From Bench to Bedside

Regulatory T Cells: Can We Make the Police Work for Us?

Sang-Mo Kang, MD
Qizhi Tang, PhD

UCSF Division of Transplantation
The Reality of Immunosuppression

*Triple Immunosuppression to Prevent Graft Rejection*
Overview

- Why does the immune system need Tregs?
- What are Tregs?
- Phenotype and function of Tregs
- Evidence for involvement of Tregs in transplantation
- Challenges for Clinical Translation
- Upcoming Trials
Why Do We Care About T cells?

- CD8
- CD4
- CTL
- Th1
- Th17
- Th2
- Tfh
- Th9

There is no solid organ allograft rejection in the absence of T cells!

- Kill targets
- Activate innate cells, kill targets
- Help B cells
Potentially self-reactive T cells are largely eliminated in the thymus via “negative selection”

BUT, self-reactive T cells are readily identified and expansion of self-reactive T cells has been demonstrated in the course of an immune response
Threat of Rogue Autoimmune T cells

- Elimination of autoreactive T cells in the thymus (central deletion) is not perfect

- T cell activation in response to foreign antigens can lead to activation of autoreactive T cells

- Deletion and anergy are imperfect mechanisms of tolerance
Tregs: Peace keepers of the immune system

1. Tregs are CD4+ CD25+ CD127lo cells
2. 1-2% of peripheral mononuclear cells are Tregs
3. Treg-deficiency, from mutations of the transcription factor Foxp3, leads to multi-organ autoimmune diseases and early lethality if untreated.
Elimination of Tregs Leads to Rapid Death

Kim JM et. al Nature Immunology 2007
## Markers of CD4+ Tregs

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Activation and memory</th>
<th>Homing and origin</th>
<th>Suppressive and effector function</th>
<th>Apoptosis, survival or other</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3</td>
<td>CD45RA</td>
<td>CD62L</td>
<td>CTLA4</td>
<td>CD27</td>
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<tr>
<td></td>
<td>CD45RO</td>
<td></td>
<td>ICOS</td>
<td>OX40</td>
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<td></td>
<td>CD25</td>
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<td>CD39–CD73</td>
<td>CD95</td>
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<td></td>
<td>HLA-DR</td>
<td></td>
<td>LAP</td>
<td>PD1</td>
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<td></td>
<td>Lack of CD127</td>
<td></td>
<td>Granzyme B</td>
<td>GITR</td>
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<td></td>
<td>CD69</td>
<td></td>
<td>Galectin 1</td>
<td>Galectin 3</td>
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<td></td>
<td>Galectin 10</td>
<td>GARP</td>
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<td></td>
<td>TRANCE</td>
<td>MS4A4B</td>
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<td></td>
<td>CD80 and CD86</td>
<td>IL-1R</td>
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<td></td>
<td>IL-10</td>
<td>CD6</td>
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<td></td>
<td>IL-17</td>
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<td></td>
<td>CD2</td>
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<td></td>
<td>Lack of CD49d</td>
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</table>

CCR, CC-chemokine receptor; CTLA4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; GARP, glycoprotein A repetitions predominant; GITR, glucocorticoid-induced TNF-receptor-related protein; ICOS, inducible T cell co-stimulator; IL, interleukin; LAP, latency-associated peptide; MS4A4B, membrane-spanning 4-domains, subfamily A, member 4B; PD1, programmed cell death 1; R, receptor; TRANCE, TNF-related activation-induced cytokine; T\textsubscript{Reg}, regulatory T.
Where Do Tregs Come From?

Interplay between “natural” Treg and “adaptive” Treg may help explain the concept of “infectious tolerance”
How Do Treg Suppress Immune Responses?

Larché et al. Nature Reviews Immunology 6, 761–771 (October 2006) | doi:10.1038/nri1934
# How Do Treg Suppress Immune Responses?

