Immunobiology 224 (2019) 307–315

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

# Expression of complement C3, C5, C3aR and C5aR1 genes in resting and activated CD4$^+$ T cells

Cecilie Bo Hansen[a], Anton Willer[a], Rafael Bayarri-Olmos[a], Claudia Kemper[b], Peter Garred[a,*]

[a] Laboratory of Molecular Medicine, Department of Clinical Immunology Section 7631, Faculty of Health and Medical Sciences, University Hospital of Copenhagen, Denmark

[b] National Heart, Lung and Blood Institute, National Institute of Health, Bethesda, MD, 20814, USA

ARTICLE INFO

*Keywords:*
T-cells
Complement expression
Intracellular complement
C3
C5

ABSTRACT

Complement activation is traditionally thought to occur in the extracellular space. However, it has been suggested that complement proteins are activated and function at additional locations. T cells contain intracellular stores of C3 and C5 that can be cleaved into C3a and C5a and bind to intracellular receptors, which have been shown to be of vital importance for the differentiation and function of these cells. However, whether the origin of the complement proteins located within T cells is derived from endogenous produced complement or from an uptake dependent mechanism is unknown.

The presence of intracellular C3 in T cells from normal donors was investigated by fluorescence microscopy and flow cytometry. Moreover, mRNA expression levels of several genes encoding for complement proteins with primary focus on C3, C3aR, C5 and C5aR1 during resting state and upon activation of CD4$^+$ T cells were investigated by a quantitative PCR technique. Furthermore, the gene expression level was evaluated at different time points.

We confirmed the presence of intracellular C3 protein in normal T-cells. However, we could not see any increase in mRNA levels using any activation strategy tested. On the contrary, we observed a slight increase in C3 and C5aR1 mRNA only in the non-activated T-cells compared to the activated T cells, and a decrease in the activated T-cells at different incubation time points.

Our results show that there is a baseline intracellular expression of the complement C3, C5, C3aR and C5aR1 genes in normal CD4$^+$ T cells, but that expression is not increased during T-cell activation, but rather down regulated. Thus, the pool of intracellular complement in CD4$^+$ T cells may either be due to accumulated complement due low-grade expression or arise from the circulation from an uptake dependent mechanism, but these possibilities are not mutually exclusive.

## 1. Introduction

The complement system is an important effector mechanism in both the innate and adaptive immune defence. It is composed of three pathways – the classical, alternative and lectin pathway, which all work by initiating a proteolytic cascade that detects and eliminates invading pathogens and altered, apoptotic and dangerous host cells (Walport, 2001a; Le and Kemper, 2009; Walport, 2001b). The three activation pathways lead to the generation of the C3 and C5 convertase enzyme complexes, which cleave C3 into the anaphylatoxin C3a and the opsonin C3b, and C5 into the anaphylatoxin C5a and into C5b, respectively. C3a and C5a are potent inflammatory mediators targeting a broad spectrum of immune and non-immune cells. C3b predominantly form ester bonds with hydroxyl groups on carbohydrates or proteins, which leads to preferential opsonization (Gadjeva et al., 1998; Law et al., 1984). Deposition of C5b onto a target initiates the formation of the C5b-9 terminal complex, which exists as the membrane attack complex (MAC) targeting cells for lysis, and an inactive soluble version named sC5b-9 (Walport, 2001a; Morgan and Gasque, 1997; Klos et al., 2009; Müller-Eberhard, 1985; Sahu et al., 1994; Ricklin et al., 2010).

The complement system does not only participate in the elimination of microbes and altered cells, but has a wide variety of functions and contributes in diverse processes such as synaptic pruning in the central nervous system (Schafer et al., 2012), bone formation (Matsuoka et al., 2014), angiogenesis (Pio et al., 2013), supports the pluripotency of human stem cells (hPSCs) (Hawksworth et al., 2014), tissue regeneration (Mastellos et al., 2001) and lipid metabolism (Ricklin et al., 2010); (Cui et al., 2007).

* Corresponding author at: Department of Clinical Immunology Section 7631, Rigshospitalet, Ole Maaloesvej 26, 2200, Copenhagen N, Denmark.
*E-mail address:* peter.garred@regionh.dk (P. Garred).

https://doi.org/10.1016/j.imbio.2018.12.004
Received 16 November 2018; Received in revised form 21 December 2018; Accepted 21 December 2018
Available online 27 December 2018

Complement activation is traditionally thought to occur exclusively in the extracellular space, however in recent years it has been suggested that complement components play additional 'non-canonical' roles in the immune system. Almost all immune cells - including T- and B-cells have receptors that can bind complement fragments and through these complement receptors activate specific cellular processes (Kolev et al., 2014; Köhl, 2006; Kolev et al., 2013; Liszewski et al., 2013). Additionally, complement molecules can be activated intracellularly and it has been shown that particular complement molecules are of vital importance for the differentiation and function of specific lymphocyte populations such as T cells (Kolev et al., 2013; Liszewski et al., 2013; Suresh et al., 2003). It has been shown that resting human $CD4^+$ T cells contain intracellular stores of C3 and the protease cathepsin L (CTSL) in endosomal and lysosomal compartments, and that CTSL is able to cleave C3 into C3a and C3b in a convertase-independent manner (Köhl, 2006). Intracellular C3a can thus bind to the lysosomal-localized receptor, C3aR, and mediate homeostatic T cell survival through basal activation of mammalian target of rapamycin (mTOR) (Liszewski et al., 2013). This autocrine interaction between the complement system and the T cell will consequently determine T cell function and activity (Köhl, 2006).

