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In vitro Differentiation of Thymic T_{reg} Cell Progenitors to Mature Thymic T_{reg} Cells

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[Abstract] Thymic T_{reg} cell differentiation occurs via a two-step process. Step one generates T_{reg} cell progenitors ($T_{reg}P$) and is driven by strong TCR interactions with antigens presented in the thymus. Step two is initiated by activation of STAT5 via IL-2, or IL-15, leading to mature T_{reg} cells capable of emigrating from the thymus and mediating immune tolerance and homeostasis in peripheral tissues. Herein we describe an *in vitro* $T_{reg}P$ cell differentiation assay that models the second, cytokine dependent, step of thymic T_{reg} cell development. It can be utilized with relative ease to determine if a population of thymocytes represents a potential progenitor population for T_{reg} cells as well as test how different cytokines or chemical inhibitors modulate the differentiation of known $T_{reg}P$ cell populations into mature T_{reg} cells.

Keywords: Thymus, Thymic selection, Regulatory T (T_{reg}) cells, T_{reg} cell development, T cell tolerance, Cytokine stimulation, STAT5, Foxp3

[Background] Regulatory T cells (T_{reg}) cells are a population of T cells that can suppress immune responses and are required to maintain immune tolerance and tissue homeostasis. While two broad classes of T_{reg} cells have been described, thymic T_{reg} cells (tT_{reg}) and peripheral T_{reg} cells (pT_{reg}), the majority of T_{reg} cells are generated during thymic selection (Asano *et al.*, 1996). Early experiments describing autoimmune manifestations in thymectomized mice, which can be rescued by T_{reg} cell transfer, confirm the importance of the thymus in generating T_{reg} cells capable of maintaining immune homeostasis (Asano *et al.*, 1996). The development of T_{reg} cells in the thymus is a two-step process (Burchill *et al.*, 2008; Lio and Hsieh, 2008). The first step is TCR mediated and gives rise to $CD25^{+}Foxp3^{-}$ or $CD25^{-}Foxp3^{lo}$ $T_{reg}P$ cells. The second step is mediated by contact of these progenitor populations with intrathymic cytokines leading to co-expression of CD25 and Foxp3 and generation of mature tT_{reg} cells.

The *in vitro* $T_{reg}P$ conversion assay models the second, cytokine dependent, step in tT_{reg} cell development. This assay builds on protocols initially described by work from Chyi Hsieh's lab and modified by our own lab (Burchill *et al.*, 2008; Lio and Hsieh, 2008). The technique described below provides a simple method to determine if an isolated thymic population represents a $T_{reg}P$, characterized by independence from TCR stimulation and upregulation of tT_{reg} markers in response to cytokine. This assay has been performed with varied stimulation conditions previously, including stimulation with high concentrations of IL-2 (200 U/ml) (Tai *et al.*, 2013) as well as IL-2 + IL-7 (Lio and Hsieh, 2008). The protocol we describe converts bona-fide $T_{reg}P$ to mature T_{reg} cells robustly and

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reproducibly with small amounts of IL-2 (Mahmud *et al.*, 2014; Owen *et al.*, 2019). As such, investigators can narrow the application of IL-2 to concentrations that are more physiologically relevant. This assay has also been able to detect conversion of $T_{reg}P$ to mature T_{reg} cells with other cytokines like IL-7, IL-15 and IL-4, as well as combination of factors such as IL-2 with TNFRSF agonists (Mahmud *et al.*, 2014). Thus, the *in vitro* $T_{reg}P$ conversion assay can be used to query various important stimulatory, or inhibitory, signals that may be encountered *in vivo* by developing $T_{reg}P$.

