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Human Seminal Plasma Fosters CD4+ Regulatory T cell Phenotype and Transforming Growth Factor- β 1 Expression

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Abstract

Problem—Semen mediates expansion of CD4+ regulatory T cells (Treg) in the murine female reproductive tract. The impact of semen on Treg in humans, however, remains unclear.

Method of Study—Using seminal plasma (SP) from 20 healthy donors, we investigated the impact of human semen on CD4+ T cell expression of CD127 and CD49d as well as CD4+ CD127^{low}CD49d^{low} Treg proliferation, apoptosis and intracellular expression of FoxP3, TGF- β 1 and IL-10.

Results—SP reduced CD4+ T cell expression of CD127 and CD49d and increased the proportion of CD127^{low}CD49d^{low} Treg. This increase was non-proliferative, mediated in part via the conversion of CD4+ helper T cells into FoxP3– but not FoxP3+ Treg. Additionally, SP induced an increase in intracellular expression of the immunosuppressive cytokine TGF- β 1 by the FoxP3– but not FoxP3+ Treg. SP had no impact on Treg intracellular expression of IL-10.

Conclusions—Human seminal plasma fosters the non-proliferative increase in the proportion and immunoregulatory activity of FoxP3– Treg.

Keywords

FoxP3; Seminal plasma; TGF- β ; Treg

INTRODUCTION

CD4+ regulatory T cells play a pivotal role in the modulation of cellular immune responses against autologous and foreign antigens ^{1–3}. This key minority population of CD4+ T cells is enriched at multiple immunological effector sites, including lymph nodes and mucosal surfaces such as the female reproductive tract ^{4–6}.

CD4+ regulatory T cells play a critical role in the immune control of pregnancy in mice and humans by dampening maternal immune reactivity to the semi-allogeneic fetus ^{5,7,8}. In both species, CD4+ regulatory T cell expansion occurs in the female reproductive tract in response to high circulating levels of estrogen during the follicular phase of the menstrual cycle and also during pregnancy ^{8–12}. In mice, presence of semen in the female reproductive tract has been shown to trigger expansion of the CD4+ regulatory T cell pool ^{13–15}, augmenting the effect of sex hormones in facilitating the induction of the maternal immunologic tolerance of conception ^{5,16,17}.

Beyond playing an important role in reproduction, CD4⁺ regulatory T cells also modulate and even exacerbate the immunopathogenesis of infectious diseases such as Leishmaniasis, tuberculosis and human immunodeficiency virus infection^{18–21}. Thus, CD4⁺ regulatory T cell mediated suppression of adaptive immune responses in the female reproductive tract may influence mucosal susceptibility to HIV and other sexually transmitted infections.

Natural CD4⁺ regulatory T cells characteristically express FoxP3, an immunoregulatory transcription factor which modulates CD4⁺ regulatory T cell development and effector function^{22–25}. CD4⁺ regulatory T cells exert their suppressive function via contact and non-contact dependent mechanisms²⁶, including secretion of the immunosuppressive cytokines TGF- β and IL-10, which in turn repress CD4⁺ helper T cell (Th) proliferation and effector function^{26,27}. In the periphery, FoxP3⁺ adaptive CD4⁺ regulatory T cells can be generated via conversion of pre-existing FoxP3[–] Th into FoxP3⁺ regulatory T cells via TGF- β mediated induction of FoxP3 expression^{25,28–30}, and more recently a third distinct class of fully functional FoxP3[–] adaptive CD4⁺ regulatory T cells has been identified that lack expression of FoxP3 and mediate Th suppression via the secretion of TGF- β but not IL-10^{25,31–33}. The relative frequency and contribution of these CD4⁺ regulatory T cell subsets to the modulation of immune responses in the female reproductive tract are the focus of active ongoing investigation⁶.

