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CD39 expression in regulatory T-cells

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Abstract

The aim of the project was to identifify molecular mechanism, which may regulate CD39 expression in $CD4^+$ T-cells and regulatory T-cells (Treg). CD39, an ectoenzyme, is expressed on a subpopulation of Treg. Interestingly, $CD39^+$ Treg show enhanced suppressive potential in comparison to $CD39^-$ Treg. For the identification of regulation mechanisms of CD39, the potential correlation of retroviral overexpression of the transcription factors (TF) Sp1, CREB, Helios and Runx1 with CD39 expression were investigated. Additionally, potential CD39-regulating TF were searched by a genomic screening approach with a $CD4^+$ T-cell derived cDNA library. Furthermore, we attempted to identify a potential stimulus inducing CD39 expression in $CD4^+$ T-cells, especially in Treg for a better understanding of the modification of CD39-expressing Treg. For that purpose, three different in vitro Treg-induction protocols were tested which included the following stimuli: all trans retinoic acid (atRA)+TGF-β, vitamin D_3 and rapamycin.
I was able to demonstrate that, against expectations, the TFs Sp1, CREB, Helios and Runx1 do not regulate CD39 expression in $CD4^+$ T-cells. Furthermore, in vitro TGF-β induced iTreg showed a highly significant increase in CD39 expression, whereas rapamycin significantly decreased CD39 expression in the primary $CD4^+$ T-cells of ten healthy donors. Using our genomic screening approach, many potential regulation factors (e.g. transcription factors) and other functional proteins were identified by mass spectrometry analysis. Potential candidates of CD39 regulation have to be verified employing additional analyses with primary $CD4^+CD39^{+/-}$ T-cells. In summary, the regulatory principles of CD39 in T-cells remain largely unknown. Data published on TFs, and stimuli which may regulate CD39 expression, remain somewhat contradictory. Continuing research in this important field will clarify these uncertainties in the near future.

Keywords:

CD39, Regulatory T-cells, $CD4^+$ T-cells, FOXP3

1. Introduction

Regulatory T-cells (Treg) are a subpopulation of $CD4^+$ T-cells, associated with immunosuppression and preservation of self-tolerance. Hence, Treg are involved in the prevention of autoimmune diseases, allergies and transplant rejection (Sakaguchi 2004). In malignant diseases, Treg also play an important role. On the one hand, they can help malignant cells escape immune responses and on the other hand, Treg can control cancer-associated inflammation, therein protecting the body against cancer (Gounaris et al. 2009). Different subtypes of Treg exist. The classical and best characterized Treg are thymus-derived regulatory T-cells (tTreg) (Sakaguchi 2004). About 7-8% of the $CD4^+$ T-cell population are Treg, phenotypically characterized as $CD4^+CD25^+FOXP3^+CD127^{low}$ (Liu et al. 2006). Treg can suppress proliferation and cytokine production of effector T-cells through various manifestations, such as secretion of anti-inflammatory cytokines and molecules, expression of suppressive receptors and the degradation of the inflammatory molecule ATP into the suppressive molecule adenosine through the ectoencymes CD39 and CD73 (Schmidt et al. 2012).

CD39 is identified in various cells including endothelia, macrophages, B-cells and a subset of tTreg (Robson et al. 2006). $CD39^+$ Treg show an enhanced suppressive potential in comparison to $CD39^-$ Treg (Rissiek et al. 2015). This is due to the fact that adenosine, generated by hydrolysis of ATP from CD39 and CD73 on Treg, binds to the A_{2A}-receptor on effector T-cells leading to a suppression of effector-functions (Haskó et al. 2008). However, the mechanisms regulating CD39 expression in $CD4^+$ T-cells and Treg are currently not known. A multitude of studies shows an association between differential CD39 expression on Treg and various diseases as well as the success rate of therapy. For example, Treg can accumulate in cancer-tissue leading to an inhibition of effector lymphocytes. As a result, Treg can support tumor-growth (Bastid et al. 2013). If the regulation-mechanism of CD39 were better understood, new potential targets for CD39-blockade in cancer or also CD39-stimulation in inflammatory diseases could be discovered and used for therapeutic purposes.