Materials and Reagents

1. Pipette tips
2. 0.22 µm 1,000 ml Stericup (Millipore Sigma, catalog number: SCGPU11RE)
3. 0.22 µm 50 ml Steriflip (Millipore Sigma, catalog number: SCGP00525)
4. LS Columns (Miltenyi Biotec, catalog number: 130-042-101)
5. 50 ml Falcon conical tube (Corning, catalog number: 352070)
6. 15 ml CentriStar conical tube (Corning, catalog number: 430790)
7. 5 ml Falcon Round Bottom test tube (Corning, catalog number: 352052)
8. 96-well round bottom, non-TC treated plates (Sarstedt, catalog number: 82.1582.001)
9. CytoOne Non-treated 6-well plates (USA Scientific, catalog number: CC7672-7506)
10. Falcon 70 µm cell stainer (Corning, catalog number: 352350)
11. Plain and frosted glass slides (VWR International, catalog number: 48312-004)
12. *Foxp3*^{GFP} (B6.Cg-Foxp3tm2(EGFP)Tch/J) mice (THE JACKSON LABORATORY, catalog number: 006772)

    The best $T_{reg}P$ cell yields come from younger (5-6 weeks old) female mice due to a generally large thymus. If younger mice are not available older mice will provide usable $T_{reg}P$ cells. However, the number of $T_{reg}P$ cells recovered could be reduced by 50% of that obtained from younger mice.
13. Recombinant Human IL-2 (R&D Systems, catalog number: 202-IL)
14. Anti-mouse CD4 (GK1.5) BV786 (1:200) (BD Biosciences, catalog number: 563331)
15. Anti-mouse CD8a (53-6.7) Biotin (1:200) (Tonbo Biosciences, catalog number: 30-0081-U500)
16. Anti-mouse CD25 (PC61.5) PerCP-Cy5.5 (1:200) (Tonbo Biosciences, catalog number: 65-0251-U100)
17. Anti-mouse CD73 (eBioTY/11.8) eF450 (1:100) (eBioscience, catalog number: 48-0731-82)
18. Anti-mouse Ter119 (Ter-119) Biotin (1:200) (BD Biosciences, catalog number: 553672)
19. (Optional) antibodies:
    a. Anti-CD11b (M1/70) Biotin (1:200) (Tonbo Biosciences, catalog number: 30-0112-U500)
    b. Anti-CD11c (N418) Biotin (1:200) (Tonbo Biosciences, catalog number: 30-0114-U500)
    c. Anti-NK1.1 (PK136) Biotin (1:200) (Tonbo Biosciences, catalog number: 30-5941-U500)
    d. Anti-γδTCR (eBioGL3) Biotin (1:200) (eBioscience, catalog number: 13-5711-85)
    e. Anti-B220 (RA3-6B2) Biotin (1:200) (Tonbo Biosciences, catalog number: 30-0452-U500)

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20. Streptavidin APC-eF780 (1:200) (eBioscience, catalog number: 47-4317-82)
21. Viability Dye Ghost^{TM} Red 780 (1:1,000) (Tonbo Biosciences, catalog number: 13-0865-T100)
22. Streptavidin Microbeads (Miltenyi Biotec, catalog number: 130-048-101)
23. AccuCheck Counting Beads (Life Technologies, catalog number: PCB100)
24. Sodium azide (NaN_3) (Ricca Chemical, catalog number: 7144.8-16)
25. 10x PBS (Fisher BioReagents, catalog number: BP399-20)
26. Ethylenediaminetetraacetic Acid (EDTA) (Fisher Scientific, catalog number: BP120-500)
27. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11150)
28. RPMI 1640 media (Corning, catalog number: 15-040-CV)
29. Penicillin-Streptomycin Solution (Corning, catalog number: 30-002-CL)
30. L-Glutamine (Corning, catalog number: 25-005-CL)
31. MEM Nonessential amino acids (Corning, catalog number: 25-025-CL)
32. 2-mercaptoethanol (MP Bio, catalog number: 02194705-CF)
33. HEPES (Corning, catalog number: 25-060-CL)
34. Sodium Pyruvate (Corning, catalog number: 25-000-CL)
35. Sort Buffer (see Recipes)
36. FACS Buffer (see Recipes)
37. cRPMI (see Recipes)

