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Regulatory T cells Suppress Zap70 Phosphorylation in Responding T cells

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Abstract

Objective: Regulatory T cells (Treg) have a critical role in controlling responding T cells (Tresp), thereby preventing autoimmune manifestations including those observed in adenosine deaminase (ADA) enzyme-deficient patients and mice (ADA-KO). Treg suppress various functions of Tresp such as proliferation, cytokine production and expression of activation markers, yet it is not known if Treg suppress the early steps of Tresp activation, such as the phosphorylation of the Zeta-associated protein (Zap)70.

Methods: Zap70 phosphorylation and CD69 expression in anti-CD3 and anti-CD28 stimulated mice $CD4^+CD25^-$ Tresp were measured by flow cytometry in the presence or absence of $CD4^+CD25^+$ Treg activated with anti-CD3 and anti-CD28 antibodies. Suppression of Zap70 phosphorylation in Tresp from normal mice cultured with Treg from ADA-KO mice, either treated with PEG-ADA enzyme replacement or untreated, was similarly measured.

Results: Zap70 phosphorylation in activated Tresp was markedly (50 ± 13%) decreased 2 hours after culture with Treg, while 51 ± 8% suppression of CD69 expression was only detected after 7 hours. Suppression of Zap70 phosphorylation in activated Tresp correlated with the ratio of Treg to Tresp. Treg from ADA-KO mice had significantly reduced (p=0.012) ability to suppress Zap70 phosphorylation (16.2 ± 16.7%) compared to Treg from healthy littermates (51.6 ± 23.4%), while PEG-ADA treatment restored Treg suppressive ability (45 ± 10%).

Conclusions: Treg suppress Zap70 phosphorylation in Tresp, a finding that might help better understand and assess Treg function.

Keywords: Zap70; Treg; Phosphorylation; Autoimmunity; Immunodeficiency

Abbreviations:

Treg: Regulatory T cells; Tresp: Responding T cells; Fox: Forkhead box; Zap: Zeta-associated protein; ADA: Adenosine Deaminase; PEG-ADA: Polyethylene glycol conjugated bovine ADA

Background

Regulatory T cells (Treg) are a subgroup of $CD4^+$ T cells capable of maintaining peripheral self-tolerance by suppressing responding T cells (Tresp). Identification of Treg abnormalities in patients suffering from autoimmunity, exaggerated inflammation, cancer, allograft rejection, immune deficiency, etc. have demonstrated the critical role of Treg in controlling self-reactive T cells [1,2]. Indeed, clinical trials assessing the ability of *ex vivo* generated Treg to suppress uncontrolled inflammation are underway [3,4].

Enumeration of Treg is often accomplished by flow cytometry measurement of cells' surface expression of CD4 and CD25 [5]. Forkhead box (Fox) P3, an intra-cellular transcription factor critical for Treg development, is the most definitive marker to identify Treg, particularly in humans, together with low or absent expression of CD127 [6]. Discordance between the numbers and capabilities of Treg has led to increased interest in assessing the actual function of the cells. Production or secretion of cytokines such as IL-10 or TGF-beta by Treg has been used as surrogate marker for the function of these cells. Treg ability to control responses of immune cells *in vitro*, particularly proliferation of T cells is considered the "gold-standard" for assessing Treg function [5]. These assays rely on the ability of $CD4^+CD25^+$ Treg to suppress the proliferation of co-cultured activated $CD4^+CD25^-$ Tresp [7]. Different methods can be used to activate Tresp, including cross-linking CD3 and CD28. Similarly, IL2 or interferon-gamma production by Tresp, CFSE dilution and $[^3H]$-Thymidine incorporation have been employed to measure the response of activated Tresp [8]. Suppression of Tresp up-regulation of CD154 (the ligand for CD40) and CD69 that can be detected 6-24 hours after activation has been shown to correlate well with the gold standard suppressor cell assays [9].

Zeta-associated protein (Zap)70 is a tyrosine kinase that is central for human and murine T-cell development and activation [10]. Following TCR stimulation a series of phosphorylation events activate the Src family Lck that lead to rapid phosphorylation of Zap70. Zap70 binds the CD3-zeta chain and phosphorylates the transmembrane protein linker-of-activated T cells. These events induce transcription of diverse gene products, culminating in cytokine secretion by T cells as well as proliferation and enhancement of other T cell functions [10]. Zap70 phosphorylation occurs within minutes after activation of T cells [11]. Accordingly, we hypothesized that activated Treg can suppress Zap70 phosphorylation in activated Tresp. We tested our

hypothesis in the adenosine deaminase (ADA) enzyme deficient mice (ADA-KO) that similar to ADA-deficient patients display autoimmunity and impaired Treg function [12].

