

# Tyrosine phosphatase SHP-1 negatively regulates suppressive potential of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells

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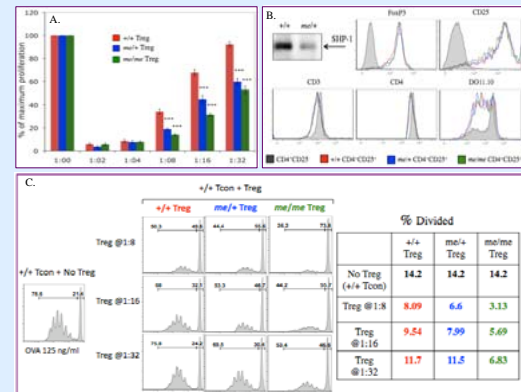


## 1. Introduction

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T (Treg) cells are well recognized as mediators of peripheral immune tolerance. However, their function remains poorly understood at the molecular and mechanistic level. We have previously shown that mice, which are deficient in the protein tyrosine phosphatase SHP-1 (*motheaten* or *me/me* mice), have increased numbers of Treg cells. Since SHP-1 is a negative regulator of TCR-mediated signaling in conventional T cells, we investigated whether SHP-1 plays a regulatory role in Treg cells. A comparison of the suppressive activities demonstrated that Treg cells derived from *me/me* or *me/+* mice suppressed *in vitro* lymphocyte proliferation more efficiently than Treg cells from wild type mice indicating that SHP-1 is a negative regulator of Treg-cell mediated suppression. Since based on microarray analyses, the differences in suppressive activities are not due to developmental disparities, our data suggest that SHP-1 directly regulates the suppressive activity of Treg cell. To gain a better mechanistic understanding of Treg cell-mediated suppression, flow cytometric and in particular ImageStream (Amnis) analyses were performed. These studies showed that in the absence of SHP-1, Treg cells express different levels of a number of surface and intracellular molecules. Moreover, SHP-1 deficiency promoted increased conjugate formation between Treg cells and antigen presenting cells, as visualized by ImageStream. Conjugate formation with SHP-1-deficient Treg cells caused a more efficient down-modulation of the co-stimulatory molecules CD80/CD86 on the dendritic cells. Taken together, our data indicate that SHP-1 regulates the function and activation status of Treg cells and that under conditions of SHP-1 deficiency, Treg cells become more potent.

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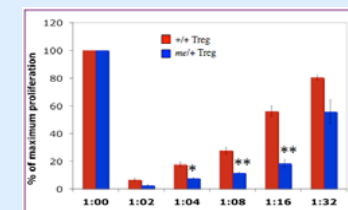
## 2. *me/me* and *me/+* CD4<sup>+</sup>CD25<sup>+</sup> Treg cells suppress the proliferation of conventional T cells more efficiently than *+/+* Treg cells



(A) CD4<sup>+</sup>CD25<sup>+</sup> conventional T cells (Tcon) and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) were isolated from lymph nodes of 18-20 days old DO11.10 TCR-Tg mice using MACS micro-beads (Miltenyi biotec). Purified Treg cells from *+/+*, *me/+* and *me/me* mice were added to 2.5x10<sup>4</sup> Tcon cells (from *+/+* DO11.10 mice) at the ratios indicated. Cells were stimulated with 125ng/ml of OVA peptide (aa323-339) in the presence of 5x10<sup>4</sup> irradiated splenocytes as APCs and cultured for 72h. Proliferation was assessed by <sup>3</sup>H-thymidine incorporation assay. Proliferation of Tcon cells in the absence of any Treg cells is set as 100%. Error bars denote ± SEM.

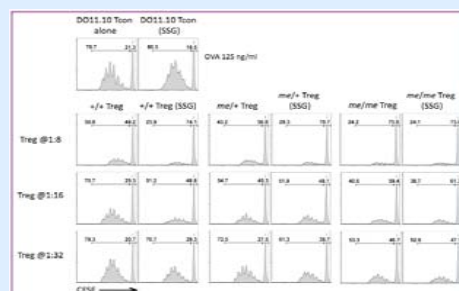
(B) To assess the protein levels, SHP-1 was immunoprecipitated from equal numbers of Treg cells purified from *+/+* and *me/+* mice. Immunoprecipitates were resolved by 10% SDS-PAGE and subjected to anti-SHP-1 immunoblotting. In addition, CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from lymph nodes of *+/+*, *me/+* and *me/me* mice were stained with antibodies recognizing indicated surface molecules followed by flow cytometric analysis. Protein expression levels on *+/+* CD4<sup>+</sup>CD25<sup>+</sup> T cells are shown for comparison. (C) As a complementary approach, proliferation of Tcon cells was assessed by CFSE dilution. Suppression assays were set up as described in A. Cells were cultured for 4 days followed by flow cytometric analyses.

