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Egr2-dependent gene expression profiling and ChIP-Seq reveal novel biologic targets in T cell anergy

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Abstract

T cell anergy is one of the mechanisms contributing to peripheral tolerance, particularly in the context of progressively growing tumors and in tolerogenic treatments promoting allograft acceptance. We recently reported that early growth response gene 2 (Egr2) is a critical transcription factor for the induction of anergy *in vitro* and *in vivo*, which was identified based on its ability to regulate the expression of inhibitory signaling molecules diacylglycerol kinase (DGK)- α and - ζ . We reasoned that other transcriptional targets of Egr2 might encode additional factors important for T cell anergy and immune regulation. Thus, we conducted two sets of genome-wide screens: gene expression profiling of wild type versus Egr2-deleted T cells treated under anergizing conditions, and a ChIP-Seq analysis to identify genes that bind Egr2 in anergic cells. Merging of these data sets revealed 49 targets that are directly regulated by Egr2. Among these are inhibitory signaling molecules previously reported to contribute to T cell anergy, but unexpectedly, also cell surface molecules and secreted factors, including lymphocyte-activation gene 3 (Lag3), Class-I-MHC-restricted T cell associated molecule (Crtam), Semaphorin 7A (Sema7A), and chemokine CCL1. These observations suggest that anergic T cells might not simply be functionally inert, and may have additional functional properties oriented towards other cellular components of the immune system.

Keywords

T cell anergy; early growth response gene 2 (Egr2); lymphocyte-activation gene 3 (Lag3); Class-I-MHC-restricted T cell associated molecule (Crtam); Semaphorin 7A (Sema7A); CCL1

1. Introduction

T cell anergy is a hyporesponsive state induced by TCR engagement in the absence of costimulation (Schwartz, 2003). Anergy induction was initially observed *in vitro* using chemically-fixed antigen presenting cells (APCs). Subsequently, it was found that anergy could be induced by immobilized anti-CD3 mAb or calcium ionophores (such as ionomycin)

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in vitro, and by superantigen and soluble antigenic peptide *in vivo*. Indirect evidence has suggested that T cell dysfunction in the tumor microenvironment and establishment of transplant tolerance is partially due to T cell anergy (Gajewski et al., 2011). T cell anergy is mainly characterized by the non-responsive state and multiple TCR signaling defects, of which, blunted Ras/MAPK activation has been consistently observed both *in vitro* and *in vivo* anergy models (Zheng et al., 2008). Further studies elucidated that the TCR signaling defects are due to presence of so called “anergy-associated factors”, which are specifically synthesized upon anergy induction (Gajewski et al., 1995; Telander et al., 1999). Several anergy-associated factors have been identified, including diacylglycerol kinase- α and - ζ (DGK- α and DGK- ζ); the E3 ubiquitin ligases Cbl-b, GRAIL, and Itch; Deltex 1 (Dtx1); and the anti-proliferative protein Tob1. In particular, we and others have demonstrated that DGK- α and DGK- ζ attenuate Ras/MAPK signaling by depleting diacylglycerol (DAG) (Olenchok et al., 2006; Zha et al., 2006).

The mechanisms leading to the generation of the anergy-associated factors have been gradually understood. TCR engagement alone activates the calcium/calcineurin/ NFAT pathway out of proportion to AP1 activation, resulting in the upregulation of early growth response gene 2 and 3 (Egr2 and Egr3). Egr2 and Egr3 are transcriptional factors containing zinc finger domains (Chavrier et al., 1988; Patwardhan et al., 1991). We and others have conducted gene-array analyses comparing anergic versus non-anergic T cells, and found that Egr2 is highly upregulated 2–3 hours after anti-CD3 treatment, which is reduced by calcineurin inhibitor cyclosporine A (Harris et al., 2004; Safford et al., 2005; Zha et al., 2006). The expression of Egr2 in anergic cells was of interest because the promoter region of the DGK- α gene contained an Egr2 binding site (Zheng et al., 2012). Forced-expression of Egr2 has been reported to suppress T cell activation as demonstrated by diminished IL-2 production and proliferation (Harris et al., 2004; Safford et al., 2005). Conversely, we recently found that Egr2-deleted T cells are largely resistant to anti-CD3-induced anergy *in vitro* with restored IL-2 production and Erk phosphorylation (Zheng et al., 2012). Similar findings were observed in superantigen staphylococcal enterotoxin B (SEB)-induced anergy *in vivo* as well. Furthermore, conditional Egr2-deficient mice demonstrated enhanced anti-tumor immunity. The necessity of Egr2 in T cell anergy is partially due to its involvement in the regulation of most identified anergy-associated genes. ChIP assays and qRT-PCR confirmed that Egr2 interacts with and directly promotes the transcription of DGK- α , DGK- ζ , Cbl-b, Itch, Dtx1, and Tob1 in anergic cells.

Despite these advances in the understanding of T cell anergy, our knowledge about the anergic phenotype remains incomplete for several reasons. First, surface markers that might be used to identify anergic T cells are lacking. Second, it has been unclear teleologically why T cells being subjected to anergy-inducing conditions are not simply deleted from the repertoire, in order to eliminate T cells of undesired specificities. In this vein it is conceivable that anergic T cells play an active functional role in peripheral tolerance and contribute to immune regulation. To further investigate these notions, we utilized the knowledge of Egr2 as a critical transcriptional regulator of anergy to identify the complete Egr2 transcriptome in the anergic state. 49 targets of Egr2 were identified by merging gene expression profiling and ChIP-Seq analyses. Interestingly, these include several cell surface molecules as well as secreted factors. Our data suggest that anergy is not just an intrinsic non-responsive state but that through these newly identified targets anergic cells might be able to interact with and influence the functions of other immune cells during peripheral tolerance.

