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Mouse CD4$^+$ CD25$^+$ T regulatory cells are protected from autologous complement mediated injury by Crry and CD59

Qing Li [a,b], Kristine Nacion [b], Hong Bu [a,*], Feng Lin [b,*]

[a] Department of Pathology, Sichuan University, Chengdu, China
[b] Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA

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ABSTRACT

Self cells depend on surface complement regulators to protect them from autologous complement-mediated attack. CD4$^+$CD25$^+$foxp3$^+$ T regulatory (Treg) cells are critical in maintaining immune homeostasis, however, which complement regulators are expressed on them and how they are protected from autologous complement attack remains unknown. We report here that mouse Treg cells express virtually no DAF or CR1. Instead, all of them express Crry and approximately half of them express CD59. Both Crry$^{-/-}$ and CD59$^{-/-}$ Treg cells exhibit greater complement mediated injury than WT Treg cells. These results clarify the status of cell surface complement regulators on mouse Treg cells and indicate that both Crry and CD59 are required to protect Treg cells from autologous complement-mediated injury. Additionally, these data also argue that different from previous assumption, at least in mice, CD4$^+$CD25$^+$foxp3$^+$ Treg cells are not homogenous and could be further divided into subgroups based on CD59 expression.

Introduction

As a pivotal part of the innate immunity, complement exists at high concentration in serum as well as in many other body fluids to fight against infections [1]. When activated, complement activation products (e.g. C3b) opsonize their targets to enable efficient phagocytosis through interactions with complement receptors on phagocytic cells [2]. At the same time, the terminal pathway of complement activation assembles membrane attack complexes (MAC), which lyses target cells by forming osmotic channels on cell surfaces [3]. Since complement activation products cannot distinguish self cells from invading pathogens, self cells depend on surface complement regulators to protect them from autologous complement mediated injury. Among known cell surface complement inhibitors, there are decay accelerating factor (DAF, CD55) [4], membrane cofactor protein (MCP, CD46) [5], complement receptor 1 (CR1, CD35) [6] and CD59 [7]. Given their physiological importance, all the cells express at least one, most times all the above complement regulators on their surfaces [8]. These cell surface complement inhibitors regulate complement activation at different steps therefore protect self cells from complement mediated injury [8].

Different from humans, mice express a unique cell surface complement inhibitor named complement receptor 1-related protein y (Crry) which possesses the same decay accelerating activity of DAF and cofactor activity of MCP in humans [9]. Since mouse MCP expression is strictly limited in testis [10] and DAF expression is widely distributed [11], Crry is considered the human MCP counterpart in mice. Crry$^{-/-}$ mice are embryonic lethal because uncontrolled complement activation attacks fetus during gestation [12]. This problem can be solved by breeding Crry$^{-/-}$ mice onto the C3 deficient background in which the resulted Crry$^{-/-}$ C3$^{-/-}$ mice are vital and fertile [12].

CD4$^+$CD25$^+$foxp3$^+$ Treg cells are a subgroup of CD4$^+$ T cells that modulate T cell activity either through direct interactions with effector T cells and antigen presenting cells (APCs) or through production of immunosuppressive soluble factors such as IL-10 and TGF-β. They are under extensive study for their potentials in treating autoimmune diseases [13] as well as transplanted grafts rejection [14]. In theory, Treg cells need cell surface complement regulators to protect them from unwanted complement mediated attack, however, which cell surface complement inhibitors are expressed on Treg cells and how important these regulators are remain unstudied. Here we report the first time that mouse CD4$^+$CD25$^+$foxp3$^+$ Tregs express Crry and CD59 but not DAF or CR1 on their cell surfaces. By comparing Treg cells isolated from respective WT, Crry$^{-/-}$ C3$^{-/-}$ [12] and CD59$^{-/-}$ mice [15], we show that both Crry and CD59 are required in protecting Treg cells from

* Corresponding author.
E-mail addresses: hongbu@scu.edu.cn (H. Bu), feng.lin@case.edu (F. Lin).

