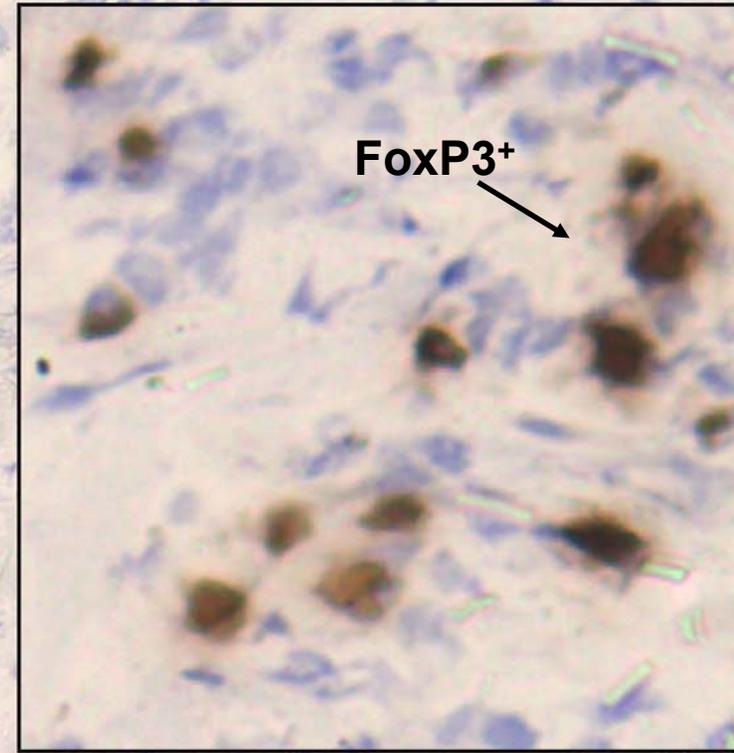


**CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> T regulatory cells  
kill autologous CD8(+) and CD4(+) T  
cells using Fas/FasL- and Granzyme B-  
mediated pathways**

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Pittsburgh, PA 15213***



# Objectives of our study were:

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

## **We considered 3 possible mechanisms:**

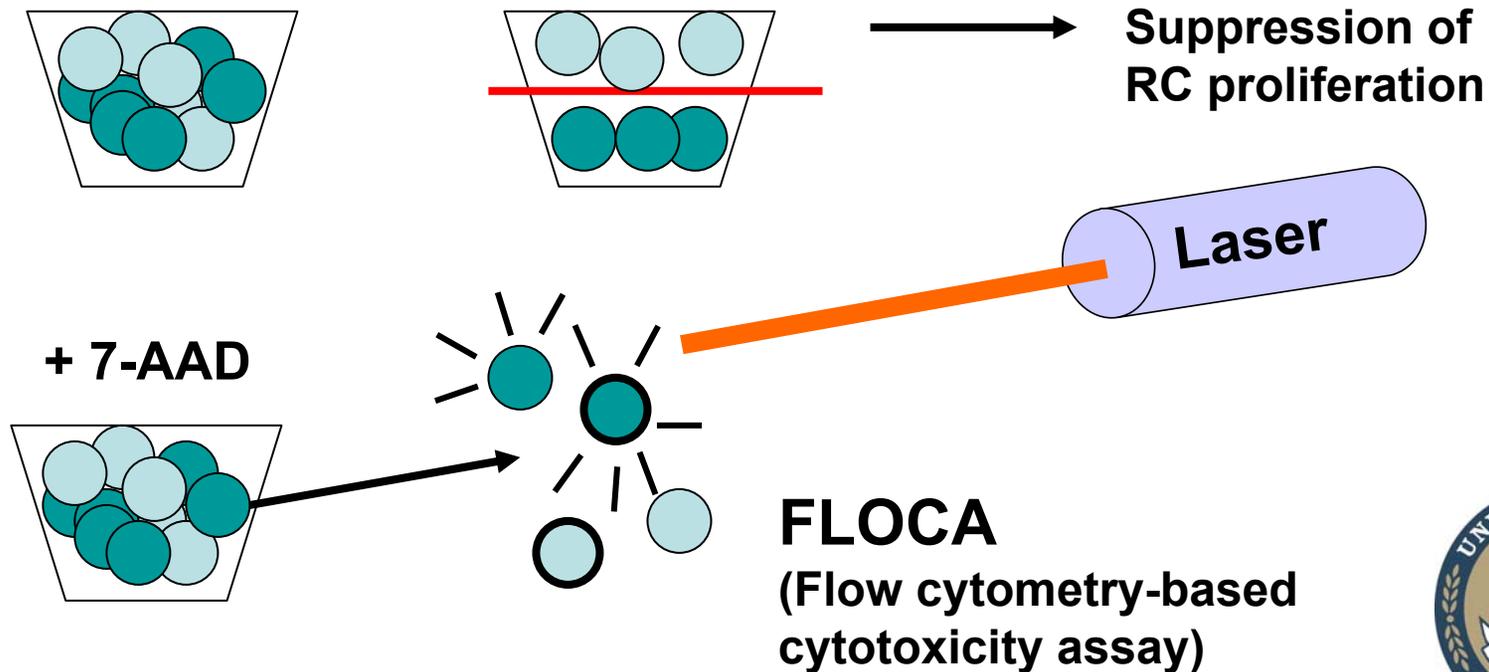
- 1. Cytokine- mediated death.**
- 2. Death receptor-mediated apoptosis.**
- 3. Cytolysis mediated by granzymes/perforin.**



