Early growth response gene 2 (Egr2) is essential for T cell anergy and its targets define dysfunctional T cells in the tumor microenvironment

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Presenter Disclosure Information

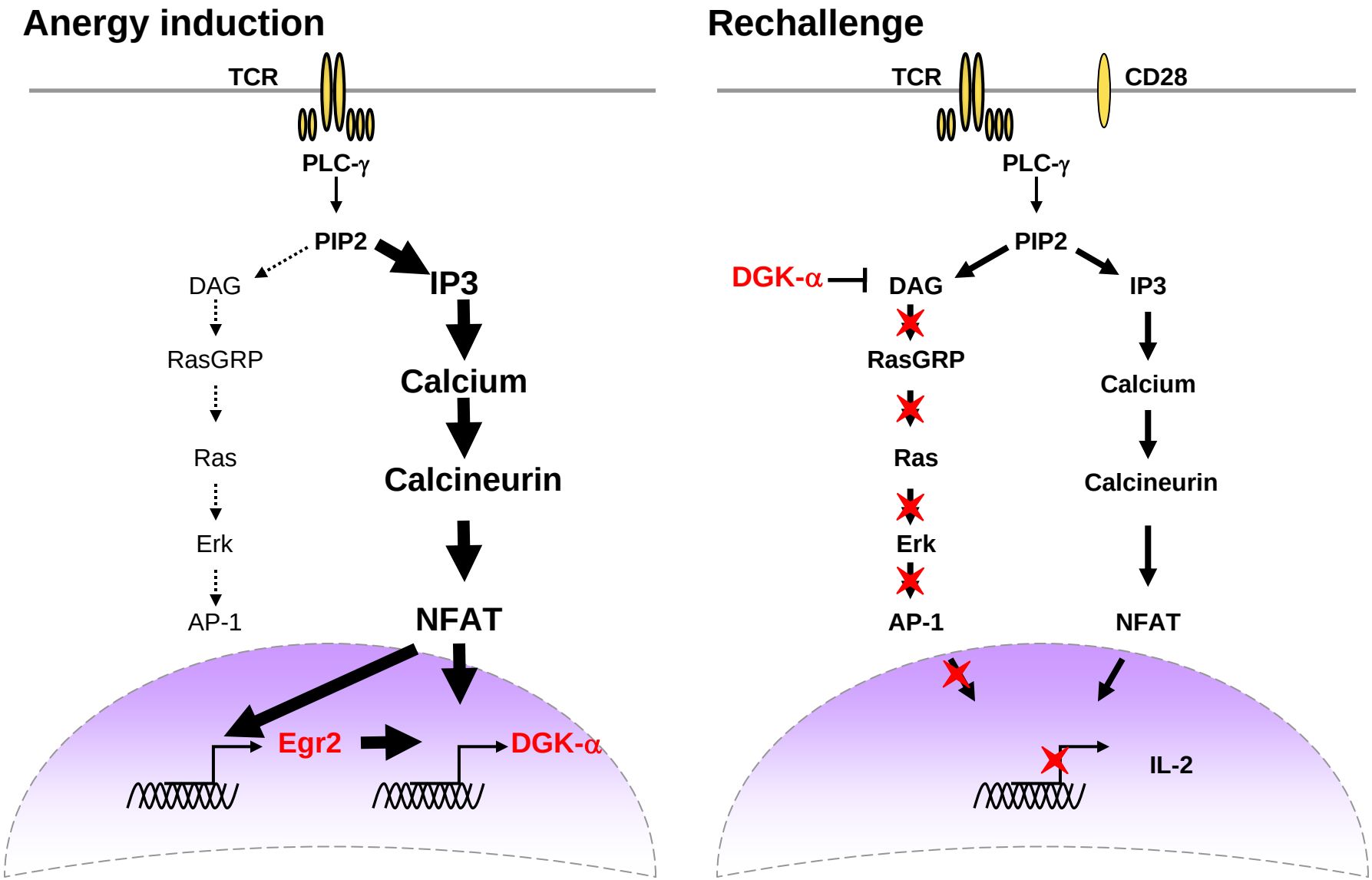
Yan Zheng

- **The following relationships exist related to this presentation**:
  - **No relationship to disclose**

T cell anergy in the tumor context

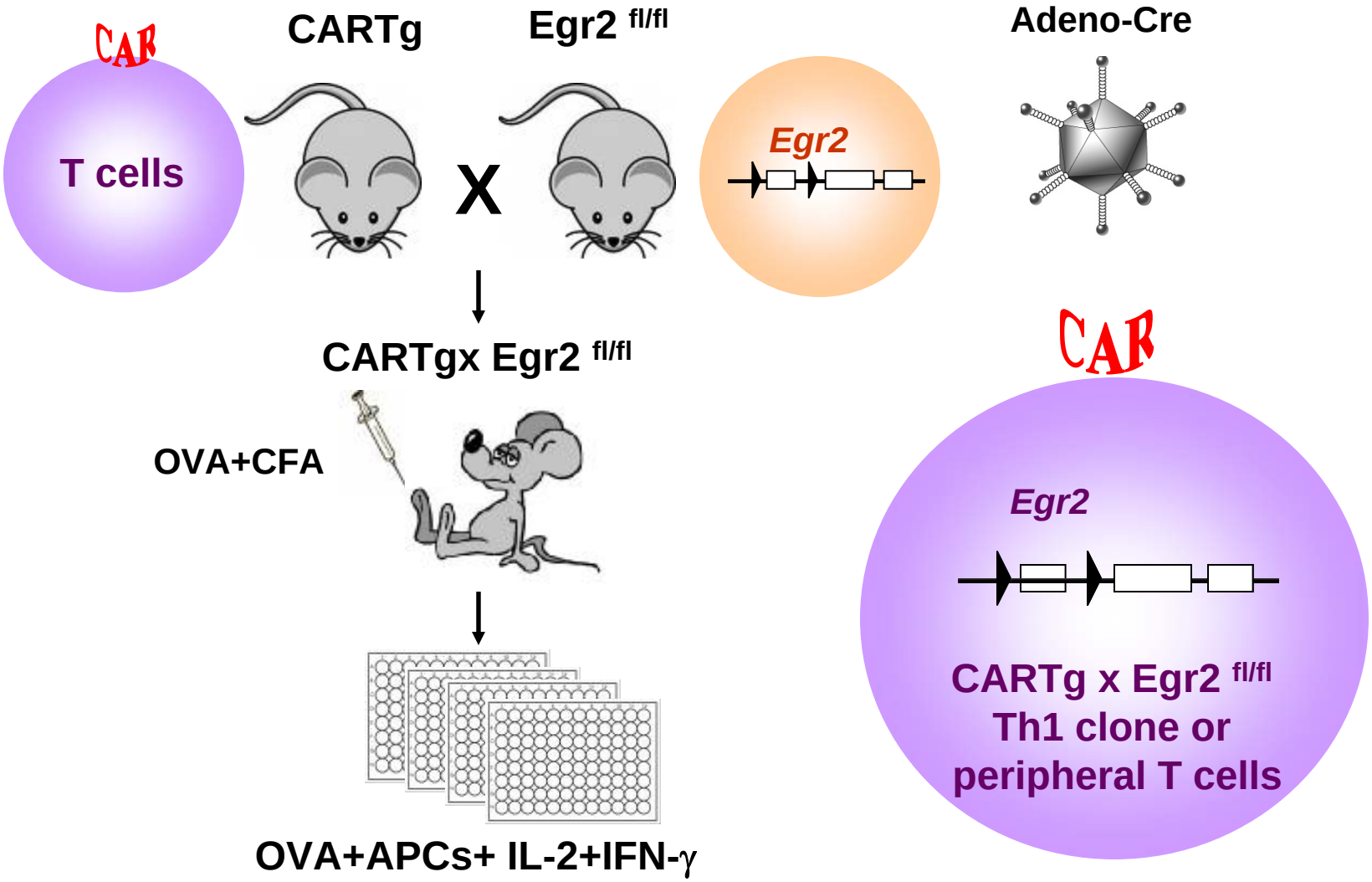
- Anergy defined as a hyporesponsive state induced by TCR engagement in the absence of costimulation
- Indirect evidence suggests that T cell dysfunction in the tumor microenvironment is partially due to anergy
  - Minimal expression of B7-1/B7-2 costimulatory molecules in the tumor microenvironment
  - T cell dysfunction can be antigen-specific *(Harlin et al., 2006)*
  - Provision of B7-1, and homeostatic proliferation, prevent anergy and promote tumor rejection *(Chen et al., 1992; Townsend et al., 1993, Kline et al., 2008)*
- Difficult to prove directly due to the lack of positive markers for this loss of function state
- We previously showed that anergy is associated with defective Ras pathway activation, and is contributed to by diacylglycerol kinases *(Fields et al., 1996; Zha et al., 2006)*

Model for DGK-α in T cell anergy



Zha et al Nature Immunol. 2006; Zheng et al EMBO Reports 2008

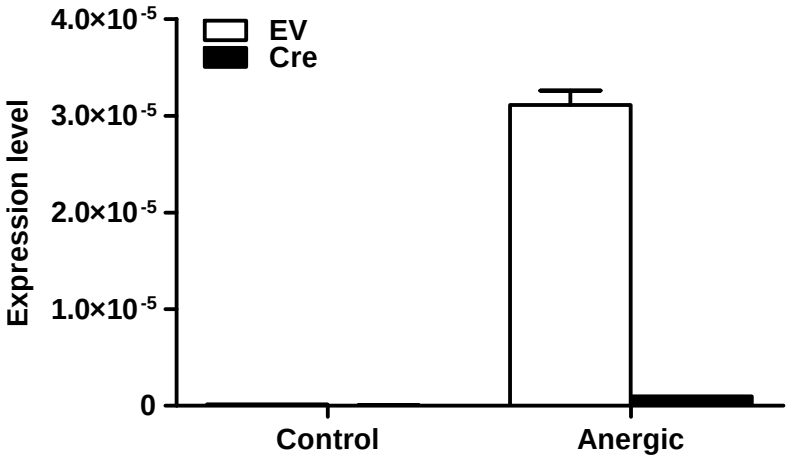
Adeno-Cre mediated Egr2 deletion in CARTg x Egr2$^{fl/fl}$ Th1 cell clones



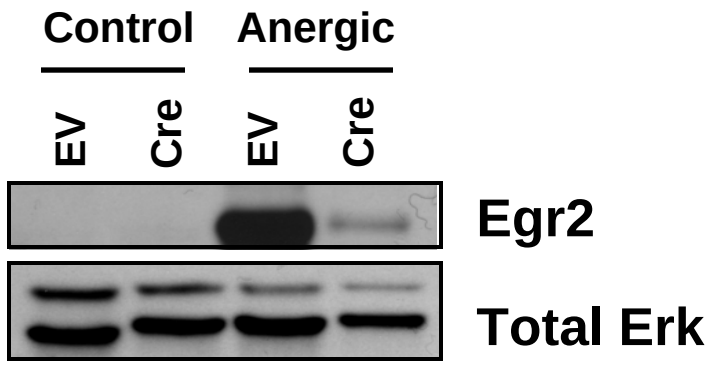
Zha et al., Journal of Immunological Methods, 2008

Adeno-Cre mediated Egr2 deletion in CARTg x Egr2$^{fl/fl}$ Th1 cell clones cont.

