

SHP-1 influences resistance of CD4⁺ T cells to regulatory T cell suppression

Mohan Sankarshanan, Tessy Iype, Ileana Mauldin and Ulrike M. Lorenz

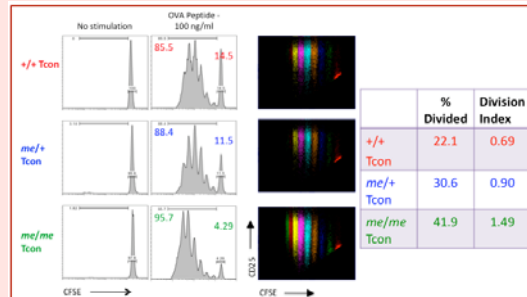
Department of Microbiology and Carter Immunology Center, University of Virginia, Charlottesville, VA 22908



1. Introduction

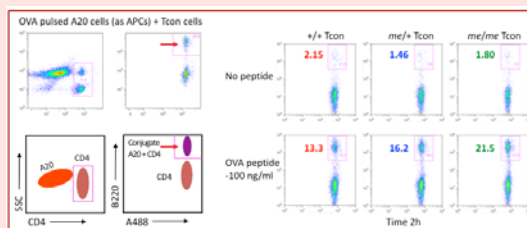
The mechanistic and molecular understanding about factors that regulate the susceptibility and/or resistance of T cells to suppression by regulatory T cells (Treg) is very limited. The protein tyrosine phosphatase SHP-1 is a negative regulator of TCR-mediated signaling. SHP-1-deficient T cells hyperproliferate in response to antigen stimulation. We therefore asked whether SHP-1 affects the susceptibility of CD4⁺ T cells to suppression by Treg cells. Our *in vitro* suppression assays show that heterozygous (*me/+*) and homozygous (*me/me*) CD4⁺ T cells are more resistant to Treg cell-mediated suppression than *+/+* CD4⁺ T cells, indicating that SHP-1 modulates the resistance to Treg cell suppression. In order to gain a better understanding of the mechanism that controls the resistance to suppression, we examined the conjugate formation between CD4⁺ T cells and the antigen presenting cells (APCs). Using flow cytometric and ImageStream (Amnis) analyses, we observed that *me/me* and *me/+* T cells form more conjugates with APCs than *+/+* T cells both in the presence or absence of Treg cells. Moreover, *+/+* T cells treated with an inhibitor of SHP-1 showed a comparable increased conjugate formation as SHP-1-deficient T cells indicating that the observed phenotype is T cell intrinsic and not due to developmental differences. Furthermore, SHP-1-deficient T cells exhibited elevated surface levels of ICAM-1. Ongoing studies are addressing whether ICAM-1 directly affects the resistance to suppression. Taken together, the increased resistance to suppression displayed by SHP-1-deficient T cells suggest that the ability of forming T cell-APC conjugates, influences the susceptibility and/or resistance of CD4⁺ T cells to suppression. Grant support: NIH, RO1 AI 048672

2. Tcon cells from *me/+* and *me/me* mice hyper proliferate in response to antigen stimulation



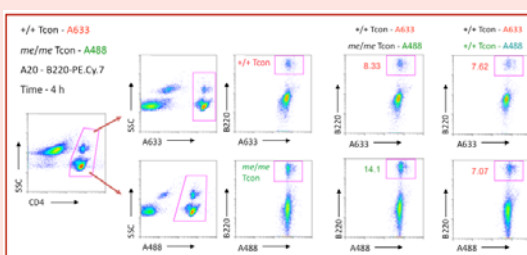
CD4⁺CD25⁻ conventional T cells (Tcon) were isolated from lymph nodes of 18-20 days old DO11.10 TCR-Tg mice using MACS micro-beads (Miltenyi biotec). 2.5x10⁴ Tcon cells from *+/+*, *me/+* and *me/me* mice were stained with CFSE and cultured with 5x10⁴ irradiated splenocytes for 4 days in the presence of 100ng/ml OVA peptide (aa323-339). Proliferation of Tcon cells was assessed by flow cytometry based on CFSE dilution.

3. SHP-1-deficient Tcon cells form more conjugates with antigen presenting cells than *+/+* Tcon cells



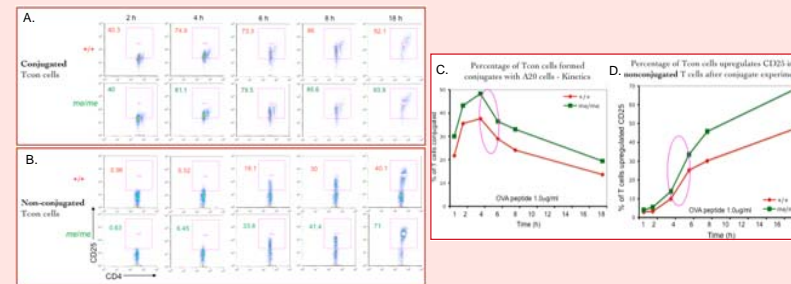
Tcon cells were stained with Alexa-488 succinimidyl ester (Molecular Probes) and incubated with OVA pre-loaded A20 cells for 2h at 37°C in a final volume of 200µl. T cells were further stained with anti-CD4 and anti-B220 antibodies for identification of CD4 T cells and A20 cells. Cells were collected on FACS Calibur and conjugate formation between A20 cells and Tcon cells was assessed. The conjugates are identified as the population being positive for CD4, Alexa-488 (Tcon cells), and B220 (A20 cells).

4. Increased conjugate formation of *me/me* Tcon cells is intrinsic to Tcon cells



In order to analyze whether SHP-1-deficient and *+/+* Tcon cells influence each other in their ability to form conjugates with APCs, *+/+* Tcon cells (stained with Alexa-633) and *me/me* Tcon cells (stained with Alexa-488) were mixed and incubated with OVA-loaded (100ng/ml) A20 cells for 4h at 37°C in a final volume of 200µl. Following incubation, T cells were stained with anti-CD4 and anti-B220 antibodies for identification of CD4 T cells and A20 cells and collected on FACS Calibur. The conjugates are identified as the population being positive for CD4 and Alexa-488 or Alexa-633 (Tcon cells) and B220 (A20 cells).

5. Kinetics of conjugate formation is not affected by SHP-1



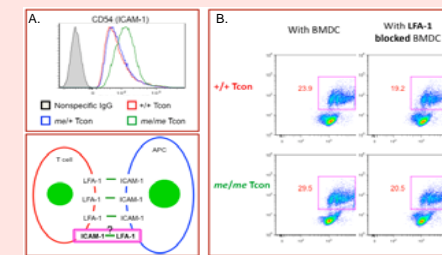
To understand whether the kinetics of conjugate formation between Tcon cells and APCs are affected by SHP-1, conjugate formation and subsequent CD25 upregulation was observed over a time course. Conjugate assays were performed for 2, 4, 6, 8, and 18h and both conjugated (A) and non-conjugated (B) cells were analyzed following staining for CD4 and CD25. The kinetics of conjugate formation between A20 cells and T cells (C) and the appearance of CD25 upregulation in the non-conjugated cells (D) are shown for *+/+* and *me/me* Tcon cells.

6. SHP-1-deficient Tcon cells form more multi-cellular conjugates with APCs than *+/+* Tcon cells - ImageStream analysis



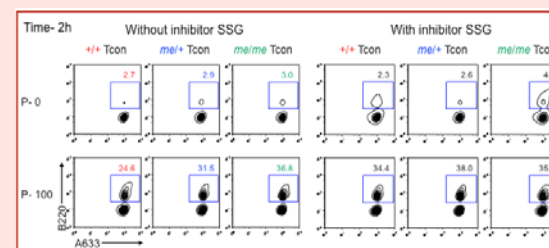
Bone marrow-derived dendritic cells (BMDCs) were differentiated in response to 10ng/ml GM-CSF and 10ng/ml IL-4 and harvested on day 5 for use in conjugate assays. Conjugation assays were set up between OVA-loaded (100 ng/ml) BMDCs and Tcon cells isolated from *+/+*, *me/+*, and *me/me* mice (DO11.10 TCR-Tg). Tcon cells, pre-stained with Alexa-488 were incubated with Alexa-405 stained-BMDC at a 1:1 ratio for 4h at 37°C in a final volume of 200µl. Following incubation, cells were stained with anti-CD4 antibody for additional identification of CD4⁺ Tcon cells. Cells were collected on the ImageStream instrument (Amnis Corporation; Seattle, WA) using the EDF (Extended Depth of Field Technology) program, and data were analyzed with IDEASTM analytical software.

7. High levels of ICAM-1 (CD54) on *me/me* T cells affect conjugate formation with BMDCs



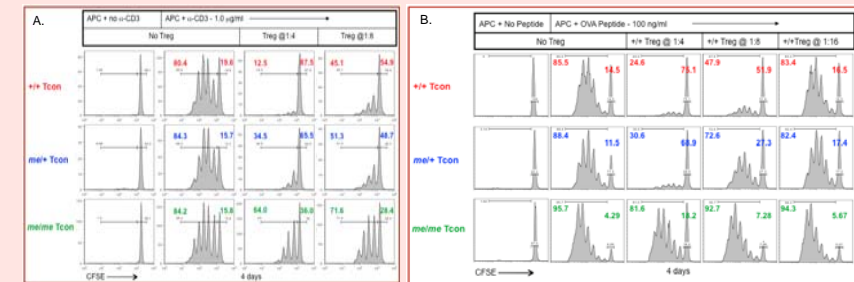
(A) *me/me* Tcon cells express elevated levels of CD54 compared to *+/+* and *me/+* mice. (B) Since ICAM-1 on the surface of T cells can interact with LFA-1 on the surface of the BMDCs, we asked whether the ICAM-1/LFA-1 interaction would facilitate conjugate formation. To inhibit the ICAM-1/LFA-1 interaction, conjugate assays were performed following antibody-mediated blocking of LFA-1 on the surface of BMDCs. The conjugate assay is set up as previously explained.

8. Sodium stibogluconate (SSG), a specific inhibitor of SHP-1, increases conjugate formation between APCs and *+/+* and *me/+*, but not *me/me* Tcon cells



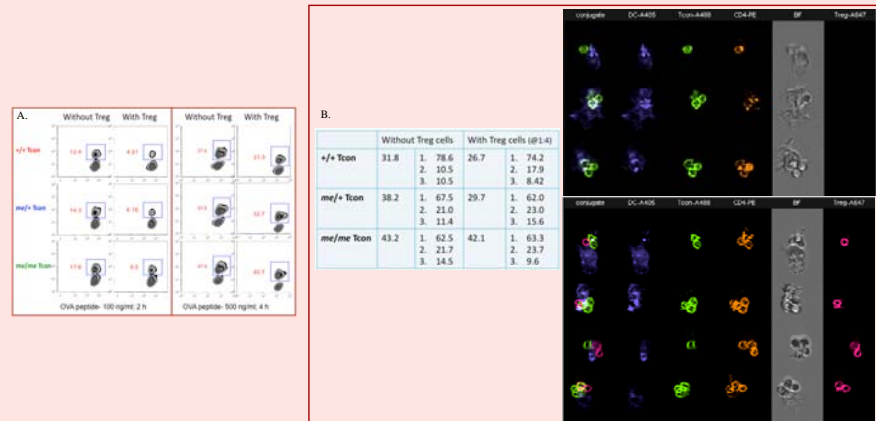
The direct effect of the inhibition of SHP-1 on conjugate formation was studied by using the SHP-1 inhibitor SSG at a concentration of 10 µg/ml, which is specific for SHP-1 inhibition. Tcon cells from *+/+*, *me/+* and *me/me* mice were treated with SSG for 1h before incubation with A20 cells for 2h at 37°C. All T cells were pre-stained with Alexa-633 and with anti-CD4 post incubation. A20 cells were identified by B220 staining.