# Methods for studies of Treg

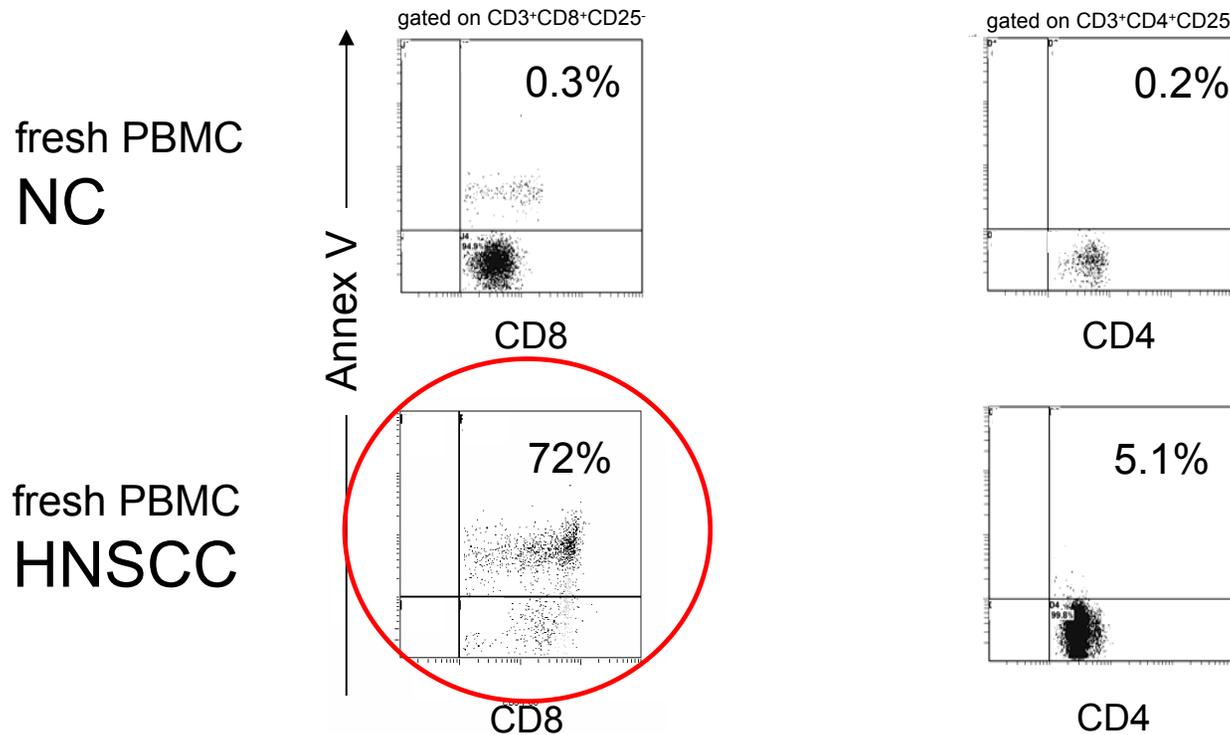
- Isolate CD4+CD25<sup>high</sup> and CD4+CD25<sup>neg</sup> or CD8+CD25<sup>neg</sup> T cells from PBMC of NC or patients with cancer by single-cell sorting (FACS)
- Multicolor flow cytometry: phenotype
- Suppressor function:

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



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

**Apoptosis in fresh T cell subsets from HNSCC patient and NC**

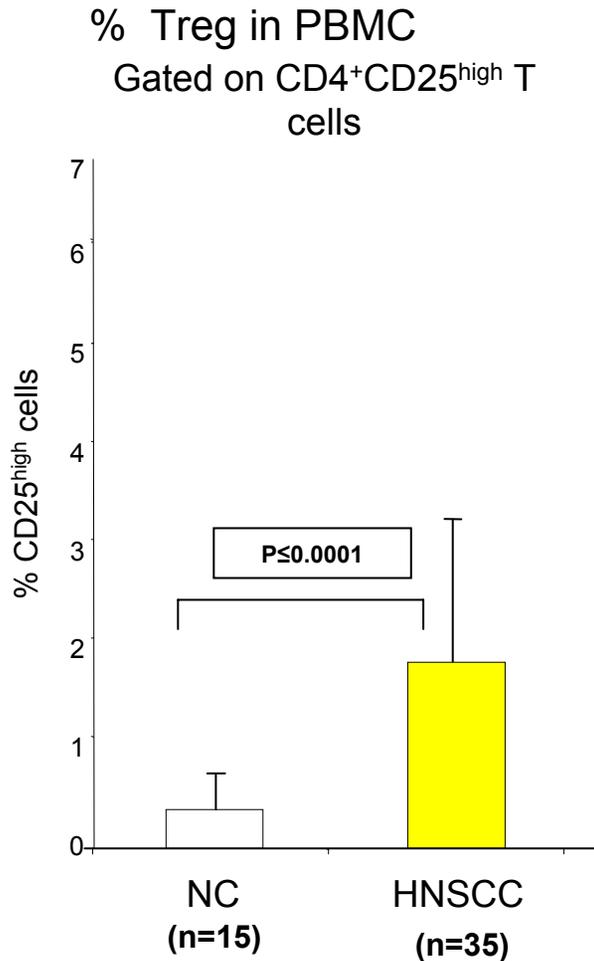


<sup>4</sup>Reichert et al., 2000

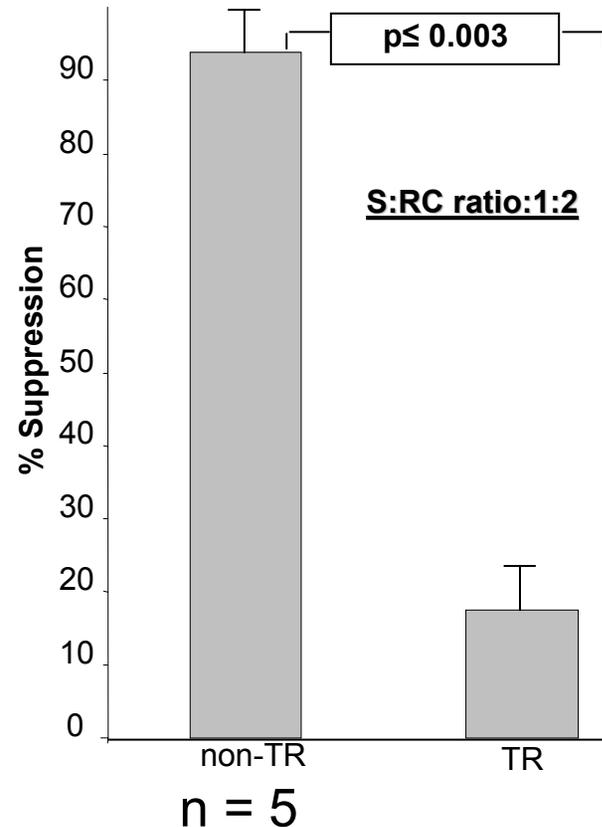
<sup>5</sup>Hoffmann et al., 2002



# Treg mediate suppression of autologous CD4<sup>+</sup> or CD8<sup>+</sup> RC proliferation via direct cell-cell contact<sup>6</sup>