## 3. Genetically SHP-1-deficient Treg cells maintain the increased suppressive potential



In order to further analyze whether SHP-1-deficient Treg cells from older mice continue to show an increased ability to suppress Tcon cell proliferation, Treg cells from 8 weeks-old *me/+* and *+/+* mice (DO11.10 TCR-Tg) were compared. MACS-separated *+/+* Tcon cells and *+/+* and *me/+* Treg cells were cultured for 72h at the ratios indicated. Irradiated splenocytes were used as APCs. Proliferation in response to 125 ng/ml of OVA peptide (aa323-339) was measured by <sup>3</sup>H-thymidine incorporation.

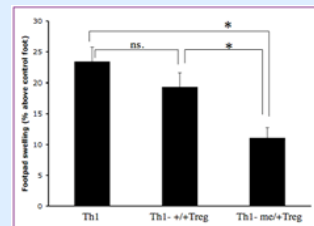
## 4. Sodium stibogluconate (SSG), a specific inhibitor of SHP-1, increases the suppressive potential of *+/+* and *me/+* Treg cells, but not *me/me* Treg cells



The SHP-1-specific inhibitor Sodium stibogluconate (SSG) was added to the *in vitro* suppression assay to study effects of direct pharmacological inhibition of SHP-1 activity on the suppressive capacity of Treg cells *+/+*, *me/+* or *me/me* Treg cells were added to the culture of 2.5x10<sup>4</sup> CFSE-labeled Tcon cells at the ratios indicated. Proliferation was assessed in response to 125 ng/ml of OVA peptide in the presence of 5x10<sup>4</sup> APCs (irradiated T cell-depleted splenocytes). The assays were set up with or without addition of 10µg/ml SSG. Cells were cultured for 4 days followed by flow cytometric analysis.

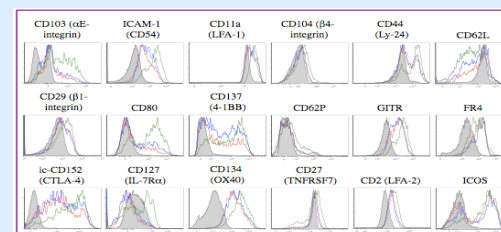
Addition of the SHP-1 inhibitor (SSG) enhances suppressive capacity of *+/+* and *me/+* Treg cells, whereas *me/me* Treg cells are insensitive.

## 5. *me/+* Treg cells are more efficient in suppressing a DTH response than *+/+* Treg cells.



Th1 cells were generated from purified CD4<sup>+</sup>CD25<sup>+</sup> T cells (from lymph nodes of DO11.10 TCR Tg mice) using a standard protocol. 1.5x10<sup>6</sup> cells were injected into the footpads of BALB/c mice along with equal numbers of irradiated T cell-depleted splenocytes and 10µg/ml OVA peptide. For conditions where *+/+* or *me/+* Tregs were included in the cell mixture, Treg cells were added at a ratio of 1:4 of Treg:Th1 cells. The final volume of injected cells was 25µl. Footpad measurements were taken before injection and 24h following the injections.

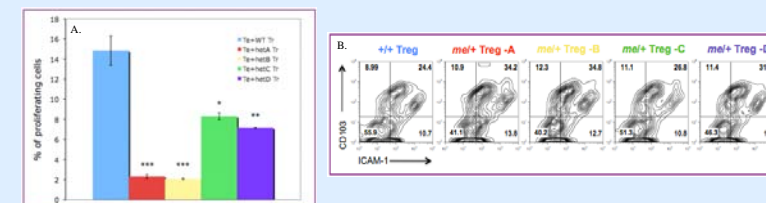
## 6. SHP-1-deficiency increases percentage of Treg cells with activated phenotype



CD4<sup>+</sup> T cells were isolated from lymph nodes of *+/+*, *me/+* and *me/me* mice and stained for flow cytometric analysis. For intracellular staining, cells were permeabilized using the cell permeabilization kit from eBioscience. Stained cells were collected on a FACS Calibur instrument and analyzed using FlowJo software. Populations of live CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells were gated and analyzed for the

