiol-proteases, as it is affected only slightly by EDTA, pepstatin A or iodoacetic acid (7-15% inhibition), whereas DFP strongly decreases TT proteolysis (58%), to an extent similar to C3 (40%). Moreover, the TT fragments which accumulate upon TT + LF incubation in the presence of DFP are similar to those observed in the presence of C3 whereas the presence of the other inhibitors leads to a different accumulation of fragments (data not shown). These results suggest that the target-protease(s) for C3 belong(s) to the serine-protease family.

3.6. *Colocalisation of TT and C3 upon cellular internalisation*

To determine if our observations made in a cell free system are physiologically relevant, we have first analysed the localisation of TT and C3b after their uptake by living cells (C3b was used instead of C3 in order to allow C3b internalisation via complement receptors). To do so, U937 were allowed to internalise Cy2- TT and Cy3-C3b at 37°C before being examined by confocal microscopy.

As it can be seen in Fig.7, some intracellular compartments contain only C3b. On the contrary and although TT is less efficiently internalised, it always colocalise with C3b as there is always an overlapping of green with red signals (yellow in merged images). One observes this phenomenon whatever the duration of the incubation, from 20min to 2h.

3.7. *Inhibitory effect of C3 upon TT proteolysis in U937*

As we demonstrated the inhibitory activity of C3 upon TT proteolysis by LF and the colocalisation of these two proteins after U937 internalisation, we wondered whether C3 could also influence TT proteolysis in living cells. After allowing U937 to internalise ^{125}I-TT alone or with C3b for different times at 37°C, we analysed TT proteolysis on SDS-PAGE. The quantification of the peak corresponding to intact TT indicates that C3b interferes with intracellular proteolytic activities in living cells as the amount of unproteolysed TT is doubled in presence of C3b, at least during the first 40min after internalisation (Fig. 8).

4. Discussion

The influence of complement component C3 on Ag presentation has been highlighted since several years. Indeed, it has been demonstrated that C3b modulates Ag processing and presentation by different APC upon covalent binding to Ag (Jacquier-Sarlin et al., 1995; Rey-Millet et al., 1994). Using macrophages from C3-deficient mice, Zhou et al. (Zhou et al., 2006) have presented evidence that C3 also modulates the potency of APC to stimulate the T-cell response, suggesting a potential effect of free C3 on immune reponse. As peptide generation through Ag degradation is a key event for both Ag presentation and T cell stimulation, we wondered whether free C3 could modulate Ag proteolysis in APC. To be able to correlate our present work with previous ones, we used the same Ag model (TT).

Our results demonstrate that proteolysis of TT by the LF from a human monocytic cell line (U937) was impaired by C3. This effect was quite specific of C3, as α2-M, another protease inhibitor belonging to the same intramolecular thioester bond-containing family (Armstrong and Quigley, 1999), did not affect TT proteolysis. The specificity of C3 inhibitory effect is strengthened by the fact that all other proteins tested were without effect although they are added at same or greater number of molecules compared to C3, depending on their MW. We further identified C3c as being responsible for inhibiting TT proteolysis by LF. It is noteworthy that C3c retained its proteolysis inhibitory activity even after disulfide disruption, although neither of its three constitutive peptides could individually be accountable for it. This observation could be related to the intrinsic structure of C3c: C3c consists of several domains, one of which is formed by parts of both the β- and α-chains (Janssen et al., 2005). This β/α intertwined domain, which is not stabilized by a disulfide bridge and is thereby preserved even after reduction of the molecule, which is confirmed by the impossibility of separating the three polypeptides by gel filtration at neutral pH (data not shown). Thus, it is likely that this β/α intertwined domain could be involved in the observed inhibitory effect of C3c.

Previous studies have illustrated the role of C3 in Ag presentation (Jacquier-Sarlin et al., 1995; Rey-Millet et al., 1994; Santoro et al., 1994), and particularly in Ag degradation: indeed, Jacquier-Sarlin et al. reported limited TT proteolysis by B cells in the presence of C3b (Jacquier-Sarlin et al., 1995). Furthermore, antigenic peptides production and loading on MHC II molecules are modified upon covalent binding of C3b to the Ag (Cretin et al., 2007; Serra et al., 1997). It has been shown that the TT-C3b interaction is fairly stable in endosomes, whereas it is gradually disrupted in lysosomes (Rey-Millet et al., 1994). Moreover, LF contains proteolytic activities leading to the generation of C3c-like fragments (Rey-Millet et al., 1993). These results, together with our present work, enable us to suggest a mechanism by which C3b binding to Ag may modulate Ag presentation: after their uptake by APC, Ag-C3b complexes are delivered to lysosomes, where C3c fragments are generated upon lysosomal proteolytic activities. These fragments then interfere with the lysosomal proteases and at least partially inhibit their activity.

