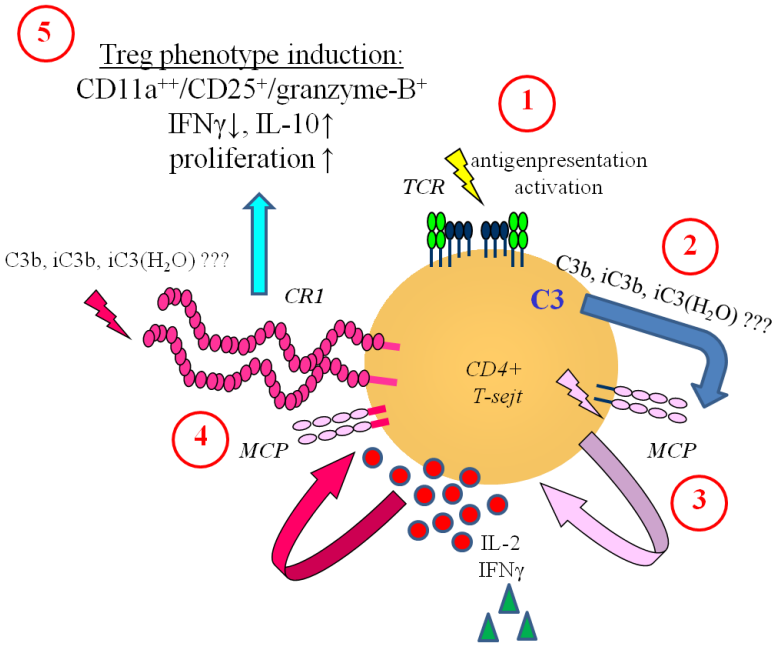
Doctoral thesis

# The expression and function of complement receptor type 1 (CR1/CD35) on human T-lymphocytes

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## Introduction

The aim of our studies is to reveal the interaction between T cells, a determining member of adaptive immunity, and the complement system, which is an important element of innate immunity. In the past few years the number of publications revealing the details of the intricate connections and regulatory links between the innate and adaptive immun systems has significantly increased.

Our group showed earlier that complement receptor type 1(CR1/CD35) - in addition to its well known complement regulatory functions - inhibits the BCR-mediated activation of B lymphocytes both under phisiological conditions and in autoimmune diseases. We proved that in SLE the expression of CR1 on the surface of memory B cells is not elevated in contrast with the healthy controls, and this phenomenon may play a role in the pathogenesis of SLE. However, while earlier it had been shown that CR1 also inhibits the proliferation of human T cells, our knowledge regarding the expression and a more detailed function of this complement receptor in the case of T cells is still not sufficient.

In addition to their effector functions in innate immunity, the elements of the complement system, particularly the activation fragments of C3, the third component - mediate important activatory and regulatory mechanisms, by binding to their relevant receptors expressed by a wide variety of cell types. These molecular mechanisms could be important both under physiological and pathological conditions, therefore could be considered as therapeutical targets in the future. Consequently, understanding the molecular mechanisms of the regulatory connections between lymphocytes and the complement system is a very important issue.

## Aims

Our group focuses on the function of the complement system and the role of complement receptors in the regulation of lymphocyte functions. Based on our earlier results and the latest publications we consider important to reveal the interactions between T cells and the complement system. My aims are to reveal the conditions of the expression of CR1 on the surface of human T lymphocytes and the generation of C3 fragments by T cells and their functions during the adaptive immune response. Furthermore, my goal is to reveal the possible connections between MCP (CD46) and CR1, mainly in the process of Treg differentiation, because the main ligand of both of these receptors is C3b. Our aims were the following:

- Demonstration of the expression of the mRNA for CR1 in human T cells by reverse-transcriptase PCR (RT PCR).
- Demonstration of the expression of CR1 at the protein level on human T cells by flow cytofluorometry (FACS) and confocal laser scanning microscopy.
- Demonstration of the expression of T cell derived C3 both at the mRNA and the protein level.
- Detection of CR1 ligands – such as C3b and iC3b - on the surface of activated T cells by FACS and immunoprecipitation.
- Establishing the role of CR1 ligation on the surface of T cells by
    - assessing the expression of the late T cell activation marker LFA-1 α-chain on the surface of T cells activated by anti-CD3 in the presence of anti-CR1 mAb or CR1 ligands – such as C3b or aggregated C3,
    - assessing the expression of Treg cell markers on Th cells activated by anti-CD3 in the presence of anti-CR1 mAb or CR1 ligands – such as C3b or aggregated C3,
    - measuring the effect of the presence of anti-CR1 mAb or CR1 ligands on the proliferation of anti-CD3 activated human T cells by $^{3}$H-thymidine incorporation
    - measuring IFNγ and IL-10 production of T cells activated by anti-CD3 in the presence of anti-CR1 mAb or CR1 ligands by *Flow Cytomix kits*,
- We also aimed to reveal the interplay between the the two cell surface membrane stuctures, namely CR1 and MCP, which bind the same ligand, C3b. Therefore

I studied how the CR1-stimulus affects the cTreg differentiation processes induced by MCP.