<table>
<thead>
<tr>
<th>Key molecule(s)</th>
<th>Function</th>
<th>Mouse</th>
<th>Human</th>
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<tbody>
<tr>
<td><strong>Mechanisms of contact-dependent suppression</strong></td>
<td></td>
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<tr>
<td>CTLA4</td>
<td>Downregulation of APC co-stimulatory function</td>
<td>76</td>
<td>–</td>
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<tr>
<td></td>
<td>Interaction with CD80 and CD86 on conventional T cells</td>
<td>101</td>
<td>–</td>
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<tr>
<td>CD73–CD39</td>
<td>Hydrolysis of inflammatory extracellular ATP</td>
<td>102</td>
<td>–</td>
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<tr>
<td>LAG3</td>
<td>Induction of inhibitory signalling through MHC class II molecules</td>
<td>103</td>
<td>–</td>
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<tr>
<td>Granzyme B (mouse) and granzyme A (human)</td>
<td>Lysis of conventional T cells</td>
<td>104,105</td>
<td>106</td>
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<tr>
<td>CD95–CD95 ligand</td>
<td>Induction of apoptosis in conventional T cells</td>
<td>–</td>
<td>107</td>
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<tr>
<td><strong>Mechanisms of cytokine-mediated suppression</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TGFβ and LAP</td>
<td>Induction of FOXP3 in conventional T cells</td>
<td>108,109</td>
<td>–</td>
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<tr>
<td>IL-10</td>
<td>Attenuation of DC function</td>
<td>–</td>
<td>20</td>
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<td>Conversion of conventional T cells to Tr1 cells</td>
<td>110,111</td>
<td>111</td>
</tr>
<tr>
<td>Galectin 1</td>
<td>Cell cycle arrest and apoptosis in conventional T cells</td>
<td>112</td>
<td>–</td>
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<tr>
<td>CD25</td>
<td>Adsorption of IL-2</td>
<td>113</td>
<td>–</td>
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<tr>
<td>IL-35</td>
<td>Induction of conventional T cell expression of IL-35 by T&lt;sub&gt;Reg&lt;/sub&gt; cells enhances suppression (IL-35 is not expressed by human T&lt;sub&gt;Reg&lt;/sub&gt; cells)</td>
<td>114</td>
<td>115</td>
</tr>
</tbody>
</table>

APC, antigen-presenting cell; CTLA4, cytotoxic T lymphocyte antigen 4; DC, dendritic cell; FOXP3, forkhead box P3; IL, interleukin; LAG3, lymphocyte activation gene 3; LAP, latency-associated peptide; TGFβ, transforming growth factor-β; T<sub>Reg</sub>, regulatory T.
Function of Tregs during an immune response

Steady state
- Preventing autoimmune pathology

Infection
- Immune activation
- Clearance of pathogens

Resolution
- Tregs
- iTregs
- Tr1
- Th3
- Th2
- Tfh
- CD8
- Th9
- Th2
- Th17
- Th1
- Tfh
- Th9
- CTL
Using Tregs to prevent rejection and induce graft tolerance

Steady state

Transplant

Immune modulation

Tolerance

Re-educating the immune system
How Do Tregs Suppress?

- TCR stimulation on Treg is required
- Cell-cell contact is required
- Once the Treg is activated specifically by antigen, the Treg can suppress Teff with different specificities
Evidence for Treg involvement in tolerance

- Depletion of Tregs abrogates established tolerance in many models
  - Also causes autoimmunity

- Augmentation of Treg activity appears to correlate with tolerance
  - Pregnancy
  - Transplantation

- Experimental administration of Treg can prevent or treat autoimmune disease in mouse models
Importance of Foxp3+ Tregs in Preventing Autoimmunity

Kim JM et al. Nature Immunology 2007
Evidence for involvement of Tregs in transplantation