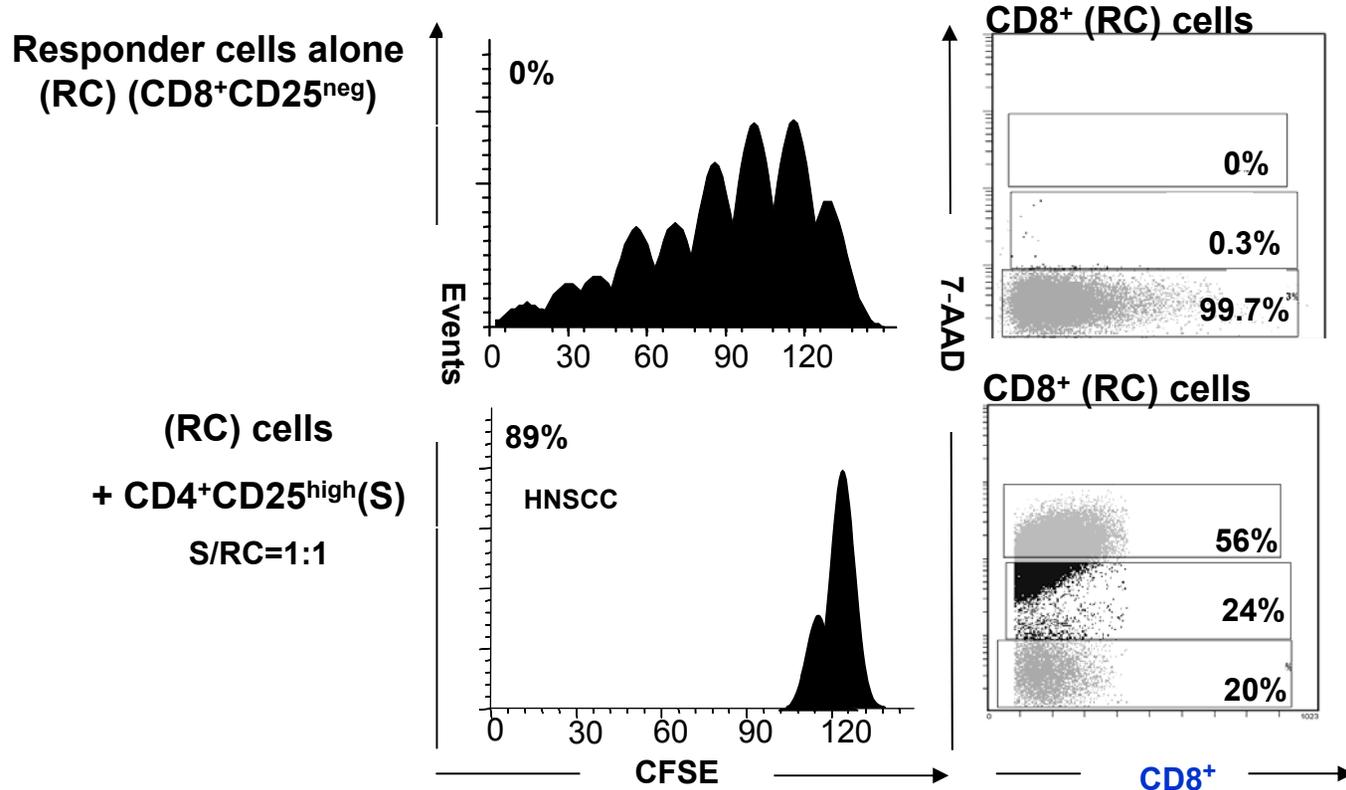


CD4<sup>+</sup>CD25<sup>high</sup> + CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup>CD25<sup>-</sup> co-cultures