Equipment

1. Pipettes
2. Sorvall Legend X1R Centrifuge (Thermo Scientific, catalog number: 75004260)
3. BD Biosciences LSR II (BD, model: LSR II)
4. BD Biosciences FACSAria II (BD, model: FACSAria II)
5. MACS Multistand (Miltenyi Biotec, catalog number: 130-042-303)
6. QuadroMACS Separator Magnet (Miltenyi, catalog number: 130-090-976)
7. Class II Biosafety Cabinet/Laminar Flow Hood
8. IncuSafe Incubator (Panasonic, catalog number: KM-CC17T0A)
9. Hemocytometer (Fisher, catalog number: 0267110)
10. Refrigerator (2-8 °C, Kenmore)
11. Panasonic -80 °C VIP Plus Freezer

Software

1. FlowJo 10 (10.5.3) (https://www.flowjo.com/)

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Procedure

A. $T_{reg}P$ cell isolation

1. Euthanize mice by CO_2 inhalation.
2. Harvest thymi from mice into 5 ml Sort buffer in 6-well plates. The number of conditions tested and replicates determine the number of mice required for each experiment. In a simple experiment, 1 mouse can be used as this should provide 5-10 x 10^4 $CD25^+$ and $Foxp3^{lo}$ $T_{reg}P$ cells.
3. Work in a clean biosafety cabinet from this point on.
4. Mechanically dissociate thymi between frosted glass slides or favored method of mechanical dissociation.
5. Transfer cells into 50 ml conical vial through 70 µm filter.
6. Spin cells at 350 *x g* for 10 min, at 4 °C (keep thymi in individual 50 ml conical tubes).
7. Resuspend cells in 1 ml depletion cocktail of biotin-labeled antibodies. This cocktail of antibodies should include anti-CD8 (1:200) and anti-Ter119 (1:200). However other optional depletion antibodies can be added to this cocktail such as anti-CD11b (1:200), anti-CD11c (1:200), anti-NK1.1 (1:200), anti-γδTCR (1:200) and anti-B220 (1:200).
8. Incubate on ice for 20 min and cover to protect from light.
9. Add 15 ml Sort buffer; spin cells at 350 *x g* for 5 min, at 4 °C.
10. Count the cells and resuspend in Strepavidin microbead cocktail (~1 µl beads per 2-3 million cells diluted in Sort buffer to a final volume of ~400 µl/thymus).
11. Incubate for 20 min at 4 °C (agitate by swirling tube gently after 10 min).
12. Add 10 ml Sort buffer; spin cells at 350 *x g* for 5 min, at 4 °C.
13. Pre-rinse LS columns (1 column/thymus) with 3 ml cold Sort Buffer.
14. Resuspend cells in 2 ml Sort buffer.
15. Apply cells from individual thymus to separate LS columns on QuadroMACS Miltenyi separator magnet through a filter (Figure 1).

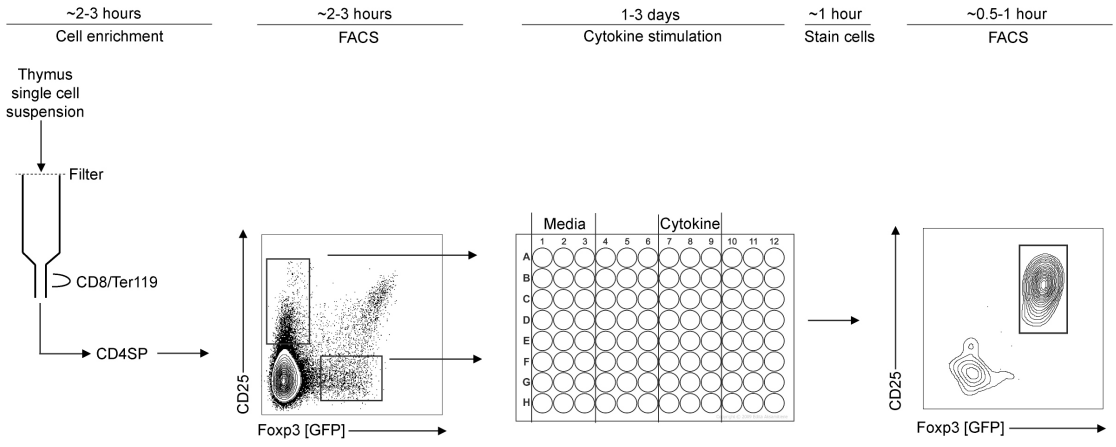

Figure 1. Schematic of *in vitro* $T_{reg}P$ differentiation assay workflow. First, thymi are

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processed and enriched for CD4 single positive (CD4SP) thymocytes. This fraction is stained for surface markers then each $T_{reg}P$ is isolated by FACS. Isolated $T_{reg}P$ are stimulated with cytokine in 96-well plate for a pre-determined time. These cells are then harvested and analyzed by flow cytometry for upregulation of Foxp3 and/or CD25.