The source of the intracellular stores of C3 and C5 has not been rigorously investigated. It has been speculated that T cells, upon activation, may be able to produce complement components endogenously due to the fact that intracellular C3 and C3a protein can be observed in patients that lack C3 circulating in serum (Liszewski et al., 2013). Hence, such existing data suggest a potential disconnection between plasma (liver-derived) C3 and (immune cell-derived) intracellular C3 expression. Additionally, the initial study describing this observation concludes that CD46 is crucial in certain T cell activation regimes, particularly those connected with Th1 induction (Köhl, 2006). CD46, or membrane cofactor protein (MCP), is a cell surface receptor, which is expressed by a variety of human nucleated cell types, including monocytes, B and T cells. CD46 functions as a cofactor for the degradation of complement fragments C3b and C4b deposited on cells and thereby protects human cells from lysis by autologous complement (Seya et al., 1986). In addition to this complement regulatory function, CD46 is a receptor for a number of pathogenic bacteria and viruses, including measles virus, human herpesvirus 6, adenovirus of different serotypes, *Streptococcus pyogenes* and pathogenic *Neisseria* (Källström et al., 1997); (Cattaneo, 2004). Furthermore, CD46 can modulate adaptive immune responses (Kemper and Atkinson, 2007; Riley-Vargas et al., 2004). In particular, CD46 functions as a co-stimulator during T cell stimulation: activation of the T cell receptor (TCR) on human $CD4^+$ T cells induces the autocrine engagement of CD46 by T cell generated C3b and this signal is critically required for T cell proliferation and successful IFN-γ secretion and Th1 induction (Astier et al., 2000); (Sánchez et al., 2004). Active crosslinking of CD46 by antibodies during TCR activation strongly potentiates this phenotype. Furthermore, CD46 also directs successful contraction of Th1 responses: CD46-mediated signals in conjunction with IL-2 receptor signalling co-induces IL-10 secretion in Th1 cells, and with this the transgression into a (self)regulative shut down phase (Kemper et al., 2003; Grossman et al., 2004; Kemper et al., 2005). Thus, patients lacking CD46 expression are unable to mount protective Th1 responses and suffer from recurrent infections, while uncontrolled activation of intracellular C3 and/or dysregulated CD46 and IL-2 receptor crosstalk contributes to the hyperactive Th1 responses observed in multiple sclerosis and rheumatoid arthritis (Cardone et al., 2010; Astier and Hafler, 2006). Impaired dendritic cell differentiation and development of memory B cell and regulatory T cell, and loss of Th1 induction have been reported in C3-deficient patients (Ghannam et al., 2008, 2014).

Since dysregulation of intracelluar C3 processing appears to contribute to autoimmune diseases and these diseases often are associated with profound cellular activation it is important to understand the regulation of immune cell expressed C3. Interestingly, in a study by Michelle Elvington et al. (2017), the authors observed by Western blot (WB) a clear distinction in the presence of C3 and C3 cleavage products between peripheral blood cells (PBC) and their respective cell lines (i.e. $CD4^+$ T cells versus Jurkat cells). A major difference between PBC and cultured cells is that the latter had not been exposed to a source of human C3 for many generations. They demonstrated that many types of human cells and cell lines including $CD4^+$ T are able to internalize the hydrolytic product of C3 ($C3(H_2O)$) from the extracellular milieu, in a manner that is rapid, saturable and sensitive to competition, indicating a specific mechanism of loading. However the precise mechanism of this internalization is currently unknown.

Moreover, it is not clear whether complement genes are induced by cellular activation and whether complement gene expression might be related to cell survival. In this study we have investigated and compared gene expression in selected complement genes in a time dependent fashion in resting and activated $CD4^+$ T cells.

## 2. Material and methods

### 2.1. Blood sampling and $CD4^+$ T cell isolation

Blood was drawn from healthy donors in EDTA tubes (VACUETTE® K3EDTA, Greiner Bio One International GmbH). All samples were handled within one hour of blood sampling. $CD4^+$ T lymphocytes were isolated from whole blood with The EasySep™ Human Whole Blood CD4 Positive Selection and RoboSep™ (STEMCELL technologies) according to the manufacturer's instructions. All donors gave informed consent. The study was approved by the regional Health Ethics Committee in the Capital Region of Denmark (reference no. H2-2011-133).

### 2.2. Antibodies

Cell-stimulating antibodies were bought from Miltenyi Biotec (T Cell Activation/Expansion Kit); anti-CD2, anti-CD3, anti-CD28 and anti-CD46 (REA312). Anti-CD4 (560158), anti-CD8 (641058), anti-CD14 (345786) were obtained from BD Biosciences and anti-CD19 (302233) from Biolegend. Intracellular C3 was detected using chicken anti-C3 (GW20073 F) from Sigma Aldrich and secondary antibody goat anti-chicken IgG (A-11039) from Life Technologies. Chicken IgY isotype control (GTX35001) was obtained from GeneTex.

### 2.3. Reagents

PBS buffer (10 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and Barbital buffer (4 mM $C_8H_{11}N_2NaO_3$, 145 mM NaCl, 2.6 mM $CaCl_2$, 2.1 mM $MgCl_2$, pH 7.4) were acquired from the hospital pharmacy (RegionH Apoteket, Rigshospitalet, Copenhagen, Denmark). RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% sterile-filtered human serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco, Thermo Fisher Scientific).

### 2.4. Confocal fluorescence microscopy

Fixed, permeabilized cells were stained with primary antibodies overnight at 4 °C and staining with secondary antibodies was performed for 2 h at room temperature. Cells were mounted with ProLong® Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific) and images were obtained in the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen by confocal fluorescence microscopy with a Zeiss LSM700 (Carl Zeiss), using the following objectives: Plan-Apochromat 63 × /1,4 Oil DIC M27 and Plan-Apochromat 20x/0,8 M27. DAPI and Alexa Fluor 488 were excited by a 405 nm diode laser and a 488 nm argon laser, respectively. The colours presented in the figures are pseudo colours (e.g., the nuclei are displayed as turquoise and C3 protein in red/green), this was done

in ZEN 2 blue edition (Zeiss).

### 2.5. Flow cytometry

Fixed, permeabilized cells were incubated with primary antibodies overnight at 4 °C and staining with secondary antibodies was performed for 2 h at room temperature. Intracellular C3 and C3 activation fragment deposition on the cells was measured as mean fluorescence intensity (MFI) by flow cytometry (Gallios, Beckman Coulter) and data was analyzed using Kaluza software (Beckman Coulter).

### 2.6. T cell activation – CD2, CD3, CD28 & CD46

$CD4^+$ T cells were purified from whole blood as previously described and cultured from 30 min to 4 days in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% sterile-filtered human serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin in the presence of beads coated with anti-CD2, anti-CD3, anti-CD28 and anti-CD46 according to manufacturer's instructions. CD4 + T cells ($2.5–3.5 \times 10^5$ cells/well) were activated in 96-well TPP® tissue culture plates (Sigma-Aldrich) coated with mAbs to CD2, CD3, CD28 with/without CD46 (2.0 μg/ml each).