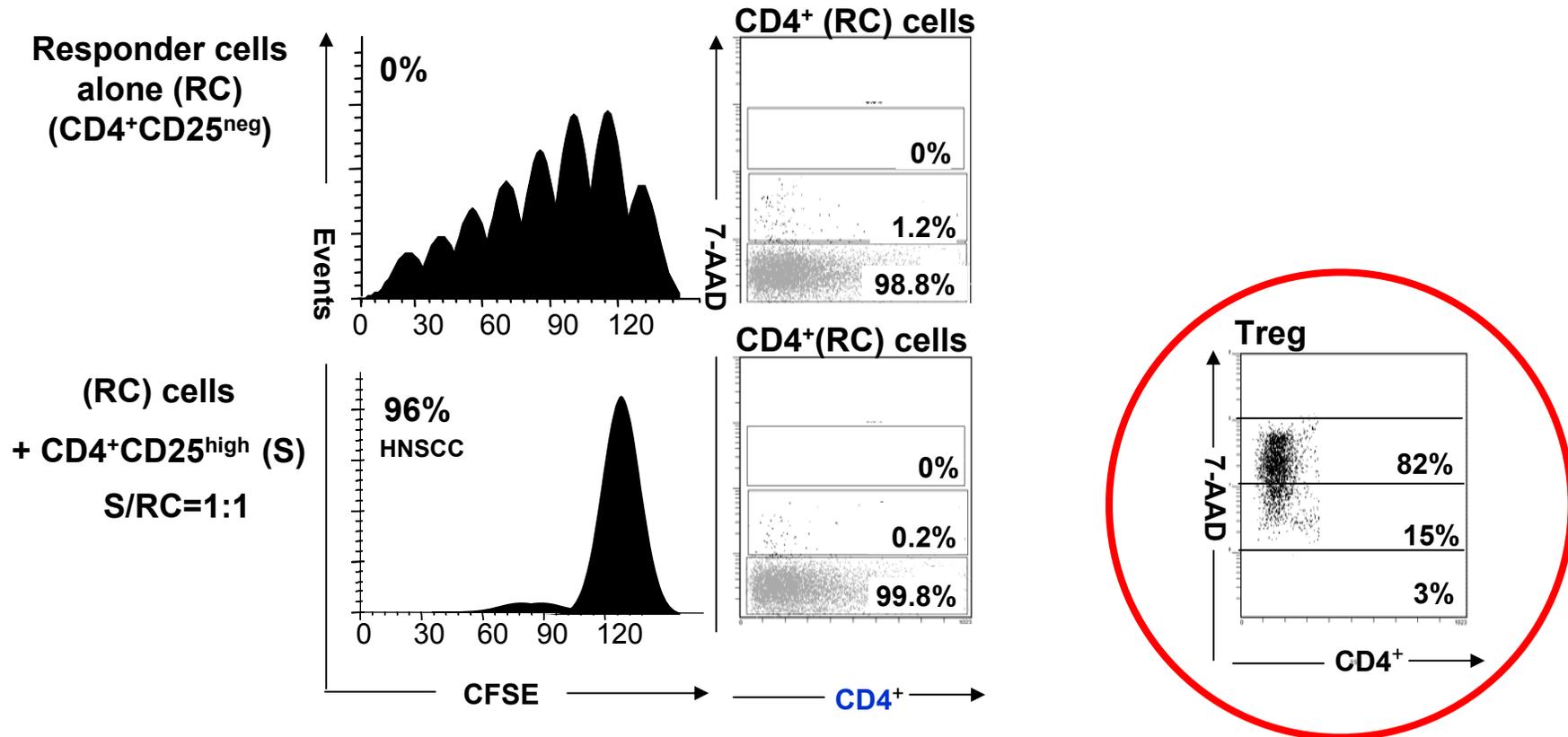
# Human CD4<sup>+</sup>CD25<sup>high</sup> Treg suppress proliferation and induce apoptosis in autologous CD8<sup>+</sup> responder cells

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

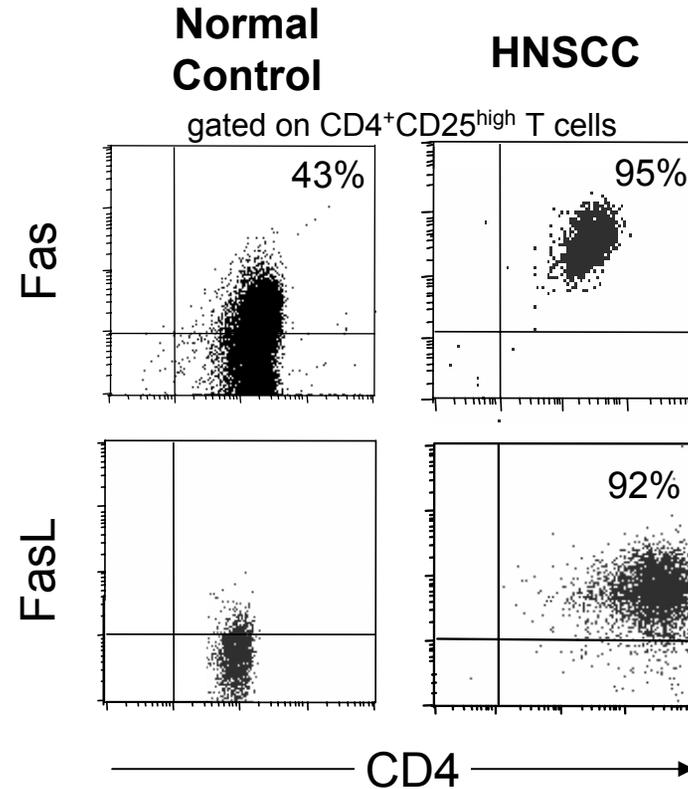
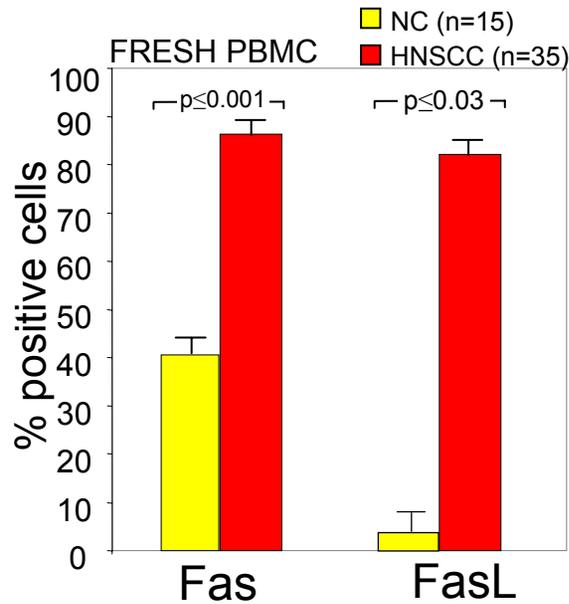


# Human CD4<sup>+</sup>CD25<sup>high</sup> Treg suppress proliferation but do not mediate apoptosis in autologous CD4<sup>+</sup> responder cells