Egr2 qRT-PCR



Immunoblot



Egr2 deletion leads to resistance to anergy induction *in vitro*

CAR Tg x Egr2$^{fl/fl}$ clones
EV and Cre

↓

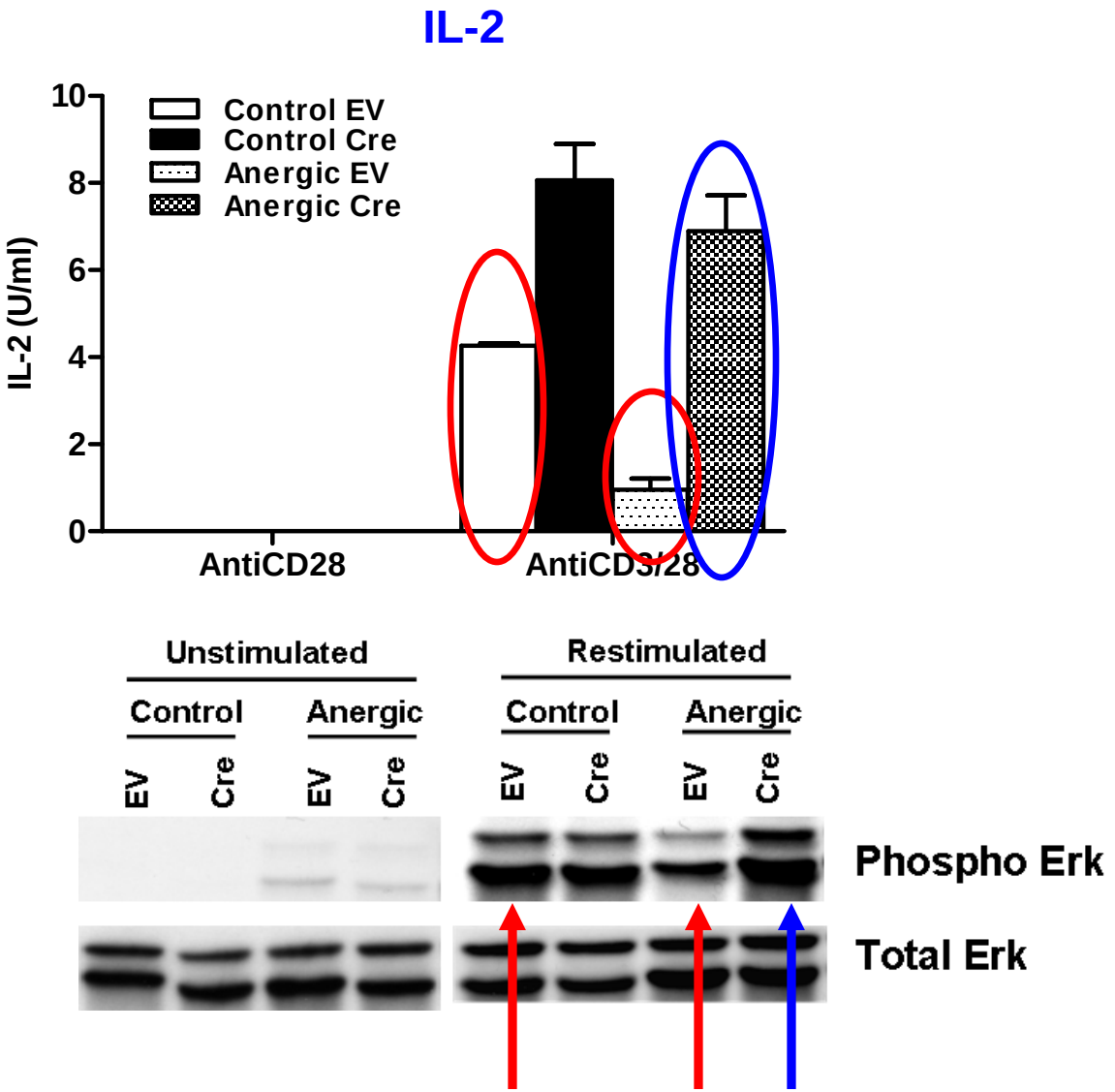
Anergy induction
No treatment (Control)
or
Plate-bound anti-CD3 (Anergic)

↓

Rest 1-2 days
In
medium

↓

Rechallenge with plate-bound anti-CD3+anti-CD28

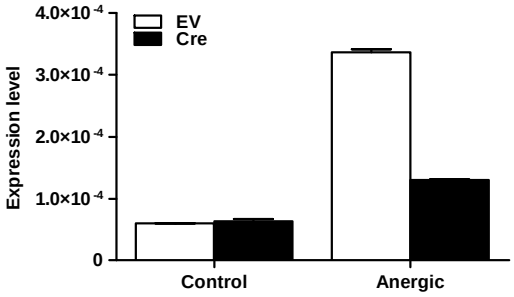


Might Egr2 be a “central regulator” of the anergic state?

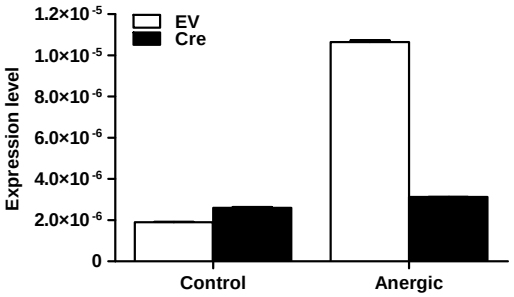
- Besides DGK-α, several other genes encoding negative regulatory molecules have been reported to be upregulated in anergic T cells
  - DGK-ζ
  - Cbl-b, GRAIL, Itch
  - Tob1, Deltex1
- Goal: to identify global transcriptional program regulated by Egr2 in anergic cells
- Strategies:
  - QRT-PCR and ChIP assays for known candidates
  - Combine gene expression profiling of conditional *Egr2*-deleted T cells with ChIP-seq analysis of genes directly binding Egr2 to identify total program