- In numerous transplant models of tolerance, allospecific Treg can be identified
  - Transfer of T cells from tolerant mice can prevent rejection by transferred Teff in secondary animals (T cell deficient)
  - Foxp3+ Treg can be identified in tolerant allografts
  - Depletion of Tregs causes rejection in tolerant animals

- Clinical data in renal transplantation
Treg Prevention and Reversal of Autoimmune Diabetes

FACS Phenotype of Treg

CD4 - PE

2.6%

CD25 - APC
Flow Sort Tregs by gating on CD4+, CD25+, CD62hi

*Foxp3 is an intracellular antigen and cannot be used to isolate Treg

*CD127 lo enriches for Tregs in humans
What are regulatory T cells

- CD8
- CD4
- Tregs
- CTL
- Th1
- Th2
- Th17
- Th9
- Tfh

Kill targets
Activate innate cells
Help B cells
What are regulatory T cells

- **CD8**
  - Kill targets

- **CD4**
  - Activate innate cells
  - Help B cells

- **Tregs**
  - Suppress unwanted immune responses

Types of regulatory T cells:
- **Th1**
- **Th2**
- **Th17**
- **Tfh**
- **Th9**
- **iTregs**
- **Tr1**
- **Th3**
<table>
<thead>
<tr>
<th>Application</th>
<th>In use</th>
<th>Under investigation</th>
<th>Side effects and caveats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection</td>
<td>Molecules inhibiting $T_{reg}$ cell function, such as CTLA4-specific antibody (ipilimumab (MDX-010; Bristol-Myers Squibb/Medarex))</td>
<td>Molecules inhibiting $T_{reg}$ cell function and differentiation</td>
<td>Low efficacy in infectious diseases</td>
</tr>
<tr>
<td>Cancer</td>
<td>Molecules that deplete $T_{reg}$ cell populations, such as DAB389–IL-2 (denileukin difitox (Ontak; Eisai))</td>
<td>Molecules that deplete $T_{reg}$ cells</td>
<td>Induction of autoimmunity</td>
</tr>
<tr>
<td>Autoimmunity</td>
<td>Molecules mimicking $T_{reg}$ cell-mediated suppression, such as CTLA4–Ig (Abatacept (Orencia; Bristol-Myers Squibb))</td>
<td>Cellular therapy based on $T_{reg}$ cell expansion*</td>
<td>Suppression of cancer surveillance and immunity</td>
</tr>
<tr>
<td>Allergy</td>
<td>Molecules mimicking $T_{reg}$ cell-mediated suppression</td>
<td>Molecules capable of maintaining or improving $T_{reg}$ cell purity and survival</td>
<td>Feasibility during pregnancy not evaluated</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Molecules that favour $T_{reg}$ cell survival and development, such as rapamycin (Sirolimus/Rapamune; Wyeth)</td>
<td>Molecules capable of maintaining or improving $T_{reg}$ cell purity and survival</td>
<td>Feasibility during pregnancy not evaluated</td>
</tr>
<tr>
<td>Transplantation*</td>
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</table>

CTLA4, cytotoxic T lymphocyte antigen 4; Ig, immunoglobulin; $T_{reg}$ regulatory T. *In limited use for some patients with graft-versus-host disease.
Overview

1. Treg therapy in mouse models of transplantation
2. Manufacturing donor-alloantigen-specific Tregs for clinical use
3. Clinical trial plans for Treg therapy in transplant patients
Tregs have very limited efficacy in preventing graft rejection

Semi-allogeneic heart transplantation
BALB/c x B6 F1 → C57BL/6

<table>
<thead>
<tr>
<th>Graft</th>
<th>Tregs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c x B6 F1</td>
<td>none</td>
</tr>
<tr>
<td>BALB/c x B6 F1</td>
<td>B6</td>
</tr>
<tr>
<td>BALB/c x B6 F1</td>
<td>4C</td>
</tr>
<tr>
<td>CBA x B6 F1</td>
<td>4C</td>
</tr>
</tbody>
</table>