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

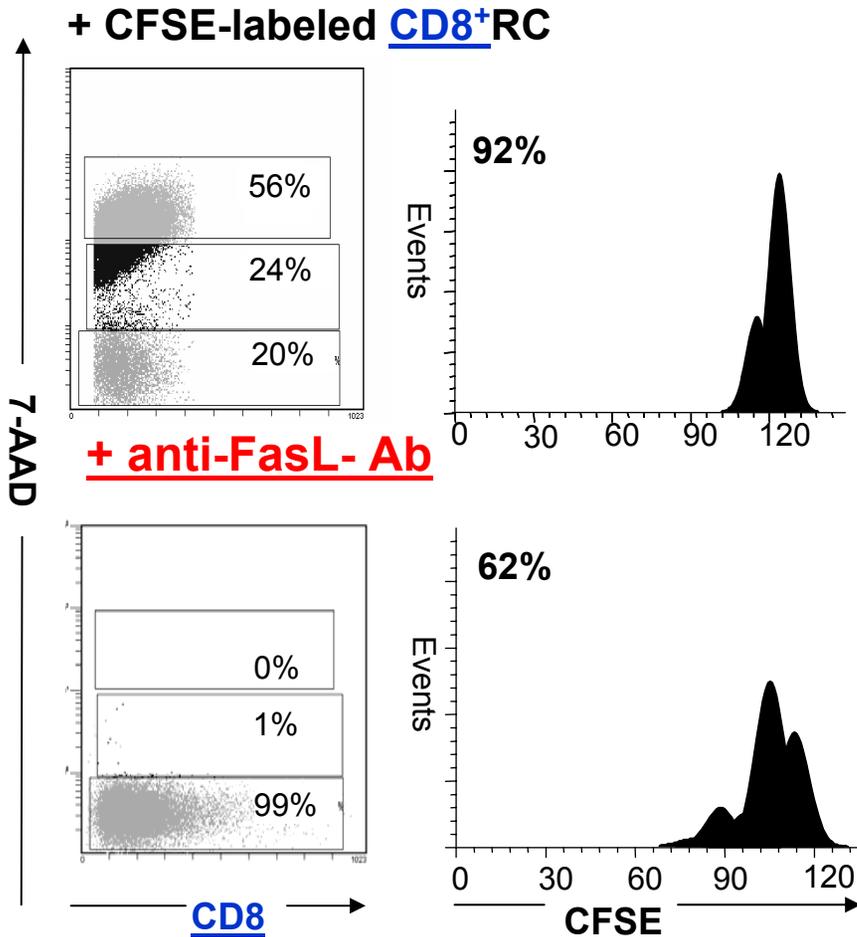


# Expression of Fas and FasL on CD4<sup>+</sup>CD25<sup>high</sup> T cells in NC or HNSCC patients

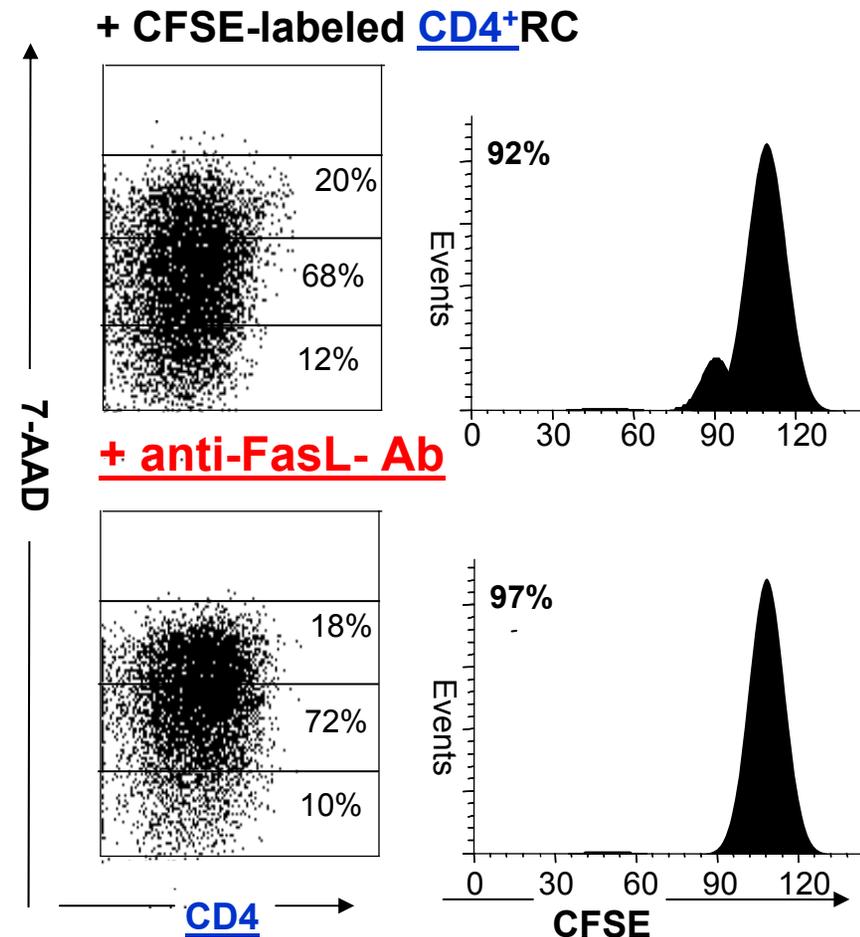


# Treg can “kill” autologous CD8<sup>+</sup>CD25<sup>-</sup> RC but not CD4<sup>+</sup>CD25<sup>-</sup> RC via the Fas/FasL pathway

CD4<sup>+</sup>CD25<sup>high</sup> Treg (FasL<sup>+</sup>)