Egr2 directly regulates most of the known anergy associated genes: qRT-PCR

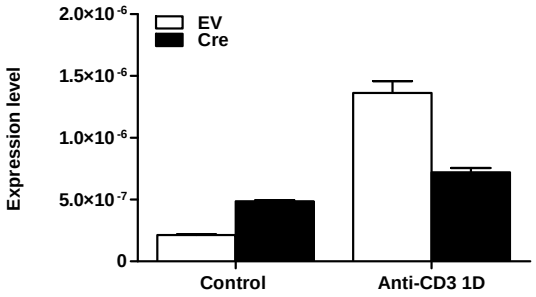
DGK-ζ



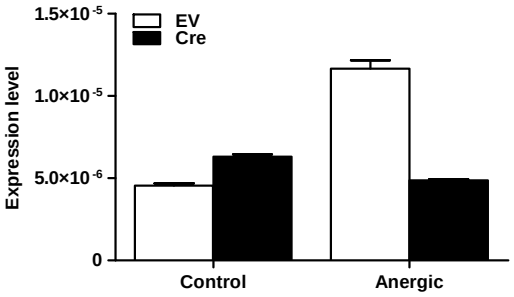
Cbl-b



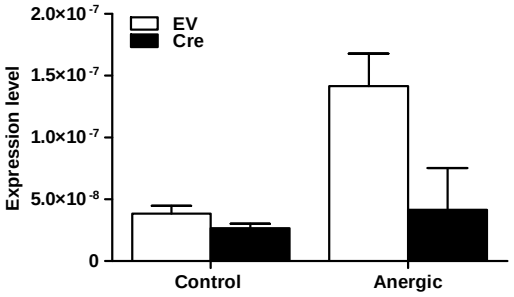
Itch



Tob1

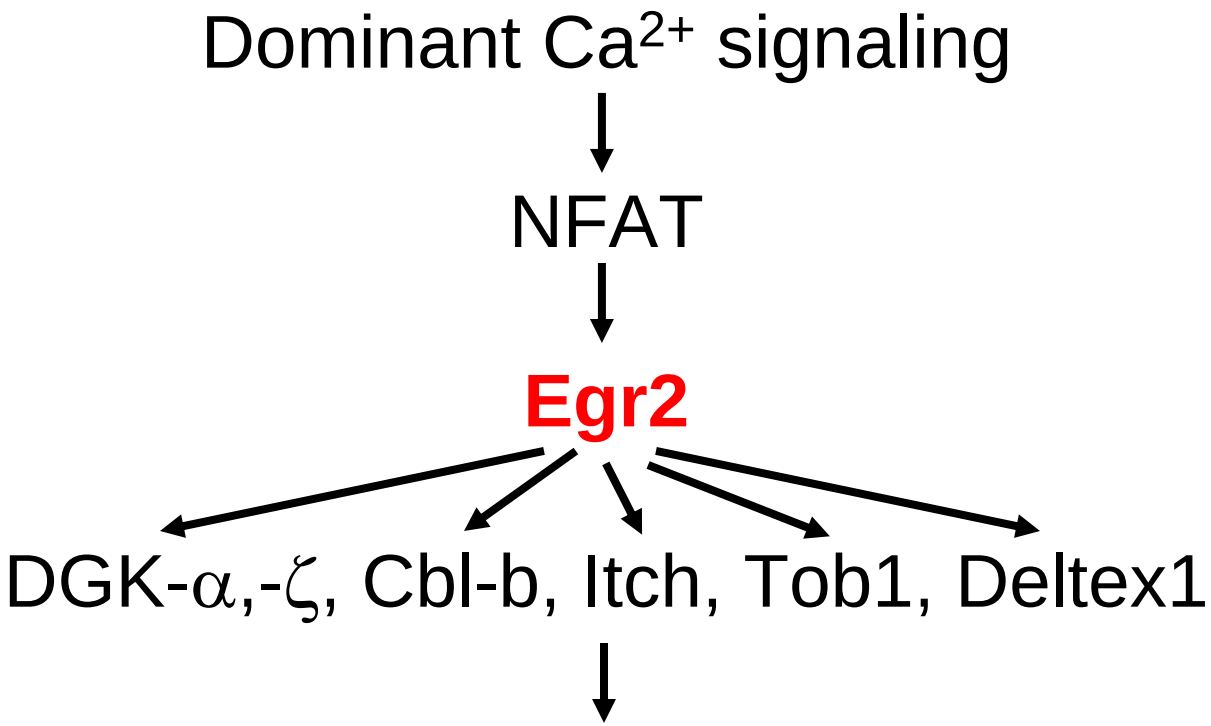


Deltex1

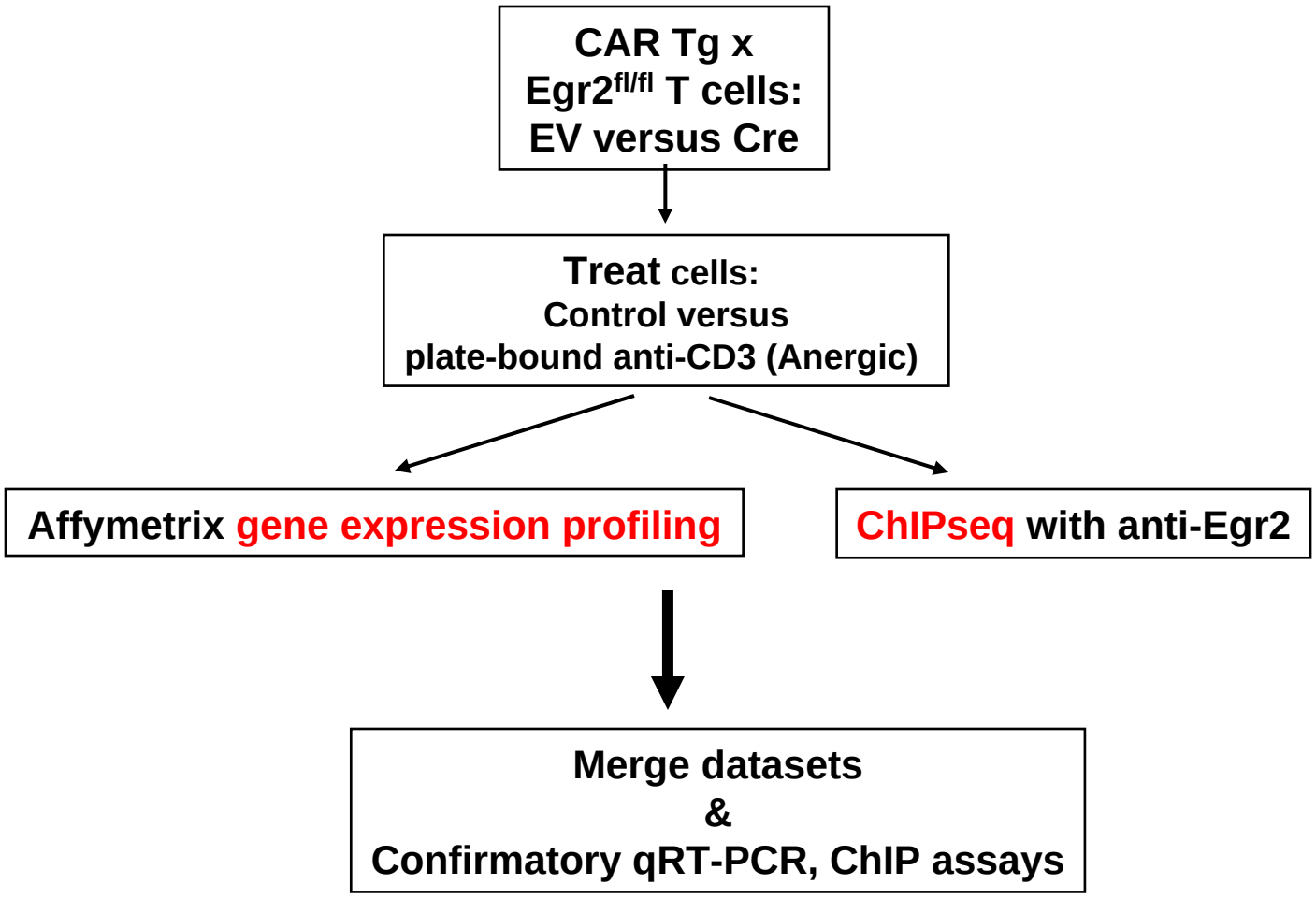


Similar results by ChIP assay

Model for Egr2 as central transcriptional regulator of T cell anergy



Strategy to determine global Egr2-driven transcriptional program in anergic T cells



46 genes identified as targets of Egr2 by gene array x ChIP-SEQ in anergy

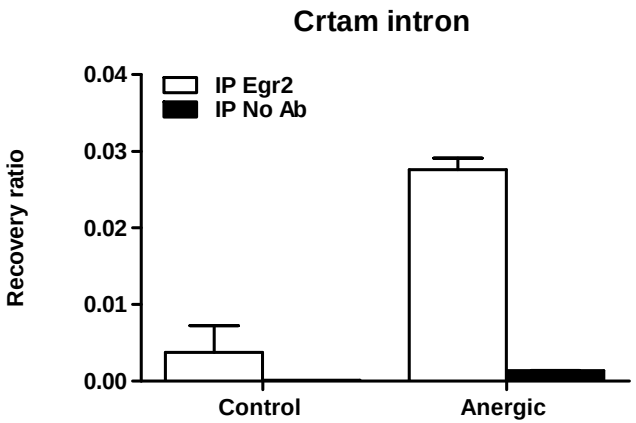
Gene
Ccl1: chemokine (C-C motif) ligand 1
Crtam: cytotoxic and regulatory T cell molecule
Egr2: early growth response 2
Rasgef1a: RasGEF domain family, member 1A
Car12: carbonic anyhydrase 12
Pscd3: pleckstrin homology, Sec7 and coiled-coil domains 3
Pacsin1: protein kinase C and casein kinase substrate in neurons 1
Tnfrsf9: tumor necrosis factor receptor superfamily, member 9
Fhl2: four and a half LIM domains 2
Bcl2l11: BCL2-like 11 (apoptosis facilitator)
Gnb5: guanine nucleotide binding protein (G protein), beta 5
short chain dehydrogenase/reductase family 39U, member 1
Nrgn: neurogranin
Crabp2: cellular retinoic acid binding protein II
Sema7a: sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A
1190002H23Rik: RIKEN cDNA 1190002H23 gene
Tnfsf11: tumor necrosis factor (ligand) superfamily, member 11
Pdk2: pyruvate dehydrogenase kinase, isoenzyme 2
Dgkz: diacylglycerol kinase zeta
Nrn1: neuritin 1
Mtss1: metastasis suppressor 1
Bach2: BTB and CNC homology 2
2310051E17Rik /// Klf9: Kruppel-like factor 9 /// RIKEN cDNA 2310051E17 gene
Rai14: retinoic acid induced 14
Cd74: CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
Lag3: lymphocyte-activation gene 3
Ptgfrn: prostaglandin F2 receptor negative regulator

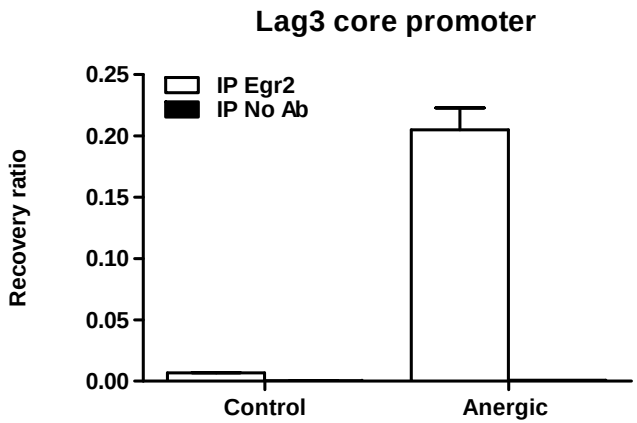
New Egr2-dependent anergy associated genes