Here we demonstrate that Treg suppression of Zap70 phosphorylation in Tresp can be detected already 2 hours after activation. We also show that Tresp Zap70 suppression by ADA-deficient Treg is improved after correction of the metabolic defect in ADA-KO mice.

Methods

Reagents

RPMI 1640 medium was supplemented with 10% heat-inactivated FCS, 1 mM glutamine, 10 mM HEPES, 100 µM nonessential amino acids, penicillin/streptomycin and 2.5×10^{-5} M 2-Mercaptoethanol, all from Invitrogen (Grand Island, NY, USA). Functional grade monoclonal antibodies anti-CD3ε (clone 145-2C11) and anti-CD28 (clone 37.51) were from ebioscience (San Diego, CA, USA). Intracellular Fixation and Permeabilization Buffer Set, anti mouse CD4 FITC (RM4-4), CD25 PE (PC61.5), CD69 APC (H1.2F3), anti-human/mouse phospho-Zap70/SYK (Y319/Y352) APC (n3kobu5) antibodies were also from eBioscience.

Animals

C57BL/6J and ADA-KO mice (FVB, 129-Adatm1Mw-TgN[PLADA]) from Jackson Laboratories (Bar Harbor, ME, USA) were maintained in pathogen free environment with strictly controlled temperature, relative humidity and a 12 hrs light/dark regimen. Mice were fed with standard lab mouse chow and provided water ad libitum. To prevent the typical 3-week lethality from early respiratory failure that uniformly affects ADA-KO mice [13], mice were rescued with polyethylene glycol conjugated bovine ADA (PEG-ADA) as we previously described [14]. Mice received high dose (2.5 unit/gram body weight) PEG-ADA [ADAGEN, Enzon Pharmaceuticals, Piscataway, NJ, USA] by intra-peritoneal injections, twice a week, from 7 days post-partum until 3 weeks of age. PEG-ADA was continued in some ADA-KO mice (defined as "treated" mice) for additional 2-3 weeks, while in other ADA-KO mice PEG-ADA was substituted with phosphate buffered saline at the same volume (defined as "untreated" mice). All procedures involving animals were approved by the institute's Animal Care Committee and performed in accordance with the guidelines of the Canadian Council for Animal Care.

Cells isolation, stimulation, activation, suppression and analysis

Cell suspensions were prepared from spleens of 4-6 weeks old C57BL/6J, ADA-KO and ADA-proficient littermates mice, minced and passed through a sterile wire mesh in phosphate buffered saline supplemented with 0.5% FBS. Following red blood cell lysis, Treg were isolated using MACS $CD4^+CD25^+$ T cell isolation kit II (Miltenyi Biotec, San Diego, CA, USA) according to the manufacturer's instructions, typically resulting in >90% $CD4^+CD25^+$ purity. Tresp ($CD4^+CD25^-$) were isolated similarly from the spleens of ADA-proficient mice only, as ADA-deficient Tresp have reduced stimulation responses [12]. Tresp were stimulated with 5 µg/ml of anti-CD3 and anti-CD28 antibodies, as described previously [9]. Zap70 phosphorylation (following fixation and permeabilization) and surface CD69 expression were determined by flow cytometry (using the FACSCalibur, Becton-Dickinson, San Jose, CA, USA) at the indicated times.

Zap70 suppression was assayed by culturing Treg (2×10^5 cells/well) activated using 5 µg/ml of anti-CD3 and anti-CD28 antibodies with Tresp (1:1 ratio) in sterile 96-well U-bottomed plates (Nunc) in 220 µl of medium, at 37°C, 5% CO_2 for the indicated time periods. Cells were then stimulated again with 5 µg/ml of anti-CD3 and anti-CD28 antibodies and stained after 3 minutes to detect Zap70 phosphorylation. Expression of phosphorylated Zap70 or CD69 were measured by flow cytometry in Tresp by gating on the $CD4^+CD25^-$ population. Analysis was performed using the CELLQuest Pro software program. Suppression (S) was calculated as: $S=100-([A/B] \times 100)$ where A is percentage of Tresp with phosphorylated Zap70 or expressing CD69 in the presence of Treg and B is the percentage of Tresp with phosphorylated Zap70 or expressing CD69 in the absence of Treg. Non-activated and non-stimulated Tresp control cultures, as well as appropriate isotype controls were included in all experiments. Results are expressed as mean ± standard deviation (SD) if not stated otherwise. Differences between groups were determined by unpaired Student's *t* test, and $p<0.05$ was considered statistically significant.