Currently, it is unclear whether semen modulates the activity of CD4⁺ regulatory T cells in humans. Taking advantage of the recent observation that CD4⁺ regulatory T cells can be purified untouched via low surface expression of the alpha chains of interleukin-7 receptor (CD127) and integrin $\alpha 4\beta 1$ (CD49d)²², we investigated the impact of human semen on the expansion and effector functions of the CD4⁺ CD127[–]CD49d[–] regulatory T cells (Treg). Understanding the impact of semen on the immunoregulatory activity of Treg in the female reproductive tract will inform the design of new strategies to improve fertility success and immune protection against the sexually transmitted infections, including HIV.

MATERIALS AND METHODS

Semen collection and processing

We collected semen from healthy male donors using a previously described research protocol³⁴. Briefly, semen was collected without the use of lubricants from 20 HIV-negative donors who gave consent in a research protocol approved by the Dartmouth Medical School Committee for the Protection of Human Subjects (CPHS). The mean age of semen donors and duration of abstinence before semen sample collection were 35 years and 3 days, respectively. All donors were asymptomatic for sexually transmitted infections and gene amplification tests for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were negative for all semen samples (Aptima Combo 2 assay, Gen-Probe Inc, San Diego, CA). Following dilution in sterile Phosphate Buffered Saline (PBS; Mediatech Inc., Manassas, VA), semen samples were centrifuged at $544 \times g$ for 10 minutes at room temperature and seminal plasma (SP) was collected from the supernatant for use fresh or after storage at -70°C . SP samples were used separately in each experiment. The final number of donor SP samples used for each experiment (n) is indicated in the figure legends.

Isolation and culture of PBMC

We isolated fresh peripheral blood mononuclear cells (PBMC) from 16 donors via ficoll density gradient centrifugation (Mediatech Inc., Manassas, VA) of platelet pheresis by-products obtained from individual HIV-negative donors different from semen donors via a research protocol approved by the Dartmouth CPHS. Subsequently, we cultured the isolated

PBMC at 37°C and 5% CO₂ in RPMI medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and amphotericin B (Mediatech Inc., Manassas, VA).

Assessment of the impact of SP on Treg FoxP3 expression, proliferation and apoptosis

To assess the impact of SP on Treg expression of FoxP3, we incubated PBMC at 37°C and 5% CO₂ in the presence or absence of 0.5% SP for 24, 48 and 72 hours. Cell cultures were not re-challenged with SP during the duration of incubation. Subsequently, cells were washed twice using sterile PBS supplemented with 1% fetal bovine serum (Mediatech Inc., Manassas, VA) and surface stained with fluorochrome-conjugated monoclonal anti-human antibodies specific for CD3 (mouse IgG1, κ ; clone UCHT1), CD4 (mouse IgG2b, κ ; clone OKT4), CD127 (mouse IgG1, κ ; clone A019D5) and CD49d (mouse IgG1, κ ; clone 9F10). Following surface staining, cells were fixed and permeabilized using the fixation and permeabilization buffers (eBioscience Inc., San Diego, CA) and stained for intracellular expression of the immunoregulatory transcriptional factor FoxP3 using a phycoerythrin-conjugated monoclonal antibody (mouse IgG1, κ ; clone 206D) in the presence of human gamma-globulin block (Sigma Aldrich, St. Louis, MO). Concurrently, cells were also stained for intracellular expression of the proliferation marker Ki67 (mouse IgG1, κ ; clone Ki-67) and pro-apoptotic marker activated caspase-3 (rat IgG; clone C92-605). All monoclonal antibodies were purchased from BioLegend (San Diego, CA), with the exception of anti-activated caspase-3 antibody (BD Biosciences, San Jose, CA). Data was acquired via multiparameter flow cytometry (Becton Dickinson FACS Canto, Sparks, MD). CD127-CD49d- regulatory T cells (Treg) were identified as CD3+CD4+ T cells lacking the surface expression of the alpha subunit of IL-7 receptor (CD127) and the alpha subunit of integrin $\alpha 4\beta 1$ (CD49d)²², and were further subdivided into FoxP3- Treg and FoxP3+ Treg based on the absence or presence of intracellular expression of FoxP3, respectively. Standard CD4+ helper T cells (Th) were identified as CD3+CD4+ T cells that were positive for both, CD127 and CD49d. Besides using CD127 and CD49d, we also identified CD4+CD25+ T cells via surface staining for the alpha chain of interleukin-2 receptor (CD25)²² using the anti-human CD25 antibody (mouse IgG1, κ ; clone BC96; BioLegend).