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complement mediated attack. Our results also suggest that $CD4^+CD25^+$ $foxp3^+$ Treg cells are not homogenous and foxp3 could regulate CR1 and DAF expression on Treg cells.

Materials and methods

Mice and reagents. Six to eight weeks old female C57BL/6 mice were purchased from Jackson Lab (Bar Harbor, ME). $CD59a^{-/-}$ mice [15] were kindly provided by Dr. BP Morgan and $Crry^{-/-}$ $C3^{-/-}$ mice [12] were kindly provided by Dr. H. Monila. Mouse $CD4^+$ T cell Easysep kits were purchased from Stem Cells Inc. (Vancouver, Canada). Mouse Treg cell staining kits were purchased from eBiosciences (San Deigo, CA). Rat anti mouse CD59 mAb (clone ERMP20) was ordered from AbCam Inc. (Cambridge, MA), rat anti CR1 (clone 8C12) and rat anti Crry mAb (clone 1F2) were purchased from BD Biosciences (San Jose, CA). Rat anti mouse DAF mAb (clone 2C6) [16] was provided by Dr. B.P. Morgan.

Mouse $CD4^+$T cell and $CD4^+CD25^+$T cell isolation. Mouse $CD4^+$ T cells were isolated from spleens and lymph nodes using mouse $CD4^+$ T cell Easyprep kits following the manufacturer's protocol. The purity of the isolated $CD4^+$ T cells was monitored by flow cytometry. To purify $CD4^+CD25^+$ T cells, $CD4^+$ T cells were first enriched using the above described kit, then stained with FITC labeled anti CD4 and APC labeled anti CD25. After washing, $CD4^+CD25^+$ T cells were flow sorted by a BD Aria sorter.

Complement mediated cell injury assay. Flow sorted $CD4^+CD25^+$ T cells ($5–8 \times 10^5$) first were incubated with 5 μM BCECF-AM (Invitrogen, CA) for 30 min at 37 °C. After this, cells were washed three times with FACS buffer (1% BSA in PBS), then incubated at 37 °C for 30 min with 10 μg/ml zymosan (Sigma, MO) and 30% mouse serum in 100 μl GVB-EGTA/Mg^{++} buffer (veronal-buffered saline supplemented with 0.1% gelatin, 5 mM EGTA and 3 mM $MgCl_2$). Following incubation, complement mediated cell injury was assessed by measuring levels of converted BCECF released from the cell. In brief, supernatants were harvested and measured by a fluorescence microtiter plate reader (Molecular Devices, CA) with excitation and emission wavelengths of 485 and 538 nm. To calculate the percentage of BCECF release after complement mediated injury, the following equation was used as reported before [17,18]: percentage of BCECF release = $[(A-B)/(C-B)] \times 100\%$; where A represents the mean experimental BCECF release, B represents the mean spontaneous BCECF release and C represents the mean maximum BCECF released which was induced by incubating cells with 0.1% Triton X-100.

Treg cells C3b deposition assay. Isolated $CD4^+$ T cells (1×10^6) were incubated at 37 °C for 20 min with 10 μg/ml zymosan and 30% mouse serum in 100 μl GVB-EGTA/Mg^{++} buffer. After this, cells were spinned down and washed with 500 μl of FACS buffer twice and then incubated with APC labeled anti-CD25 and FITC labeled anti-mouse C3 mAbs. After washing, cells were analyzed by flow cytometry (LSR I, BD Bioscience, CA) and gated on the $CD4^+CD25^+$ population.