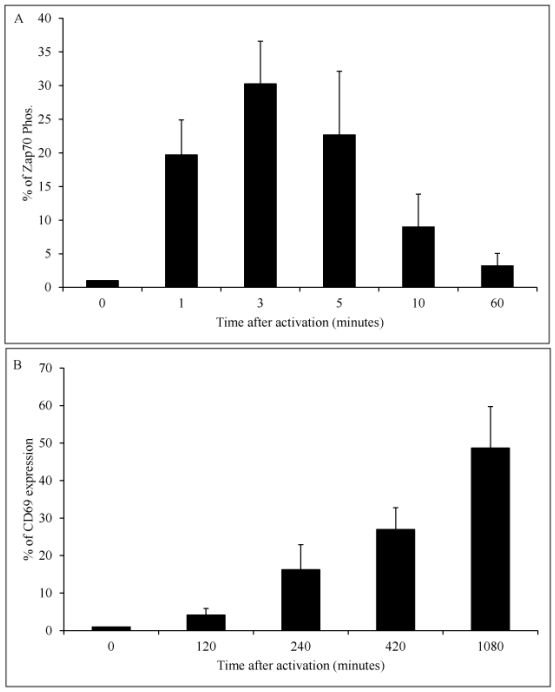

Figure 1: Zap70 phosphorylation and CD69 expression in stimulated Tresp cells. Tresp ($CD4^+CD25^-$) were stimulated for the indicated times with anti-CD3 and anti-CD28 mAbs. The percentages of cells with either **(A)** Phosphorylated Zap70 or **(B)** CD69 expression were determined by flow cytometry among Tresp. Results, expressed as mean ± standard deviation are from 3 different experiments with N=3-5 mice in each experiment.

Results

We initially determined the rate and percentage of Tresp with phosphorylated Zap70 after stimulating the cells with anti-CD3 and anti-CD28 antibodies. Zap70 phosphorylation was evident already 1

minute after the stimulation of Tresp and was eventually detected in as many as 30 ± 7% of the cells at 3 minutes (Figure 1A). Yet, the phosphorylated Zap70 increase was transient, as at 10 minutes after stimulation, phosphorylation significantly (p=0.002) decreased and was identified in only 14 ± 5% of the cells. Therefore, in subsequent assays, Zap70 phosphorylation was assessed at 3 minutes after Tresp stimulation. Measuring CD69 expression in Tresp, albeit at much later times, provided confirmation of cells' stimulation (Figure 1B). A marked increase in CD69 expression was noted 240 minutes after stimulation of Tresp, which reached 49 ± 11% at 1040 minutes.

The ability of activated Treg to suppress Zap70 phosphorylation in stimulated Tresp (cultured at 1:1 ratio) was then determined (Figure 2A). Reduced Zap70 phosphorylation was evident already 1 hour after activation, which was even more pronounced 2 and 4 hours after activation (50 ± 13% and 60 ± 18% suppression, respectively). In contrast, reduced CD69 expression was detected only 4 hours after activation of Tresp, with 51 ± 8% suppression achieved after 7 hours (Figure 2B). To validate Treg ability to suppress Tresp Zap70 phosphorylation, the effects of Treg to Tresp ratio on Zap70 phosphorylation in activated Tresp were also assessed. The ability of Treg to suppress Zap70 Tresp declined as the Treg to Tresp ratio was reduced (Figure 3).