- Finally I also aimed to localize $CR1^+$ Th cells by immuno-histochemistry in a secondary lymphoid organ by employing human tonsills-sections.

## Methods

- isolation of T cells from human blood and tonsills (fluorescent cell sorting or MACS), *in vitro* T-cell activation
- differentiation of MDC-s *in vitro* from human blood derived monocytes
- allogeneic -antigenpresentation
- flow cytofluoromtery (FACS)
- conjugate formation between T cells and U937 human monocyte-derived cells
- measurment of cytokine production by T cells using Flow Cytomix kits
- measuring T cell proliferation by $^3$H-thymidine incorporation
- reverse-transcriptase polymerase chain reaction (RT PCR)
- isolation of C3 protein from human sera by FPLC
- heat-aggregation of C3 protein
- isolation of mAbs from hybridoma's supernatant
- biotinylation of T-cell surface proteins
- immunoprecipitation, SDS-PAGE, Western blot
- investigation of tonsils-section by immuno-histochemistry

**Results and conclusions I:**

## The expression of CR1 on the surface of human T cells

The expression of CR1 on the surface of human T cells has been first described more than thrity years ago, nevertheless the results are still controversial. Therefore we decided to re-investigate the expression and function of CR1 on human T cells using up-to date techniques, and taking into consideration the latest published experimental results. We clearly demonstrated the expression of CR1 both at the mRNA and the protein level in the case of helper (Th) as well as cytotoxic (Tc) T-lymphocytes. However, we found remarkable differences between the percentage of $CR1^+$ T cells both in the case of non-activated and *in vitro* activated cells, strengthening earlier data. Although these differences are thought to be donor dependent by some authors, in our opinion other factors contribute to these individual differences. Namely, several times we experienced a slight shift of the $CR1^+$ population, while in other cases we could not clearly identify $CR1^+$ T cell populations by FACS. Therefore we decided to calculate ΔMFI (which is the difference of the geomean values of fluorescence dispersions of specific fluorescent mAb and isotype control adjusted with autofluorescence values) and made statistical analyses using these values, instead of following the changes of CR1 positive T cell numbers through *in vitro* activation. It is important to point out that we investigated CR1 expression on the surface of T cells by using several different monoclonal and policlonal antibodies, and all these experiments gave similar results.

Based on our earlier results and data of other groups, it was plausible to assume that activated T cells produce C3b - the ligand of CR1 -, which may affect the detection of CR1 through binding to this complement receptor. We showed that adding exogenous C3b to *in vitro* activated T cells reduced the binding of anti-CR1 mAb by nearly 50 percent, when used at the concentration of 10 μg/ml. Therefore we investigated whether T cells can produce C3 and bind back to the cells' surface. We proved that activated T cells produce C3 which - binds back to the cell surface - mainly in the form of iC3b.

*Thus, our results clearly prove that endogenous iC3b binds to CR1 which is upregulated upon activation of T cells, and this interaction influences the detectability of CR1 by antibodies.*

**Results and conclusions II:**

## The function of activated T cell derived C3-fragments

After proving C3 expression by activated T cells, we found a strong iC3b deposition on the surface of activated T cells by the use of a mAb specific to iC3b neo-epitope. The activation fragment, iC3b is the main ligand of CR3 and CR4, therefore we examined the presence of these integrins on the surface of activated T cells and found that CR3 expression is elevated upon activation, while CR4 expression is totally reduced. Based on these results we assumed that mainly CR3 has functional significance on activated T cells. Next we investigated if iC3b present on the surface of activated T cells might be involved in cellular adhesion by using cells of the $CR3^+$ U937 human monocitic cell line. We found conjugate formation between the two cell types, which could be inhibited by mAbs specific to the iC3b neo-epitope or with TMG-6-5, the CR3 ligand-binding site specific antibody. Our results thus clearly suggest that endogenous iC3b may act like an adhesion molecule. Because DCs, the most efficient antigen-presenting cells express CR3 in high numbers, next we studied if endogenous iC3b has any function in the process of antigen pesentation.

When monocyte-derived DCs were cultured together with pre-activated allogeneic T cells, a strong stimuation could be observed, which could be inhibited by a coctail of mAbs specific to the iC3b neo-epitope, the ligand-bindingsite of CR3 and a $F(ab')_2$ fragment of policlonal anti-C3.

*These results show that the activated human T cell derived iC3b fragments which are deposited on the cell surface behave as adhesion molecules and are involved in the enhancement of the proliferation of allogenic T cells.*

**Results and conclusions III:**

## The function of CR1 on the surface of human T cells

It has been shown earlier that MCP, which also belongs to the RCA-family like CR1, and is expressed constitutively on T cells can induce differentiation of activated T cells into Treg phenotype. Since C3b is the main ligand of both CR1 and MCP, we aimed to reveal whether CR1 may also act similarly on activated human T cells.