Results

$CD4^+CD25^+foxp3^+$ Treg cells express Crry and CD59 but not DAF or CR1

To detect cell surface complement regulators on $CD4^+CD25^+$ $foxp3^+$ Treg cells, we isolated $CD4^+$ T cells from C57BL/6 mice and checked their purity by flow cytometry (>98.5%, data not shown). After staining the isolated mouse $CD4^+$ T cells with respective rat anti Crry, DAF, CD59 and CR1 mAbs and FITC conjugated goat anti rat IgG, we stained the cells again using APC conjugated anti CD25 mAb. Following fixation and permeabilization, we stained intracellular foxp3 by PE conjugated anti foxp3 mAb and analyzed the samples by flow cytometry (BD LSR I). These assays showed that while all CR1, DAF, CD59 and Crry were present on $CD4^+CD25^+foxp3^-$ T cells (Fig. 1, upper left quarters), only Crry

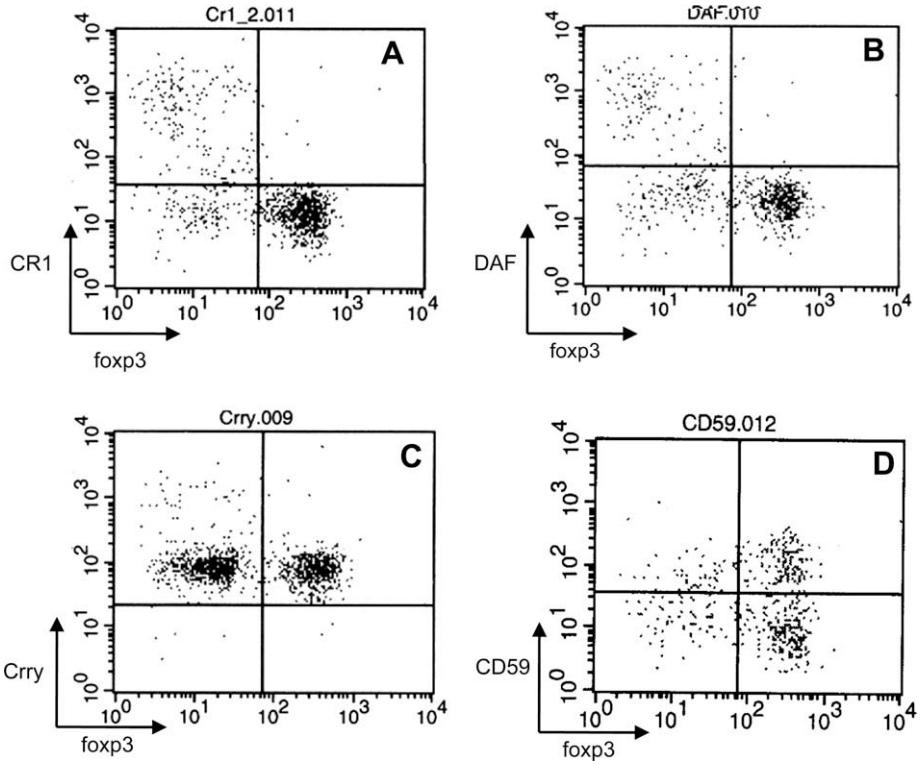

Fig. 1. Murine $CD4^+CD25^+foxp3^+$ Treg cells express Crry and CD59 but not CR1 or DAF. Isolated $CD4^+$ T cells (>98.5% pure) were first stained for CR1 (a), DAF (b), Crry (c) and CD59 (d), then stained for CD25 and foxp3. After this, the samples were analyzed on a flow cytometer (BD LSRI).

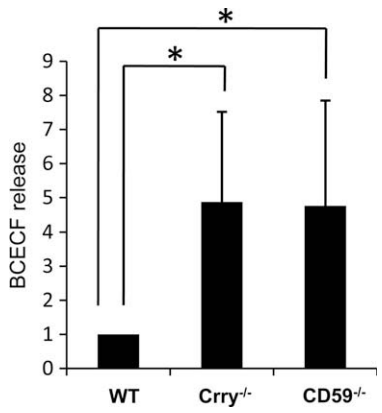

Fig. 2. Both Crry and CD59 are required for efficient protection of Treg cells from autologous complement attack. Flow sorted Treg cells from WT, Crry$^{-/-}$ C3$^{-/-}$ and CD59$^{-/-}$ mice were first loaded with BCECF-AM, and then incubated with zymosan and serum at 37 °C for 30 min. After incubation, percentages of BCECF release were detected and normalized against that of WT Treg cells. $^{*}p < 0.05$.