Assessment of the impact of SP on Treg expression of TGF- β 1 and IL-10

We determined the impact of SP on Treg expression of TGF- β and IL-10 using the Intracellular Cytokine Staining (ICS) protocol³⁵. PBMC were incubated with or without 0.5% SP for 24, 48 and 72 hours after which cells were treated for 4 hours with Brefeldin-A. Cell cultures were not re-challenged with SP during the duration of incubation. Subsequently, cells were washed, stained for surface markers CD3, CD4, CD127 and CD49d, fixed, permeabilized and stained with monoclonal antibodies for the intracellular expression of TGF- β 1 (mouse IgG1, κ ; clone TWE-2F8), IL-10 (rat IgG1, κ ; clone JES3-9D7) and FoxP3. The appropriate mouse anti-human IgG1, κ - Fc antibody (clone MOPC-21) was used as an isotype control for TGF- β 1 staining. All antibodies were purchased from BioLegend. Flow cytometry data were acquired with a BD FACS Canto (Sparks, MD). Percentage intracellular expression of TGF- β and IL-10 was compared between SP treated and media controls among both Treg and Th cells, and also between FoxP3- Treg and FoxP3+ Treg.

Statistical analyses

Flow cytometry data were analyzed using the FlowJo 8.8.6 software (Tree Star Inc., Ashland, OR) and statistical analyses were conducted using the Prism 4 software (Graph Pad Software, La Jolla, CA). We used Mann Whitney *U* tests for statistical comparisons. Two sided *P* values of <0.05 were considered statistically significant. All experiments were repeated at least three times.

RESULTS

SP induces a non-proliferative increase in the proportion of T cells that are Treg

Using staurosporine as a positive control, we observed a dose-dependent increase in CD4⁺ T cell apoptosis following 24 hour incubation with SP (percentage CD4⁺ T cell intracellular expression of activated caspase-3 of 21.63% (standard deviation [SD] 7.16) vs. 1.86% (SD 0.31) between 2.5% SP and media controls, respectively; $P = 0.0088$). However, no difference in CD4⁺ T cell expression of activated caspase-3 was noted at the lower 0.5% SP concentration (1.54% [SD 0.35]) vs. 1.86% [SD 0.31] between 0.5% SP and media, respectively; $P = 0.3045$). Thus, we subsequently used 0.5% SP in all experiments to remove the confounding presence of dying cells in the cultures. Additionally, this lower SP concentration likely mimics the translationally-relevant amount of SP that impacts immune cells after dilution by reproductive tract secretions and tissue diffusion.

Among CD4⁺ T cells, we defined CD4⁺ helper T cells (Th) as positive for CD127 and/or CD49d, and CD4⁺ regulatory T cells (Treg) as negative for CD127 and CD49d (Figure 1). Twenty-four hour incubation of PBMC with 0.5% SP induced a significant suppression of Th surface expression of both CD127 (Figure 2A) and CD49d (Figure 2B). This effect was maintained after 72 hours, with CD127 mean fluorescence intensity [MFI] among T cells cultured with SP vs. medium alone being 3,437 (SD 334) vs. 3,960 (SD 57), respectively, $P = 0.0003$; and CD49d MFI of 2,464 (SD 160) vs. 2,963 (SD 41), respectively, $P = 0.0003$. Similarly, SP reduced overall ungated CD4⁺ T cell expression of both CD127 and CD49d at all time points (data not shown).