### 2.7. qPCR

Isolation of mRNA from T cells was performed using the KingFisher Technology (Thermo Labsystems Oy) and the MagAttract Direct mRNA M48 kit (Qiagen) according to the manufacturer's instructions. mRNA was quantified using a Eppendorf BioSpectrometer® (Eppendorf) and complementary DNA (cDNA) was then generated by reverse transcription using the MuLV Reverse transcriptase (Applied Biosystems #N808-0018). Complement and cytokine gene expression in T cells were assessed by quantitative real-time PCR with TaqMan probes against *C3* (Hs00163811_m1), *C3aR* (Hs00269693_s1), *C5* (Hs01004342_m1), *C5aR1* (Hs00704891_s1), *IFNG* (Hs00989291_m1), *IL10* (Hs00961622_m1) obtained from Applied Biosystems. Forward primer 5′-GGTGGTTCCTGCTGCCC-3′, reverse primer 5′-CGCCCCAGATAG GCA-AACT-3′, and internal probe 5′-/ 56-FAM/CAA GGT CGT /ZEN/ GCG TCT GAA GCC TAC AAG /3IABkFQ/-3′ were designed using Primer Express 1.0 (Applied Biosystems) and purchased from Integrated DNA Technologies. Quantitative Real-time PCR was performed in duplicates and carried out on the Stratagene Mx3005 P Real-Time PCR system (Agilent Technologies). PCR runs included no-template controls. The target genes were normalized to the reference gene, ribosomal protein L13 a (*RPL13A*), and results are shown as delta Ct values.

### 2.8. Statistical analysis

All gene expression data was analyzed using GraphPad Prism Software, version 6 (GraphPad Software Inc). Comparisons between groups of non-activated and activated T cells were calculated using the paired Student's *t*-test and $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Intracellular C3 in $CD4^+$ T cells assessed by flow and fluorescence microscopy

We aimed to confirm the previous findings of intracellular complement in a variety of immune cells as described in the article by Liszewski MK et al. (Liszewski et al., 2013). We were able to confirm detection of intracellular C3 in $CD4^+$ T cells, as well as $CD8^+$ T cells, B-lymphocytes and monocytes in resting states by flow cytometry and fluorescence microscopy (Fig. 1).

### 3.2. Validation of activation of $CD4^+$ T cells

To assess whether T cell activation by anti-CD2, anti-CD3 and anti-CD28 induces cell proliferation we used a validated assay using carboxy fluorescein diacetate succcinimidyl ester (CFSE) stain on cells prior to activation. When a CFSE-labeled cell divides, its progeny have half the amount of fluorescence, which can thereby be used to assess cell division. Supplementary figure shows the flow cytometric evaluation of the cell activation from day one to four. As the cells proliferate, different generations can be observed as distinct peaks due the decreasing concentration of CFSE (supplementary figure, lower panel). Cell activation was confirmed by measuring the mRNA levels of cytokines IFN-γ and IL-10 (Fig. 4) which are both secreted upon activation of the T cell receptor (TCR) (Saraiva and O'Garra, 2010; Billiau and Matthys, 2009).

### 3.3. mRNA expression in $CD4^+$ T cells upon activation

In the present study, we activated healthy $CD4^+$ T cells isolated by antibody coupled beads (α-CD2, α-CD3, α-CD28 with/without α-CD46) and quantified the difference in C3, C3aR, C5, C5aR, IFN-γ and IL-10 mRNA expression levels between activated and non-activated $CD4^+$ T cells. We measured the expression levels during the first 24 h post activation at time points 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h (Fig. 2) and in the course of 4 days assessing the gene expression levels at 24 h, 48 h, 72 h and 96 h with two different activation strategies (Figs. 3 and 4). Expression levels are measured as ΔCt values ($Ct_{targetgene}$ - $Ct_{referencegene}$), a low ΔCt value equals a high mRNA expression level. We found no increase in mRNA coding for C3, C5, C3aR or C5aR mRNA levels using any of the two activation strategies, but rather the opposite phenomenon. Within the first 24 h we observed no significant difference between complement transcripts in the activated and non-activated cells in the time points 30 min-12 h, but found a significantly increased mRNA expression of C3 and C5aR in non-activated $CD4^+$ T cells compared to activated $CD4^+$ T cells at the 24 h time point (Fig. 2). The significant difference at 24 h in C5aR mRNA expression between activated and non-activated cells appears to be a product of both down regulation in the activated cells and increased expression in the non-activated cells. Figs. 3 and 4 illustrate that the difference in mRNA levels was persistent throughout the course of 4 days. This led us to the conclusion that there is no increased endogenous biosynthesis of C3, C5, C3aR or C5aR upon activation of $CD4^+$ T cells using standard T cell activation protocols.

## 4. Discussion

In the present study we investigated the mRNA expression of different complement molecules, with our primary focus on C3, C3aR, C5 and C5aR during the resting state and upon activation in $CD4^+$ T cells. We started out by validating previous findings of intracellular C3 in resting human T cells by means of flow cytometry and fluorescence microscopy. Preceding literature has uncovered an intracellular pathway and cleavage of C3 and C5 into C3a and C5a and shown that these anaphylatoxins are able to bind intracellular receptors (Liszewski et al., 2013). These receptors are involved in processes such as differentiation, proliferation and survival of T cells (Kolev et al., 2013). To our knowledge it has not been shown, but speculated, that the amounts of complement components and receptors are changed upon activation of the T cell receptor (TCR) and specifically through stimulation of CD46 which functions as a co-stimulatory molecule in conjunction with TCR activation and induces $CD4^+$ T cell proliferation and Th1 induction and contraction (Astier et al., 2000; Zaffran et al., 2001).

In the present study we activated T cells in two ways, the classic anti-CD3/CD28 stimulation of the TCR, which is a known and well tested method of T cell activation and proliferation and the anti-CD3/CD28/CD46 which have been shown to induce drastic morphological changes and induces initially preferential Th1 cells in order to obtain