4C = donor alloantigen-Tregs

Not effective on:
- fully allogeneic heart
- islet
- skin

High frequency of alloreactive T cells may reduce the efficacy of Tregs

Brennan et al 2011
A strategy to induce long-term allograft survival using Treg therapy

Day 0
- DST
- Donor Splenocytes (20 x 10^6)
- Kill Proliferating Cells (100 or 200 mg/Kg)

Day 2
- CY
- STZ (streptozotocin)

Day 4
- Tregs (5 x 10^6)

Day 6
- iTx
- (450~500 islets)

Treg Transplantation
Donor-Reactive T cell Deletion
T+Cy efficiently deletes donor-reactive T cells

**CD4**

- Naïve: 1.0
- DST+CY100: 0.6
- DST+CY200: 0.3

57% 82%

**CD8**

- Naïve: 1.0
- DST+CY100: 0.6
- DST+CY200: 0.3

38% 67%
Deletion alone is not sufficient to prolong islet allograft survival.
Minor-specific Tregs combined with deletion can induce long-term allograft survival

BALB/c islets

- None (n=7)
- DST+CY100+4C Treg (n=9)
- DST+CY200+4C Treg (n=22)

Days after transplantation
nor-specific Tregs combined with deletion can induce long-term allograft survival
Summary 1

- Tregs alone are not sufficient to protect graft rejection in lymphoreplete hosts.
- In mice, 70-80% reduction of donor-reactive T cells is required to create a therapeutic window for Tregs.
- Donor-specific Tregs works better than polyclonal Tregs, however, polyclonal Tregs can work.
Translating to humans

- Manufacturing Tregs for therapeutic use
- Designing clinical protocols
- Planning mechanistic studies
What Tregs to use - Expansion or not?

May work without expansion in
- GvHD, Tregs from donor
- Organ transplant with lymphodepletion
  isolate and bank autologous Tregs prior to depletion
What Tregs to use - Expansion or not?

May work without expansion in
• GvHD, Tregs from donor
• Organ transplant with lymphodepletion
  isolate and bank autologous Tregs prior to depletion

<table>
<thead>
<tr>
<th></th>
<th>in circulation</th>
<th>total</th>
</tr>
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<tbody>
<tr>
<td>CD4</td>
<td>2 - 3 x 10⁹</td>
<td>200 - 300 x 10⁹</td>
</tr>
<tr>
<td>Treg</td>
<td>50 – 100 x 10⁶</td>
<td>5 - 10 x 10⁹</td>
</tr>
</tbody>
</table>

There is a limit of how many Tregs one can give without expansion, ~100 x 10⁶, which is most likely not enough for preventing graft rejection.
What Tregs to expand, polyclonal or donor-reactive?

<table>
<thead>
<tr>
<th>Pro</th>
<th>Polyclonal</th>
<th>Donor-reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Manufacturing process established</td>
<td>• Universally more effective than polyclonal Tregs in various preclinical models;</td>
</tr>
<tr>
<td></td>
<td>• Prior experiences in patients, reported in two trials in GvHD</td>
<td>• Targeted to graft alloantigens - more specific immune regulation</td>
</tr>
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<td></td>
<td>• Ongoing T1D trial at UCSF, with an open IND</td>
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<tr>
<th>Con</th>
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<tbody>
<tr>
<td></td>
<td>• May suppress non-specifically</td>
</tr>
<tr>
<td></td>
<td>• May be less efficacious</td>
</tr>
<tr>
<td></td>
<td>• No established protocol for large scale expansion of donor-reactive Tregs</td>
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</tbody>
</table>
Human donor-reactive Treg manufacturing