9. SHP-1-deficient Tcon cells are more resistant to suppression by regulatory T cells than *+/+* Tcon cells



Since conjugate formation with APCs is thought to affect susceptibility to Treg cell-mediated suppression, we assessed whether SHP-1 plays a role in resistance to Treg cell-mediated suppression. CD4⁺CD25⁻ Tcon and CD4⁺CD25⁺ Treg cells were isolated from lymph nodes of 18-20 days old DO11.10 TCR-Tg mice using MACS micro-beads. 2.5x10⁴ Tcon cells from *+/+*, *me/+*, and *me/me* mice were stained with CFSE and cultured either with or without Treg cells (from wild type TCR-Tg mice) at the indicated ratios. Cells were stimulated with 1.0µg/ml of anti-CD3 antibody (2C11) (A) or 100ng/ml OVA peptide (B) in the presence of 5x10⁴ irradiated T cell-depleted splenocytes for 4 days. Proliferation of conventional T cells was assessed by flow cytometry based on CFSE dilution.

10. SHP-1 increases susceptibility to Treg-mediated inhibition of conjugate formation with APC



Conjugation assays were set up with the following cells: Tcon cells were isolated from *+/+*, *me/+* and *me/me* mice (DO11.10 TCR-Tg). Treg cells were purified from wild type DO11.10 TCR-Tg mice. BMDCs, which had been differentiated with 10ng/ml GM-CSF and 10ng/ml IL-4 for 5 days, were used as APCs. Tcon cells pre-stained with Alexa-488 succinimidyl ester (Molecular Probes) were incubated with OVA peptide-loaded BMDC for 2h or 4h at 37°C in a final volume of 200µl at 1:2 (left panel) or 1:1 (right panel) ratio of DC:T cell. In order to study the effect of Treg cells on the conjugate formation between Tcon cells and APCs, Tregs pre-stained with Alexa-633 were added at a 1:2 ratio with Tcon cells. Cells were stained with anti-CD4 and anti-CD11c for additional identification of CD4 T cells and CD11c⁺ BMDCs followed by flow cytometric analysis. (A) Conjugates formed between BMDCs and Tregs were assessed using FlowJo software. The conjugates are identified as the population being triple positive for CD4 and Alexa-488 (Tcon cells) and CD11c (BMDCs). (B) To visualize individual cell complexes using ImageStream instrumentation, BMDCs from day 5 were pre-stained with Alexa-405 and conjugate assays were set up as described above for 4h at 37°C. Cells were collected on a ImageStream instrument (Amnis Corporation; Seattle, WA) followed by data analysis with IDEASTM analytical software. The samples collected on ImageStream instrument were acquired using the EDF (Extended Depth of Field Technology) program. Percentages of conjugates formed by Tcon cells in the presence or absence of Tregs are given in the table. 1, 2 and 3 indicates % of conjugates with one, two or three or more Tcon cells per BMDCs.

11. Summary

- SHP-1-deficient conventional T cells (*me/+* and *me/me*) have increased proliferative potential compared to *+/+* T cells.
- SHP-1-deficient conventional T cells show increased ability to form conjugates with antigen presenting cells compared to *+/+* Tcon cells. This effect is T cell intrinsic.
- Kinetics of conjugate formation is not affected by SHP-1.
- When cells complexes were visualized using ImageStream technology, SHP-1-deficient Tcon cells had more numbers of cells conjugated to an individual APC than wild type Tcon cells.
- Increased levels of ICAM-1 on the surface of *me/me* Tcon cells seem to facilitate conjugate formation based on antibody-blocking experiments.
- Direct inhibition of SHP-1 using Sodium stibogluconate (SSG), a pharmacological inhibitor of SHP-1, increases conjugate formation of *+/+* and *me/+* Tcon cells with A20 cells, but does not affect *me/me* Tcon cells.

SHP-1-deficient Tcon cells are more resistant to suppression mediated by regulatory T cells, as evidenced by proliferation and conjugate formation.