Crtam

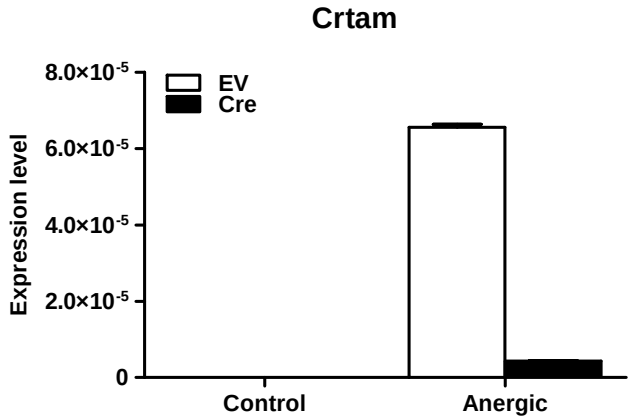
Lag3

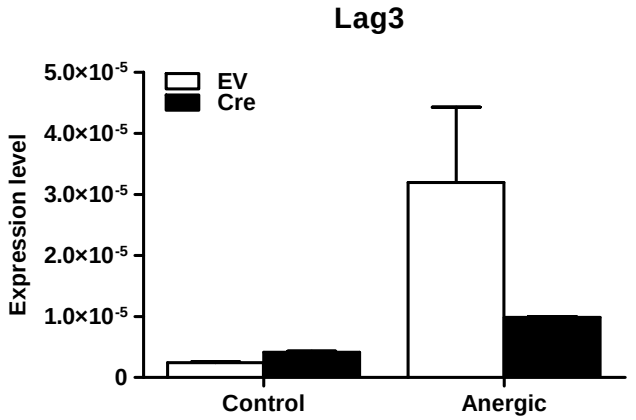
Chip Assay





qRT-PCR

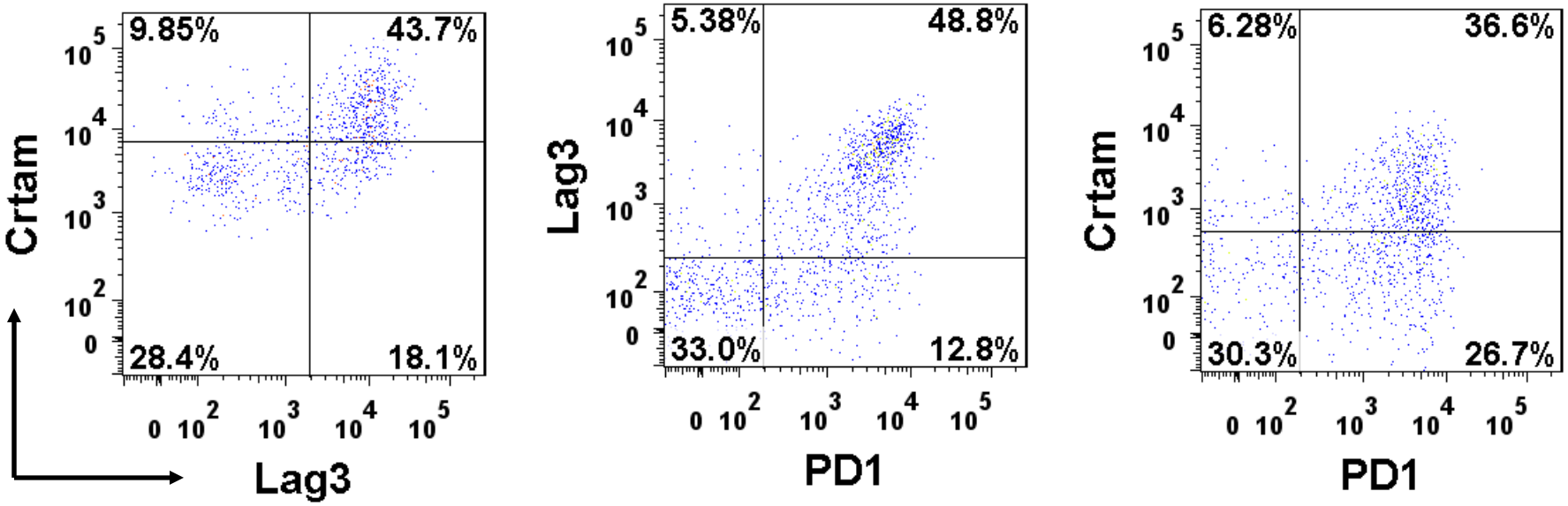




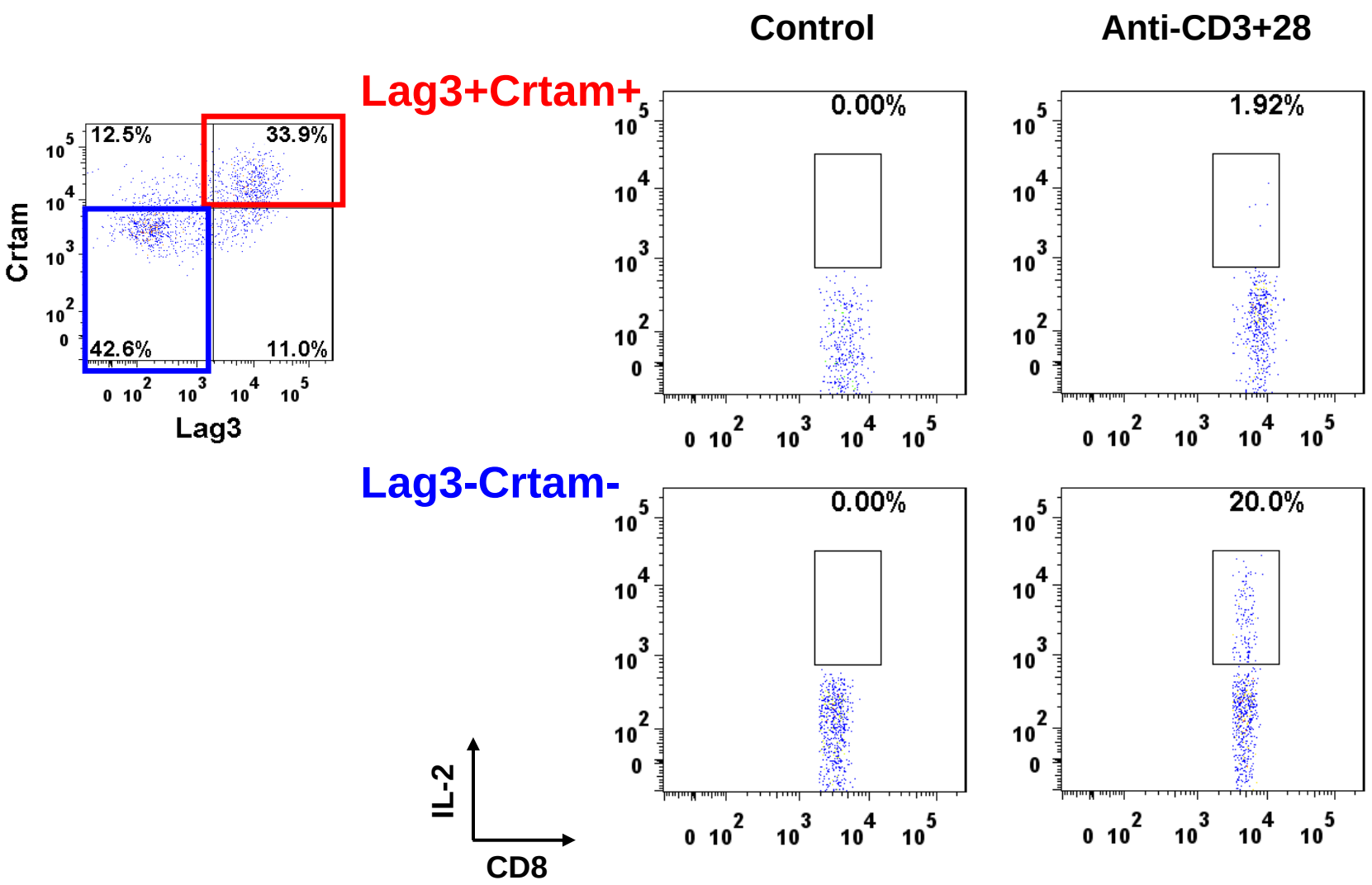
Can Egr2-driven cell surface molecules mark the anergic T cells in the tumor microenvironment ?

- Crtam (Class-I-MHC-restricted T cell associated molecule)
  - A transmembrane protein, and expressed mainly on T cells and NK cells
  - Reported to maintain T cell polarity during the late phase of T cell activation, and T cell retention in lymph nodes *(Yeh et al., Cell, 2008; Takeuchi et al., 2009).*
- Lag3 (lymphocyte-activation gene 3)
  - CD4-related transmembrane protein, and binds to MHC class II on APCs with higher affinity than CD4
  - Deletion of Lag3 in T cells led to enhanced anti-tumor response *(Grosso et al., 2007).*

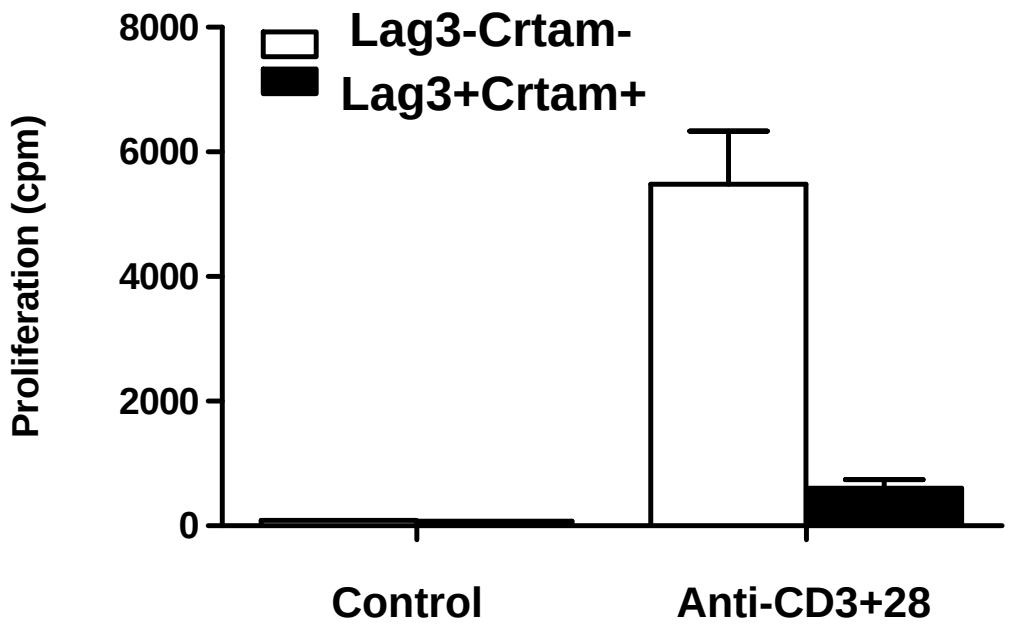
PD1, Lag3 and Crtam are highly upregulated on CD8$^+$ tumor-infiltrating lymphocytes (TILs) in the context of B16 melanoma



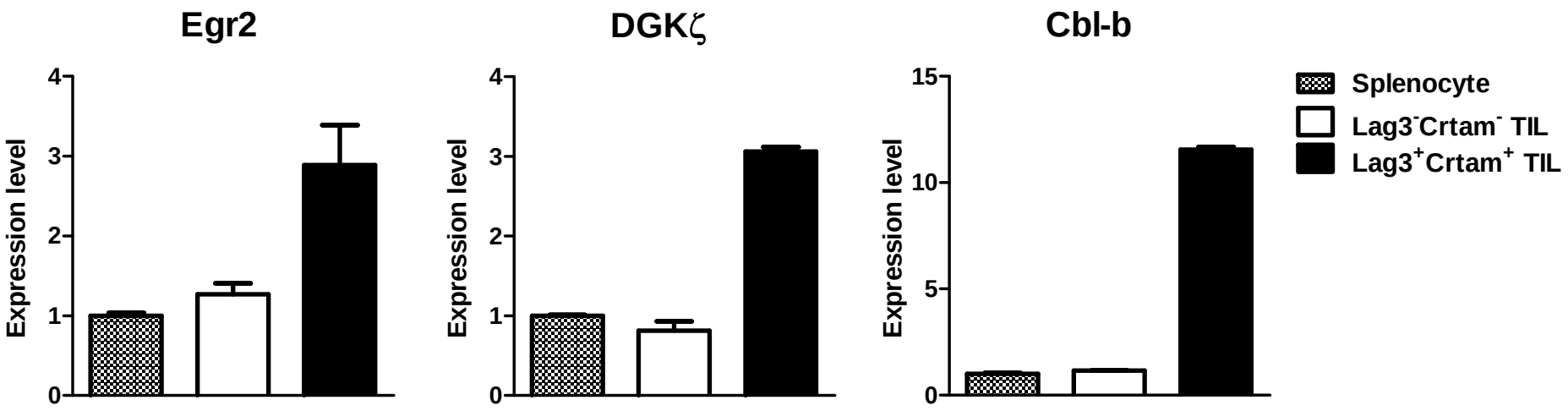
Lag3^{+}Crtam^{+} CD8^{+} TILs are defective in IL-2 production upon *ex vivo* stimulation