Despite the increase in the proportion of T cells that are Treg following incubation with SP (Figure 3A), SP also reduced the percentage of CD127[−]CD49d[−] Treg expressing FoxP3 (Figure 2B) and, among FoxP3⁺ Treg, SP induced a modest and transient increase in FoxP3 MFI (Figure 2C). To reconcile the apparent discordance between the observed SP mediated increase in the proportion of Treg and reduction in the percentage of Treg expressing FoxP3, we separately analyzed the effect of SP on FoxP3[−] compared to FoxP3⁺ Treg subset. Here, we found that SP induced a preferential increase in the percentage of FoxP3[−] Treg but not FoxP3⁺ Treg (Figure 3D). Thus, the apparent reduction in the percentage of Treg expressing FoxP3 was due to SP mediated preferential increase in the proportion of FoxP3[−] but not FoxP3⁺ Treg. SP did not alter the proportion of CD4⁺CD25⁺ T cells (Figure 3E) but induced a moderate and transient increase in the expression of FoxP3 within the CD4⁺CD25⁺ T cells (Figure 3F). Importantly, incubation with 0.5% SP had no impact on Th or Treg proliferation by Ki67 staining (Figure 4A and 4B) or apoptosis by activated caspase-3 expression (Figure 4C and 4D), supporting the hypothesis that SP-mediated increase in the proportion of FoxP3[−] Treg occurred not through proliferation or deletion but at least in part via ongoing conversion of Th cells into FoxP3[−] Treg.

SP induces FoxP3[−] Treg secretion of TGF- β 1 but not IL-10

We assessed the impact of SP on the intracellular expression of the key Treg immunosuppressive cytokines TGF- β and IL-10^{26,27}. Incubation with SP increased intracellular expression of TGF- β 1 by Treg but not Th cells (Figure 5A), an increase observed primarily within FoxP3[−] Treg (Figure 5B), consistent with the observation that suppressive FoxP3[−] Treg secrete TGF- β ^{25,31–33}. Importantly, the observed SP-mediated induction of TGF- β 1 expression among FoxP3[−] Treg increased progressively over time, becoming maximal at 72 hours of incubation with SP (Figure 6). Much like the previous reports of FoxP3[−] Treg^{31,32}, SP did not impact IL-10 expression by either FoxP3[−] or FoxP3⁺ Treg (SP- vs. media-treated, 0.47% vs. 0.42% among FoxP3[−] Treg, $P = 0.8413$; and 0.58% vs. 0.76% among FoxP3⁺ Treg, $P = 0.5476$).

DISCUSSION

To date, despite a clear population of sex steroid-responsive Treg at mucosal immune surfaces^{10–12}, and the demonstration of semen-mediated modulation of Treg expansion in mice^{13–15}, the role of semen in the modulation of human Treg activity has been unclear. Here, we show that human SP induces a non-proliferative increase in the proportion of Treg via the conversion of Th into predominantly FoxP3– Treg which exhibit enhanced expression of the immunosuppressive cytokine TGF- β . We hypothesize that human SP promotes the immunologic tolerance of conception, and potentially the modulation of immune responses to sexually transmitted infections, via enhancement of the suppressive activity of FoxP3– Treg in the female reproductive tract. Understanding the mechanisms of semen induction of Treg activity in the female reproductive tract will inform the design of new strategies to improve fertility success and immune protection against sexually transmitted infections, including HIV.

In mice, semen mediates the expansion of the Treg population in the female reproductive tract^{13–15}, partly contributing to the induction of the maternal immunologic tolerance of conception^{5,16,17}. To our knowledge, our study is first to demonstrate SP-mediated increase in Treg in humans, and we newly show that the observed increase in Treg occurred partly via the non-proliferative conversion of Th into FoxP3– Treg that express the cardinal immunosuppressive cytokine TGF- β .