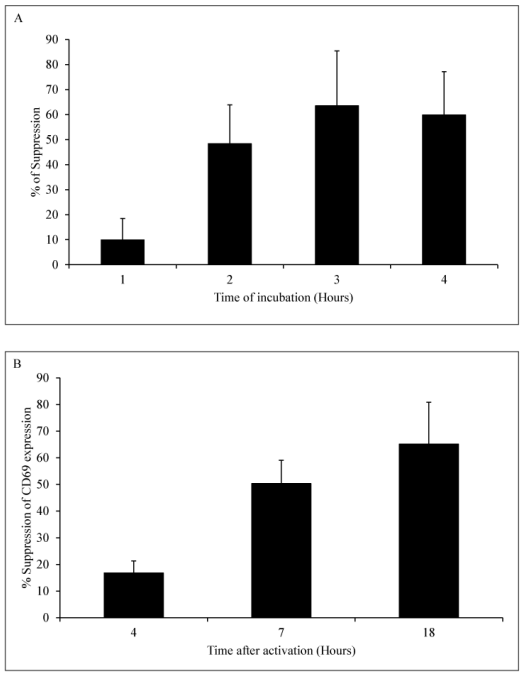

Figure 2: Suppression of Zap70 phosphorylation and CD69 expression in stimulated Tresp following incubation with Treg. Tresp ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+$) incubated at 1:1 ratio were stimulated and activated with anti-CD3 and anti-CD28. The percentages of Tresp with either **(A)** Phosphorylated Zap70 or **(B)** CD69 expression were determined by flow cytometry at the indicated times among Tresp. Suppression was calculated as the percentage of fluorescent Tresp in the presence or absence of Treg. Results, expressed as mean ± standard deviation, are from 3 different experiments with N=3-5 mice in each experiment.

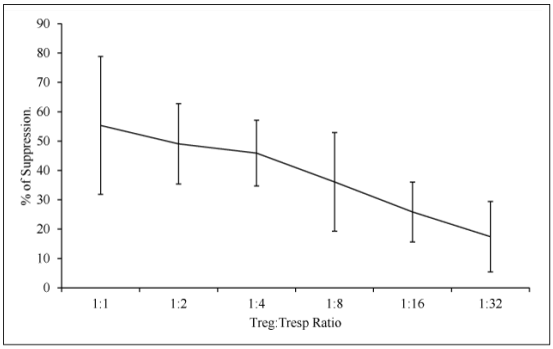

Figure 3: Effect of Treg to Tresp ratio on Zap70 expression in Tresp. Tresp ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+$) activated with anti-CD3 and anti-CD28 were incubated for 2 hours at the indicated ratios. The percentages of Tresp with phosphorylated Zap70 were determined by flow cytometry 3 minutes after stimulation. Suppression of Zap70 suppression was calculated as the percentage of fluorescent Tresp in the presence or absence of Treg. Results, expressed as mean ± standard deviation, are from 3 different experiments with N=3-5 mice in each experiment.

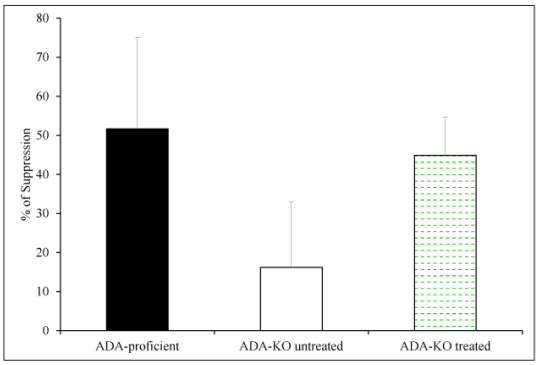

Figure 4: Suppression of Zap70 phosphorylation in Tresp by Treg from untreated or PEG-ADA treated ADA-deficient mice. Treg ($CD4^+CD25^+$) from untreated adenosine deaminase- deficient (ADA-KO) mice, or from ADA-KO mice treated with polyethylene glycol conjugated ADA (PEG-ADA) or from ADA-proficient littermate mice were co-incubated for 2 hours at 1:1 ratio with anti-CD3 and anti-CD28 stimulated ADA-proficient Tresp ($CD4^+CD25^-$). The percentages of Tresp with phosphorylated Zap70 were determined by flow cytometry 3 minutes after stimulation. Suppression of Zap70 phosphorylation in Tresp was calculated as the percentage of fluorescent Tresp with or without Treg. Results, expressed as mean ± standard deviation, are from 3 different experiments with N=3-5 mice in each experiment.

To further test Treg suppression of Tresp Zap70 phosphorylation, we used the ADA-KO mouse model, as ADA-deficient Treg have been shown to have reduced ability to suppress activated Tresp [12]. Indeed, Treg from untreated ADA-KO mice suppressed Zap70 phosphorylation in activated Tresp from ADA-proficient mice by only 16.2 ± 16.7%, which was significantly (p=0.012) lower than the 51.6 ± 23.4% Zap70 suppression induced by Treg from healthy ADA-

proficient littermates (Figure 4). PEG-ADA treatment significantly (p=0.021) improved ADA-KO Treg ability to suppress phosphorylated Zap70 expression in Tresp to 45 ± 10%, which was not different (p=0.772) than the expression in Tresp from ADA-proficient littermates (Figure 4). The number of viable spleen cells was not significantly different (p=0.130) between untreated ADA-KO mice (7.1 ± 4.6 × 10^6) and PEG-ADA treated ADA-KO mice (12.8 ± 3.3 × 10^6), suggesting that the improvement of Treg ability to suppress Zap70 expression was independent of other effects of PEG-ADA on the immune system.