This hypothesis is strengthened by our experiments using living cells. First, analysis by confocal microscopy of U937 having internalised TT and C3 showed that C3 is always found in subcellular

compartments containing TT, which makes possible their interaction. The great efficiency of C3 uptake compared to TT is probably due to the presence of C3 receptors at the surface of U937 (CD11b, CD11c, (Lopez-Rodriguez and Corbi, 1997)). Thus, whereas TT is internalised only via pinocytosis and phagocytosis, C3 could enter the cells using both receptor –independent (colocalisation with TT) and receptor -dependent pathway which may lead in this case to a different subcellular localisation (C3 alone) as shown previously on B cells (Perrin-Cocon et al., 2004). Second, the analysis of the intracellular proteolysis of TT by living APC such as U937 demonstrates that C3 is able to exert its inhibitory effect in physiological conditions. This effect decreases with time, but persists at least during the first hour after internalisation. It has been demonstrated that modulation of Ag proteolysis may interferes with Ag presentation, either by increasing or by decreasing production of antigenic peptides suitable for MHC binding (Delamarre et al., 2006; Polla et al., 2007).

The proteases involved in Ag degradation and the mechanisms that control their activities are not yet fully understood and differ according to the Ag and the cells involved (Burster et al., 2005). Cathepsins are often associated with Ag proteolysis (Hewitt et al., 1997; Hsieh et al., 2002; Ruckrich et al., 2006; Shi et al., 1999), but other proteases such as asparagine endopeptidase (AEP) also play an important role (Manoury et al., 1998). In our hands, C3c inhibited TT proteolysis in a way similar to DFP, indicating that the target protease(s) belongs to the serine-protease family; this conclusion is strengthened by the fact that all other protease inhibitors tested had less effect on TT proteolysis. A previous study by Rey-Millet et al. (Rey-Millet et al., 1993) pointed out the presence of a serine-protease activity within LF, but its involvement in Ag processing had not been established. Most attempts to identify the proteases accounting for intracellular Ag proteolysis are usually indirect, consisting in the analysis of Ag presentation to T cells in the presence of different inhibitors. Ag degradation has either a creative or a destructive effect, mainly depending on MHC restriction (Puri and Factorovich, 1988; Takahashi et al., 1989). Nevertheless, inhibition of Ag degradation is often related to increased Ag presentation, as demonstrated using protease inhibitors (Vidard et al., 1991), antigen resistant to degradation (Delamarre et al., 2006) or modulation of intravacuolar pH (Savina et al., 2006). Indeed, reduced intracellular proteolysis avoids the destruction of T cell epitopes, favoring the production of peptide-MHC classs II complexes. This is particularly important in cells such as macrophages which possess elevated degradation capacity due to their high protease content. Here, we provide direct evidence for the control of Ag proteolysis by C3c, which could be related to the previously described C3-mediated enhancement of Ag presentation and, more generally, of the establishment of a specific immune response. C3 is therefore directly involved in Ag degradation by APC through at least two distinct mechanisms: i) C3b modulates Ag trafficking within the APC (Perrin-Cocon et al., 2004) thereby potentially affecting Ag processing through modified protease activities located within the intracellular compartments, and ii) C3 directly inhibits Ag proteolysis by lysosomal proteases, as illustrated by our results. The C3 present in APC could be of extracellular origin, via C3b binding to exogenous Ag, as well as of intracellular origin, since C3 is synthesized by various APC such as dendritic cells (Peng et al., 2006) or macrophages (Fischer et al., 1998). Whether endogenous C3 could influence Ag proteolysis in the same way as exogenous C3 or not remains to be established. Nevertheless, the relevance of the role of C3 in Ag presentation “in vivo” has been highlighted by Zhou et al.(Zhou et al., 2006), who demonstrated that stimulation of alloreactive T cells is impaired in C3-deficient mice.

The capacity of C3c to control lysosomal degradation could provide an explanation as to why induction of an increased Ab response in mice requires injection of three C3d molecules bound to Ag (Dempsey et al., 1996) versus a single molecule of C3b (Villiers et al., 1999). Indeed, only C3b allows local production of C3c which could control Ag proteolysis favouring Ag presentation, while Ag uptake can be ensured by both C3b and C3d.

In conclusion, our results, by revealing a novel role for C3, supplement the integrative view on the modulatory role of C3 in acquired immune response, and especially on Ag processing: C3 acts at different levels such as Ag uptake (Villiers et al., 1996), intracellular Ag trafficking and targeting (Perrin-Cocon et al., 2004), proteolytic machinery of Ag processing (these results), Ag peptide editing (Cretin et al., 2007) and loading on MHC II molecules (Serra et al., 1997), all of these increasing Ag presentation efficiency (Jacquier-Sarlin et al., 1995) and thus the quality of the acquired immune response (Dempsey et al., 1996; Villiers et al., 1999).

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Figure Captions

Fig. 1. TT proteolysis by LF. ^{125}I-TT (5 µg) was incubated for 18 h at 37°C in the presence or absence of LF from U937 cells (2.5 x 10^6 cells) and of C3 (5 µg). The mixture was analyzed by SDS-PAGE under non reducing conditions (A). The radioactivity associated with the protein bands was quantified using a phosphorimager (B).

Fig. 2. Kinetic analysis of TT proteolysis by LF in the presence or absence of C3. ^{125}I-TT (5 µg) was incubated at 37°C for different times in the presence of LF from U937 cells (2.5 x 10^6 cells) and of C3 (5 µg). TT proteolysis was analyzed by SDS-PAGE and quantified as described in Materials and Methods. Results are given as means ±SD from at least three separate experiments.

Fig. 3. Specificity of the C3 inhibitory effect and identification of the C3 fragments involved. ^{125}I-TT (5 µg) was incubated for 18 h at 37°C in the presence of LF from U937 cells (2.5 x 10^6 cells) and of 5 µg of various proteins: A) TT, C3, ovalbumine (Ova), BSA, hemoglobine (Hem), IgG2a, α2-M and B) C3 fragments. ^{125}I-TT proteolysis was analyzed by SDS-PAGE and quantified as described in Materials and Methods. (A) 100% proteolysis was determined using an assay including only ^{125}I-TT + LF (Ctl). (B) 100% proteolysis was determined using an assay including ^{125}I-TT + LF and unlabelled TT (Ctl). Results are given as means ±SD from at least three separate experiments.

Fig. 4. Loss of the C3 inhibitory effect after C3c cleavage. (A) SDS-PAGE analysis of C3c (5 µg) in non reducing (NR) and reducing (R) conditions before and after cleavage by trypsin (see Materials and Methods). (B) TT proteolysis by LF in the presence of intact or trypsin-proteolyzed C3c. ^{125}I-TT (5 µg) was incubated for 18 h at 37°C in the presence of LF from U937 cells (2.5 x 10^6 cells) and C3c (5 µg) treated or not by trypsin. TT proteolysis was analyzed by SDS-PAGE and quantified as described in Materials and Methods. 100% proteolysis was determined using an assay including ^{125}I-TT + LF and unlabelled TT. Results are given as means ±SD from at least three separate experiments.

Fig. 5. Identification of C3c domains involved in proteolysis inhibition. After reduction and alkylation of C3c, the different polypeptides were purified by reverse phase-HPLC on a Vydac C4 column (see Materials and Methods). (A) Elution profile. (B) TT proteolysis by LF in the presence of reduced or non reduced C3c or each of its constitutive peptides. ^{125}I-TT (5 µg) was incubated for 18 h at 37°C in the presence of LF from U937 cells (2.5 x 10^6 cells) and C3c or its peptides (5 µg). TT proteolysis was analyzed by SDS-PAGE and quantified as described in Materials and Methods. 100% proteolysis was determined using an assay including ^{125}I-TT + LF and unlabelled TT (Ctl). Results are given as means ±SD from at least three separate experiments.

Fig. 6. Characterization of the lysosomal protease family involved in TT proteolysis. ^{125}I-TT (5 µg) was incubated for 18 h at 37°C in the presence of LF from U937 cells (2.5 x 10^6 cells) and C3 (5 µg) or different protease inhibitors (DFP, EDTA, iodoacetic acid (5mM) or pepstatin A (1.5µM)). TT proteolysis was analyzed by SDS-PAGE and quantified as described in Materials and Methods. 100% proteolysis was determined using an assay including ^{125}I-TT + LF and unlabelled TT (Ctl). Results are given as means ±SD from at least three separate experiments.

Fig. 7. Localisation of internalised TT and C3b by confocal microscopy. U937 were incubated at 37°C for 2h in the presence of Cy3-C3b and Cy2-TT. Merged images of red (C3b) and green (TT) fluorescence are shown.

Fig. 8. TT proteolysis by U937 in the presence or absence of C3b. ^{125}I-TT (100µg) was internalised by U937 (10 x 10^6 cells) at 37°C for the indicated times in the presence or not of C3b (100µg). After cell disruption, samples were treated as described in Materials and Methods and analysed by SDS-PAGE. Residual intact TT was quantified after 20, 40 and 60min incubation as described in Materials and Methods. Results are given as means ±SD from at least three separate experiments.

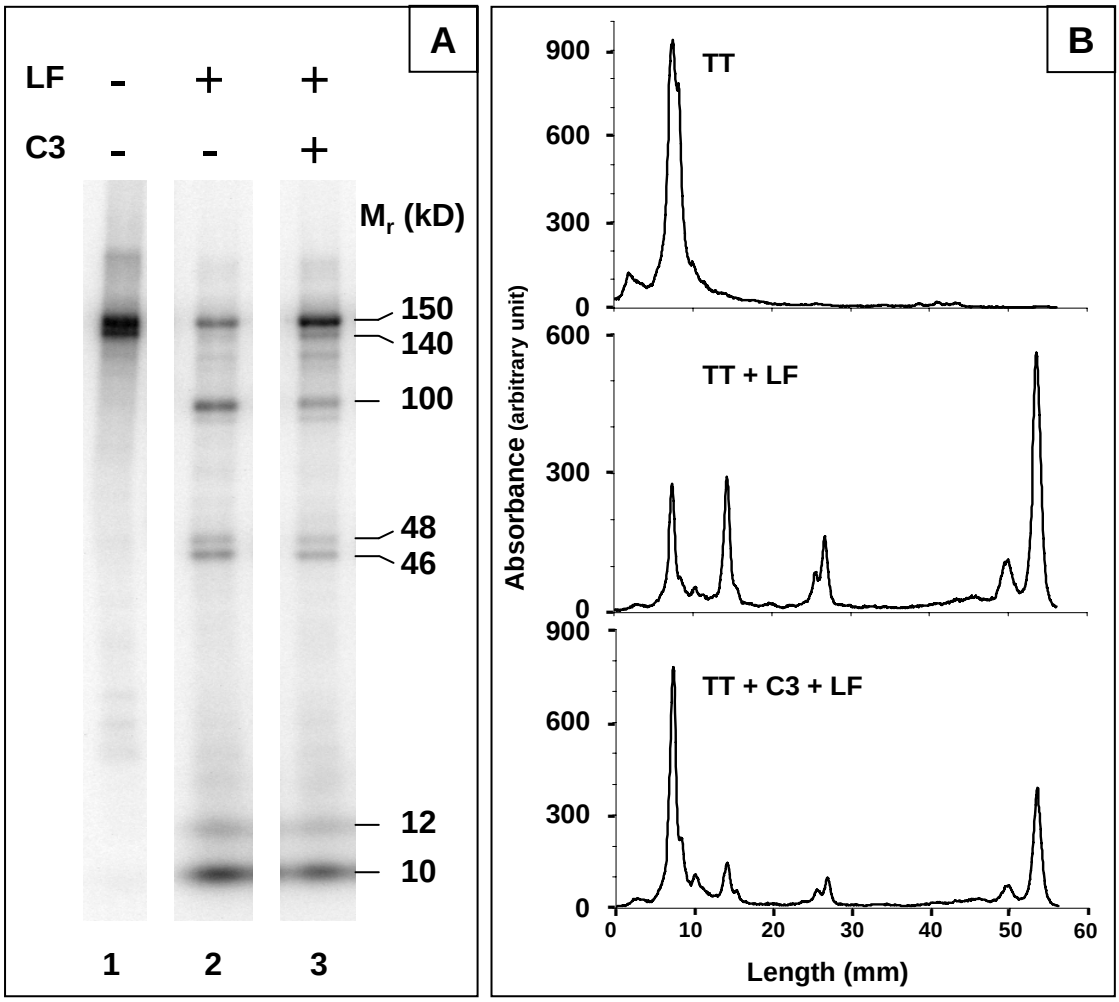

A
LF - + +
C3 - - +
Mr (kD)
150
140
100
48
46
12
10
1 2 3
B
TT
TT + LF
TT + C3 + LF
Absorbance (arbitrary unit)
900
600
300
0
0 10 20 30 40 50 60
Length (mm)