We observed a profound impact of SP on the FoxP3– but not FoxP3+ Treg. The increase in the proportion of FoxP3– Treg may represent an intermediate step towards conversion into FoxP3+ Treg^{25,28–30}, and alternatively, since FoxP3– Treg demonstrate full immunoregulatory capability via secretion of TGF- β , it is possible that SP promotes the preferential increase in the proportion of a new and distinct population of Treg^{25,31–33}. Like the previously described FoxP3– adaptive Treg^{25,31–33}, we observed that the FoxP3– Treg increased after incubation with SP are CD4+ and secrete TGF- β but not IL-10. Interestingly, these phenotypic attributes are also shared with another type of suppressor cells, the CD4+ T helper 3 (Th3) cells, which plays a pivotal role in the induction of tolerance to foreign antigens^{36,37} and are also enriched at mucosal surfaces such as the female reproductive tract^{5,6}. It is therefore possible, as previously postulated³¹, that distinct populations of Treg divide immunoregulatory labor, with FoxP3+ Treg predominantly preventing autoimmunity while FoxP3– Treg suppress overly-exuberant immune responses against foreign antigens. Accordingly, while the recently described lack of semen impact on the expansion of FoxP3+ Treg *in vivo* in humans conforms to our findings³⁸, our data suggest SP does impact FoxP3– Treg exhibiting TGF- β expression. The observed enhancement by SP of FoxP3– but not FoxP3+ Treg also explains the apparent reduction in the percentage of Treg expressing FoxP3 after SP impact. It is therefore intriguing to postulate that human semen modulates mucosal adaptive immune responses partly via enhancement of the activity of FoxP3– adaptive Treg and/or Th3 cells in the female reproductive tract.

Although no expansion of FoxP3+ Treg/CD4+CD25+ T cell population was noted following incubation with SP, treatment with SP induced a modest and transient enhancement of FoxP3+ Treg/CD4+CD25+ T cell expression of the immunoregulatory transcriptional factor FoxP3. This suggests that human semen mediates enhancement of the activity of the FoxP3+, as well as the FoxP3– Treg, although we saw no impact of SP on FoxP3+ cytokine secretion thus leaving open the question of whether SP impacts the suppressor function of FoxP3+ Treg. The temporal dynamics of the observed SP-mediated increase in the proportion and TGF- β 1 expression by the FoxP3– Treg suggest that these responses occur via the direct impact of soluble factors in SP on CD4+ T cells. However, it is possible that

the antigen presenting cells such as monocytes and B-cells present in PBMC act as cellular intermediaries in mediating the observed SP effects on Treg.

SP contains innumerable immunomodulatory factors^{16,17}. Among these, TGF- β and testosterone are highly enriched, and are known to facilitate the expansion of the Treg pool in both the murine male and female reproductive tracts^{14,39}. It is therefore intriguing to postulate that seminal TGF- β and/or testosterone are potential mediators of the observed SP-mediated modulation of Treg activity.

We acknowledge that our *in-vitro* model cannot capture the rich interplay of multiple cell types found at the mucosal surfaces of the female reproductive tract. Our data do correspond well with *in-vivo* observations made by Sharkey *et al* in humans³⁸ including lack of semen effect on the expansion of FoxP3+ Treg and the previously reported SP-mediated reduction of T cell expression of CD4³⁴. The presence of semen in the female reproductive tract has been shown to alter immune cell phenotype *in-vivo* using animal models^{13,14}, which along with prior observations that human semen remains in the female reproductive tract for days following deposition^{40,41} makes our observation of SP-mediated modulation of FoxP3–Treg activity imminently plausible and a logical extension of our earlier findings of the impact of SP on the HIV infectivity of CD4+ T cells³⁴. In the experiments reported here, we used PBMC from HIV-negative donors distinct from our semen donors, which does raise the question of whether SP would have the same impact on autologous PBMC. Most importantly, to thoroughly evaluate the impact of human semen on the activity of the different CD4+ regulatory T cell subsets in the female reproductive tract, our experiments suggest it will be high priority to confirm our findings on mononuclear cells from the female reproductive tract including FoxP3+ and FoxP3– Treg using the elegant *in vivo* approaches recently described by Sharkey *et al*³⁸.