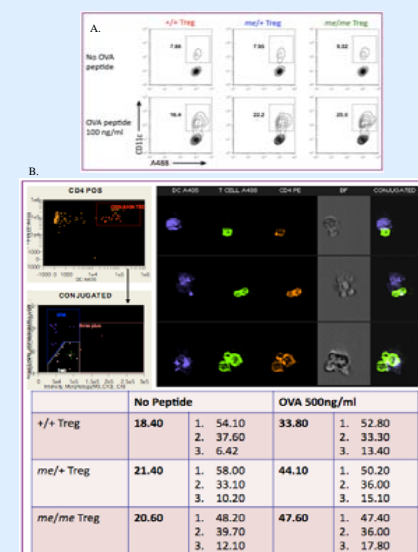
expression of each individual surface or intracellular molecule. Examples of molecules, whose expression pattern is changed between *+/+*, *me/+*, and *me/me* Treg cells, are depicted. Shaded histograms represent CD4<sup>+</sup>CD25<sup>+</sup> derived from *+/+* mice.

## 7. Suppressive activity of Treg cells correlates with surface expression of CD103 and ICAM-1



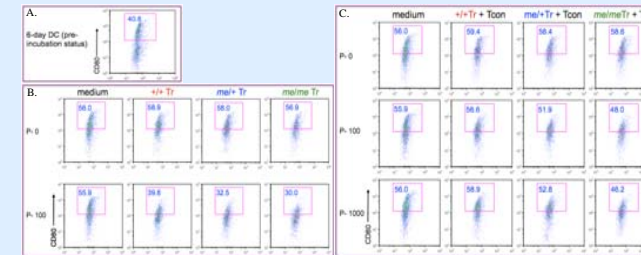
Treg cells were isolated from individual *me/+* mice, and the suppressive activities were compared to *+/+* Treg cells. (A) Treg cells of *me/+* (A, B, C and D) or *+/+* genotype (DO11.10 TCR-Tg) were added to 2.5x10<sup>4</sup> *+/+* Tcon cells at Treg : Tcon ratio of 1:8, along with 5x10<sup>4</sup> APCs and OVA peptide (125ng/ml). Percentages of proliferation were calculated based on Tcon proliferation in the absence of regulatory T cells. Each data point represents an individual mouse. (B) Surface expressions of CD103 and CD54 (ICAM-1) (BD Biosciences) on Treg cells of the indicated *me/+* mice are shown.

## 8. SHP-1 deficient Treg cells form more conjugates with BMDC than *+/+* Treg cells



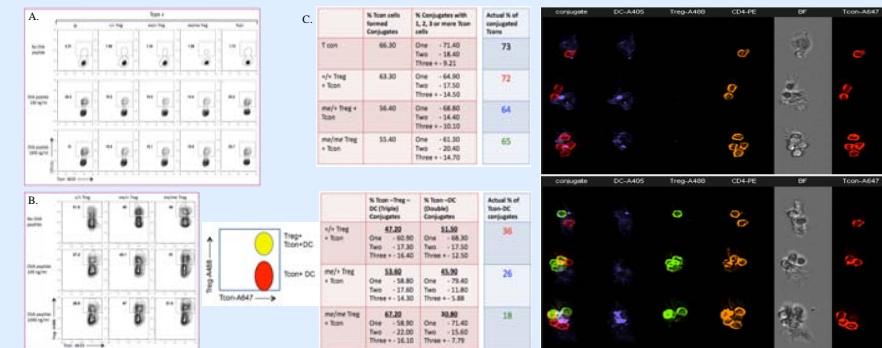
Bone marrow-derived dendritic cells (BMDCs) were differentiated in the presence of 10ng/ml GM-CSF and 10ng/ml IL-4. BMDCs were harvested on day 5 and set up in a conjugation assay with Treg cells isolated from *+/+*, *me/+*, and *me/me* mice (DO11.10 TCR-Tg) at 1:1 ratio. Treg cells pre-stained with Alexa-488 succinimidyl ester (Molecular Probes) were incubated with BMDC for 4h at 37°C in a final volume of 200µl followed by staining with anti-CD4 and anti-CD11c for additional identification of CD4 T cells and CD11c<sup>+</sup> BMDCs. Cells were collected either on a FACS Calibur or on an ImageStream instrument (Amnis Corporation; Seattle, WA). Samples collected on ImageStream instrument were acquired using the EDF (Extended Depth of Field Technology) program. Data analyses were performed using FlowJo or IDEAS<sup>TM</sup> analytical software respectively with a focus on assessment of conjugate formation between BMDCs and Treg cells. On the FACS Calibur, the conjugates are identified as the population being positive for CD4 and Alexa-488 (Treg cells) and CD11c (BMDCs) (A). For ImageStream analyses, BMDCs were stained with Alexa-405 and the conjugates are identified as being positive for CD4, Alexa-488 (Tregs) and Alexa-405 (BMDCs) (B).