Figure 1

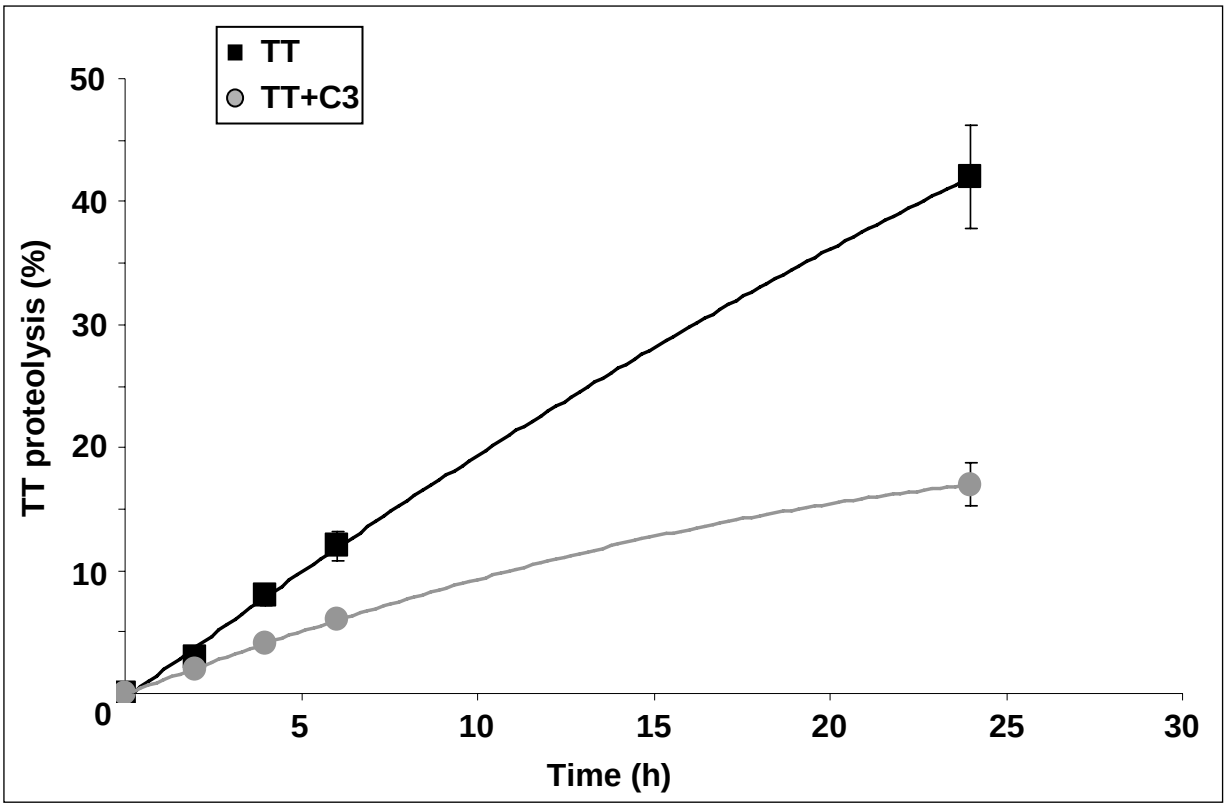


Figure 2

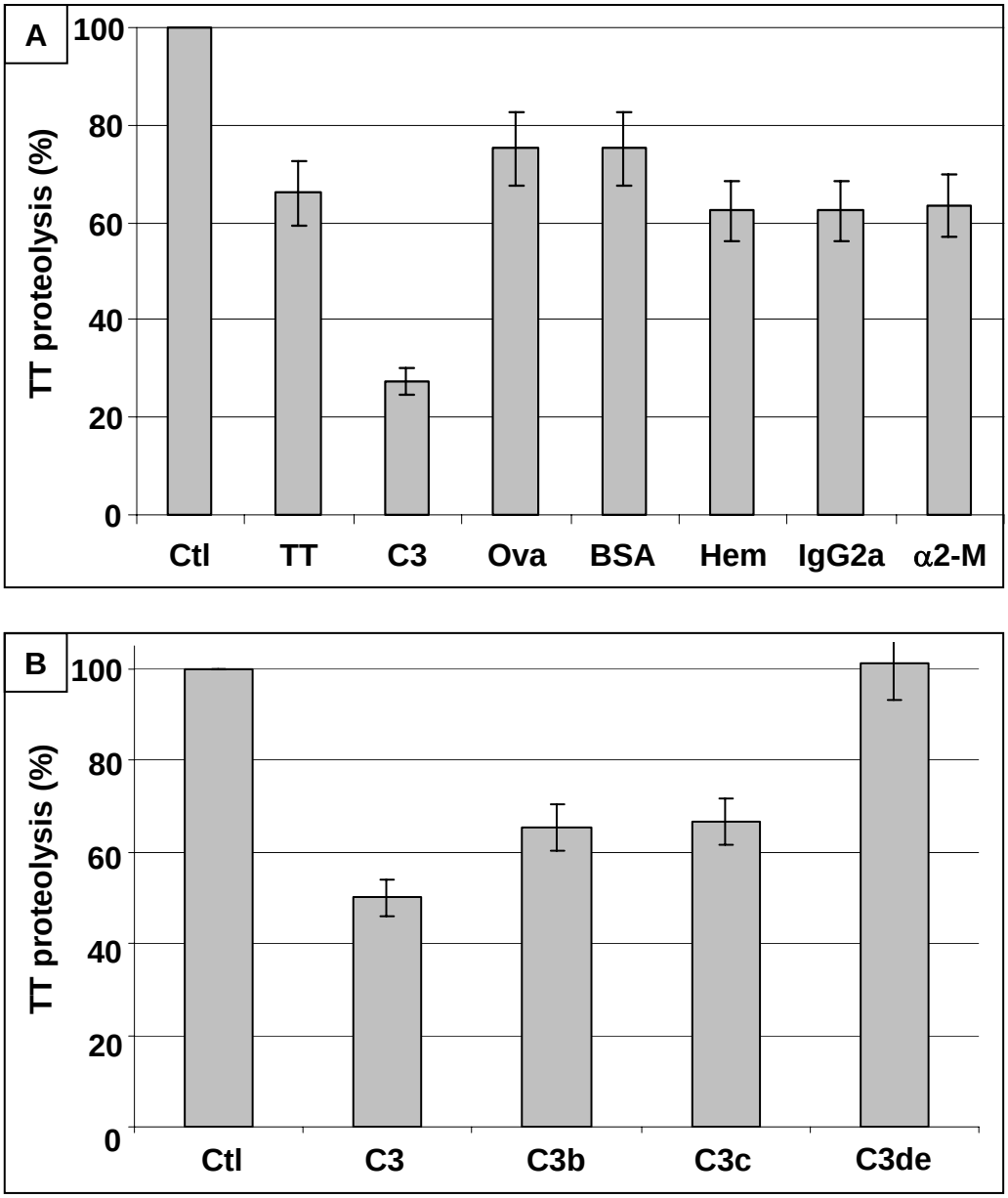

A
TT proteolysis (%)
100
80
60
40
20
0
Ctl
TT
C3
Ova
BSA
Hem
IgG2a
α2-M
B
TT proteolysis (%)
100
80
60
40
20
0
Ctl
C3
C3b
C3c
C3de