In conclusion, we found that human SP promotes the activity of T^{reg} in part via facilitating the non-proliferative conversion of Th into FoxP3– T^{reg} which exhibit enhanced TGF- β secretion. These data suggest that human SP modulates the immunoregulatory activity of T^{reg} in the female reproductive tract and could have potential application in reproductive medicine in key areas of fertility and the prevention against the sexually transmitted infections, including HIV.

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References

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 1995; 155:1151–1164. [PubMed: 7636184]
2. Baecher-Allan C, Hafler DA. Human regulatory T cells and their role in autoimmune disease. *Immunological reviews.* 2006; 212:203–216. [PubMed: 16903916]
3. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. Peripheral education of the immune system by colonic commensal microbiota. *Nature.* 2011; 478:250–254. [PubMed: 21937990]
4. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25^{high} regulatory cells in human peripheral blood. *J Immunol.* 2001; 167:1245–1253. [PubMed: 11466340]

5. Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? Human reproduction update. 2009; 15:517–535. [PubMed: 19279047]
6. Robertson SA. Control of the immunological environment of the uterus. Reviews of reproduction. 2000; 5:164–174. [PubMed: 11006166]
7. Jasper MJ, Tremellen KP, Robertson SA. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. Molecular human reproduction. 2006; 12:301–308. [PubMed: 16574699]
8. Jin LP, Chen QY, Zhang T, Guo PF, Li DJ. The CD4+CD25 bright regulatory T cells and CTLA-4 expression in peripheral and decidual lymphocytes are down-regulated in human miscarriage. Clin Immunol. 2009; 133:402–410. [PubMed: 19766059]
9. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of CD4+CD25+ and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. J Immunol. 2007; 178:2572–2578. [PubMed: 17277167]
10. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenberg AA, Ziegler SF, Offner H. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. J Immunol. 2004; 173:2227–2230. [PubMed: 15294932]
11. Polanczyk MJ, Hopke C, Huan J, Vandenberg AA, Offner H. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. Journal of neuroimmunology. 2005; 170:85–92. [PubMed: 16253347]
12. Trowsdale J, Betz AG. Mother's little helpers: mechanisms of maternal-fetal tolerance. Nature immunology. 2006; 7:241–246. [PubMed: 16482172]
13. Guerin LR, Moldenhauer LM, Prins JR, Bromfield JJ, Hayball JD, Robertson SA. Seminal fluid regulates accumulation of FOXP3+ regulatory T Cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. Biol Reprod. 2011; 85:397–408. [PubMed: 21389340]
14. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod. 2009; 80:1036–1045. [PubMed: 19164169]
15. Robertson SA, Guerin LR, Moldenhauer LM, Hayball JD. Activating T regulatory cells for tolerance in early pregnancy - the contribution of seminal fluid. Journal of reproductive immunology. 2009; 83:109–116. [PubMed: 19875178]
16. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res. 2005; 322:43–52. [PubMed: 15909166]
17. Robertson SA, Sharkey DJ. The role of semen in induction of maternal immune tolerance to pregnancy. Semin Immunol. 2001; 13:243–254. [PubMed: 11437632]
18. Bernardes SS, Borges IK, Lima JE, Milanez PA, Conchon-Costa I, Felipe I, Saridakis HO, Watanabe MA. Involvement of regulatory T cells in HIV immunopathogenesis. Current HIV research. 2010; 8:340–346. [PubMed: 20353390]
19. Fazekas de St Groth B, Landay AL. Regulatory T cells in HIV infection: pathogenic or protective participants in the immune response? Aids. 2008; 22:671–683. [PubMed: 18356596]
20. Rai AK, Thakur CP, Singh A, Seth T, Srivastava SK, Singh P, Mitra DK. Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis. PLoS One. 2012; 7:e31551. [PubMed: 22347492]
21. Trinath J, Maddur MS, Kaveri SV, Balaji KN, Bayry J. Mycobacterium tuberculosis promotes regulatory T-cell expansion via induction of programmed death-1 ligand 1 (PD-L1, CD274) on dendritic cells. J Infect Dis. 2012; 205:694–696. [PubMed: 22238465]
22. Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, Rotzschke O, Falk K. CD49d provides access to “untouched” human Foxp3+ Treg free of contaminating effector cells. Blood. 2009; 113:827–836. [PubMed: 18941119]
23. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299:1057–1061. [PubMed: 12522256]
24. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology. 2003; 4:330–336. [PubMed: 12612578]