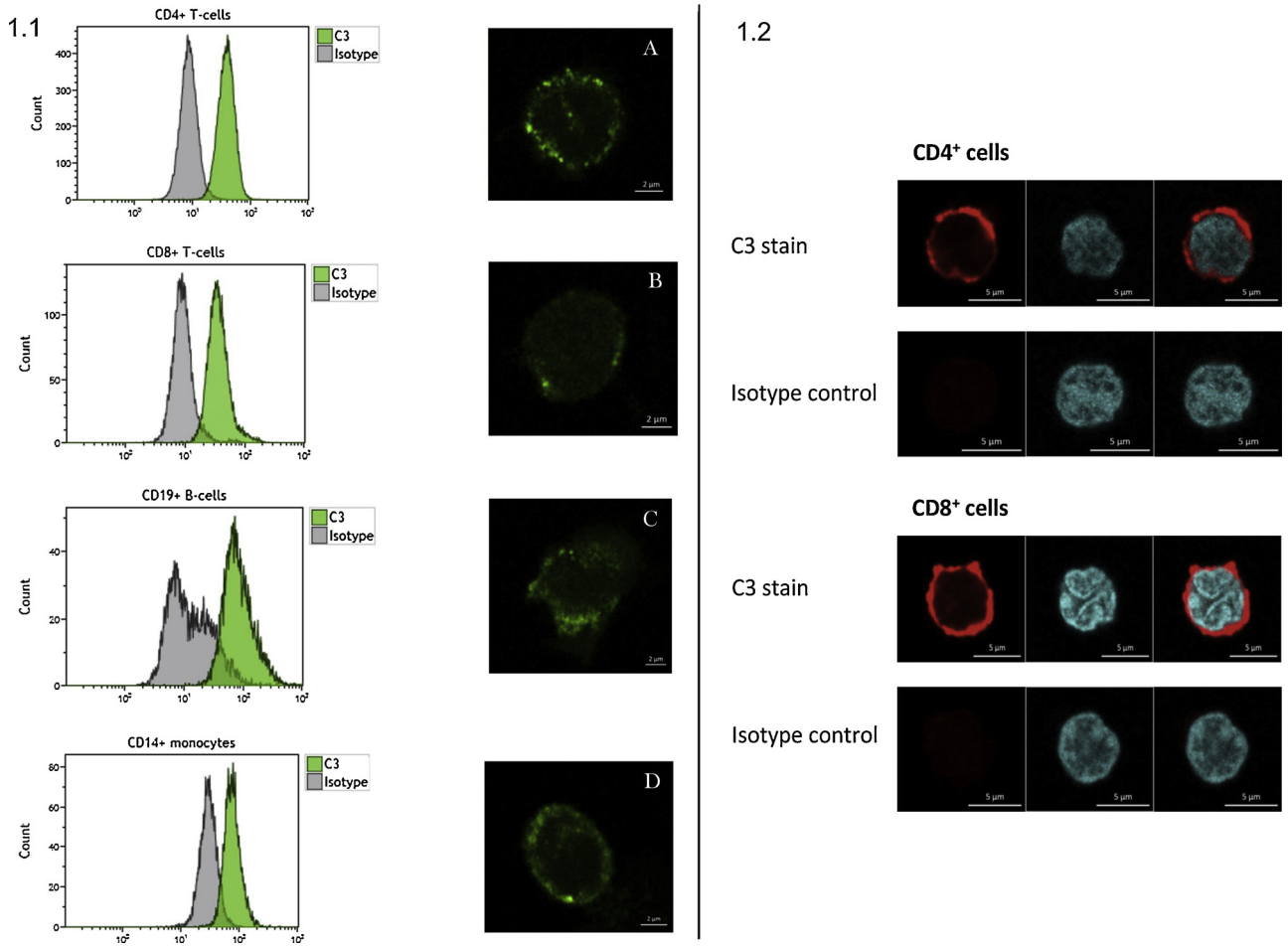


**Fig. 1.** (1.1) Freshly isolated $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes were assessed for presence of intracellular C3 by flow cytometry and fluorescence microscopy (A = $CD4^+$ T cells, B = $CD8^+$ T cells, C = $CD19^+$ B cells, D = $CD14^+$ monocytes). Results shown are representative of three independently performed experiments (n = 3). (1.2) Freshly isolated $CD4^+$ T cells and $CD8^+$ T cells were assessed for presence of intracellular C3 by confocal microscopy image analyses in the resting state. The colours presented in the figures are pseudo colours (e.g., the nuclei are displayed as turquoise and C3 protein in red). Upper panel = C3, lower panel = isotype control. Results shown are representative of three independently performed experiments (n = 3). Scale bar represents 5 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

maximal T cell activation (Zaffran et al., 2001). We validated our induction of activation/proliferation of the T cells by flow cytometry and measured mRNA levels of known cytokines which are upregulated and secreted upon activation. We found no increase in mRNA C3, C5, C3aR or C5aR using any of the two activation strategies. On the contrary, we actually observed a significant decrease in mRNA level during cellular activation. We evaluated the gene expression levels at different time points both within a 24 h window and during the course of 4 days. Within the first 24 h we found no significant difference between complement molecules in the activated and non-activated cells. When observing the complement mRNA levels during a time course of 4 days, we discovered a significant increase in C3 and C5aR mRNA in the non-activated cells compared to the activated ones, both with and without CD46 stimulation. This led us to the conclusion that there is no increased endogenous biosynthesis of C3, C5, C3aR or C5aR upon activation of $CD4^+$ T cells using standard T cell activation protocols. In additional experiments we observed that the expression of C3 mRNA was virtually undetectable in T cells from patients will T cell acute lymphocytic leukemia (unpublished). Further strengthen the notion that endogenous expressed C3 is not vital for T cell turnover.

It came as a surprise to us to find a significant increase in C3 and C5aR mRNA in the non-activated T cells compared to the activated T cells throughout the course of the 4 day experiment. We observed no significant difference between the mRNA levels in the two groups 30 min after activation; the mRNA levels were either equal (Fig. 2: C5) or there was a slight tendency towards a higher mRNA level in the activated T cells (Fig. 2: C3, C3aR and C5aR). In the course of the first 24 h we detected a switch in the mRNA levels, now with a tendency towards increased mRNA levels of C3, C3aR and C5aR. At 24 h after activation there was a significant higher mRNA of C3 and C5aR in the non-activated cells compared to the activated cells, which appeared to be a product of both down regulation in the activated cells and increased expression in the non-activated cells. We asked ourselves the question why we would detect higher levels of C3 and C5aR in the non-activated T cells; cells which are just kept in media without any kind of stimulation? In the periphery, T cells can die by several mechanisms: e.g. by extrinsic pathways involving death receptors and caspase activation or by intrinsic mitochondria- and caspase-dependent or caspase-independent apoptosis. Autonomous cell death arises in the absence of proper survival signals. This type of cell death is known as death by cytokine deprivation or by neglect, or activated T cell autonomous death (ACAD) (Arnold et al., 2006; Hildeman et al., 2002; Krammer et al., 2007). In our experiment the non-activated T cells were kept in a low stimulatory milieu, which could have induced a degree of apoptosis. Still the activated and non-activated cells were both kept in buffer with 10% human serum, which contain cytokines and therefore a stimulatory capacity. In a study by M Martin et al (Martin et al., 2016) they detect a non-significant but yet slightly increased C3 mRNA level