150 IU/mL IL-2

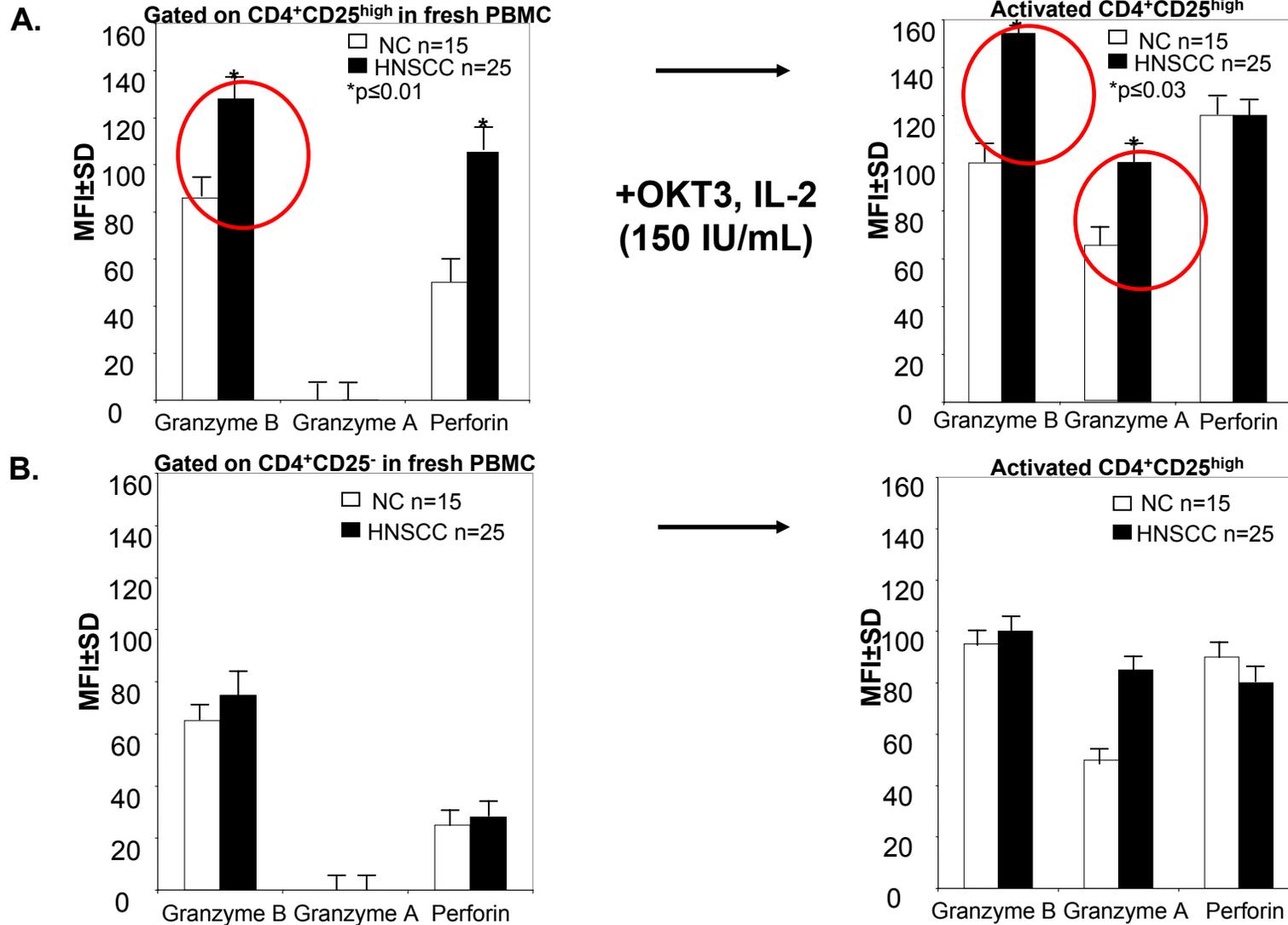


1000 IU/mL IL-2

Results of cell death obtained in FLOCA



# Expression of Granzymes and Perforin in fresh and activated peripheral T-cell subsets

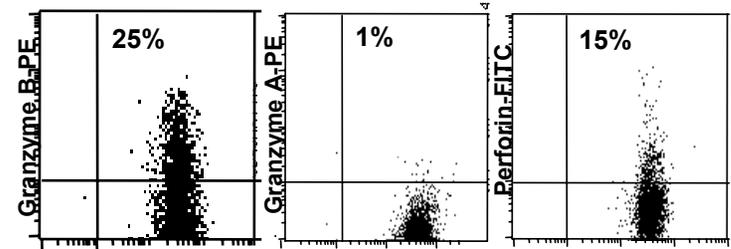
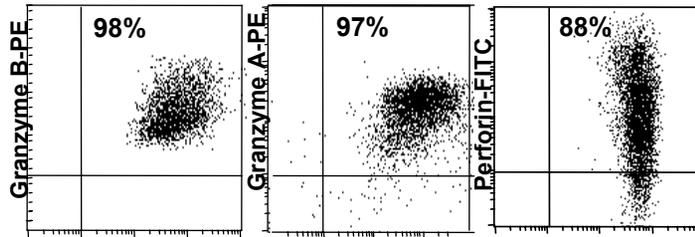


# Expression of cytotoxins is regulated by IL-2 in the presence of the “partner” T cell

(150 IU IL-2/mL)

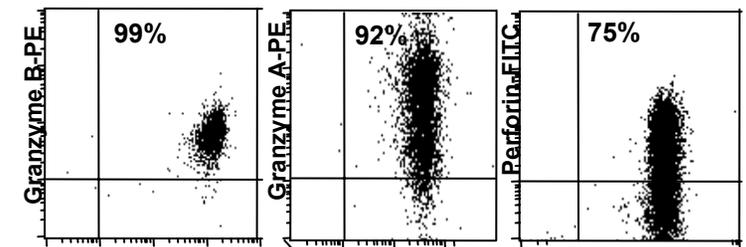
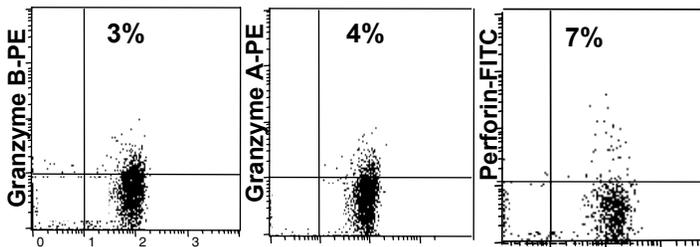
(1000 IU IL-2/mL)

**CD4<sup>+</sup>CD25<sup>neg</sup>**  
after co-incubation  
with  
Treg cells  
1S:1 (RC)



**R**

**CD4<sup>+</sup>CD25<sup>high</sup>**  
after co-incubation  
with  
CD4<sup>+</sup> RC cells  
1S:1 (RC)



**S**

CD4 →

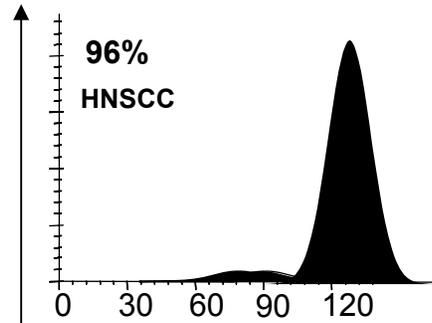
5-day co-cultures of Treg and autologous RC