25. Rothstein DM. Immunosuppression and regulation: cast in new light? *Journal of the American Society of Nephrology*. 2006; 17:2644–2646. [PubMed: 16971654]
26. von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nature immunology*. 2005; 6:338–344. [PubMed: 15785759]
27. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*. 2002; 2:389–400. [PubMed: 12093005]
28. Chen W, Jin W, Hardegen N, Lei K, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003; 198:1875–1886. [PubMed: 14676299]
29. Kim BS, Kim IK, Park YJ, Kim YS, Kim YJ, Chang WS, Lee YS, Kweon MN, Chung Y, Kang CY. Conversion of Th2 memory cells into Foxp3+ regulatory T cells suppressing Th2-mediated allergic asthma. *Proc Natl Acad Sci U S A*. 2010; 107:8742–8747. [PubMed: 20421479]
30. Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, Vollmar P, Stritesky GL, Kaplan MH, Waisman A, Kuchroo VK, Oukka M. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2008; 105:18460–18465. [PubMed: 19015529]
31. Xu Q, Lee J, Jankowska-Gan E, Schultz J, Roenneburg DA, Haynes LD, Kusaka S, Sollinger HW, Knechtle SJ, VanBuskirk AM, Torrealba JR, Burlingham WJ. Human CD4+CD25low adaptive T regulatory cells suppress delayed-type hypersensitivity during transplant tolerance. *J Immunol*. 2007; 178:3983–3995. [PubMed: 17339499]
32. Kochetkova I, Thornburg T, Callis G, Pascual DW. Segregated regulatory CD39+CD4+ T cell function: TGF-beta-producing Foxp3– and IL-10-producing Foxp3+ cells are interdependent for protection against collagen-induced arthritis. *J Immunol*. 2011; 187:4654–4666. [PubMed: 21967895]
33. Otsubo K, Kanegane H, Kamachi Y, Kobayashi I, Tsuge I, Imaizumi M, Sasahara Y, Hayakawa A, Nozu K, Iijima K, Ito S, Horikawa R, Nagai Y, Takatsu K, Mori H, Ochs HD, Miyawaki T. Identification of FOXP3-negative regulatory T-like (CD4(+)-CD25(+)-CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Clin Immunol*. 2011; 141:111–120. [PubMed: 21802372]
34. Balandya E, Sheth S, Sanders K, Wieland-Alter W, Lahey T. Semen protects CD4+ target cells from HIV infection but promotes the preferential transmission of R5 tropic HIV. *J Immunol*. 2010; 185:7596–7604. [PubMed: 21059891]
35. Lovelace P, Maecker HT. Multiparameter intracellular cytokine staining. *Methods Mol Biol*. 2011; 699:165–178. [PubMed: 21116983]
36. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunological reviews*. 2001; 182:207–214. [PubMed: 11722636]
37. Carrier Y, Yuan J, Kuchroo VK, Weiner HL. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *J Immunol*. 2007; 178:179–185. [PubMed: 17182553]
38. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *J Immunol*. 2012; 188:2445–2454. [PubMed: 22271649]
39. Fijak M, Schenider E, Klug J, Bhushan S, Hackstein H, Schuler G, Wygrecka M, Gromoll J, Meinhardt A. Testosterone replacement effectively inhibits the development of experimental autoimmune orchitis in rats: Evidence for a direct role of testosterone on regulatory T cell expansion. *J Immunol*. 2011; 186:5162–5172. [PubMed: 21441459]
40. Hooft PJ, van de Voorde HP. Bayesian evaluation of the modified zinc test and the acid phosphatase spot test for forensic semen investigation. *The American journal of forensic medicine and pathology*. 1997; 18:45–49. [PubMed: 9095300]
41. Willott GM, Allard JE. Spermatozoa--their persistence after sexual intercourse. *Forensic Sci Int*. 1982; 19:135–154. [PubMed: 7040183]