1. Collect patient PBMC
2. Cryo-preserve PBMC
3. Collect or spl or LN
4. Isolate donor B cells
5. Make donor stimulated B cells, bank
6. 10K rads
7. Banked cGMP 62-hCD40Leder cells
8. Isolate donor B cells
9. Treg culture with donor B cells
10. Day 11: Treg re-stimulation with anti-CD3/28 beads
11. Day 16: Harvest
12. Day 16: Review and approval
13. Day 16: Release assays
14. Day 16: Infusion
15. 1 wk after release: post release assays
Two-step donor-reactive Treg expansion

Using this approach, we can routinely generate 1-5 billion donor-reactive Tregs from one unit of blood.
or-specific Tregs are highly potent suppressors

![Graph showing Treg: Responder ratio for polyTreg and dsTreg](image)
Summary 2

- Combining initial antigen selective expansion and polyclonal restimulation of FACS purified Tregs
- 200-1000 expansion of donor-specific Tregs in less than 20 days
- >95% CD4+ and >60% Foxp3+; >95% have demethylated Foxp3 promoter – stable Tregs
- Highly donor-reactive and highly suppressive in vitro
Moving to the clinic

1. Phase I trial in adult liver transplant – R34 planning grant funded by NIAID
2. Phase I trial in adult living donor kidney transplant – funded by EU One Study
3. Phase I/II trial adult kidney transplant with subclinical inflammation on protocol biopsy
4. Phase I/II trial in pediatric liver transplant for facilitating immunosuppression withdrawal in non-tolerance patients
Schematic for the adult liver trial

Donor

Generate and bank donor B cell line from donor spleen

Man manufacture donor-specific Tregs

Bank PBMCs

Sample banking: blood, serum, biopsy

Sample analyses: flow, gene/protein expression in blood & tissue, allo T cell frequencies & functions, allo Ab, pathology/IHC/IF

Recruit patient

Liver Tx

Thymoglobulin

Conversion to Sirolimus-based IS

Treg infusion

Follow-up 1-2 yrs

PIs: Sandy Feng, Sang-Mo Kang, Jeff Bluestone, Qizhi Tang
Schematic for the One Study: adult living-donor kidney

UCSF investigators: Sang-Mo Kang, Flavio Vencentí, Jeff Bluestone, Qizhi Tang
Prolongation of F1 → B6 Heart Transplant Using Cultured Tregs

- Relatively weak in unmanipulated animals—works better in T cell depleted animals
- Unlikely to be efficacious alone in transplantation
Foxp3 in Urine: Correlation with Reversal of Rejection?

Figure 2. Correlation between Levels of FOXP3 mRNA in Urinary Cells and Reversal of an Episode of Acute Rejection.

Box plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles for levels of mRNA for FOXP3, CD25, CD3e, and perforin in urine samples obtained from 26 subjects with successful reversal of acute rejection (classified as reversible and defined by the return of serum creatinine levels to within 15 percent of prerejection levels within four weeks after the initiation of antirejection treatment) and 10 patients without reversal of acute rejection (nonreversible). The levels of mRNA for FOXP3 but not for CD25, CD3e, and perforin were significantly higher in subjects with reversible acute rejection than in subjects with nonreversible acute rejection. Two-tailed P values are based on the Mann–Whitney test. In all cases, log-transformed levels, normalized for 18S rRNA, are shown.

Muthukumar et. al NEJM, 2005
Frequency of Tregs pre-transplant may correlate with rejection

- **Non-Rejectors**
  - $n=18$
  - Frequency: 6.95%

- **Rejectors**
  - $n=5$
  - Frequency: 4.29%

$p=0.03$
A Treg-Centric View of Transplant

- Utilize immunosuppressants/immunomodulatory agents that do not BLOCK Treg formation, and ideally use those that may induce Tregs
  - Sirolimus
  - Low dose thymo
  - Belatacept?
  - Avoid calcineurin inhibitors
  - Remarkably, corticosteroids may help induce Treg

- Additional studies to understand how immunosuppressants and immunomodulatory agents affect Treg numbers and function are needed
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