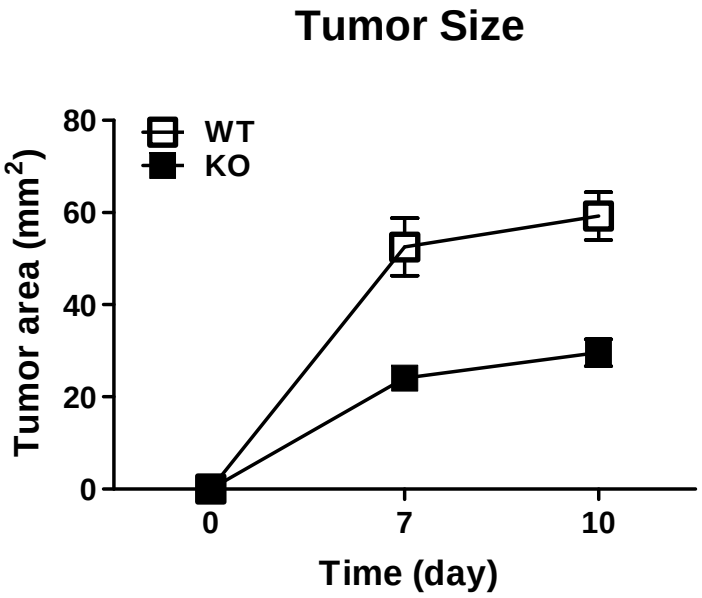
Sorted Lag3^{+}Crtam^{+} CD8^{+} TILs are hypoproliferative upon *ex vivo* stimulation

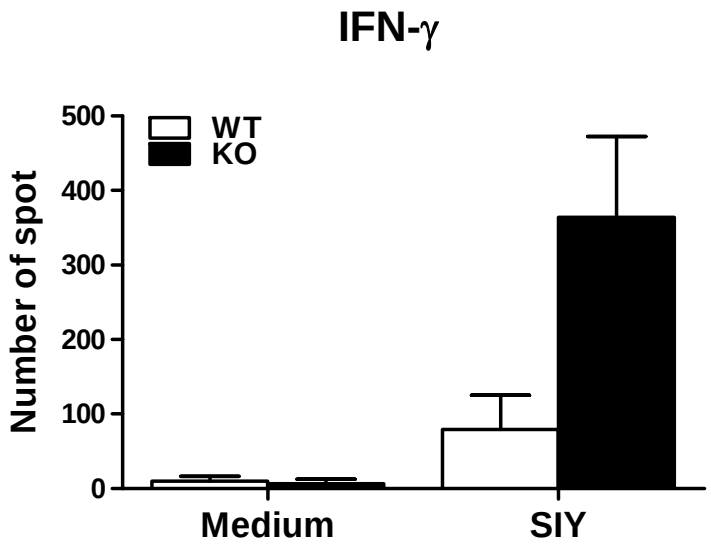


Anergy-associated genes are enriched in Lag3^{+}Crtam^{+} CD8^{+} TILs



Conditional deletion of Eg2 in T cells leads to enhanced anti-tumor immune response and slowed tumor growth





Conclusions

- Egr2 is a major transcriptional regulator of the anergic state
- Egr2-deleted T cells are relative anergy-resistant in vitro and also to SEB in vivo (data not shown)
- Combined gene expression profiling and ChIP-seq has identified the knowable Egr2 transcriptome in T cell anergy
- New identified anergy-associated genes are surface markers
- Crtam and Lag3 may identify the population of anergic T cells from the tumor microenvironment *ex vivo*

Acknowledgments

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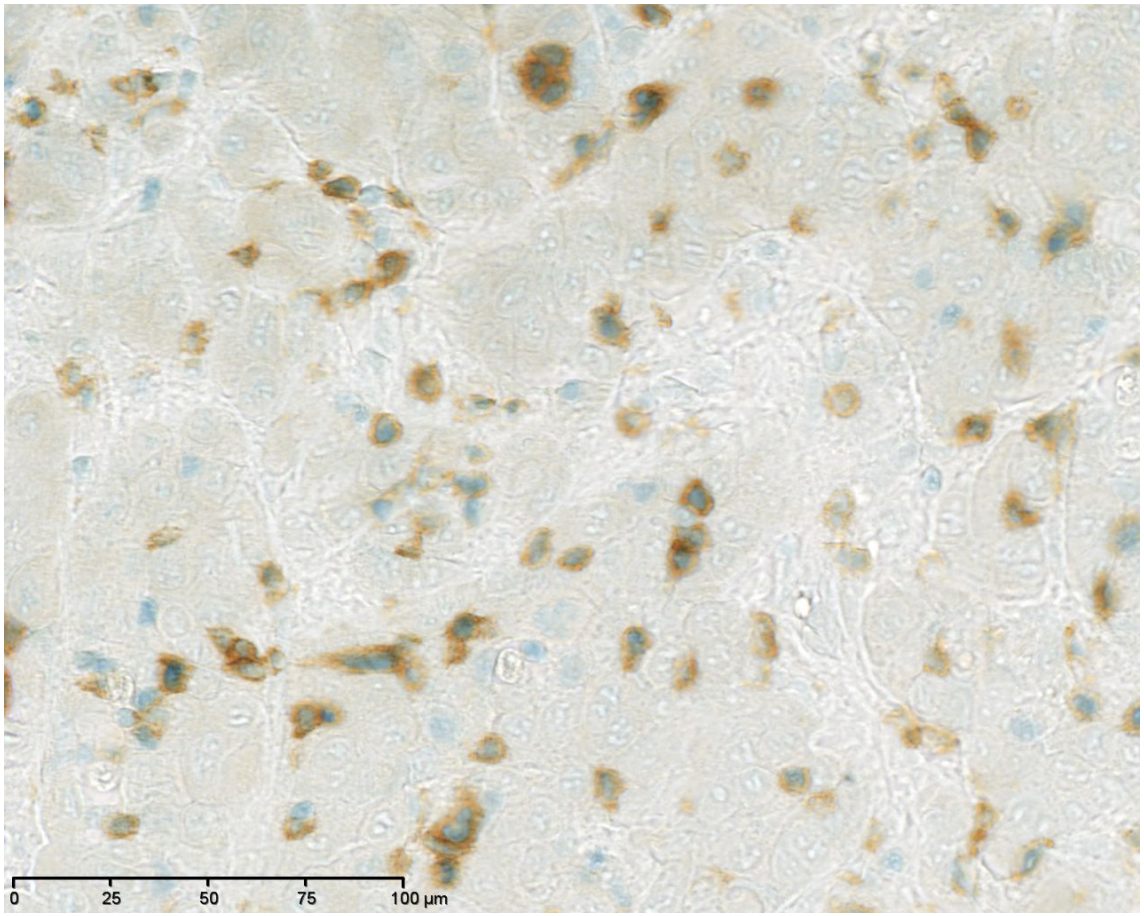
- Chauncey Spooner

- **Albert Bendelac Laboratory**
- Rebecca Mathew

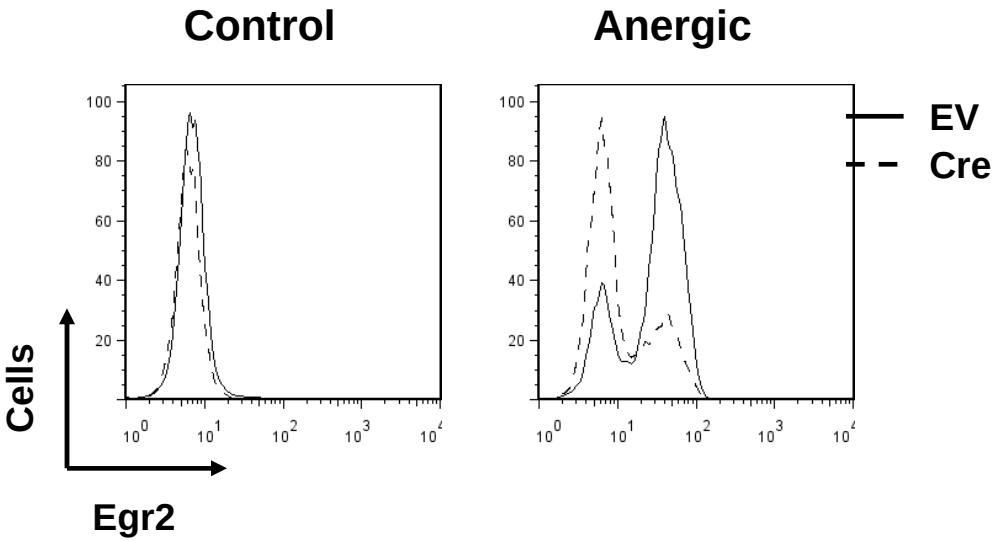
Tumor-infiltrating $CD8^+$ T cells (brown) in human melanoma are $EGR2^+$ (blue)