Figure 3

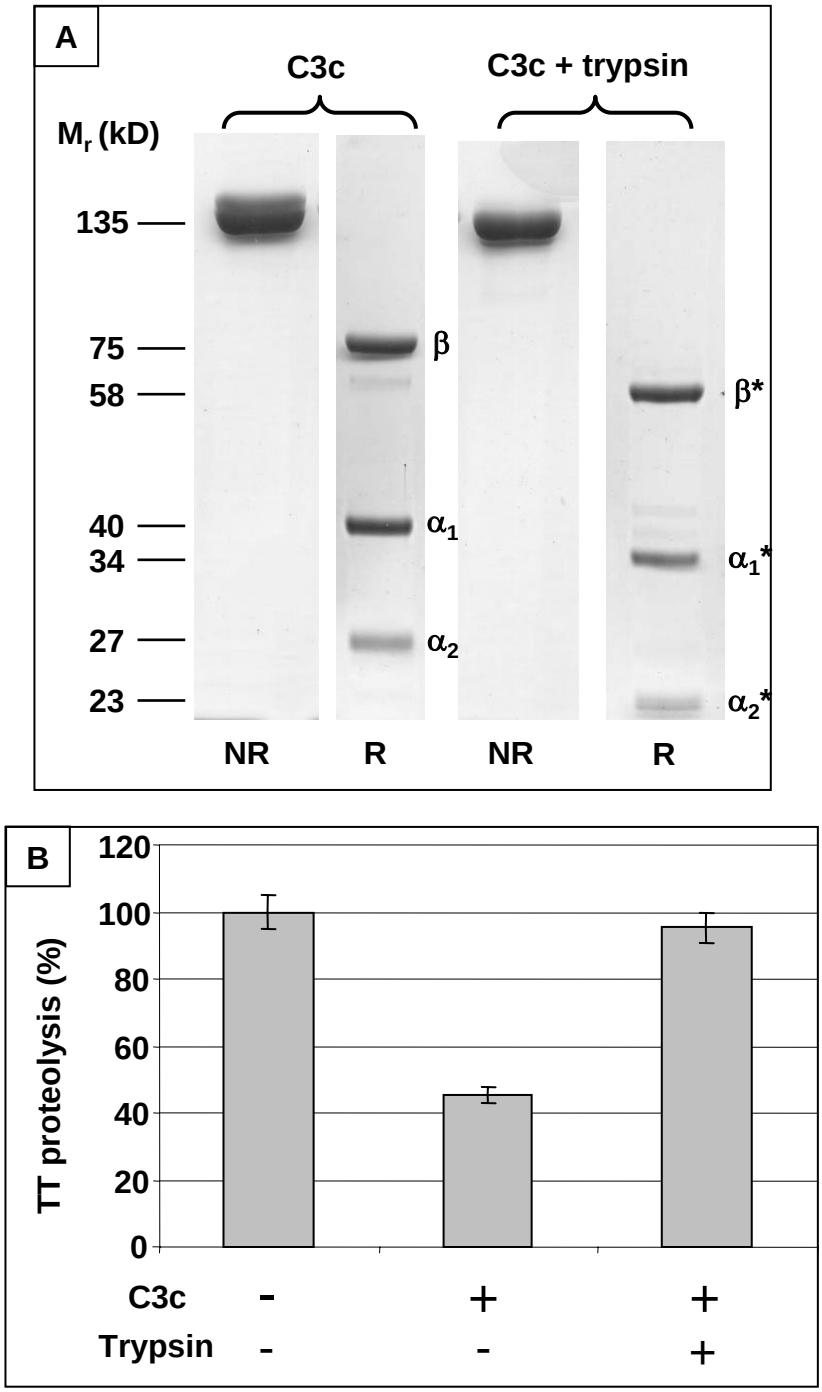
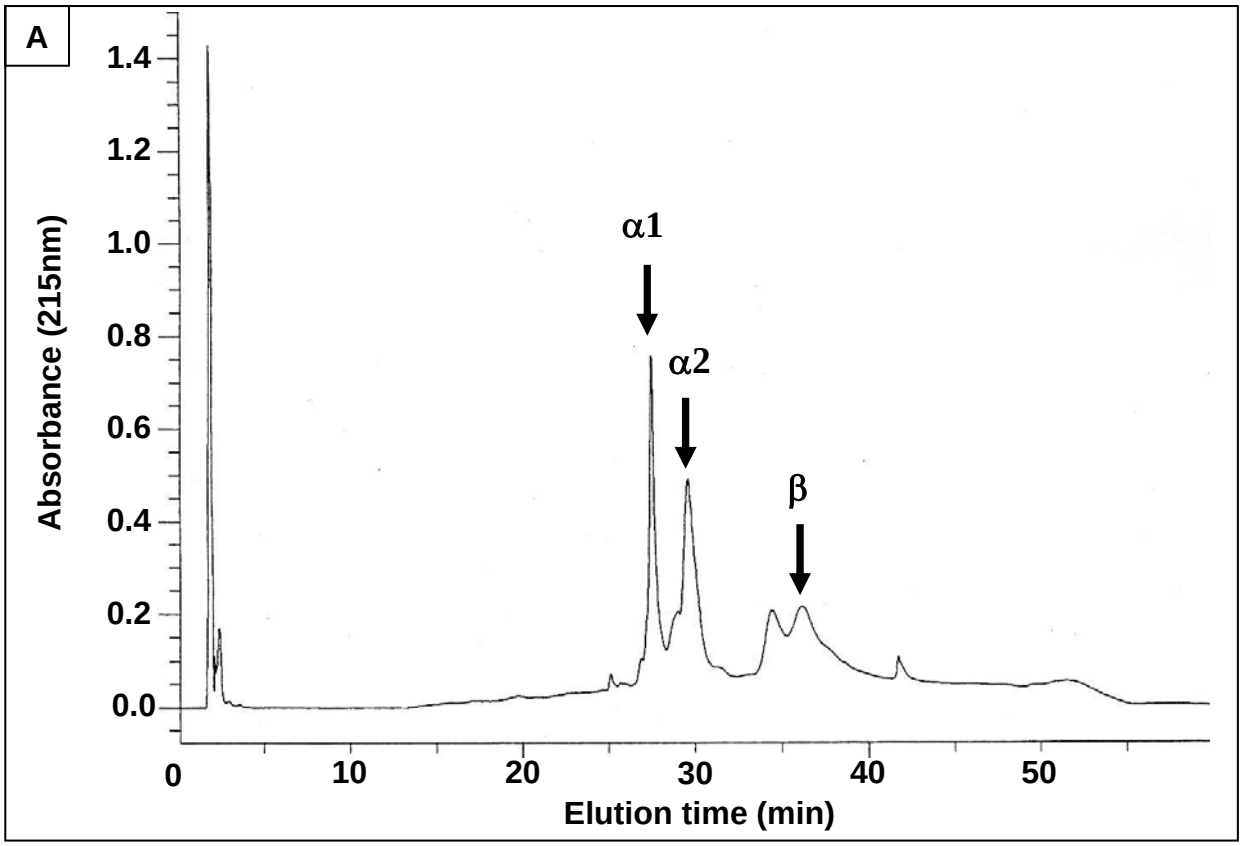