and CD59 were expressed by CD4^{+}CD25^{+}foxp3^{+} Treg cells (Fig. 1, upper right quarters). Interestingly, CD59 was only present on approximately half of the CD4^{+}CD25^{+}foxp3^{+} Treg cells (Fig. 1d)

Absence of Crry or CD59 leads to heightened complement mediated injury to Treg cells

To determine the importance of Crry and CD59 on Treg cells, we isolated Treg cells from WT, Crry$^{-/-}$ C3$^{-/-}$ and CD59$^{-/-}$ mice using magnetic beads negative selection followed by flow sorting. Because these knockout mice were not on the foxp3 GFP knockin background, we were only able to sort live CD4^{+}CD25^{+} cells as Treg cells for experiments. After labeling the purified WT, CD59$^{-/-}$ and Crry$^{-/-}$ Treg cells with BCECF-AM, we incubated them with mouse serum and zymosan, which activated complement via the alternative pathway. After incubation, we quantitated complement mediated Treg cell injury by measuring levels of converted BCECF that was released from injured cells into the media. These assays (Fig. 2) showed that compared to WT Treg cells, Crry$^{-/-}$ and CD59$^{-/-}$ Treg cells had ~5-fold increased BCECF release into the media, which indicated ~5-fold more severe cell injury.

Crry$^{-/-}$ Treg cells had greater C3b deposition on their cell surfaces than WT Tregs

To determine whether Crry$^{-/-}$ Treg cells exhibited changes in C3 convertase regulation, we compared deposited C3b levels on different Treg cells after incubation with zymosan activated complement. In brief, we repeated the above experiments and instead of measuring released BCECF levels, we quantitated C3b deposition on respective Treg cell surfaces by flow cytometry. The assays showed that compared to WT Treg cells, Crry$^{-/-}$ Treg cells had elevated levels of C3b deposition while CD59$^{-/-}$ Treg cells had similar C3b deposition on their cell surfaces (Fig. 3).

Discussion

In this report we provided demonstration the first time that mouse CD4^{+}CD25^{+}foxp3^{+} Treg cells expressed Crry and CD59 but not DAF or CR1. Crry$^{-/-}$ or CD59$^{-/-}$ Treg cells suffered more severe autologous complement mediated injury than WT Treg cells. While Crry$^{-/-}$ Treg cells had increased levels of C3b deposition on their surfaces, CD59$^{-/-}$ Treg cells had similar levels of C3b deposition as WT Treg cells which is consistent with CD59's regulatory role on later MAC formation.

Complement cannot distinguish self cells from invaded pathogens because after activation, C3b binds covalently to any surface with free hydroxyl or amino groups [19]. As indicated in the Introduction, to protect self cells from autologous complement mediated attack, complement activation is tightly regulated by cell surface complement inhibitors, i.e. DAF, CR1, MCP and CD59. Both DAF and CR1 accelerate the decay of C3/C5 convertases [4,20], while MCP only serves as a cofactor for factor I to convert the deposited C3b to its inactive form iC3b [5]. Although CD59 has no effect on C3/C5 convertases, it functions by inhibiting the insertion of C9 at the final step of MAC assembly, thus prevent MAC mediated cell injury/lysis [21]. The important roles of these cell surface complement regulators in protecting self cells against complement mediated injury are well documented in both human and animal studies e.g. DAF and CD59 deficient erythrocytes in paroxysmal nocturnal hemoglobinuria patients are lysed by autologous complement activation and MAC formation, leading to hemoglobinuria and anemia [22]. MCP gene mutations predispose to development of atypical hemolytic uremic syndrome in which hemolytic anemia and kidney failure occur due to excessive complement activation [23]. DAF deficient mice develop elevated levels of proteinuria because of complement mediated renal injury in NTS-induced nephritis [24] and severe muscle weakness due to complement mediated endplates damage in passive experimental myasthenia gravis [25]. CD59 deficient mice develop spontaneous complement mediated intravascular hemolysis and hemoglobinuria [15] and Crry deficient mice are embryonic lethal due to placental destruction mediated by the maternal complement system [12].