16. Wash columns 3 times each with 3 ml cold Sort buffer.
17. Collect unbound fraction in 15 ml conical vials; spin cells at 350 *x g* for 5 min, at 4 °C.
18. Combine cells into a single 15 ml conical tube; apply surface staining cocktail [CD4 (1:200), Live-Dead (1:1,000), CD25 (1:200), CD73 (1:100) and Streptavidin-APC-eF780 (1:200)]. The Streptavidin-APC-eF780 is used to detect biotin-conjugated antibodies used for magnetic depletion, including CD8, Ter119 and any other optional antibodies included from Step 7 above.
19. Incubate on ice for 20 min and cover to protect from light.
20. Add 14 ml Sort buffer; spin cells at 350 *x g* for 5 min, at 4 °C.
21. Resuspend cells in 1 ml Sort buffer and filter cells through a 70 µm filter into a new, pre-chilled 15 ml conical; wash the filter with Sort buffer to bring cells to appropriate final volume.
22. Set up gates on FACS Aria II for sorting $T_{reg}P$ cells as described in Figure 2.
23. Collect $CD25^+$ and/or $Foxp3^{lo}$ $T_{reg}P$ cells into separate 5 ml round bottom tubes with 1.5 ml Catch buffer (1x PBS + 50% FBS).

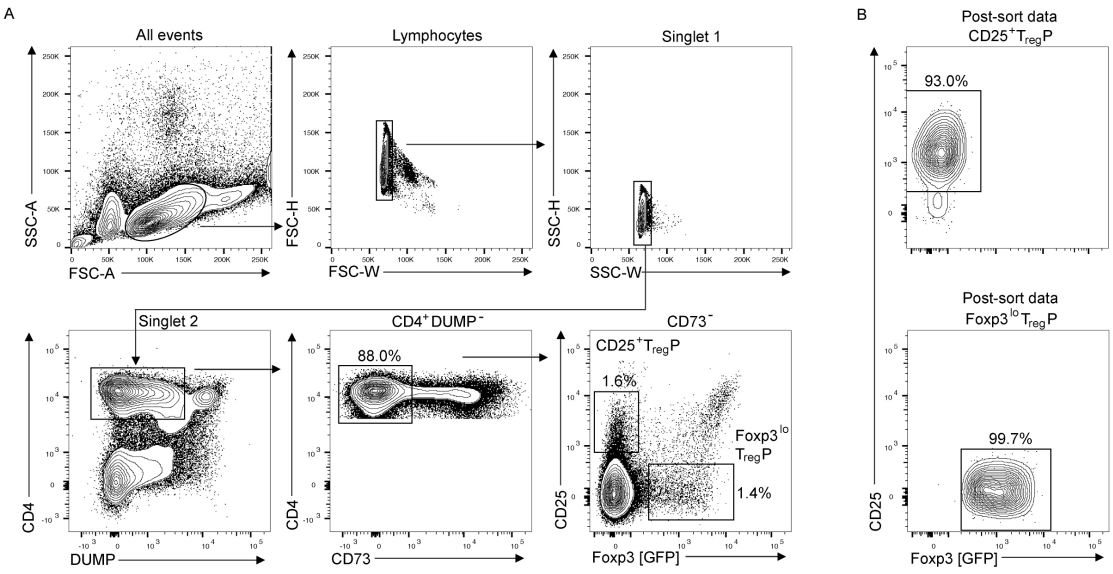

Figure 2. Flow cytometry gating strategy for sorting $T_{reg}P$. A. Flow cytometry gating scheme for sorting $T_{reg}P$ from CD8/Ter119 depleted thymocytes. DUMP channel contains CD8, Ter119 (these are labeled with biotin conjugated antibodies during magnetic depletion that are probed with APC-eF780-conjugated streptavidin) and Live-Dead. $CD73^+$ cells represent recirculating and long-term resident $T_{reg}P$ and T_{reg}. Thus sorting $CD73^-$ thymocytes ensures

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analysis of *de novo* developing $T_{reg}P$. B. Post-sort purity analysis of $CD25^+$ $T_{reg}P$ (top) and $Foxp3^{lo}$ $T_{reg}P$ (bottom).

B. Stimulation of $T_{reg}P$ cells
   1. Make stimulation media (example: 2 U/ml IL-2 in cRPMI for a final concentration of 1 U/ml).
   2. Plate 100 µl of stimulation media into each well of non-TC treated round bottom 96-well plate.
   3. Place the 96-well plate in 37 °C incubator.
   4. Fill 5 ml round bottom tubes containing sorted $T_{reg}P$ cells with cRPMI, spin cells at 350 *x g* for 5 min, at 4 °C.
   5. Resuspend cells at 1-2 x 10^5 cells/ml in cRPMI.
   6. Add 100 µl of cells to 100 µl stimulation media in 96-well plates (plate 1-2 x 10^4 cells/well).
   7. Place the 96-well plates into 37 °C incubator.
   8. Incubate for 24-72 h (we perform these assays for 72 h typically but CD25 and/or Foxp3 upregulation can be observed at earlier timepoints).

C. Analysis of *in vitro* $T_{reg}P$ differentiation assay
   1. Add 100 µl FACS buffer to each well of stimulated $T_{reg}P$.
   2. Spin cells at 350 *x g* for 5 min at 4 °C.
   3. Decant supernatant and add 200 µl FACS buffer.
   4. Spin cells at 350 *x g* for 5 min at 4 °C.
   5. Resuspend cells in 50 µl staining cocktail [anti-CD4 (1:200), anti-CD8 (1:200), anti-CD25 (1:200), Live-Dead (1:1,000)].
   6. Incubate on ice for 20 mins and cover to protect from light.
   7. Add 200 µl FACS buffer.
   8. Spin cells at 350 *x g* for 5 min at 4 °C.
   9. Resuspend each sample in FACS buffer/counting bead master mix (190 µl FACS buffer + 10 µl counting beads/sample).
   10. Analyze differentiation of $T_{reg}P$ to mature T_{reg} cells (typically co-expression of CD25 and Foxp3).

Data analysis

1. Data analysis was performed in FlowJo 10.
2. Gating scheme for quantification of T_{reg} differentiation is shown in Figure 3. Briefly, lymphocytes were gated followed by singlets. $CD4^+$, $CD8/Live\text{-}Dead^-$ singlets were gated and displayed for expression of CD25 and Foxp3. Representative data can be found in Owen *et al.* (2019) in Figure 7A, Supplemental Figure 1 and Supplemental Figure 7.

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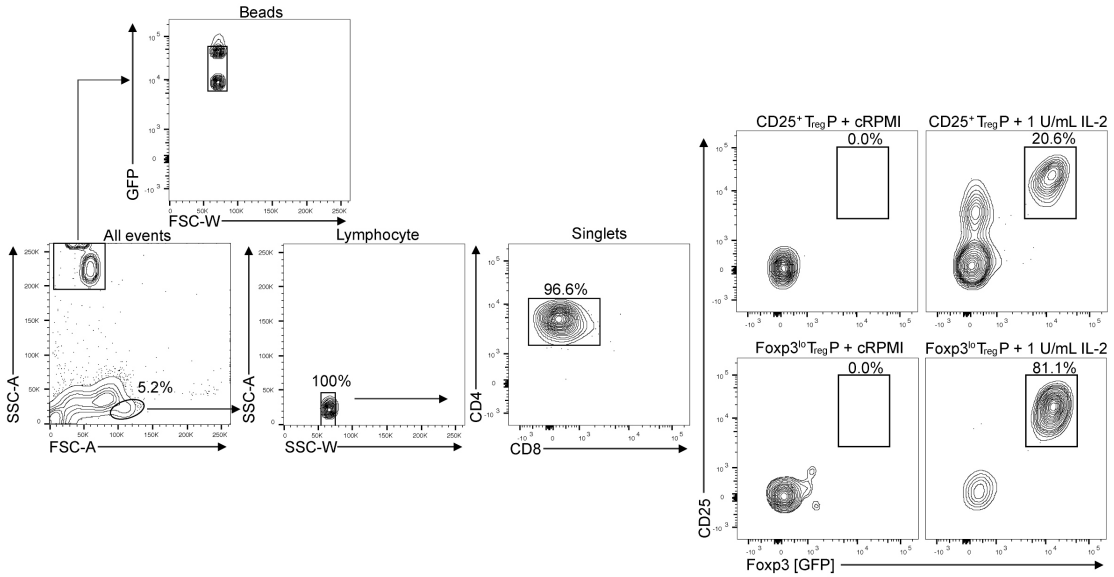