A
C3c
C3c + trypsin
Mr (kD)
135
75
58
40
34
27
23
β
α1
α2
β*
α1*
α2*
NR
R
NR
R
B
TT proteolysis (%)
120
100
80
60
40
20
0
C3c
Trypsin
-
+
+
-
-
+

Figure 4


A
Absorbance (215nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
α1
α2
β
0
10
20
30
40
50
Elution time (min)

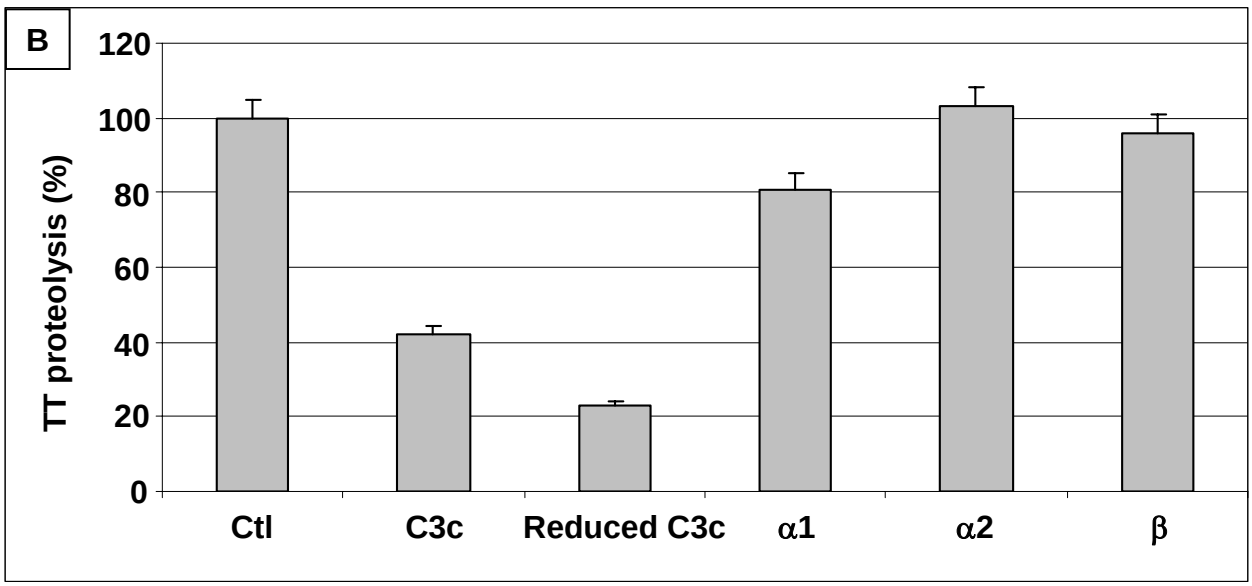

B
TT proteolysis (%)
120
100
80
60
40
20
0
Ctl
C3c
Reduced C3c
α1
α2
β

Figure 5

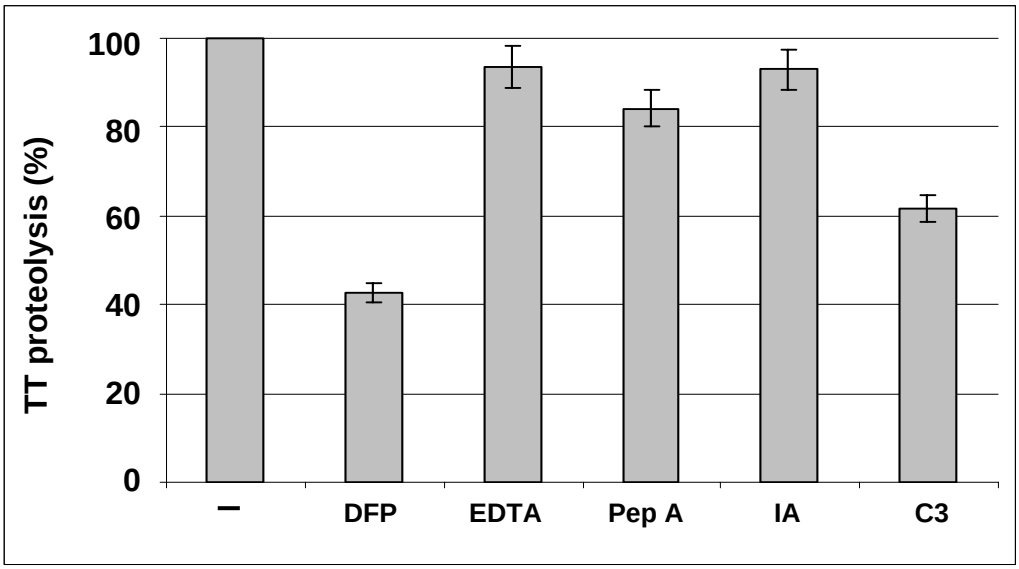


Figure 6

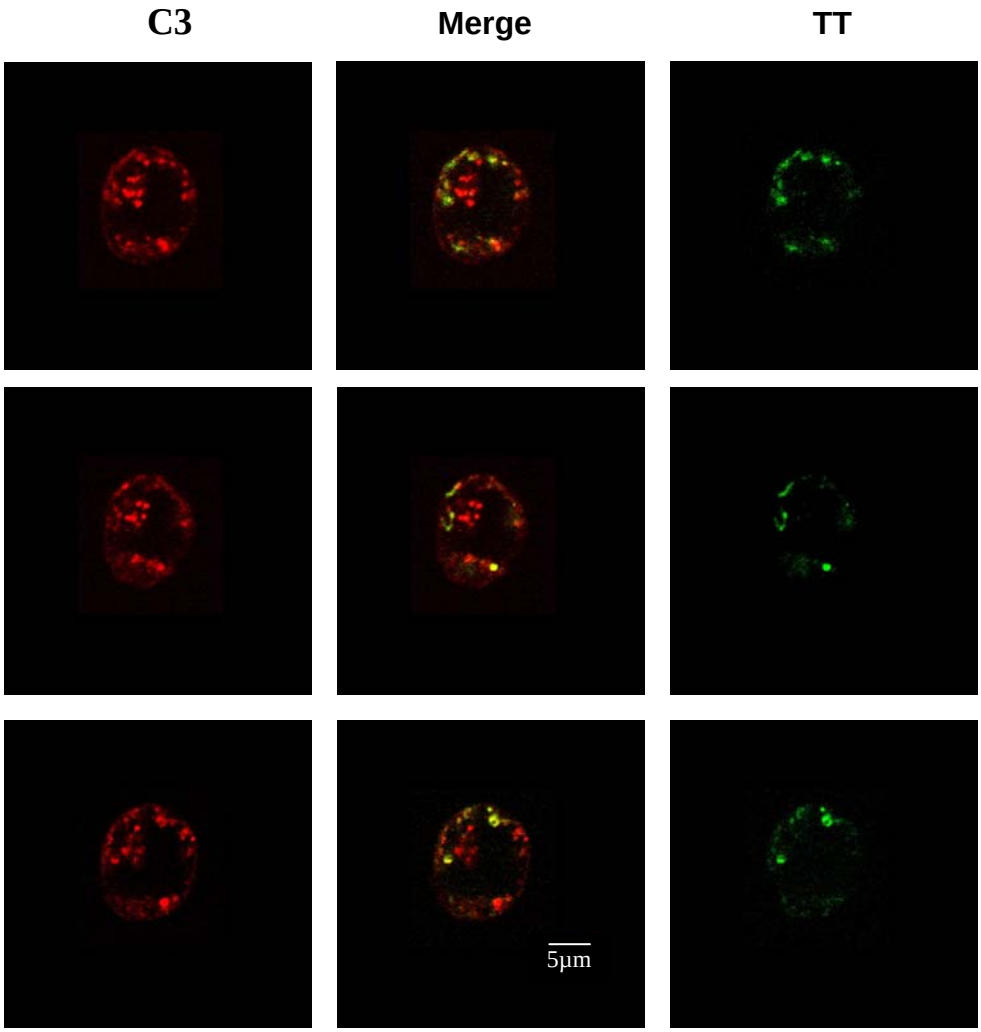


Figure 7

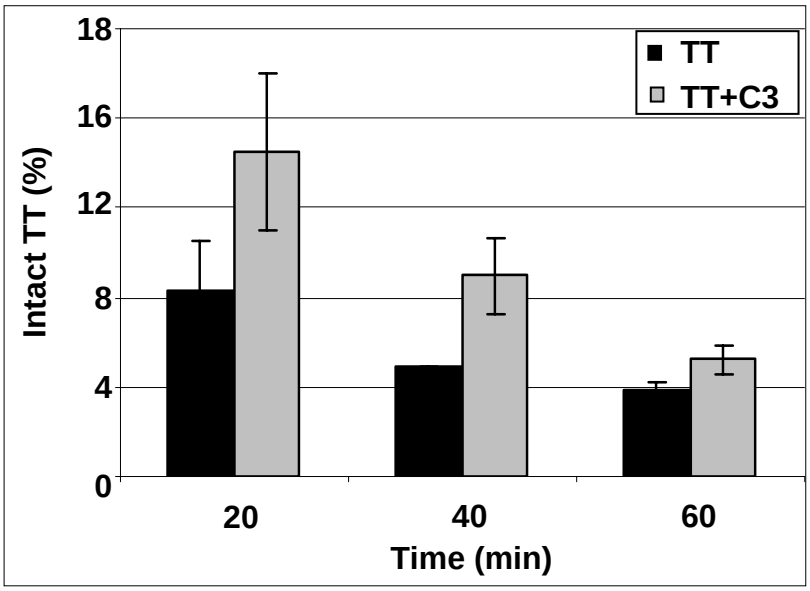


Figure 8