Figure 1. Gating strategy for Th and Treg

We initially identified lymphocytes within the peripheral blood mononuclear cells (PBMC), followed by the identification of CD4⁺ T cells as lymphocytes that were positive for CD3 and CD4 (A). Subsequently, we identified CD127⁺CD49d⁺ Th cells as CD4⁺ T cells that were positive for the alpha chains of IL-7 receptor (CD127) and integrin $\alpha 4\beta 1$ (CD49d), and CD127⁻CD49d⁻ Treg as CD4⁺ T cells that were negative for both, CD127 and CD49d (B). The identity of Treg and Th was further confirmed via presence or absence of intracellular FoxP3 expression, respectively (C and D, $n=20$).

Figure 2. SP reduces Th expression of CD127 and CD49d

We incubated PBMC with or without 0.5% SP followed by assessment of Th surface expression of CD127 and CD49d. Incubation with SP for 24 hours triggered a decrease in Th surface expression of both CD127 (A, $n=20$) and CD49d (B, $n=20$). *** $P < 0.001$

Figure 3. SP increases the proportion of FoxP3- Treg and enhances FoxP3+ Treg expression of FoxP3

72 hours incubation with SP significantly increased the percentage of Treg (A, $n=20$). However, SP reduced the percentage of Treg expressing FoxP3 (B, $n=20$). Within the FoxP3+ Treg, SP induced a small and transient increase in FoxP3 expression (C, $n=20$). Upon separate analysis of the FoxP3+ compared to FoxP3- Treg, we observed that the SP-mediated increase in the percentage of Treg was primarily confined to the FoxP3- but not FoxP3+ Treg (D, $n=10$). Similarly, SP did not impact the proportion of CD4+CD25+ T cell (E, $n=20$) and induced a small and transient increase in CD4+CD25+ T cell expression of FoxP3 (F, $n=20$). NS = Not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 4. SP mediates a non-proliferative increase in the proportion of FoxP3⁺ Treg

We assessed whether the observed SP mediated increase in the percentage of Treg was mediated via increased proliferation or ongoing conversion of Th into Treg by comparing the intracellular expression of Ki67 and activated caspase-3 between Treg and Th, also between Treg subsets after 72 hours of incubation with 0.5% SP. No difference was observed in the intracellular expression of Ki67 (A and B, $n=6$) or activated caspase-3 (C and D, $n=10$) between Treg and Th, or between FoxP3⁻ and FoxP3⁺ Treg, suggesting an increase in Treg via ongoing conversion of the pre-existing Th into Treg. NS = Not significant.

Figure 5. SP induces Treg secretion of TGF-β1

We examined the impact of SP on the suppressive function of the Treg via assessment of Treg expression of the immunosuppressive cytokines TGF-β1 via intracellular cytokine staining (ICS), 72 hours after incubation with or without 0.5% SP. SP induced a profound and significant induction of Treg but not Th intracellular expression of TGF-β1 (A, $n=5$). Importantly, this increase was mostly observed within the FoxP3- Treg compared to FoxP3+ Treg (B, $n=5$). * $P < 0.05$, ** $P < 0.01$

Figure 6. SP induces a time-dependent increase in FoxP3+ Treg secretion of TGF-β1

We investigated the time dynamics of SP mediated induction of FoxP3+ Treg intracellular expression of TGF-β1. SP induced a time-dependent increase in TGF-β1 expression by the FoxP3+ Treg, peaking at 72 hours ($n=5$). Error bars represent standard error of mean, NS = Not significant, ** $P < 0.01$