## 9. SHP-1-deficient Treg cells are more effective inhibitors of BMDC function than wild type Treg cells



(A) The level of CD80 expression on BMDCs on day 6. (B) Treg cells from *+/+*, *me/+* and *me/me* mice were added to the BMDCs for 24h at the indicated ratios. OVA peptide was added as indicated. Cells were harvested and stained for CD11c (eBiosciences), CD4 and CD80 (BD Bioscience) followed by flow cytometric analyses. CD4<sup>+</sup> cells if any, still conjugated to CD11c<sup>+</sup> cells were excluded. Profiles of CD80 expression on gated CD11c<sup>+</sup> BMDCs are shown. (C) The same experimental set-up as in B, but *+/+* Tcon cells are added to the BMDC/Treg culture to assess the effect of Treg cells under conditions of Tcon activation.

## 10. SHP-1-deficient Treg cells are more efficient in inhibiting conjugate formation between Tcon cells and BMDCs



BMDCs were differentiated by culturing with 10ng/ml GM-CSF and 10ng/ml IL-4 and were harvested on day 5 and set up in a conjugation assay with *+/+* Tcon cells either alone or along with Tregs isolated from *+/+*, *me/+* and *me/me* mice (DO11.10 TCR-Tg) at the ratios indicated. Tcon cells pre-stained with Alexa-633 were incubated with BMDC and OVA peptide for 4h at 37°C in a final volume of 200µl. Tregs pre-stained with Alexa-488 were included at a 1:1 ratio with Tcons. Following incubation, cells were stained with anti-CD4 and anti-CD11c for additional identification of CD4 T cells and CD11c<sup>+</sup> BMDCs. Conjugate formation between BMDCs and Tcon cells were assessed using flow cytometry and FlowJo software. (A) Tcon/BMDC conjugates were identified as being positive for CD4 and Alexa-633 (Tcon cells) and CD11c (BMDCs). (B) Triple conjugates were identified as the population positive for CD4, Alexa-488, Alexa-633 and CD11c<sup>+</sup>.

(C) For the analysis of conjugates on the ImageStream, BMDCs from day 5 were stained with Alexa-405 and used for the conjugate assays as described above. Cells were collected on an ImageStream instrument using the EDF program for sample acquisition followed by data analysis with IDEAS<sup>TM</sup> analytical software. The actual percentages of Tcon-DC conjugates were calculated after adjusting for the multiple conjugates formed by these cells. Tcon cells, which are found in conjugation with BMDCs, but not with Tregs, are considered Tcon cells with proliferation potential.

## 11. Summary

- Treg cells genetically deficient for SHP-1 (*me/+* and *me/me*) have increased suppressive activity compared to *+/+* Treg cells, independent of age.
- The SHP-1-specific inhibitor SSG enhances the suppressive activity of *+/+* and *me/+*, but not *me/me* Treg cells, further indicating that SHP-1 is a negative regulator of Treg function. Therefore SHP-1 might be a potential drug target for increasing Treg cell function.
- SHP-1-deficiency increases percentage of Treg cells with activated phenotype as assessed by expression of surface and intracellular proteins and suppressive activity directly correlates with increased surface expression of CD103 and ICAM-1.
- me/me* and *me/+* Treg cells form more conjugates with BMDC than *+/+* Treg cells at sub-optimal peptide concentration and at early time points, which might at least partially explain the increased suppressive potential of SHP-1-deficient Treg cells.
- me/me* Treg cells are more effective in down-modulating CD80 and CD86 on BMDC, which might contribute to their increased suppressive activity.
- SHP-1-deficient Treg cells are efficient in inhibiting Tcon cells from conjugate formation with BMDCs. Furthermore, the majority of Tcon/BMDC conjugates formed in the presence of Treg cells contain Treg cells, which inhibits their activation.
- Increased signaling through the TCR heightens the suppressive efficiency of *+/+* and *me/+* Treg cells, but not *me/me*. This indicates that SHP-1 is a negative regulator of TCR-mediated signaling in Treg cells, thereby affecting the suppressive potential of Treg cells. However, increasing the signaling via the TCR in *+/+* or *me/+* Treg cells never increases their suppressive activity to the level of *me/me* Treg cells indicating that SHP-1 regulates additional pathways in Treg cells.

SHP-1 negatively regulates the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells.