FOXP3: il “peacekeeper” del sistema immunitario

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Outline

- **FOXP3**: FOXP3 and regulatory T cells structure, regulation of expression and function
  
  - FOXP3 mutations and IPEX syndrome

  - Treg cell immunotherapy in IPEX syndrome
Human $\text{CD}4^+\text{CD}25^+\text{FOXP3}^+$ regulatory T cells

- **Tregs**: CD4+ T cell subset devoted to maintenance of immunological tolerance
- **Thymic-derived**: $t$Tregs
- **Anergy**: hypo-proliferative upon TCR activation in vitro
- **Markers**: Constitutive expression of FOXP3, CD25 (IL-2Rα), CTLA4, GITR and negative/low for CD127 (IL-7Rα); Resting CD45RA+, activated CD45RA-; Helios
- **Mechanism**: suppressive in vitro & in vivo vs APCs and Teff through cell-cell contact mechanism (+ soluble factors)
- **Function**: mainly involved in the maintenance of tolerance to self Ags
- **FOXP3 expression**: is fundamental for full maturation of Tregs (FOXP3-dependent and -independent features)

Curotto de Lafaille et al., Immunity 2009
tTreg Cells and FOXP3

Control of Regulatory T Cell Development by the Transcription Factor Foxp3
Shohei Hori, Takashi Nomura, Shimon Sakaguchi
Science Vol 299 14 February 2003

Crucial role of FOXP3 in the development and function of human CD25^+CD4^+ regulatory T cells
Haruhiko Yagi, Takashi Nomura, Kyoko Nakamura, Sayuri Yamazaki, Tosio Kitawaki, Shohei Hori, Michiyuki Maeda, Masafumi Onodera, Takashi Uchiyama, Shingo Fujii and Shimon Sakaguchi
International Immunology, Vol. 16, No. 11, pp. 1643–1656

CD4^+CD25^+ Tregs ‘discovered’

FOXP3 is the master gene regulator in mice and human Tregs

Il gene Forkhead Box P3 (FOXP3): codifica per un fattore di trascrizione fondamentale per il funzionamento delle cellule T-regolatorie
Forkhead box P3 (FOXP3)

✓ transcription factor (*forkhead family* of TFs)

✓ **Structure**: proline-rich repressor domain (transcriptional activity), and zinc finger and leucine zipper domains (protein-protein interactions and homodimerization), forkhead DNA-binding domain at the C terminus of FOXP3

✓ interacting with other transcription factors (NFAT, NFkB, Runx1, RORs, IRF4, STAT3 and Jun)

✓ **repressor/activator** of target genes (ex. IL-2, IL2RA, CTLA4, IL7RA), via multiple mechanism (co-repressor, epigenetic remodeling, ...)

✓ In humans: **expressed in Treg cells** and **activated Teff cells** (function?)
FOXP3 regulation

✓ **Transcriptional regulation**: TCR-responsive elements, STAT5 binding sites and a TGF-β-responsive region;

✓ **Epigenetic modifications**: DNA-methylation and histone acetylation also control the activity of TFs

✓ **Post-translational**: miR regulation (miR24, 31 and 210 negative regulators, miR 95 and 21 positive regulation)

✓ **Post-transcriptional regulation**: acetylation, ubiquitination (STUB1), phosphorylation modulate subcellular localization, protein interaction and degradation

*(reviewed in van Loosdreg, Trends in Immunol 2014)*
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*(reviewed in van Loosdreg, Trends in Immunol 2014)*
Epigenetic regulation of FOXP3

CNS1: important for pTreg cell induction, recruits factors downstream TCR engagement and TGFβ

CNS2: comprises the TSDR, region specifically demethylated in tTregs, recruits TFs activated by TCR/γ-chain cytokine receptor engagement to maintain constant FOXP3 expression

CNS3: enhancer, pioneer element, bound by c-Rel (NFκB pathway- TCR activation)

PROMOTER: PIAS1 restrains foxp3 expression by regulating accessibility and binding of the TFs (TCRand IL2R signaling)

Modified from F.R. Santoni de Sio, From Int Reviews Immunol 2013
Treg Specific Demethylated Region allows precise quantification of circulating tTregs

1) Bisulfite modification of DNA
2) PCR with demethylation specific primer sets
3) Normalization to nucleated cells

La TSDR è: una regione regolatoria del gene FOXP3 (c)

2) PCR with demethylation specific primer sets
3) Normalization to nucleated cells

Wieczorek G, Cancer Res 2009
✓ **Transcriptional regulation**: TCR-responsive elements, STAT5 binding sites and a TGF-β-responsive region;

✓ **Epigenetic modifications**: DNA-methylation and histone acetylation also control the activity of TFs

✓ **Post-transcriptional**: miR regulation (miR24, 31 and 210 negative regulators, miR 95 and 21 positive regulation)

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(Reviewed in van Loosdreg, Trends in Immunol 2014)
Foxp3 mutation is reported to be the cause of the murine scurfy phenotype (Brunkow)

First description of a new clinical entity later designated as IPEX (Powell)

FOXP3 mutation is reported to be the cause of IPEX (Wildin & Bennett)

X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy

The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3

Craig L. Bennett¹*, Jacinda Christie²*, Fred Ramsdell³, Mary E. Brunkow⁴, Polly J. Ferguson⁴, Luke Whitesell⁵, Thaddeus E. Kelly⁶, Frank T. Saulsbury⁶, Philip F. Chance¹ & Hans D. Ochs²

nature genetics • volume 27 • January 2001
The IPEX Syndrome is a genetic disorder characterized by multiple autoimmune manifestations, caused by mutations in the FOXP3 gene.
Treg cell development in IPEX patients

- FOXP3+ T cells may be present in peripheral blood

- Bona fide Tregs (TSR demethylation) are detectable in all IPEX patients (FOXP3mut allow tTreg development)
Treg cell dysfunction in IPEX patients

In vitro suppression assay day+6
Activation aCD3/28/2 coated beads
R:Tr=3:1
R: ND PBMC
CD4 cell gate

- R activated
- Tr FOXwt

- R activated
- Tr FOXmut

% divided cells

- R
- R+beads
- +Tr ND
- +Tr Pt24

6%
59%
Immune dysregulation in IPEX syndrome

FOXP3 mutations lead to different degree of biological abnormalities:

- **impaired suppressive function by Treg cells**
  Bacchetta R. et al., JCI, 2006
  Moes N. et al., Gastroent, 2010
  d’Hennezel E. et al., NEJM, 2009

- **increased frequency of Th17 cells and Treg instability**
  Passerini L. et al., JACI, 2011

- **altered cytokine production by Teff cells**
  Chatila T.A. et al., JCI, 2000
  Nieves D.S. et al., Arch Dermatol, 2004
  Bacchetta R. et al., JCI, 2006
  d’Hennezel E. et al., NEJM, 2009

- **accumulation of autoreactive B cells**
  Kinnunen et al, Blood 2012

Reviewed in: Barzaghi et al, Frontiers in Immunology 2012;
Passerini et al., Curr Gene Therapy 2014
**FOXP3 and Treg function.** FOXP3 is essential for acquisition and maintenance of suppressive function
- Stable overexpression induces Treg-like cells
- FOXP3 mutation/KD causes loss of suppressive function

**FOXP3 and Treg transcriptional program.** FOXP3 per se does NOT activate the complete Treg signature
- Analysis of foxp3gfpKO mice demonstrated that most of the Treg signature is maintained in the absence of foxp3 *(Gavin, Nature 2007)*

**FOXP3 and Treg development.** FOXP3 is NOT necessary for tTreg development
- Emergence of tTregs from IPEX thymus
- tTreg development is achieved through establishment of specific epigenetic changes and the induction of Foxp3 expression. The two events are independent *(Okhura, Immunity 2012)*
- foxp3gfpKO model: foxp3-independent thymic emergence of Tregs *(Gavin, Nature 2007)*

**FOXP3 and Teff cells.** FOXP3 is expressed in activated Teff cells (human)
- Function poorly defined
- Expression associated with suppression of cytokine production, proliferation and development of Th17 cells *(McMurchy, Blood 2013)*
**Treg cell immunotherapy for IPEX syndrome?**

Current therapeutic approaches for IPEX syndrome

- **Immunosuppression**
  
  only partial efficacy and side effects in long-term treatment

- **Haematopoietic stem cell transplantation (HSCT)**
  
  curative treatment BUT suitable donors are not available for all patients (no donor available for 1/3)

**IPEX as candidate disease for Treg-cell immunotherapy?**

✓ The presence of functional Tregs is sufficient to control the disease:

- remission post HSCT with full donor chimerism in Treg cells only
  

- skewed vs random expression of the wt **FOXP3** allele in nTreg vs Teff cells from healthy female carriers

  *(Di Nunzio, Blood 2009)*
FOXP3-Engineered Human CD4+ T Cells (CD4FOXP3)

Generation of Potent and Stable Human CD4+ T Regulatory Cells by Activation-independent Expression of FOXP3

Sarah E Allan1,2, Alicia N Alstad1,2, Natacha Merindo1,2, Natasha K Crellin1,2, Mario Amendola3,4, Rosa Bacchetta6, Luigi Naldini6,7, Maria Grazia Roncarolo6,7, Hugo Soudeyns3,5 and Megan K Levings1,2

Transduction of CD4+ T cells with Bd.LV.FOXP3 generates potent and stable human Tregs

Transduction protocol

Day 28
CD4FOXP3 T cells acquire Treg-like phenotype, anergy and suppressive activity

Day 10 beads sorting
CD4+NGFR+ T cells

Day 14 restimulation and expansion

LV-FOXP3/ΔNGFR

MOI20
1 day

9 days
IL-7 (10 ng/ml)
IL-2 (100 U/ml)

4 days
IL-15 (10 ng/ml)
IL-2 (100 U/ml)

16hrs
alloAPCs+aCD3
IL-7 & IL-2

Total PBMC
Regulatory T cell immunotherapy

1. wtFOXP3 over-expression in conventional CD4+ T cells (memory/Ag-experienced)
2. In vitro expansion and selection of transduced cells
3. Re-infusion of purified autologous suppressive T cells
4. Cell therapy with LV-engineered T cells to restore tolerance in IPEX?

In vitro/In vivo validation of the purified population
LV-mediated FOXP3 gene transfer in IPEX CD4+ T cells

IPEX Tconv can be efficiently transduced with LV.FOXP3

Passerini et al, Sci Transl Med 2013
**IPEX CD4<sup>FOX</sup>P<sup>3</sup> acquire expression of Treg markers**

- CD4<sup>FOX</sup>P<sup>3</sup> T cells upregulate Tre-related markers
- IPEX CD4<sup>FOX</sup>P<sup>3</sup> display phenotype superimposable to Healthy donors’ CD4<sup>FOX</sup>P<sup>3</sup>
**CD4^{FOXP3} acquire Treg-like in vitro functions**

Inhibition of Responder cell (R) proliferation upon in vitro co-culture

- **Healthy subjects (n=11)**
- **Patients (n=5)**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>% Suppression</th>
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</thead>
<tbody>
<tr>
<td>[S]</td>
<td></td>
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<tr>
<td>[R:S]</td>
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</tr>
<tr>
<td>[1:1]</td>
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<td>[2:1]</td>
<td></td>
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<tr>
<td>[4:1]</td>
<td></td>
</tr>
</tbody>
</table>

**UT**

- **CD4^{NGFR}**
- **CD4^{FOXP3}

Proliferative capacity upon polyclonal activation

- **Healthy subjects (n=11)**
- **Patients (n=5)**

<table>
<thead>
<tr>
<th></th>
<th>cpm (x10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>169%</td>
</tr>
<tr>
<td>CD4^{NGFR}</td>
<td>180%</td>
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</table>

**Patients’ CD4^{FOXP3} are anergic and suppressive in *in vitro* functional assays**
Lethal xenogeneic Graft-versus-host disease model (xenoGvHD)

In vivo function of $CD4^{FOXP3}$ T cells

- d0
- ¥ irradiation
- Teff cell transfer (2x10^6)
  $\pm$ $CD4^{NGFR}/CD4^{FOXP3}$ [1:1]
  @ day0 or day6
- d+3
- monitor weight (3 times/week)
- day +40
- monitor hu-chimerism in PB (once/week)

NSG

GvHD:
weight loss >20%
human chimerism >10%
IPEX CD4^{FOXP3} are functional in vivo

Pt#21

LV-FOXP3/NGFR

CD4^{FOXP3} CD4^{NGFR}

CD4

IPEX CD4^{FOXP3} efficiently arrest a xeno-GvHD reaction
FOXP3 gene transfer in FOXP3-mutated T cells is feasible

High and stable FOXP3 expression can be induced in FOXP3mut T cells isolated from IPEX patients

CD4^{FOXP3} from Pts acquire Treg-like phenotype and potent in vitro and in vivo suppressor function

CD4^{FOXP3} Tregs are stable in inflammatory environment and do not induce per se xenoGvHD

LV-mediated overexpression of wtFOXP3 can restore impaired Treg cell function in IPEX: clinical translation?