The results that CR1 and DAF presented on CD4^{+}CD25^{+}foxp3^{-} cells but not CD4^{+}CD25^{+}foxp3^{+} Treg cells argue that the transcription factor foxp3 inhibited the expression of CR1 and DAF in Treg cells. Although the underlying mechanism remains unknown and previous studies [26] have shown that foxp3 modulates expression of many genes associated with immunity including CTLA-4, CD25

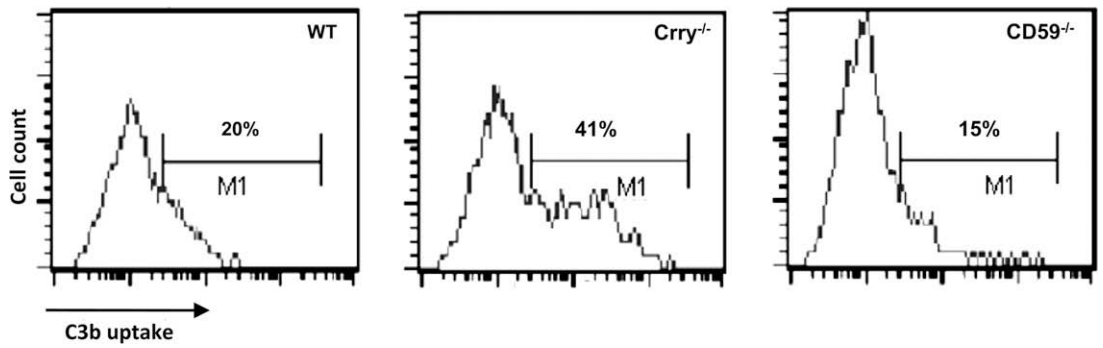

Fig. 3. Crry$^{-/-}$ Treg cells have elevated levels of C3b deposition on their surfaces after bystand complement activation by zymosan.

and ICOS, to our knowledge this is the first evidence suggesting that foxp3 is associated with complement gene regulation.

The significance of DAF absence on $CD4^+CD25^+foxp3^+$ Treg cells is unclear. Previous studies showed that during T cell activation, DAF expression is downregulated on activated T cells [27]. Our recent work also found that DAF is downregulated on both antigen presenting cells (APCs) and T cells during their cognate interactions to allow efficient T cell activation [28]. Another subset of lymphocytes that do not carry DAF on their surfaces are nature killer (NK) cells [29]. Incorporation of purified DAF protein on NK cells *in vitro* inhibits their cytotoxicity [30], suggesting that DAF may regulate NK cell function via a non-complement dependant mechanism. Further studies are needed to clarify whether overexpressing/incorporating DAF on Treg cells modulates their immunoregulatory function.

$CD4^+CD25^+foxp3^+$ Treg cells are generally considered to be homogenous. Our results that approximately half of the Treg cells expressed CD59 while another half did not argue that they could be divided into at least 2 subgroups: the $CD59^+$ Treg cells and the $CD59^-$ Treg cells. However, the physiological relevance of CD59 presence and absence on Treg cells requires further investigation.

Similar to other cells, Treg cells need cell surface complement regulators to protect them from autologous complement mediated attack. The studies reported here clarified the distribution of cell surface complement regulators on mouse Treg cells, showed that both Crry and CD59 are critical in protecting Treg cells from complement mediated injuries. These data also suggested that foxp3 could regulate CR1 and DAF expression in Treg cells, and at least in mice, $CD4^+CD25^+foxp3^+$ Treg cells could be further divided into 2 subgroups based on their cell surface CD59 expression.

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