Figure 3. Flow cytometry gating scheme for analysis of $T_{reg}P$ following 3 days of stimulation. Media alone (left) fails to convert $T_{reg}P$ to T_{reg} cells while 1 U/ml IL-2 stimulation (right) converts $T_{reg}P$ to T_{reg} cells. Beads are used to calculate total numbers of T_{reg} cells generated in each sample. The formula used is Total cells = Cells detected x (Known Bead number added/Beads detected).

3. Published results (Burchill *et al.*, 2008; Lio and Hsieh, 2008; Vang *et al.*, 2008; Mahmud *et al.*, 2014; Owen *et al.*, 2019) have indicated successful conversion of $CD25^+$ or $Foxp3^{lo}$ $T_{reg}P$ is represented by dual expression of CD25 and Foxp3 in response to IL-2. However, other cytokines we have tested, such as IL-4 (Owen *et al.*, 2019), have led to generation of $CD25^+$ $Foxp3^+$ T_{reg} cells and the maintenance of $CD25^-Foxp3^{lo}$ cells. Interestingly, IL4 stimulation of $CD25^-Foxp3^{lo}$ $T_{reg}P$ cells results in the development of $CD25^+Foxp3^+$ T_{reg} cells that express distinctly lower levels of CD25 than observed with IL-2 stimulation. Thus, depending on the condition, it is relevant to also consider the proportion of $CD25^-Foxp3^+$ cells as well as the mean fluorescence intensity of CD25 and Foxp3, as shown in Owen *et al.* (2019) Supplemental Figure 7.

Notes

1. In our hands, $T_{reg}P$ cells are a delicate population and quite susceptible to cell death. Given this, gentle processing throughout the cell isolation protocol will yield higher amounts of $T_{reg}P$ cells from thymi.
2. A typical yield from 1 thymus is ~50,000-100,000 $T_{reg}P$ depending on age (5-6 weeks being ideal) and sex (female having larger thymi).
3. We make fresh cRPMI for each assay to help maintain viability of $T_{reg}P$ cells *in vitro*.
4. Our greatest success has been using round bottom, non-TC treated 96-well plates. Other

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96-well formats led to increased cell death in our hands.

Recipes

1. Sort Buffer (store at 4 °C, ~55-60 ml/sample)
   1x PBS
   2% FBS
   2 mM EDTA
2. FACS Buffer (store at 4 °C, ~1 ml/well)
   1x PBS
   2% FBS
   2 mM EDTA
   0.05% NaN_3
3. cRPMI (filter sterilize, store at 4 °C)
   RPMI 1640 Medium
   10% Fetal Bovine Serum
   1% Penicillin/Streptomycin
   1% L-Glutamine
   1% Sodium Pyruvate
   1% Non-essential amino acids
   10 mM HEPES
   50 µM 2-mercaptoethanol

Acknowledgments

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Competing interests

The authors declare no competing financial or non-financial interests.

Ethics

All animal experimentation in this protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee (most recent IACUC protocol 1904-36975A, valid 5/16/2019-5/15/2022).

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