CD4⁺CD25<sup>high</sup>Foxp3⁺ T regulatory cells kill autologous CD8(+) and CD4(+) T cells using Fas/FasL- and Granzyme B-mediated pathways

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Objectives of our study were:

- To define mechanisms employed by Treg to mediate suppression of proliferating T responder cells (RC)

We considered 3 possible mechanisms:

1. Cytokine-mediated death.
2. Death receptor-mediated apoptosis.
3. Cytolysis mediated by granzymes/perforin.
Methods for studies of Treg

- Isolate CD4+CD25^{high} and CD4+CD25^{neg} or CD8+CD25^{neg} T cells from PBMC of NC or patients with cancer by single-cell sorting (FACS)
- Multicolor flow cytometry: phenotype
- Suppressor function:

  - CFSE-labeled RC (+ OKT3+ IL-2) + Unlabeled S
  - 5-day co-culture

  - Suppression of RC proliferation

  - FLOCA
    (Flow cytometry-based cytotoxicity assay)

  - Laser
We have shown before that CD8⁺ T effector cells in the circulation of patients with HNSCC (but not NC) are highly sensitive to apoptosis ⁴,⁵

Apoptosis in fresh T cell subsets from HNSCC patient and NC

[Diagram showing flow cytometry analysis]

[Legend]
- fresh PBMC
- NC
- HNSCC

⁴Reichert et al., 2000
⁵Hoffmann et al., 2002
Treg mediate suppression of autologous CD4\(^+\) or CD8\(^+\) RC proliferation via direct cell-cell contact\(^6\)

\(^6\)Strauss et al., 2007, Clinical Cancer Res, 1:13 (15):4352
Human CD4⁺CD25<sup>high</sup> Treg suppress proliferation and induce apoptosis in autologous CD8<sup>+</sup> responder cells

5-day co-cultures in the presence of 150 IU/mL IL-2 + OKT3

Responders cells alone (RC) (CD8⁺CD25<sup>neg</sup>)

(RC) cells + CD4⁺CD25<sup>high</sup>(S)
S/RC=1:1

CD8<sup>+</sup> (RC) cells

- 0%
- 0.3%
- 99.7%

7-AAD

CD8<sup>+</sup> (RC) cells

- 56%
- 24%
- 20%

CFSE

HNSCC
Human CD4⁺CD25<sup>high</sup> Treg suppress proliferation but do **not** mediate apoptosis in autologous CD4⁺ responder cells.

5-day co-cultures in the presence of 150 IU/mL IL-2 + OKT3.

- **Responder cells alone (RC)** (CD4⁺CD25<sup>neg</sup>)
- **(RC) cells + CD4⁺CD25<sup>high</sup> (S)**
  - S/RC = 1:1

**CD4⁺ (RC) cells**
- 7-AAD: 0%
- 1.2%
- 98.8%

**CD4⁺(RC) cells**
- 7-AAD: 0%
- 0.2%
- 99.8%

**CD4⁺ (S) cells**
- 7-AAD: 82%
- 15%
- 3%
Expression of Fas and FasL on CD4^+CD25^{high} T cells in NC or HNSCC patients

- Fas
  - NC (n=15): 5.5%
  - HNSCC (n=35): 43%
- FasL
  - NC (n=15): 95%
  - HNSCC (n=35): 92%

FRESH PBMC

% positive cells

- p<0.001
- p<0.03
Treg can “kill” autologous $\text{CD8}^+\text{CD25}^-$ RC but not $\text{CD4}^+\text{CD25}^-$ RC via the Fas/FasL pathway

**CD4$^+$CD25$^{\text{high}}$ Treg (FasL$^+$)

- + CFSE-labeled $\text{CD8}^+$RC
  - + anti-FasL- Ab
    - 7-AAD
      - 56%
      - 24%
      - 20%
    - CFSE
      - 62%

- + CFSE-labeled $\text{CD4}^+$RC
  - + anti-FasL- Ab
    - 7-AAD
      - 92%
      - 20%
      - 12%
    - CFSE
      - 97%

Results of cell death obtained in FLOCA

150 IU/mL IL-2

1000 IU/mL IL-2
Expression of Granzymes and Perforin in fresh and activated peripheral T-cell subsets

A. Gated on CD4+CD25\textsuperscript{high} in fresh PBMC

\begin{itemize}
  \item NC n=15
  \item HNSCC n=25
  \item *p≤0.01
\end{itemize}

+OKT3, IL-2 (150 IU/mL)

B. Gated on CD4+CD25\textsuperscript{low} in fresh PBMC

\begin{itemize}
  \item NC n=15
  \item HNSCC n=25
  \item *p≤0.03
\end{itemize}
Expression of cytotoxins is regulated by IL-2 in the presence of the “partner” T cell

CD4+CD25neg after co-incubation with Treg cells 1S:1 (RC)

CD4+CD25high after co-incubation with CD4+ RC cells 1S:1 (RC)

5-day co-cultures of Treg and autologous RC
Treg suppress CD4+ RC at low and high dose of IL-2, but can kill RC only at high IL-2 concentrations.

At low IL-2 doses, Treg induce suppression of RC proliferation and then undergo apoptosis. This type of suppression does not involve death of RC.
Mechanisms responsible for RC death and Treg survival in these co-cultures are IL-2 dependent
Treg-mediated killing of CD4+ RC is GranzymeB-dependent, but Perforin-independent.
CD4+ RC-mediated killing of Treg is GranzymeB and Perforin-dependent.
Conclusion 2

The GranzymeB-mediated reciprocal killing mediated by RC or Treg is IL-2-dependent
Expression of proteins that protect from apoptosis is regulated by IL-2

At low IL-2 concentrations, Treg co-cultured with RC do not up-regulate PI-9 and are sensitive to GrB-mediated apoptosis.
Conclusions

- Treg can suppress RC via three different mechanisms
- Tr1 largely use IL-10 and TGFβ1 to suppress RC proliferation (a contact independent process)
- CD8+RC expansion is suppressed by the Fas/FasL-mediated apoptosis
- CD4+RC proliferation is suppressed via a contact-dependent GrB/perforin pathway which is regulated by the IL-2 concentration in the microenvironment
- Resistance or sensitivity to death of Treg vs. RC is dependent on IL-2-mediated T cell activation