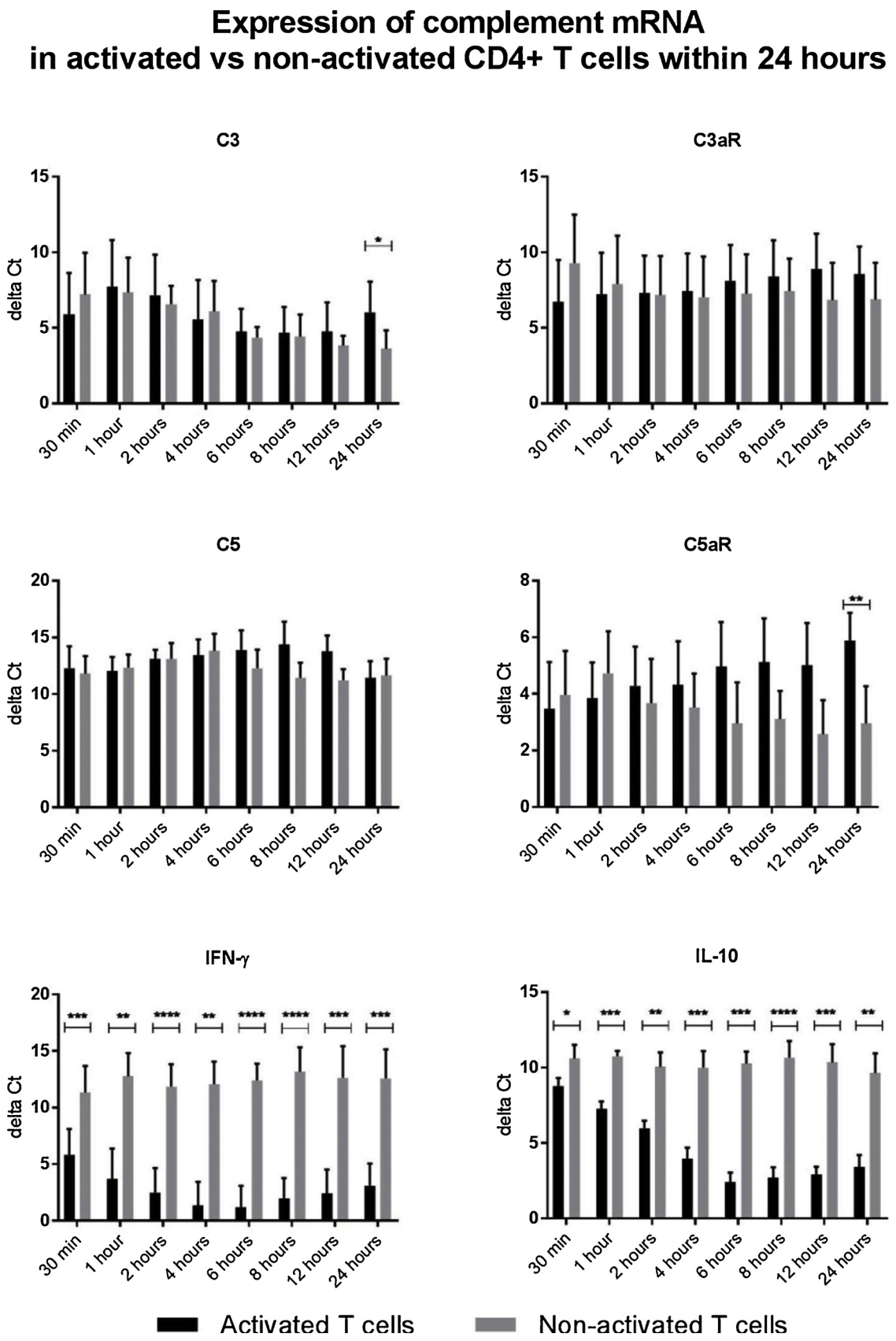


**Fig. 2.** A time dependent measurement of mRNA expression (C3, C3aR, C5, C5aR, IL-10 and IFN-γ) in α-CD2 + α-CD3 + α-CD28 activated (black) and non-activated (grey) $CD4^+$ T cells. The Y-axis represents ΔCt-values and the X-axis represents the different time points of collection and analysis (0,5, 1, 2, 4, 6, 8, 12, 24 h). The ΔCt is calculated $Ct_{targetgene}$ - $Ct_{referencegene}$. A low ΔCt value = high mRNA level. Data ± 95% CI are from four independent experiments (n = 4).