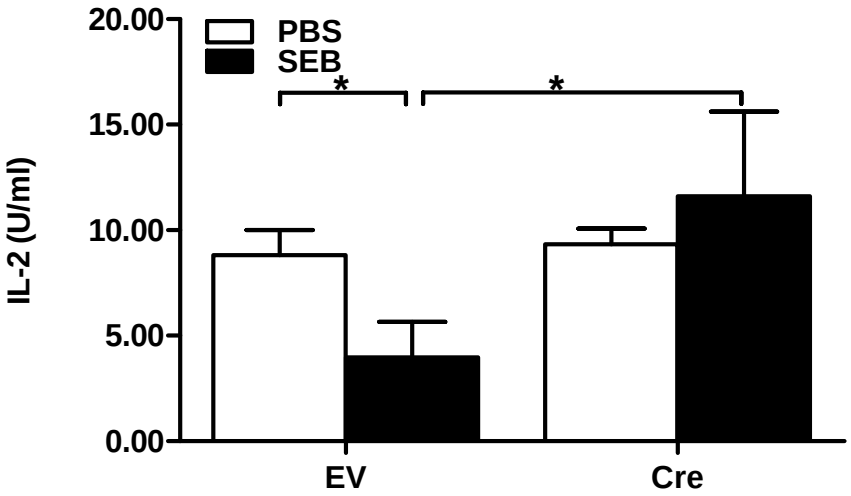
Implies that strategies to inhibit EGR2 pathway or target genes may have the potential to improve T cell function in tumor context

Egr2 deletion with Cre Adenovirus followed by adoptive transfer leads to resistance to superantigen induced anergy *in vivo*

Egr2 deletion confirmation



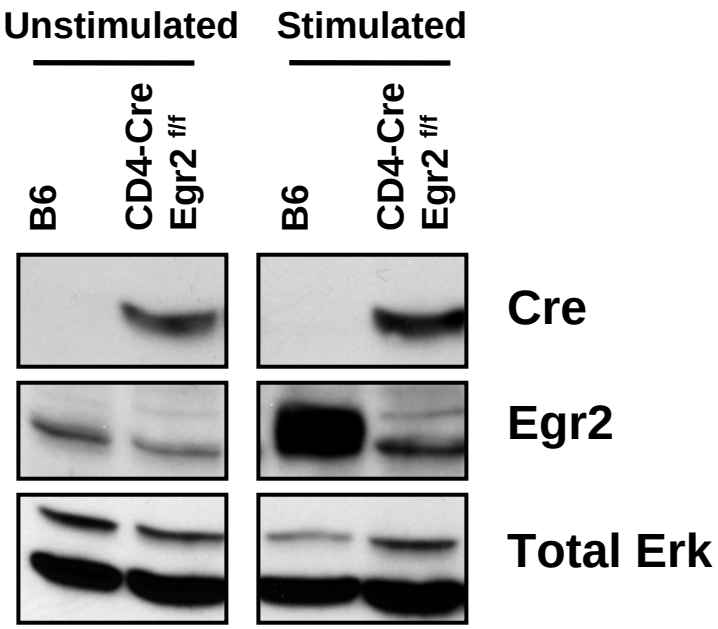
Rechallenge with SEB



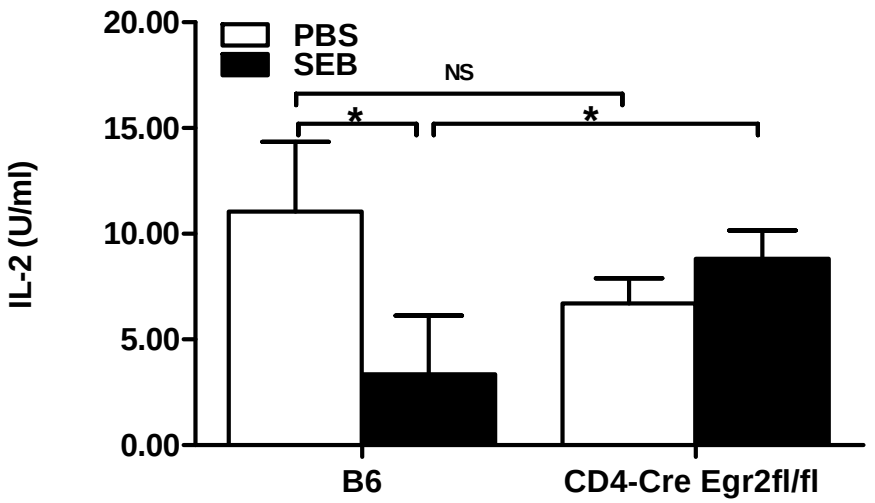
Infect with EV or Cre - adenovirus → 3 days → Adopt transfer into B6 mice → 1 day → I.P. mice with SEB → 7 days → Harvest spleen, and rechallenge with PBS or SEB

Egr2 deletion leads to resistance to superantigen induced anergy *in vivo*

Egr2 deletion confirmation



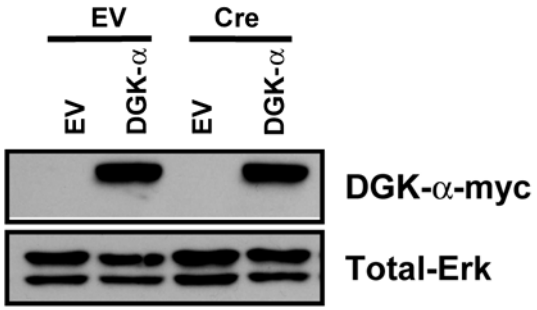
Rechallenge with SEB



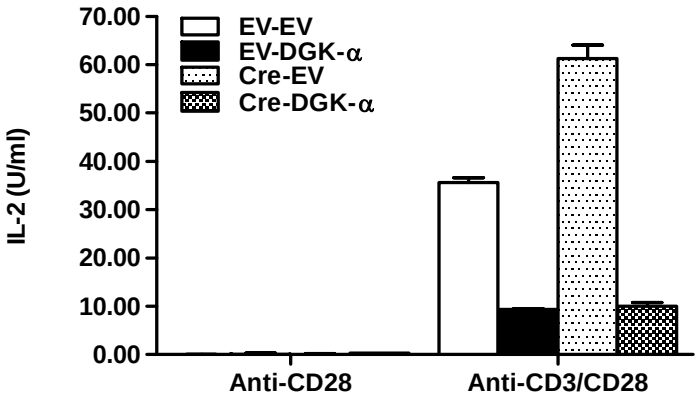
Inject mice intraperitoneally with SEB → 7 days → Harvest spleen, and rechallenged with PBS or SEB