Passerini et al, Sci Transl Med 2013
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www.ipexconsortium.org

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Thank you
CD4^{FOXp3} are stable in vivo

- d0: irradiation
- Teff cell

- d6: CD4^{NGFR}/CD4^{FOXp3}

- d13: \texttt{\textasciitilde} in vitro assays

**Treg phenotype @ day+13** (NGFR+ gate)

**Cytokine production** (TPA/iono) @ day+13 (NGFR+ gate)

\textbf{CD4^{FOXp3}} recovered after 7 days in vivo in inflammatory conditions maintained stable Treg phenotype and did not produce cytokines
CD4^{FOXP3} do not proliferate in vivo and do not induce per se xenoGvHD

- CD4^{FOXP3} cells do not induce xenoGvHD when injected alone
- CD4^{FOXP3} cells do not expand in vivo
Assessing the transcriptome of CD4^{FOXP3} T cells

Analysis (q<0.25) revealed:
- 73 genes up in CD4^{FOXP3} vs CD4^{NGFR} T cells.
- 94 genes down in CD4^{FOXP3} vs CD4^{NGFR} T cells.

RNA-seq: Illumina HiSeq 2000 2x101, Mapped to hg19 reference genome (no pre-amplification), analysis was performed using TopHat and Cufflinks software tools (Trapnell et al, Nature Protocols 2012).
### Top 20 genes overexpressed in CD4<sup>FOXP3</sup> T cells (vs CD4<sup>NGFR</sup>, q<0.25)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene description</th>
<th>log2 (fold_change)</th>
<th>q_value</th>
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<tbody>
<tr>
<td>FOXP3</td>
<td>forkhead box P3</td>
<td>4.65845</td>
<td>0.0158537</td>
</tr>
<tr>
<td>LGMN</td>
<td>legumain</td>
<td>3.67259</td>
<td>0.0158537</td>
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<td>ANK1</td>
<td>ankyrin 1, erythrocytic</td>
<td>1.77368</td>
<td>0.0158537</td>
</tr>
<tr>
<td>IL6R</td>
<td>interleukin 6 receptor</td>
<td>2.12484</td>
<td>0.0158537</td>
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<tr>
<td>COL5A3</td>
<td>collagen, type V, alpha 3</td>
<td>2.19715</td>
<td>0.0158537</td>
</tr>
<tr>
<td>TBC1D4</td>
<td>TBC1 domain family, member 4</td>
<td>1.46488</td>
<td>0.0158537</td>
</tr>
<tr>
<td>CTLA4</td>
<td>cytotoxic T-lymphocyte-associated protein 4</td>
<td>1.69929</td>
<td>0.0158537</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular cell adhesion molecule 1</td>
<td>2.97911</td>
<td>0.0158537</td>
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<tr>
<td>SLC6A20</td>
<td>solute carrier family 6 (proline imino transporter), member 20</td>
<td>2.85798</td>
<td>0.0158537</td>
</tr>
<tr>
<td>CCR7</td>
<td>chemokine (C-C motif) receptor 7</td>
<td>1.9468</td>
<td>0.0158537</td>
</tr>
<tr>
<td>CTSL1</td>
<td>cathepsin L1</td>
<td>3.56997</td>
<td>0.0158537</td>
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<tr>
<td>MEGF6</td>
<td>multiple EGF-like-domains 6</td>
<td>1.17033</td>
<td>0.0158537</td>
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<tr>
<td>IKZF3</td>
<td>IKAROS family zinc finger 3 (Aiolos)</td>
<td>0.97284</td>
<td>0.0158537</td>
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<tr>
<td>IL1R1</td>
<td>interleukin 1 receptor, type 1</td>
<td>2.71248</td>
<td>0.0254902</td>
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<tr>
<td>DENND5B</td>
<td>DENN/MADD domain containing 5B</td>
<td>2.98437</td>
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<tr>
<td>ZDHHC23</td>
<td>zinc finger, DHHC-type containing 23</td>
<td>2.2809</td>
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<td>STON2</td>
<td>stonin 2</td>
<td>3.37441</td>
<td>0.0254902</td>
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<tr>
<td>SETD7</td>
<td>SET domain containing (lysine methyltransferase) 7</td>
<td>1.14561</td>
<td>0.0254902</td>
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<tr>
<td>PLCL1</td>
<td>phospholipase C-like 1</td>
<td>1.30084</td>
<td>0.0254902</td>
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<tr>
<td>KLRC2</td>