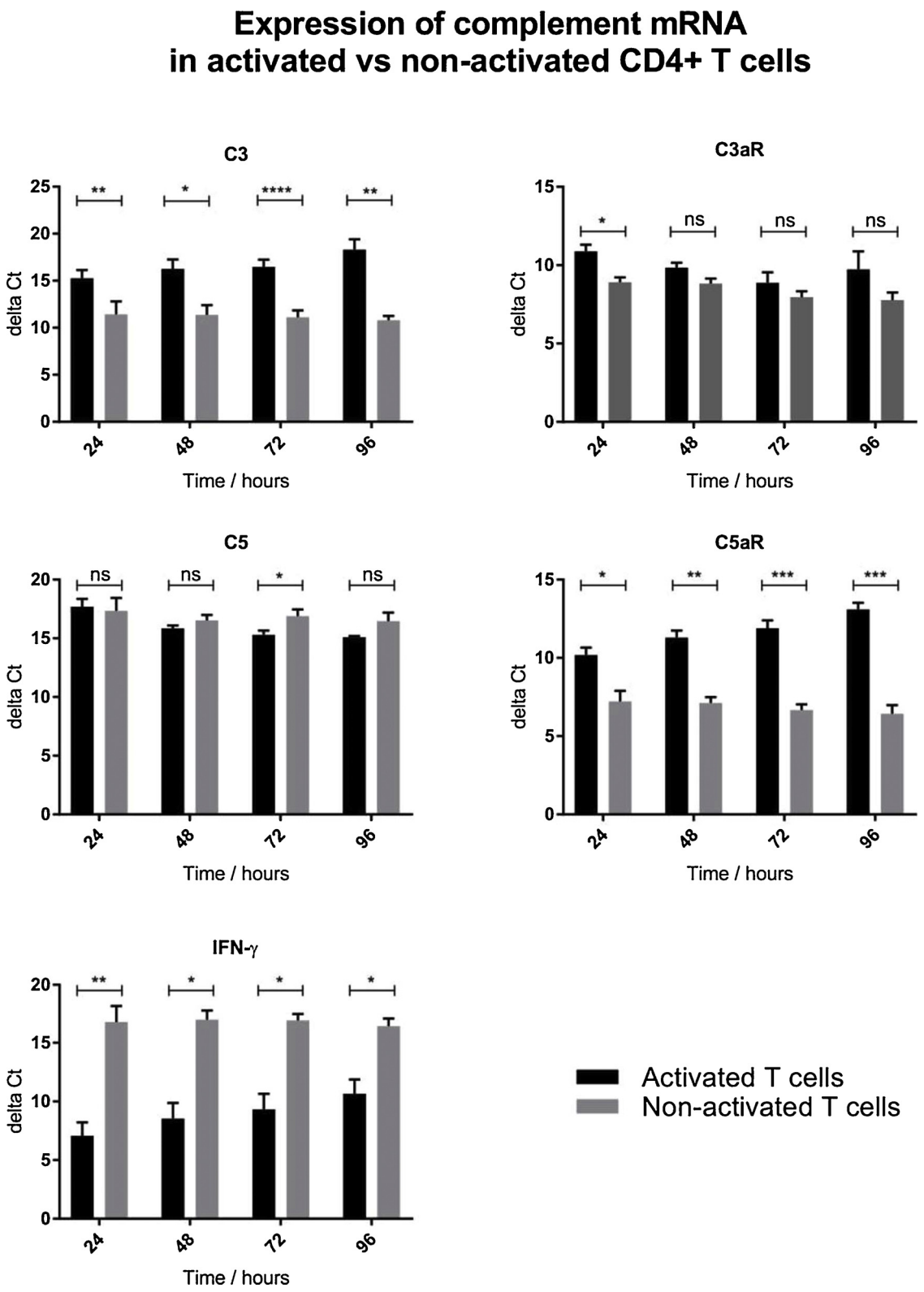


**Fig. 3.** A time dependent measurement of mRNA expression (C3, C3aR, C5, C5aR and IFN-γ) in α-CD2 + α-CD3 + α-CD28 activated (black) and non-activated (grey) $CD4^+$ T cells. The Y-axis represents ΔCt-values and the X-axis represents the different time points of collection and analysis (24, 48, 72 and 96 h). The ΔCt is calculated $Ct_{targetgene}$ - $Ct_{referencegene}$. A low ΔCt value = high mRNA level. Data ± 95% CI are from three independent experiments (n = 3).

in Jurkat T cells upon apoptosis induction compared to living Jurkat T cells. Furthermore they showed that cell surface C3/C3d deposition increased dramatically on cells rendered apoptotic (Martin et al., 2016; Martin and Blom, 2016). The study confirmed that C3 is endogenously present in Jurkat T cells and that it is cleaved into the opsonin iC3b, which becomes exposed on the surface upon apoptosis induction. Further studies have to be conducted to explore the role of intracellular complement during the apoptotic process. Michelle Elvington et al. demonstrated that different cell types, including T and B cells, are able to internalize hydrolysed C3 ($C3(H_2O)$) from the extracellular milieu

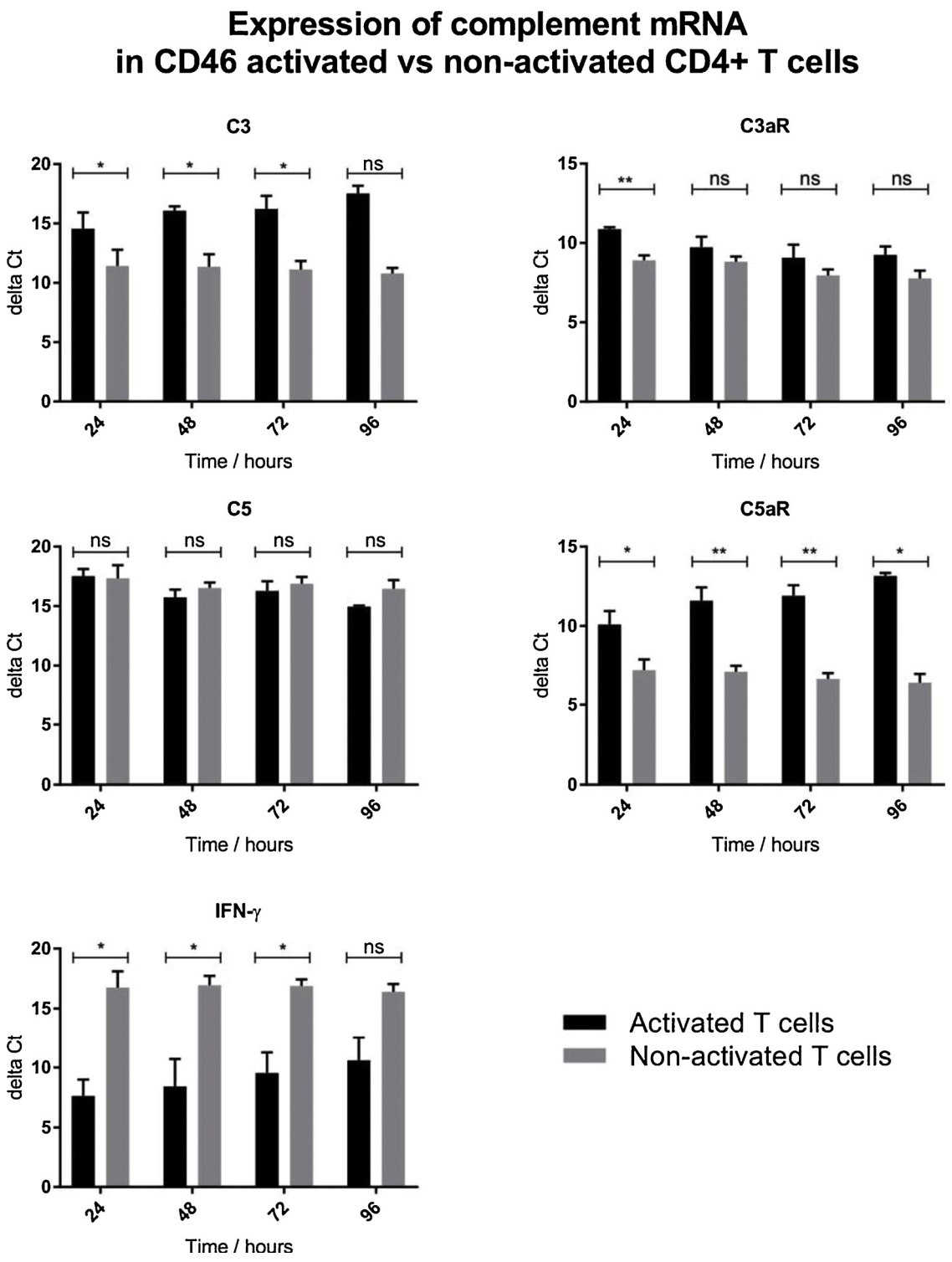


**Fig. 4.** A time dependent measurement of mRNA expression (C3, C3aR, C5, C5aR and IFN-γ) in α-CD2 + α-CD3 + α-CD28 + α-CD46 activated (black) and non-activated (grey) CD4 + T cells. The Y-axis represents ΔCt-values and the X-axis represents the different time points of collection and analysis (24, 48, 72 and 96 h). The ΔCt is calculated $Ct_{targetgene}$ - $Ct_{referencegene}$. A low ΔCt value = high mRNA level. Data ± 95% CI are from three independent experiments (n = 3), except measurements at 96 h which are from two independent experiments (n = 2).

and store the proteins in endosomes in a rapid, selective and saturable fashion (Elvington et al., 2017). They also observed that cell lines, which have not been exposed to complement proteins for several generations, lacked detectable stores of intracellular C3. They illustrate how $CD4^+$ T cells and different cell lines (e.g. Farage cells and Jurkat cells) are able to secrete the intracellular stores of C3($H_2O$) promptly within 24 h when kept in C3-free media. The study establishes a recycling process where several cell types in vivo are homeostatically internalizing, processing, and secreting C3($H_2O$), suggesting that this process is of extensive significance. They also show that C3a production can occur from C3($H_2O$) following uptake and that the C3a production is increased under activation conditions in $CD4^+$ T cells.