DGK-α triumphs Egr2 deletion and inhibits T cell activation

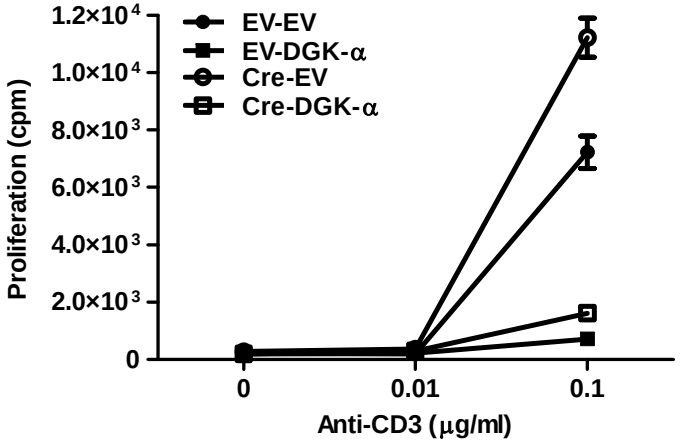
Immunoblot



IL-2

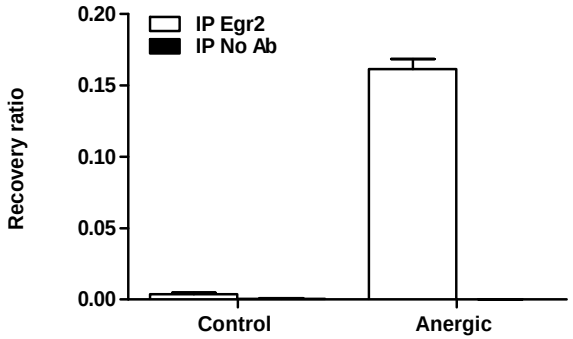


Proliferation

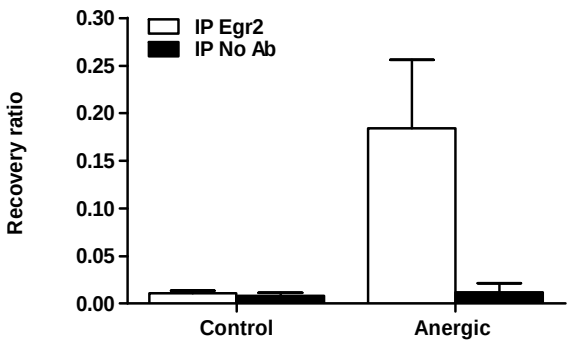


Egr2 directly regulates the most known anergy factors: ChIP

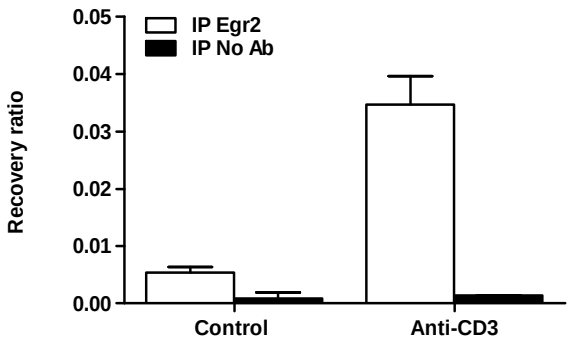
DGK-ζ



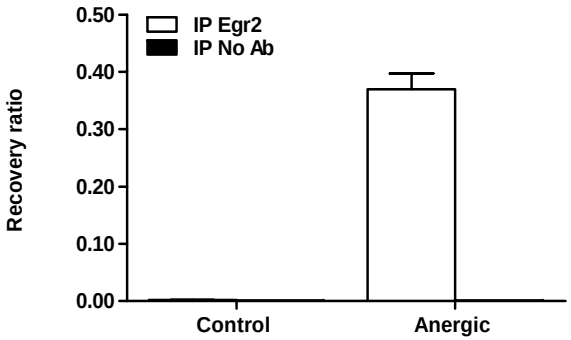
Cbl-b



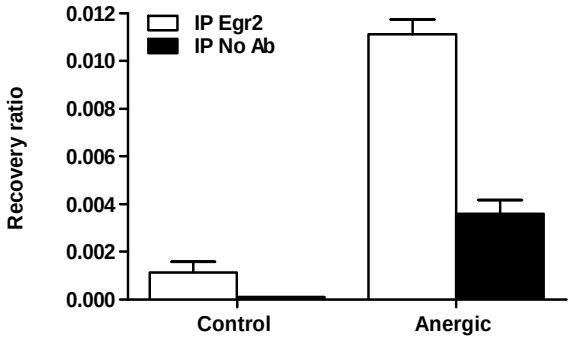
Itch

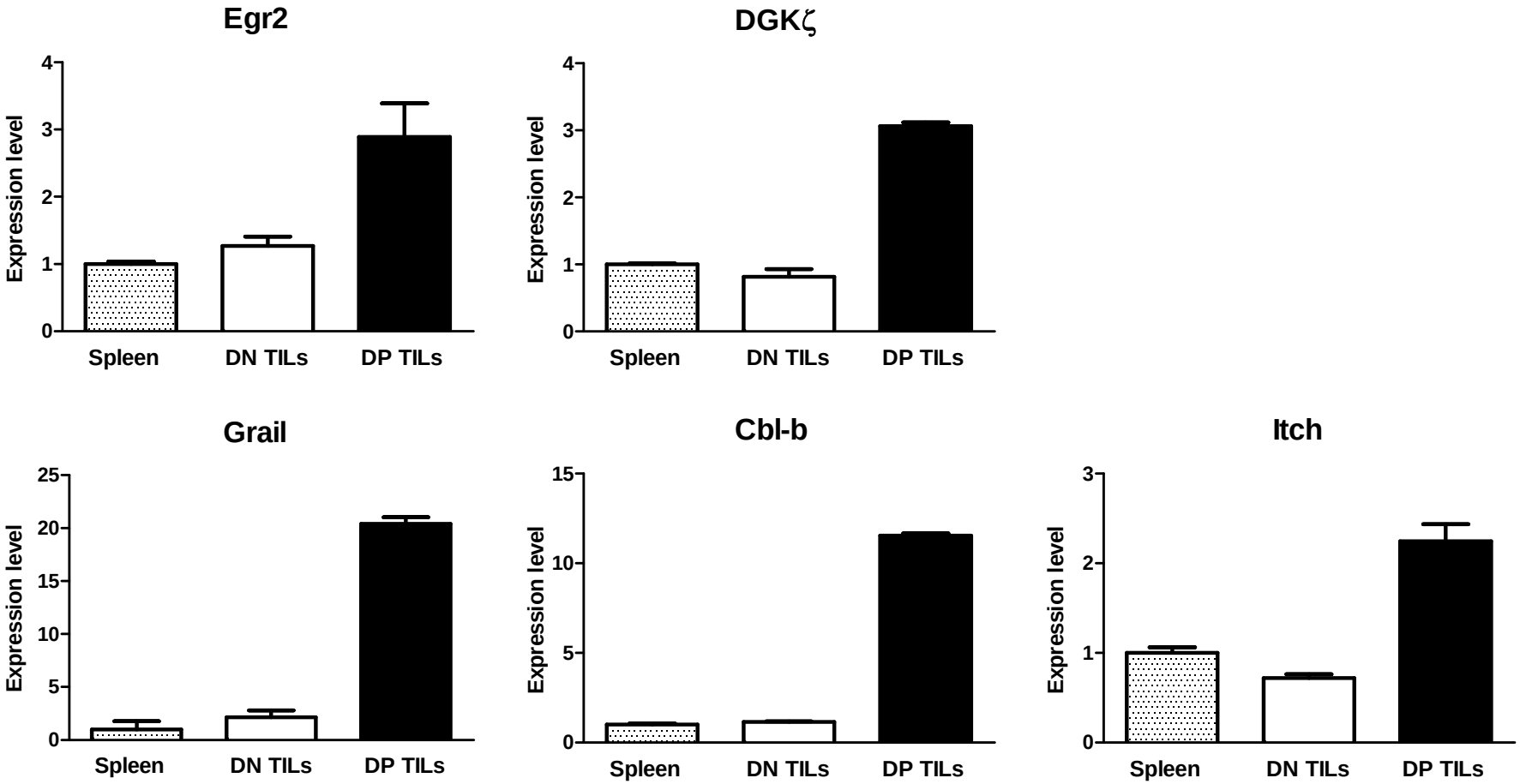


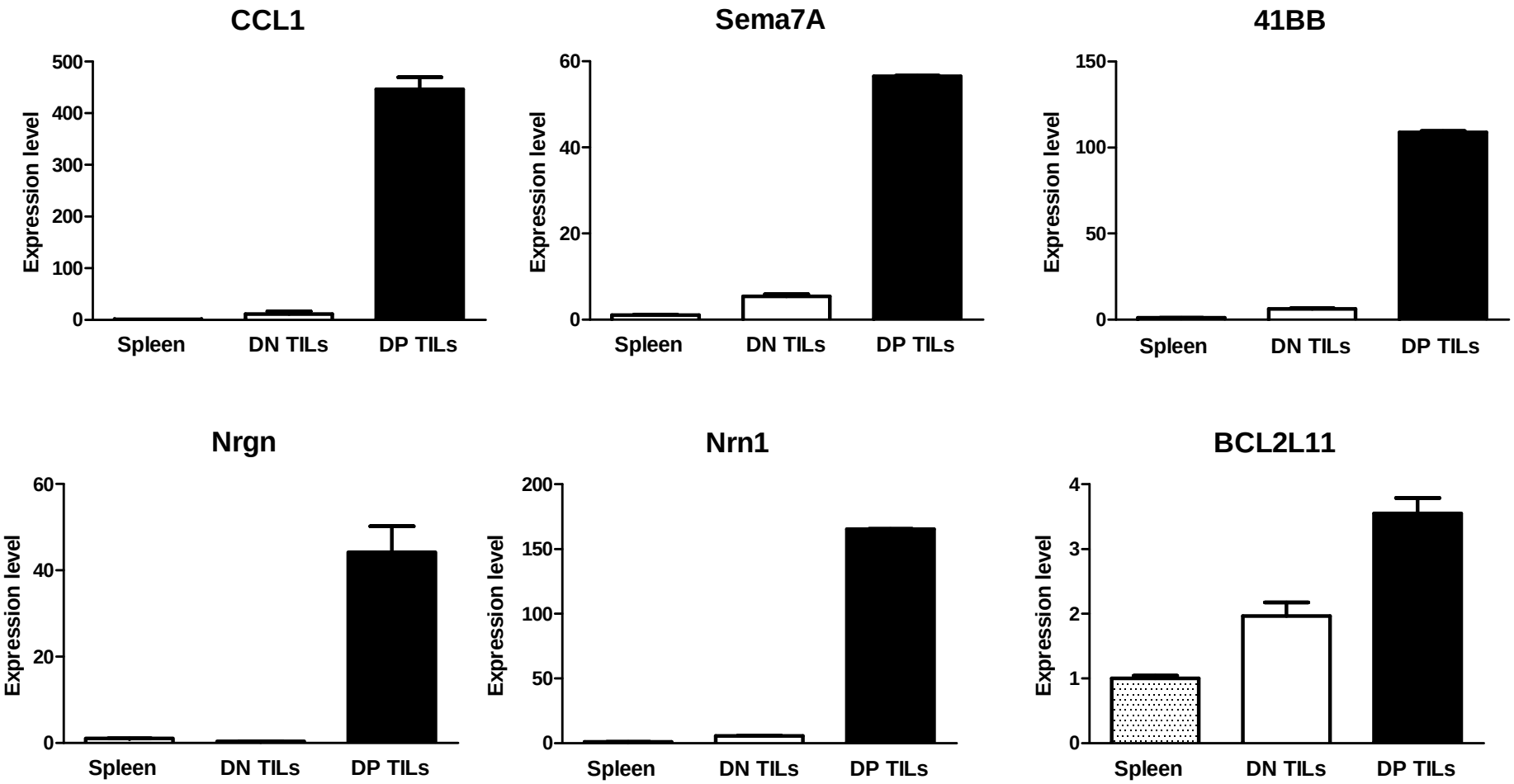
Tob1



Deltex1



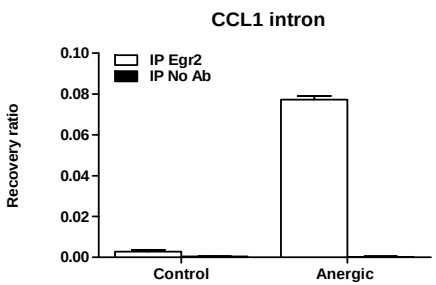

Egr2
Expression level
0
1
2
3
4
Spleen
DN TILs
DP TILs
DGKζ
Expression level
0
1
2
3
4
Spleen
DN TILs
DP TILs
Grail
Expression level
0
5
10
15
20
25
Spleen
DN TILs
DP TILs
Cbl-b
Expression level
0
5
10
15
Spleen
DN TILs
DP TILs
Itch
Expression level
0
1
2
3
Spleen
DN TILs
DP TILs


CCL1
Expression level
500
400
300
200
100
0
Spleen
DN TILs
DP TILs
Sema7A
Expression level
60
40
20
0
Spleen
DN TILs
DP TILs
41BB
Expression level
150
100
50
0
Spleen
DN TILs
DP TILs
Nrgn
Expression level
60
40
20
0
Spleen
DN TILs
DP TILs
Nrn1
Expression level
200
150
100
50
0
Spleen
DN TILs
DP TILs
BCL2L11
Expression level
4
3
2
1
0
Spleen
DN TILs
DP TILs

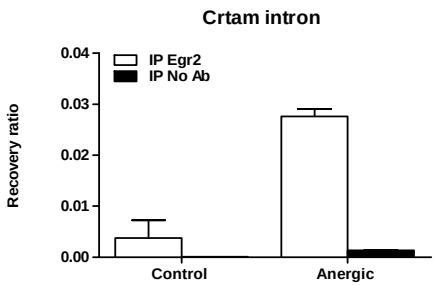
New Egr2-dependent anergy associated genes