Discussion

The results presented here establish that Treg inhibit Zap70 phosphorylation in activated Tresp. Phospho-flow has been shown to be an excellent tool to detect intracellular signaling in complex populations of cells [15] including Zap70 phosphorylation in $CD4^+$ T cells [16]. We show that Zap70 phosphorylation occurs within few minutes of Tresp stimulation and is detected for only 10 minutes, similar to the short period of Zap70 phosphorylation previously reported [11]. Treg were reported to require several hours for exerting their effect on Tresp [9], therefore we employed a "2-stages approach" for activating Treg and then measuring Zap70 phosphorylation in Tresp using anti-CD3 and anti-CD28 antibodies for both stages. Whether alternative methods, such as initial activation of Tresp with PMA and ionomycin that act downstream of Zap70, followed by stimulation of the T cell synapse through CD3 and CD28, IL2 or other T cell stimulators, will produce similar or more robust responses than observed here, will need to be studied. Interestingly, although Treg have been shown to increase Zap70 phosphorylation after stimulation [10], and Tresp isolation purity in the current study was >90%, Zap70 phosphorylation was not detected in all cells, possibly because of "contamination" with non-responsive cells. Alternatively, the anti-CD3 and anti-CD28 used here might have not been sufficient to stimulate all Tresp, as also suggested by the limited CD69 expression on only approximately 50% of the cells.

The ability to readily follow Treg activation by measuring Zap70 phosphorylation in Tresp, enabled determining the time required for Treg effects. Suppression of Zap70 phosphorylation in activated Tresp was evident already 1 hour after co-culture with Treg and the suppression of Zap70 phosphorylation continued to increase over the following hours. Thus, detection of Zap70 suppression as early as 1 hour may provide further insight into the mechanisms of Treg action, as completion of de novo gene transcription-translation and protein synthesis requires longer time. Hence Treg early effects might be due to rapid release of preformed cytokines. Further studies will be required to identify the precise mechanisms by Treg exert their effects, as well as the relevance of these *ex vivo* findings to the actual suppressive functions of Treg.

Alterations in adenosine metabolism disrupt Treg function in ADA-deficient SCID and may contribute to the autoimmunity in ADA-KO mice [12]. Therefore, we used the ADA-KO mouse model to further demonstrate Treg suppression of Zap70 phosphorylation and the effects of correcting the metabolic abnormalities on Treg function. Indeed, Treg from untreated ADA-KO mice had markedly reduced the ability to suppress Zap70 phosphorylation. Yet, in contrast to the previous report [12], in the current study PEG-ADA treatment normalized Treg function. The differences between the studies might be due to the higher PEG-ADA dose and shorter treatment period used here.

Our study has several potential caveats. Similar to previous reports [5], Treg were distinguished from Tresp based on expression of CD4 and CD25 without inclusion of FoxP3, CD127 or other Treg characteristics in the isolation panel. In mice there is >95% correlation between CD4/CD25 positivity and FoxP3 [8], hence for the "proof-of-concept" purposes of the current study, the CD4/CD25 distinction was deemed sufficient. Additional cell identifiers might be required when analyzing other effects of Treg on Tresp. Although the expression of CD25 is upregulated on activated T cells, this expression can be detected in flow cytometry only 15 hours after T cells activation [17], making the possibility that our assay selected non-activated or non-responsive cells among Tresp cells unlikely. Also, while we assessed the effects of Treg on Tresp Zap70 phosphorylation, Treg have many additional roles that may not correlate with Zap70, including effects on antigen-presenting cells, de novo cytokine production, etc. Further studies are needed to show that differences in Zap70 phosphorylation correlate with the actual suppression of T cell responses such as Tresp proliferation and/or cytokine production.

Conclusions

This study demonstrates that Treg suppress Zap70 phosphorylation in activated Tresp, a finding that might help better understand and assess Treg function.

Declarations

- All procedures involving animals were approved by the Hospital for Sick Children's Animal Care Committee and performed in accordance with the guidelines of the Canadian Council for Animal Care.
- The manuscript does not contain any individual person's data in any form.
- All authors declare that there are no competing interest with interpretation of data or presentation of information. Authors also do not have any financial or non-financial competing interests.
- YF made substantial contributions to conception and important intellectual contribution to the design as well as acquisition of data, analysis and interpretation of data. YF also drafted the manuscript. WM made substantial contributions to design and acquisition of data. EG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
- The data set supporting the results of this article are included within the article and its additional files.

Acknowledgments

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