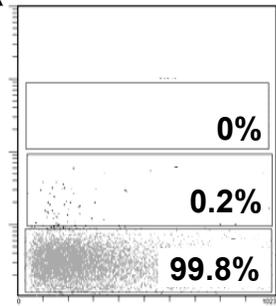
# Treg suppress CD4+ RC at low and high dose of IL-2, but can kill RC only at high IL-2 concentrations

S/RC=1:1

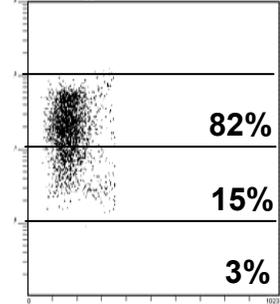
(150 IU IL-2/mL) →



CD4+(RC) cells

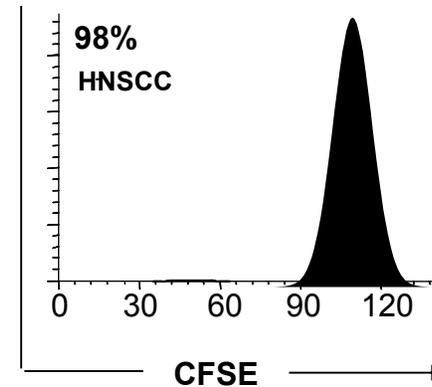


Treg



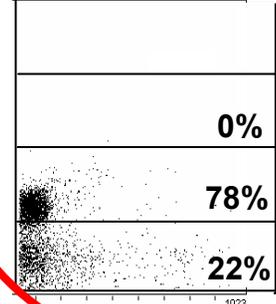
Events

(1000 IU IL-2/mL) →

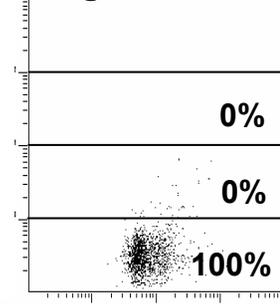


7-AAD

CD4+(RC) cells



Treg



CD4



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.

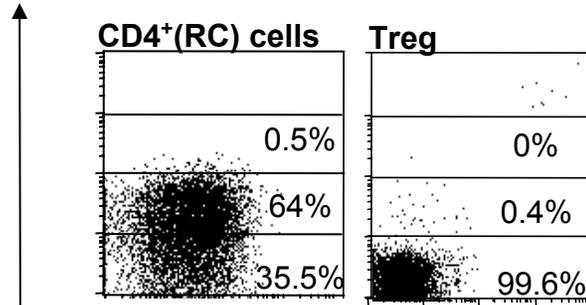
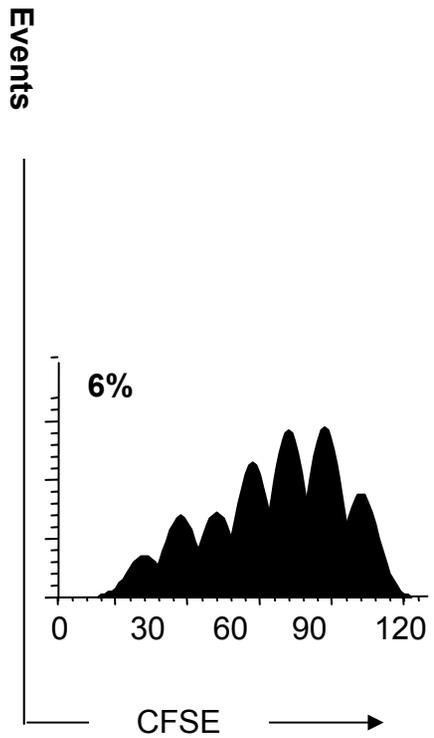
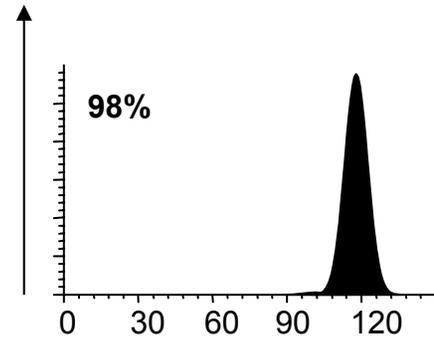
# Conclusion 1

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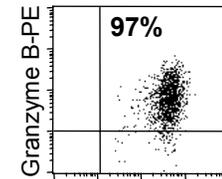
Mechanisms responsible for RC death and Treg survival in these co-cultures are IL-2 dependent



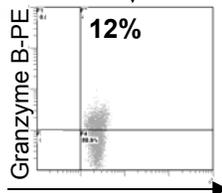
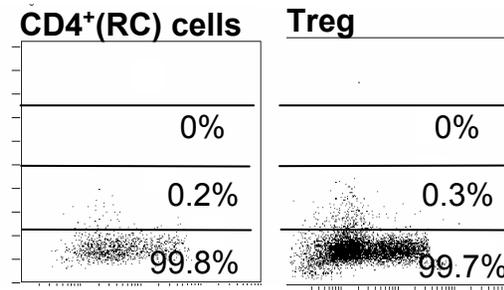
# Treg- mediated killing of CD4<sup>+</sup> RC is GranzymeB- dependent, but Perforin-independent