This paradigm shift in our understanding of the function of the complement system has opened the doors to a whole new way of

perceiving the interplay between the innate and the adaptive immune system. For example the significance of intracellular C3a generation is underscored in the study by M. Kathryn Liszewski et al (Liszewski et al., 2013) showing that T cells from the synovial fluid of autoimmune arthritis patients had an increased generation of C3a compared with healthy controls due to an overactive intracellular complement system. By blocking CTSL-dependent cleavage of C3 to C3a they were able to "normalize" the increased production of proinflammatory cytokines by the T cells.

In the present study we confirm that there is a baseline intracellular generation of several complement proteins in $CD4^+$ T cells (Figs. 2–4) but upon activation and acute generation of complement cleavage products within T cells, it is not endogenous biosynthesis that appears to be the central mechanism that sustains the pools of this intracellular system, but we cannot exclude the possibility that the low grade expression of the components might contribute to the pool. Future work will need to define what the single contributions of endogenous complement expression versus its uptake from serum to normal cell activities are. Moreover, it will be critical to determine what regulates the expression of complement genes within immune cells during migration and/or residency into tissues as the extracellular levels of complement components will be sparse there.

## Author contributions

Cecilie Bo Hansen and Peter Garred conceived the project. Cecilie Bo Hansen, Anthon Willer and Rafael Bayarri-Olmos performed the experiments. Claudia Kemper and Peter Garred overlooked the progression of the project and came with critical inputs. Cecilie Bo Hansen wrote the first draft of the manuscript. All others contributed to critical revision of the manuscript.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Funding

This work was supported by grant fronts from the Danish Research Foundation of Independent Research (DFF-6110-00489), the Sven Andersen Research Foundation, Novo Nordisk Research Foundation and Rigshospitalet. The funding sources had no involvement in study design, the collection, analysis, interpretation of data, writing of the report or in the decision to submit the article for publication.

## Acknowledgements

We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen, for their expertise and help during the imaging process.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2018.12.004.

## References

Arnold, R., Brenner, D., Becker, M., Frey, C.R., Krammer, P.H., 2006. How T lymphocytes switch between life and death. Eur. J. Immunol. 36 (7), 1654–1658. https://doi.org/10.1002/eji.200636197.

Astier, A.L., Hafler, D.A., 2006. Alterations in CD46- mediated Tr1 regulatory T cells in patients with multiple sclerosis. J. Clin. Invest. 116 (12). https://doi.org/10.1172/JCI29251.

Astier, A., Trescol-Biemont, M.-C., Azocar, O., Lamouille, B., 2000. Rabourdin-combe C. Cutting edge: CD46, a new costimulatory molecule for T cells, that induces p120CBL and LAT phosphorylation. J. Immunol. 164 (12), 6091–6095. https://doi.org/10.4049/jimmunol.164.12.6091.

Cardone, J., Le Friec, G., Vantourout, P., et al., 2010. Complement regulator CD46 temporally regulates cytokine production by conventional and unconventional T cells. Nat. Immunol. 11 (9), 862–871. https://doi.org/10.1038/ni.1917.

Cattaneo, R., 2004. GUEST COMMENTARY four viruses, two bacteria, and one receptor: membrane cofactor protein (CD46) as pathogens' magnet. J. Virol. 78 (9), 4385–4388. https://doi.org/10.1128/JVI.78.9.4385–4388.2004.

Cui, W., Paglialunga, S., Kalant, D., et al., 2007. Acylation-stimulating protein / C5L2-neutralizing antibodies alter triglyceride metabolism in vitro and in vivo. Am. J. Physiol. Endocrinol. Metab. 5, 1482–1491. https://doi.org/10.1152/ajpendo.00565.2006.

Billiau, A., Matthys, P., 2009. Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev . 20 (2), 97–113.

Elvington, M., Liszewski, M.K., Bertram, P., Kulkarni, H.S., Atkinson, J.P., 2017. A C3(H20) recycling pathway is a component of the intracellular complement system. J. Clin. Invest. 127 (3), 970–981. https://doi.org/10.1172/JCI89412.

Gadjeva, M., Dodds, A.W., Taniguchi-Sidle, A., Willis, A.C., Isenman, D.E., Law, S.K., 1998. The covalent binding reaction of complement component C3. J. Immunol. 161 (2), 985–990.

Ghannam, A., Pernollet, M., Fauquert, J.-L., et al., 2008. Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells. J. Immunol. 181 (7), 5158–5166.

Ghannam, A., Fauquert, J.-L., Thomas, C., Kemper, C., Drouet, C., 2014. Human complement C3 deficiency: Th1 induction requires T cell-derived complement C3a and CD46 activation. Mol. Immunol. 58 (1), 98–107. https://doi.org/10.1016/j.molimm.2013.11.010.

Grossman, W.J., Verbsky, J.W., Barchet, W., Colonna, M., Atkinson, J.P., Ley, T.J., 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 21 (4), 589–601. https://doi.org/10.1016/j.immuni.2004.09.002.

Hawksworth, O.A., Coulthard, L.G., Taylor, S.M., Wolvetang, E.J., Woodruff, T.M., 2014. Brief report: complement C5a promotes human embryonic stem cell pluripotency in the absence of FGF2. Stem Cells 32 (12), 3278–3284. https://doi.org/10.1002/stem.1801.

Hildeman, D.A., Zhu, Y., Mitchell, T.C., Kappler, J., Marrack, P., 2002. Molecular mechanisms of activated T cell death in vivo. Curr. Opin. Immunol. 14 (3), 354–359. https://doi.org/10.1016/S0952-7915(02)00335-7.