The aim of the project was to identify molecular mechanisms which regulate CD39 expression in $CD4^+$ T-cells and Treg. Due to numerous studies that illustrate the role of FOXP3 as main transcription factor of Treg, one could assume that FOXP3 is involved in the expression of CD39. However, $CD39^-$ Treg also express FOXP3, and the existence of $CD39^+$ T-cells, which are negative for the expression of FOXP3 has been demonstrated (Borsellino et al. 2007). These findings suggest that it is improbable that FOXP3 acts on its own as the main regulator of CD39 expression in T-cells. It was demonstrated, that the transcription factor (TF) Sp1 regulates CD39 in endothelia in response to hypoxia (Eltzschig et al. 2009). CREB may regulate CD39-expression in macrophages (Liao et al. 2010) and HELIOS and Runx1 play an important role in the regulation of suppressive mechanisms in Treg (Lu et al. 2014), (Grzanka et al. 2013). In this thesis, the TF listed were investigated in T-cells for potential correlations with CD39 expression. We also searched for potential CD39-regulating TF by a genomic screening approach. Additionally, for the purpose of finding a potential stimulus of CD39 expression in $CD4^+$ T-

cells, different *in vitro* Treg-induction protocols were tested including TGF-β, *all trans* retinoic acid, vitamin D_3 and rapamycin (reviewed in Schmetterer et al. 2012):

2. Methods

All experiments described were performed at the Department of Laboratory Medicine, Medical University of Vienna. Human T-cells were derived from peripheral blood mononuclear cells (PBMCs) from healthy donors, provided by "Rotes Kreuz Wien", in accordance with the Ethics Commission of the Medical University of Vienna (EK Nr.: 1150/2015).

2.1. Testing of Sp1, CREB, Runx1 and Helios

We investigated the effects of the TFs Sp1, CREB, Runx1 and Helios on CD39-expression in $CD4^+$ T-cells. To that end, all TFs were cloned into the pMMP-IRES-GFP vector. After a generation of retroviral particles, Jurkat wildtype and Jurkat $FOXP3^+$ cells were transduced as well as primary $CD4^+$ T-cells including additional co-transduction with FOXP3. 72h after transduction, the cells were marked for CD39 surface expression and were analyzed by flow cytometry. GFP fluorescence indicated positively transduced cells.

2.2. Genomic screening approach

In addition, we also searched for potential CD39-regulating TF by a genomic screening approach. In that pursuit, a cDNA library derived from $CD4^+$ T-cells was transduced into a Jurkat-$FOXP3^+$ test-cell line. It can be assumed, that library coverage includes most relevant genes expressed in $CD4^+$ T-cells. Thus a distinct number of cells which had taken up the putative regulation factor of CD39 expression, were supposed to show CD39 expression on their surface. These $CD39^+$ cells were separated from $CD39^-$ cells by FACS-sorting and single cell clones (SCC) were generated to obtain homogenous $CD39^+$ cell lines. In figure 1, the procedure of genomic screening is described. For an improved validity of genomic screening, the protein-profile of following cells was analyzed by mass spectrometry (Q Exactive Orbitrap ^{TM}). For that purpose, nuclear extracts and cytoplasmic fraction were generated from the following cells:

- Jurkat wildtype: Human $CD4^+$ CD39- T-cell line, originated from an acute T-cell leukaemia
- Jurkat FOXP3: Jurkat with constitutive FOXP3 expression, created by retroviral transduction
- Two different single cell clones of the Jurkat $FOXP3^+$ $CD39^+$ cell line which showed a CD39 expression after transduction with the T-cell library and where CD39 itself as retroviral cDNA insert was excluded by PCR.