1000 IU/mL IL-2



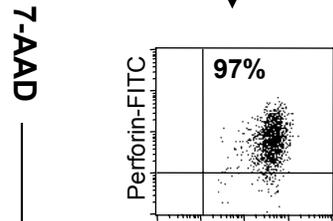
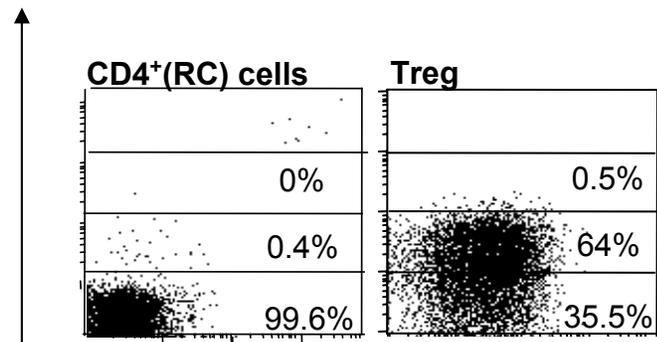
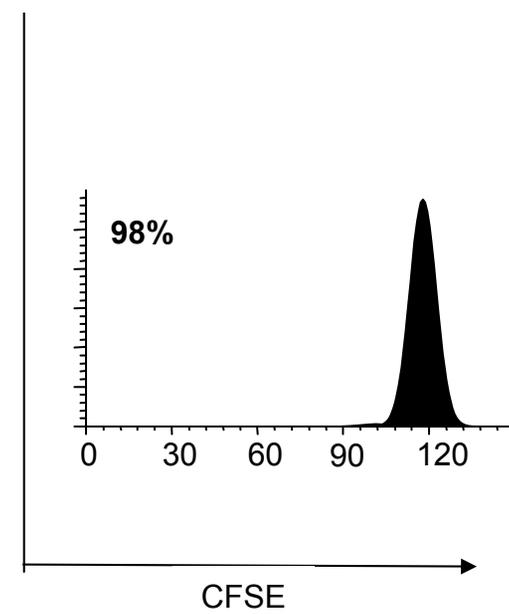
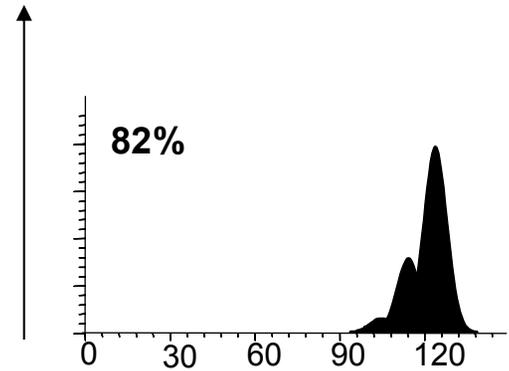
+ siRNA GrB



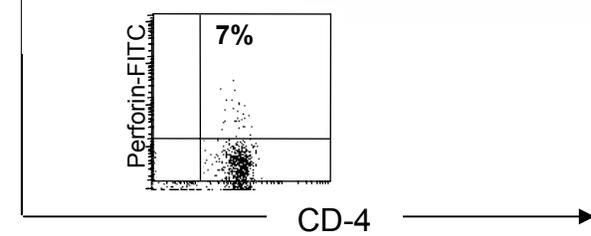
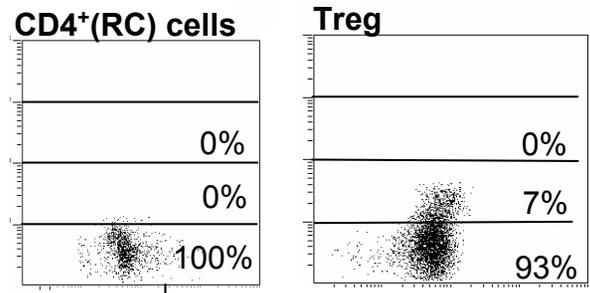
CD-4



# CD4<sup>+</sup> RC- mediated killing of Treg is GranzymeB and Perforin-dependent



## +ConcanamycinA



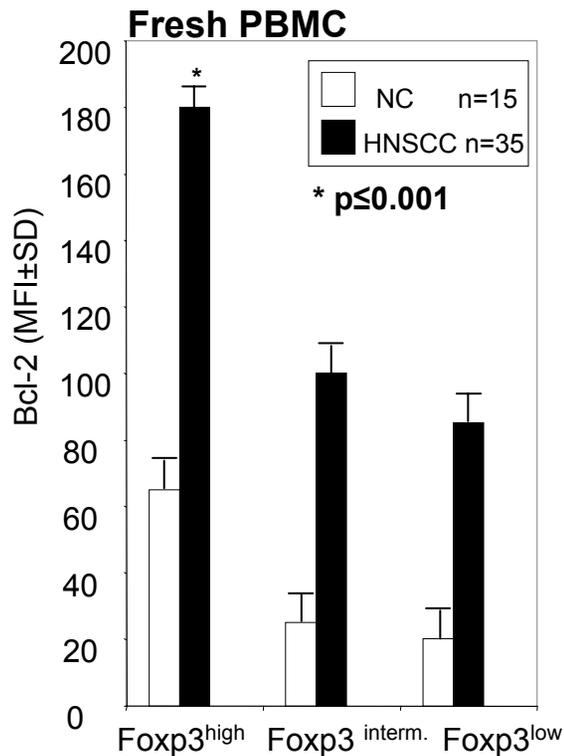
# Conclusion 2

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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



## PI-9 expression in Treg and RC after co-culture + IL-2

PI-9	NC	AD	NED
<u>1000 IU/mL IL-2:</u> MFI ± SD			
CD4 <sup>+</sup> CD25 <sup>high</sup>	83 ± 5*	115 ± 10*	150* ± 12
CD4 <sup>+</sup> CD25 <sup>-</sup>	25 ± 12	25 ± 10	32 ± 2.5
CD8 <sup>+</sup> CD25 <sup>-</sup>	12 ± 1.8	28 ± 4.5	65 ± 5.8
*p ≤ 0.001 for differences between Treg and RC			
<u>150 IU/mL IL-2:</u>			
CD4 <sup>+</sup> CD25 <sup>high</sup>	22 ± 6.7*	18 ± 4.58*	18 ± 2.5*
CD4 <sup>+</sup> CD25 <sup>-</sup>	85 ± 6.5	68 ± 12	58 ± 10
CD8 <sup>+</sup> CD25 <sup>-</sup>	95 ± 5.9	64 ± 8	54 ± 12

\*p ≤ 0.03 for differences between Treg and RC

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



# Conclusions

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- Treg can suppress RC via three different mechanisms
- Tr1 largely use IL-10 and TGF $\beta$ 1 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