Since earlier it has been proven that CR1 inhibits CD3-induced T cell activation, first we examined whether this phenomenon occurs in a direct or indirect manner. Monitoring the expression of LFA-1 α-chain (CD11a) however, we didn't find a decrease in the expression of this late activation marker of T cells, suggesting that CR1 inhibits CD3-induced T cell activation through an indirect mechanism. Thus next we examined whether Treg cell markers are expressed on Th cells upon CR1 stimulus.

Our results show that the CR1-mediated stimulus of CD3-activated Th cells induces a signifinatly enhanced CD25 and CD11a expression on the $CD25^{+}CD11a^{++}$ population compared to the $CD25^{-}CD11a^{++}$ cells. These results prove the presence of Treg cells. Similraly, we found an elevated Granzyme-B expression, but the expression of the Foxp3 transcription factor needs further investigations.

Measuring citokine production we detected a reduced IFNγ and an elevated IL-10 level in the case when CR1 and MCP was targetted by mAbs or by the natural ligand. *Studying the effect of the concomitant engagement of CR1 and MCP we found that the the function of these two receptors are connected.* Namely, stimulation of these structures simultaneously resulted in mean values of the data which were obtained when the receptors were stimulated seapartely. Consequently CR1 is involved in the MCP-mediated generation of the cytokine profile of Treg cells.

Investigating T cell proliferation by $^{3}$H-thymidine incorporation an *elevated activation could be observed when CR1 or MCP was targetted specifically via mAbs or the natural ligand.*

**Results and conclusions IV:**

## Localization of $CR1^+$ Th cell in the tonsills

Finally, it was important to reveal what the *in vivo* functional significance of the *in vitro* found results could be. Therefore we investigated the localization of the $CR1^+CD4^+$ cells in human tonsillar sections by immunohistochemical staining, employing double fluorescence labeling. *We found that $CR1^+$ T helper lymphocytes are localized in the folliculi and in the mantle zone of the secondary lymphoid organ. These are the locations where direct contact of Th cells and B lymphocytes is of great importance to exert the regulatory roles found in our in vitro assays.*

**Summary**

Based on our results we suggest the following model for the induction and function of complement-induced Treg cells. T cells are activated as a consequence of antigen-presentation in the secondary lymphoid organs, and - in addition to their well-known functions – these lymphocytes produce C3. From the C3 molecule various activation fragments – such as C3b, iC3b or iC3($H_2O$) - can be generated either through spontaneous hidrolysis, or by the activation of the complement cascade at the site of inflammation, or by enzymes present on the cell surface or inside the cells. These ligands may then bind to complement receptors expressed on the surface of T-lymphocytes. iC3b may interact with CR3 and/or MCP. By binding to the integrin-type receptor it can stimulate the proliferation of the T cells, while through MCP this C3-fragment can stimulate the production of IFNγ and IL-2 cytokines and maintain activation until local IL-2 concentration reaches a relatively high treshold. At this point CR1 expression becomes more excessive and by ligand binding it promotes switching to the Treg phenotype induced by MCP. CR1 has a short intracellular tale with only one tyrosine phosphorylation site in contrast to MCP which has two different cytoplasmic regions with more phosphorylation sites. Thus it can be assumed that MCP and CR1 might use the same signal transduction pathway. In the next step Treg cells differentiated at the site of inflammation may promote the extension of the B cell response, while they suppress activated, bystander T cells, thus inhibiting the expansion of chronic inflammatory processes. Finally, in the absence of IL-2 cytokine Tregs become anergic and die.

By their virtue of being able to mediate T cell modulatory effects, CR1 and MCP could be considered as therapeutical targetsly in the future to treat chronic inflammatory processes, autoimmune diseases and transplant rejection.

**Publications**

Publications connected to the PhD thesis

K. Török, M. Kremlitzka, N. Sándor, E.A. Tóth, Zs. Bajtay, A Erdei. *Human T cell derived, cell-bound complement iC3b is integrally involved in T cell activation* IMMUNOLOGY LETTERS 143:(1) pp. 131-136. (2012)

A Erdei., A. Isaák, K. Török, N. Sándor, M. Kremlitzka, J. Prechl, Zs Bajtay. *Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions.* MOLECULAR IMMUNOLOGY 46:(14 Special Issue) pp. 2767-2773. (2009)

K. Török, A. Bencsik, B Uzonyi, A Erdei. *The role of complement receptor type 1 (CR1/CD35) in Treg differentiation of CD4+ human T lymphocytes,* sent for publication, (2014)

Other publications

A Balogh, M Ádori, K Török, J Matkó, G László *A closer look into the GL7 antigen: Its spatio-temporally selective differential expression and localization in lymphoid cells and organs in human* IMMUNOLOGY LETTERS 130:(1-2) pp. 89-96. (2010)

Published abstracts

A Erdei, K Torok. *Modulation of human T cell function by exogenous and endogenous C3* IMMUNOBIOLOGY 217:(11) p. 1188. 1 p. (2012)