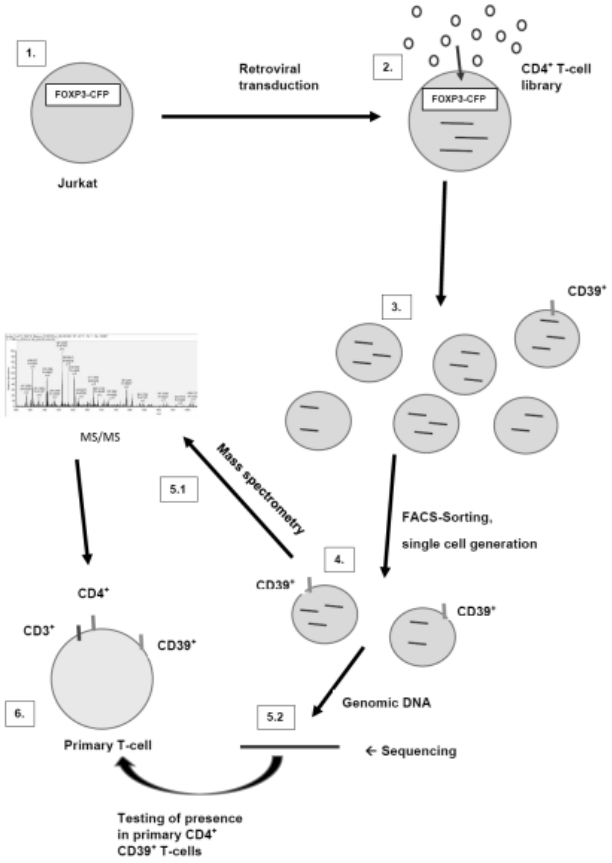

Figure 1: Procedure of genomic screening. *First, Jurkat cells are transduced with FOXP3 (1), followed by the transduction with cDNA from a CD4$^+$ T-cell library (2). Due to an uptake of the regulation factor for CD39, a few cells should express CD39 (3). Using a FACS-Sorter, CD39-expressing cells are sorted and single cell clones are generated (4) Protein-expression is measured using mass spectrometry for the identification of up- and down-regulated proteins including regulation factors (5.1). In this project, we succeed with step 5.1. In step 5.2, cDNA is identified by sequencing and finally, all identified regulation factors are tested in primary T-cells (level of expression and overexpression approaches).*

2.3. Treg-induction protocols

In the quest for a potential stimulus which induces CD39 expression in CD4$^+$ T-cells, especially in Treg, we tested different *in vitro* Treg-induction protocols: repetitive activation of primary T-cells in the presence of *all-trans* retinoic acid (atRA) + TGF-β1, vitamin D_3 and rapamycin. In that process, we used primary CD4$^+$ T-cells from ten different donors. For a reference-value (control), cells were also cultivated with a plain medium. All protocols took 14 days in culture. On day 14, cells were marked for surface expression of Treg-marker (CD3, CD4, CD25, CD127) as well as CD39. Statistical analysis was performed using the software “IBM SPSS statistics 21”. For comparison of the CD39 expression-state between different Treg-Induction protocols and the negative control, we used One-Sample T-test and Shapiro-Wilk test.

3. Results and discussion

Our experiments do not show a correlation between overexpression of the TFs Sp1, CREB, Runx1 and Helios with enhanced CD39 expression in Jurkat cells, Jurkat FOXP3$^+$ cells as well as in primary

$CD4^+$ T-cells. These results lead to the suggestion that CD39 is regulated differently in different cell types. The TFs analyzed may thus act rather only as co-regulatory factors or are not involved in the regulation of CD39 in T-cells at all.

Additionally, we searched for potential CD39-regulating TF by a genomic screening approach. For that purpose, Jurkat $FOXP3^+$ cells were transduced with a $CD4^+$ T-cell derived cDNA library. We expected that as a result of an uptake of a cDNA encoding regulation factor for CD39, a few cells should express CD39. Using a FACS-Sorter, CD39-expressing cells were isolated and single cell clones were generated. The uptake of the cDNA-insert leads to an overexpression of the encoded protein. For the identification and relative quantification of the proteins, protein expression of two different $CD39^+$ Jurkat FOXP3 SCC were compared to the original Jurkat $FOXP3^+$ cells as well as Jurkat wildtype cells using mass spectrometry (Q Exactive Orbitrap). We decided to analyze the protein expression of our cells, because of the improved sensitivity compared to PCR alone, which may also detect DNA which is not translated to proteins. All above listed TFs were successfully identified in all tested cells. CREB and ATF-2 proteins were measured in similar amounts in both, $CD39^+$ and $CD39^-$ Jurkat $FOXP3^+$ SCC leading to the assumption that both proteins cannot be the main regulation factors of CD39-expression. Furthermore, all family members of the Sp- transcription factor family (Sp1, Sp2, Sp3 and Sp4) were detected in both, $CD39^+$ and $CD39^-$ Jurkat $FOXP3^+$ cells. Protein amounts of Sp1 and Sp2 appeared to be increased in $CD39^+$ single cell clones (5 times to 8 times) compared to the protein amount of original Jurkat $FOXP3^+$ cells. Helios was slightly increased (4 times) in both $CD39^+$ Jurkat $FOXP3^+$ SCC. Furthermore, the Runx transcription factor family was represented with Runx1, Runx2 and Runx3. Only Runx2 seemed to be increased (6 times) in both $CD39^+$ Jurkat $FOXP3^+$ SCC. Due to these results, we assume that Sp1, CREB, Helios and Runx1 may serve as important co-regulatory factors for CD39 expression, but nevertheless may not directly regulate CD39 expression.