<td>killer cell lectin-like receptor subfamily C, member 2</td>
<td>2.44852</td>
<td>0.0354545</td>
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</table>
### Top 20 genes downregulated in CD4<sup>FOXP3</sup> T cells (vs CD4<sup>NGFR</sup>, q<0.25)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene description</th>
<th>log2 (fold_change)</th>
<th>q_value</th>
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<tr>
<td>IL7R</td>
<td>Interleukin 7 Receptor</td>
<td>-1.91428</td>
<td>0.0158537</td>
</tr>
<tr>
<td>CFH</td>
<td>complement factor H</td>
<td>-2.41263</td>
<td>0.0158537</td>
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<tr>
<td>P2RY1</td>
<td>purinergic receptor P2Y, G-protein coupled, 1</td>
<td>-4.67849</td>
<td>0.0158537</td>
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<tr>
<td>CD300A</td>
<td>CD300a molecule</td>
<td>-1.81308</td>
<td>0.0158537</td>
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<tr>
<td>CDC42BPA</td>
<td>CDC42 binding protein kinase alpha (DMPK-like)</td>
<td>-3.21946</td>
<td>0.0158537</td>
</tr>
<tr>
<td>PDE7B</td>
<td>phosphodiesterase 7B</td>
<td>-1.54689</td>
<td>0.0158537</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>arachidonate 5-lipoxygenase-activating protein</td>
<td>-1.42923</td>
<td>0.0158537</td>
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<tr>
<td>CCL1</td>
<td>chemokine (C-C motif) ligand 1</td>
<td>-2.14766</td>
<td>0.0158537</td>
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<td>HPGDS</td>
<td>hematopoietic prostaglandin D synthase</td>
<td>-2.53194</td>
<td>0.0158537</td>
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<td>ATP9A</td>
<td>ATPase, class II, type 9A</td>
<td>-2.37648</td>
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<tr>
<td>MIR210HG</td>
<td>MIR210 host gene (non-protein coding)</td>
<td>-3.16741</td>
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<td>AK4</td>
<td>adenylate kinase 4</td>
<td>-1.90385</td>
<td>0.0158537</td>
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<tr>
<td>STAP1</td>
<td>signal transducing adaptor family member 1</td>
<td>-1.49093</td>
<td>0.0158537</td>
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<td>FRY</td>
<td>furry homolog (Drosophila)</td>
<td>-1.44387</td>
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<td>SYTL2</td>
<td>synaptotagmin-like 2</td>
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<td>GCNT1</td>
<td>glucosaminyl (N-acetyl) transferase 1, core 2</td>
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<td>HIST1H3B</td>
<td>histone cluster 1, H3b</td>
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<td>IL17RB</td>
<td>interleukin 17 receptor B</td>
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<td>0.0158537</td>
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<td>CLU</td>
<td>clusterin</td>
<td>-1.72368</td>
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<tr>
<td>PION</td>
<td>Gamma-Secretase Activating Protein</td>
<td>-1.47872</td>
<td>0.0158537</td>
</tr>
</tbody>
</table>
Do $\text{CD}^{4\text{FOXP3}}$ T cells acquire a FOXP3-Treg-like signature?

The most significant ($q<0.06$) DEGs in $\text{CD}^{4\text{FOXP3}}$ T cells were compared to DEGs in Treg cells.
STAT5 activation positively affects FOXP3 expression in Tregs and activated Teff cells.

**CD4+CD25+ Tregs**

ACTIVATION: αCD3+αCD28 mAbs +
- aIL-2Rα 10 µg/ml
- IL-2 100U/ml
- IL-4 10ng/ml + aIL-2Rα 10µg/ml
- IL-7 10ng/ml + aIL-2Rα 10µg/ml
- IL-15 10ng/ml + aIL-2Rα 10 µg/ml

**CD4+CD25- Teff cells**

ACTIVATION: αCD3+αCD28 mAbs +
- medium
- IL-2 100U/ml
- aIL-2Rα mAb 10µg/ml

Passerini et al., Int Immunology 2008
**FOXP3 knock-down reverts Treg suppressive capacity**

LV-mediated siFOXP3 reverts Treg suppressive activity...

and increases IL-17 production by CD4⁺CD25⁺ Treg

*Amendola et al., Mol Ther 2009*