A ChIP Assay

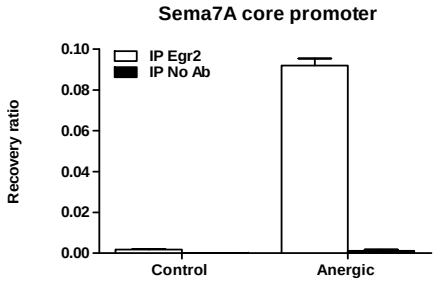
CCL1



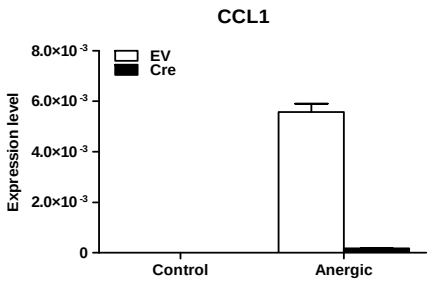
Crtam

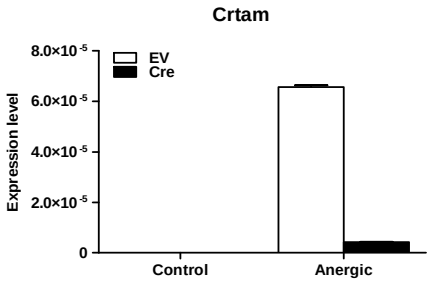


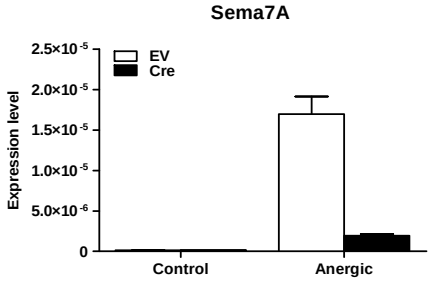
Sema7A



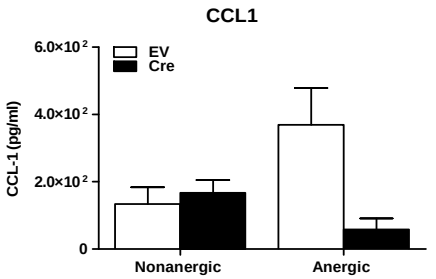
B Real-time RT-PCR

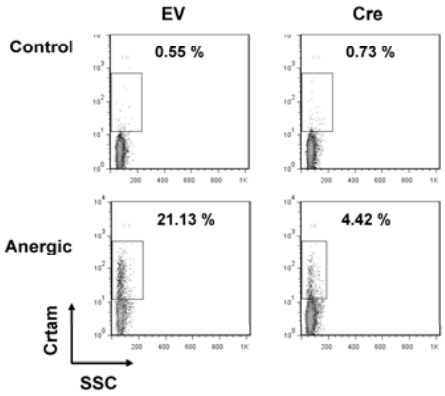


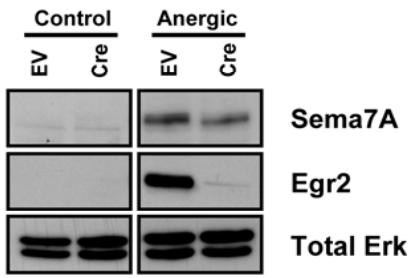




C Protein expression







T cell anergy and tumor conti.

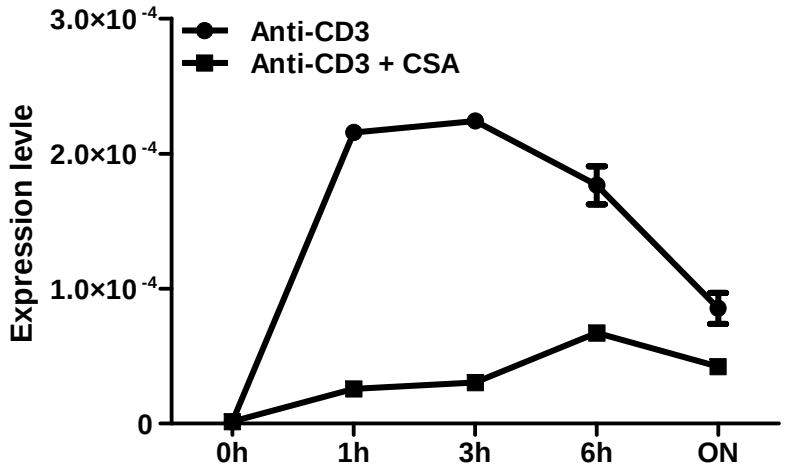
- Characteristics of anergic T cells
  - Defective TCR/CD28-induced Ras pathway activation
  - Defective proximal TCR signaling
- Mechanisms of anergy induction
  - Depends on new protein synthesis (*Gajewski et al., 1995; Telander et al., 1999*)
  - Unbalanced activation of NFAT over AP-1 pathway (*Heissmeyer et al., 2004*)

What regulates DGK-α gene? Anergic cells also express transcriptional regulator Egr2

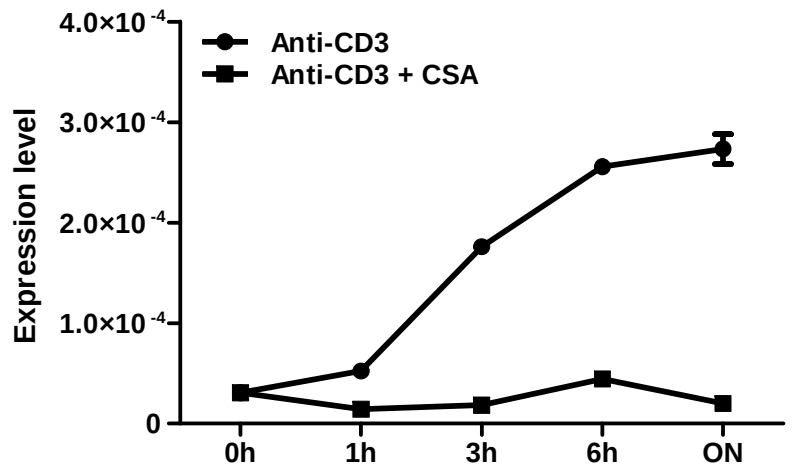
- Early growth response gene
- Transcription factor with 3 zinc finger DNA binding motifs
- Expressed in anergic T cells
- NFAT-dependent
- Reported as a negative regulator of T cell activation *(Safford et al., 2005; Zhu et al., 2008)*
- Hypothesis: Egr2 might regulate the expression of DGK-α and perhaps other anergy-associated genes.

Sequential upregulation of Egr2 then DGK-α mRNA during anergy induction

Egr2

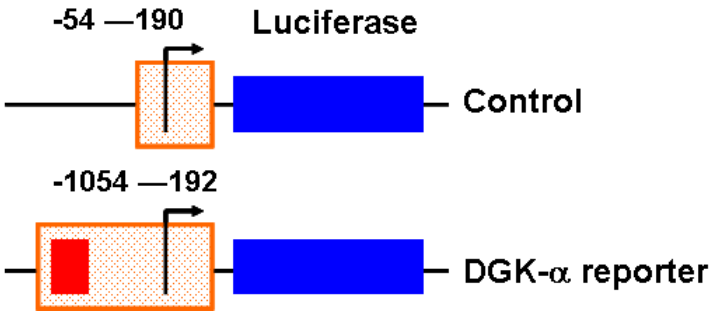


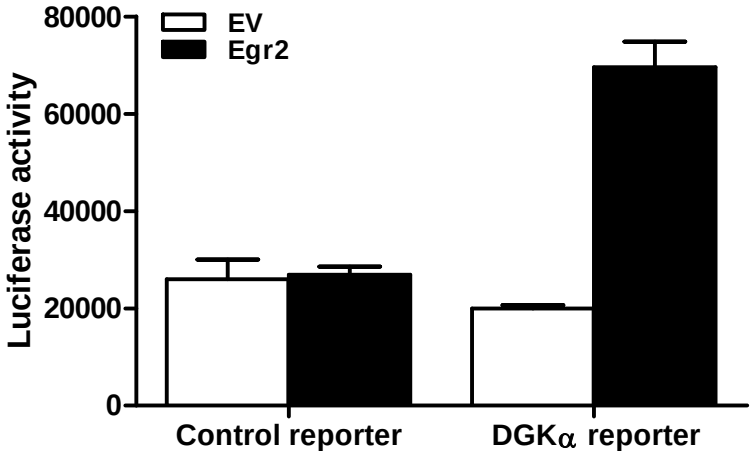
DGK-α



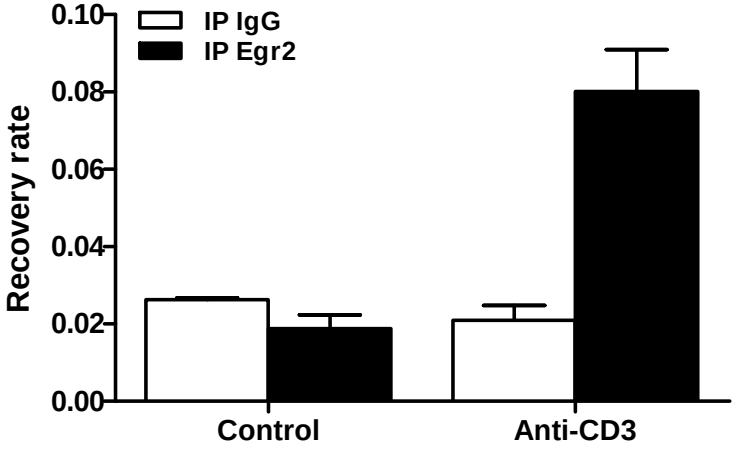
Egr2 can regulate DGK-α gene expression and is associated with its promoter upon anergy induction

Reporter Assay



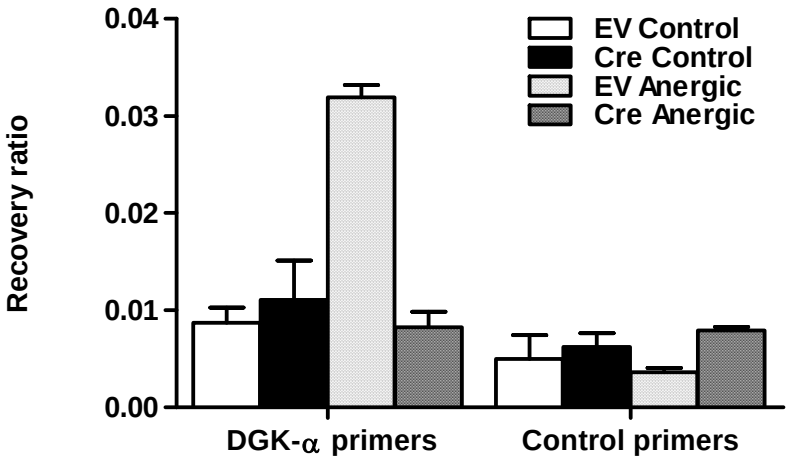


ChIP



Egr2 deletion results in reduced DGK-α upregulation upon anergy induction

ChIP



Real-time RT-PCR