Källström, H., Liszewski, M.K., Atkinson, J.P., Jonsson, A.B., 1997. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. Mol. Microbiol. 25 (4), 639–647. https://doi.org/10.1046/j.1365-2958.1997.4841857.x.

Kemper, C., Atkinson, J.P., 2007. T-cell regulation: with complements from innate immunity. Nat. Rev. Immunol. 7 (1), 9–18. https://doi.org/10.1038/nri1994.

Kemper, C., Chan, A.C., Green, J.M., Brett, Ka, Murphy, K.M., Atkinson, J.P., 2003. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature 421 (6921), 388–392. https://doi.org/10.1038/nature01315.

Kemper, C., Verbsky, J.W., Price, J.D., Atkinson, J.P., 2005. T-cell stimulation and regulation: with complements from CD46. Immunol. Res. 32 (1-3), 31–43. https://doi.org/10.1385/IR:32:1-3:031.

Klos, A., Tenner, A.J., Johswich, K.O., Ager, R.R., Reis, E.S., Köhl, J., 2009. The role of the anaphylatoxins in health and disease. Mol. Immunol. 46 (14), 2753–2766. https://doi.org/10.1016/j.molimm.2009.04.027.

Köhl, J., 2006. The role of complement in danger sensing and transmission. Immunol. Res. 34 (2), 157–176. https://doi.org/10.1385/IR:34:2:157.

Kolev, M., Le Friec, G., Kemper, C., 2013. The role of complement in CD4+ T cell homeostasis and effector functions. Semin. Immunol. 25 (1), 12–19. https://doi.org/10.1016/j.smim.2013.04.012.

Kolev, M., Le, Friec G., Kemper, C., 2014. Complement — tapping into new sites and effector systems. Nat. Rev. Immunol. 14 (12), 811–820. https://doi.org/10.1038/nri3761.

Krammer, P.H., Arnold, R., Lavrik, I.N., 2007. Life and death in peripheral T cells. Nat. Rev. Immunol. 7 (7), 532–542. https://doi.org/10.1038/nri2115.

Law, S.K., Minich, T.M., Levine, R.P., 1984. Covalent binding efficiency of the third and fourth complement proteins in relation to pH, nucleophilicity, and availability of hydroxyl groups. Biochemistry 23 (14), 3267–3272.

Le, Friec G., Kemper, C., 2009. Complement: coming full circle. Arch. Immunol. Ther. Exp. (Warsz.) 57 (6), 393–407. https://doi.org/10.1007/s00005-009-0047-4.

Liszewski, M.K., Kolev, M., Le Friec, G., et al., 2013. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. Immunity 39 (6), 1143–1157. https://doi.org/10.1016/j.immuni.2013.10.018.

Martin, M., Blom, A.M., 2016. Complement in removal of the dead - balancing inflammation. Immunol. Rev. 274 (1), 218–232. https://doi.org/10.1111/imr.12462.

Martin, M., Leffler, J., Smoląg, K.I., et al., 2016. Factor H uptake regulates intracellular C3 activation during apoptosis and decreases the inflammatory potential of nucleosomes. Cell Death Differ. 23 (5), 903–911. https://doi.org/10.1038/cdd.2015.164.

Mastellos, D., Papadimitriou, J.C., Franchini, S., Tsonis, P.A., Lambris, J.D., 2001. A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. J. Immunol. 166 (4), 2479–2486. https://doi.org/10.4049/jimmunol.166.4.2479.

Matsuoka, K., Park, K., Ito, M., Ikeda, K., Takeshita, S., 2014. Osteoclast-derived complement component 3a stimulates osteoblast differentiation. J. Bone Miner. Res. 29 (7), 1522–1530. https://doi.org/10.1002/jbmr.2187.

Morgan, B.P., Gasque, P., 1997. Extrahepatic complement biosynthesis: where, when and why? Clin. Exp. Immunol. 107 (1), 1–7. https://doi.org/10.1046/j.1365-2249.1997.d01-890.x.

Müller-Eberhard, H.J., 1985. The killer molecule of complement. J. Invest. Dermatol. 85

(1 Suppl), 47s–52s. https://doi.org/10.1111/1523-1747.ep12275445.
Pio, R., Ajona, D., Lambris, J.D., 2013. Complement inhibition in cancer therapy. Semin. Immunol. 25 (1), 54–64. https://doi.org/10.1016/j.smim.2013.04.001.
Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 11 (9), 785–797. https://doi.org/10.1038/ni.1923.
Riley-Vargas, R.C., Gill, D.B., Kemper, C., Liszewski, M.K., Atkinson, J.P., 2004. CD46: expanding beyond complement regulation. Trends Immunol. 25 (9), 496–503. https://doi.org/10.1016/j.it.2004.07.004.
Sahu, A., Kozel, T.R., Pangburn, M.K., 1994. Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation. Biochem. J. 436 (Pt 2), 429–436. https://doi.org/10.1016/0161-5890(93)90354-E.
Sánchez, A., Feito, M.J., Rojo, J.M., 2004. CD46-mediated costimulation induces a Th1-biased response and enhances early TCR/CD3 signaling in human CD4+ T lymphocytes. Eur. J. Immunol. 34 (9), 2439–2448. https://doi.org/10.1002/eji.200324259.
Saraiva, M., O'Garra, A., 2010. The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 10 (3), 170–181. https://doi.org/10.1038/nri2711.
Schafer, D.P., Lehrman, E.K., Kautzman, A.G., et al., 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74 (4), 691–705. https://doi.org/10.1016/j.neuron.2012.03.026.
Seya, T., Turner, J.R., Atkinson, J.P., 1986. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. J. Exp. Med. 163 (4) (Accessed 10 July 2017). http://jem.rupress.org/content/163/4/837.
Suresh, M., Molina, H., Salvato, M.S., Mastellos, D., Lambris, J.D., Sandor, M., 2003. Complement component 3 is required for optimal expansion of CD8 T cells during a systemic viral infection. J. Immunol. 170 (2), 788–794. https://doi.org/10.4049/jimmunol.170.2.788.
Walport, M.J., 2001a. Complement-first of two parts. N. Engl. J. Med. 344, 1058–1066. https://doi.org/10.1056/NEJM200104053441406.
Walport, M.J., 2001b. Complement. Second of two parts. N. Engl. J. Med. 344 (15), 1140–1144. https://doi.org/10.1056/NEJM200104123441506.
Zaffran, Y., Destaing, O., Roux, A., et al., 2001. CD46/CD3 costimulation induces morphological changes of human T cells and activation of vav, rac, and extracellular signal-regulated kinase mitogen-activated protein kinase. J. Immunol. 167 (12), 6780–6785. https://doi.org/10.4049/jimmunol.167.12.6780.