In summary, using our genomic screening approach, many interesting regulation factors and other proteins were identified by mass spectrometry analyses of two $CD39^+$ Jurkat $FOXP3^+$ SCC compared to original Jurkat $FOXP3^+$ cells as well as Jurkat wildtype cells. Potential candidates of CD39 regulation have to be verified employing additional $CD39^+$ Jurkat $FOXP3^+$ SCC for an improved reliability of statement as well as additional analysis with primary $CD4^+CD39^{+/-}$ T-cells. In another approach, we will also aim to identify cDNA-inserts of $CD39^+$ Jurkat $FOXP3^+$ SCC which eventually lead to the expression of CD39 by sequencing. To that end, the insert cDNAs have to be amplified with $CD4^+$ T-cell library-specific primers from the genomic DNA of the clones followed by sequencing of the inserts.

Furthermore, we attempted to find a potential stimulus, which induces CD39 expression in $CD4^+$ T-cells, especially in Treg for a better understanding of modification of CD39-expressing Treg due to various diseases including cancer, chronic infections and autoimmune disorders. In that process, three different *in vitro* Treg-induction protocols were tested which included the following stimuli: atRA+TGF-β, vitamin D_3 and rapamycin. In summary, 10 donors were tested (see figure 2). The highest CD39-

expression was observed following the protocol with atRA+TGF-β. The vitamin A metabolite atRA is involved in the differentiation and proliferation of various cells and also increases the expression and phosphorylation of Smad3 (Xiao et al. 2008). In our results, atRA alone did not increase CD39-expression. We therefore suggest that CD39 is not regulated solely by Smad3-signalling alone. In contrast, TGF-β alone stimulated induction of CD39-expression significantly (p=0.006), leading to the assumption that TGF-β signaling regulates CD39-expression. However, TGF-β has multiple signaling functions including phosphorylation of Smads as well as MAPK-signalling (Prud'homme / Piccirillo 2000), (Derynck / Zhang 2003). So far, it is not understood which TGF-β triggered pathway contributes to the regulation of CD39. Vitamin D_3 did not show a significant difference in CD39 expression probably due to degradation of vitamin D_3. Interestingly, the first donor tested, in whose case we used freshly acquired vitamin D_3, showed an increase in CD39-expression in vitamin D_3 induced iTreg. Hence, further tests with stable vitamin D_3 are needed. Due to these results, we suggest that CD39 expression may rely on mTOR-signalling (rapamycin is an mTOR inhibitor). Further tests will be needed in this respect, as well as functional tests for the investigation of suppressive functions of the *in vitro* generated Treg.

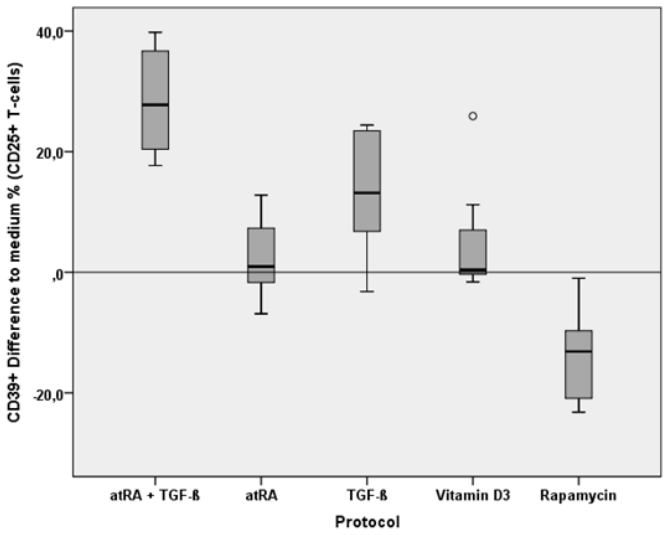

Figure2: CD39-expression of different Treg-induction protocols in comparison to medium (baseline). atRA+TGF-β showed a statistically highly significant increase in CD39-expression. (p<0,01; n=10) TGF-β significantly increased the CD39-expression (p= 0.006) in comparison to atRA alone (p=0.335) (both n=8). Rapamycin showed a statistically significant decrease (p<0.01) in CD39-expression (n=10) Vitamin D_3 did not show a difference, because the protocol did not give consistent results, most likely due to the degradation of Vitamin D_3 (n=7)

4. Conclusion

The regulatory principles of CD39 in T-cells remain largely unknown. Data published on TFs, and stimuli which may regulate CD39 expression are still contradictory. Continuing research in this important field will clarify these uncertainties in the near future.

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