2. Material and Methods

2.1. Mice and T Cell Clones

Egr2^{flox/flox} mice were a gift from Dr. Harinder Singh (University of Chicago, Chicago, IL). Coxsackie/adenovirus receptor (CAR) Tg mice expressing the extracellular domain of CAR under control of a Lck promoter/CD2 enhancer were generated as previously described (Wan et al., 2000). All mice were housed in pathogen-free conditions at the University of Chicago, and all animal protocols were approved by the Institutional Animal Care and Use Committee. To generate CAR Tg x Egr2^{flox/flox} Th1 clones, CAR Tg x Egr2^{flox/flox} mice were immunized in the hind footpads with chicken ovalbumin (OVA; A5503, Sigma) emulsified in complete Freund's adjuvant (F5881, Sigma). Seven days later, the draining lymph nodes were harvested, and CD4⁺ Th1 cell clones were derived and maintained as we recently described (Zheng et al., 2012).

2.2. Adenovirus Transduction

A Cre-expressing adenovirus was produced as described (Zha et al., 2006; Zha et al., 2008). For T cell transduction, cells were suspended at high density of 10×10^6 /mL in DMEM with 2% FBS, incubated with an EV or the Cre adenovirus at 37°C for 50 minutes, transferred to DMEM with 10% FBS, and cultured for another 16 hours at low density of 1×10^6 /mL.

2.3. Anergy Induction *In vitro*

In vitro anergy was induced by treating cells overnight with immobilized anti-CD3 mAb (1 µg/mL; 145-2C11, BioXCell). The cells were then harvested, washed, and rested for 1–2 days prior to analysis.

2.4. ChIP-Seq Analysis

100 ng of DNA from Egr2 ChIP and Input were used to generate ChIP-seq library according to Illumina's protocols. Specifically, the DNA was end-repaired using a combination of T4 DNA polymerase, *E. coli* DNA Pol I large fragment (Klenow polymerase) and T4 polynucleotide kinase. The blunt, phosphorylated ends were treated with Klenow fragment (32 to 52 exo minus) and dATP to yield a protruding 3'- 'A' base for ligation of Illumina's adapter oligo mix which have a single 'T' base overhang at the 3' end. After adapter ligation, DNA was PCR amplified with Illumina primers for 16 cycles, and DNA fragments between 200–400 bp (insert plus adaptor and PCR primer sequences) were band isolated from a 2% agarose gel (Qiagen). 8 pmoles of the isolated DNA was captured on an Illumina flow cell for cluster generation. Libraries were sequenced on an Illumina GAII sequencer following the manufacturer's protocols. For data analysis, images obtained from the sequencer were processed by Illumina image extraction pipeline software. Eland Extended was used to align sequences to mouse genome (NCBI 37/mm9). Non-unique sequences that aligned to more than two different locations were discarded prior to subsequent analysis. QuEST (Valouev et al., 2008) was used to identify enriched binding regions or peaks. MEME (Bailey et al., 2009) was used for motif identification by searching the sequences composed of 200 bp upstream and 200 bp downstream of each peak. Two independent ChIP-Seq experiments were performed, and genes present in both datasets were considered as positive.

2.5. Gene Expression Profiling Analysis

All RNA samples used for gene array analysis had RNA Integrity Number > 8.0, OD260/280 and OD260/230 ratio >1.8. The RNA was labeled, fragmented and hybridized to Affymetrix mouse genome 430 2.0 expression arrays at the Functional Genomics Core Facility of the University of Chicago (Chicago, IL). The arrays were then scanned and CEL

intensity files were generated by MicroArray Suite 5.0. The gene array analysis was performed three times using three sets of independently manipulated samples. Results from the three gene arrays were combined and analyzed using dChip software. Specifically, the genes scored as “absent” or with signal intensity <100 were first filtered out. Among the remaining genes, those with greater than or equal to a 2-fold increase in expression upon anergy induction were considered as anergy-associated genes, and those with more than a 1.5-fold reduction upon Egr2 deletion were considered as Egr2-dependent genes. Genes were considered as positive when the average fold changes of the three sets of samples met the thresholds listed above. Genes were classified using Ingenuity software.

2.6. ChIP Assay

ChIP assays were conducted following the manufacturer’s protocol (Millipore, 17-259). Briefly, 2.5×10^6 cells were lysed in 500 μ L SDS lysis buffer, and cellular DNA was sheared 6 times with a 15-second pulse plus 60-second rest using a Misonix Sonicator 3000 (Qsonica). For immunoprecipitation, 200 μ L cell lysate supernatant (corresponding to 1×10^6 cells) was diluted 5 fold in ChIP dilution buffer, and anti-Egr2 Ab was added at a final concentration 10 μ g/mL (PRB-236P, Covance). SYBR Green qRT-PCR was conducted using primers specific for *CCL1* intron (forward 5′-AATGGCCACATGGAAAATC-3′, reverse 5′-CCAAACATACCTCGAATACGC-3′); *Crtam* intron (forward 5′-TCTGGACAGGAGGGGATGT-3′, reverse 5′-AGGAAACACCCACAGCAAAG-3′); *Sema7A* core promoter (forward 5′-GCTTCTGCTGGTGTCTGG-3′, reverse 5′-CGCCTACCTTTCCAGACG-3′); *Lag3* core promoter (forward 5′-CTCCAGACCCAGTCCTTCTG-3′, reverse 5′-ACACTTCCACTGCGAAGC-3′); *4-1BB* 5′ UTR (forward 5′-AATCTCTTAAGTCAGGAGAGACGTG-3′, reverse 5′-TTCCCACCACAGTGACATTC-3′); *Nrgn* intron (forward 5′-GGCTTGGCTCAGATCAGG-3′, reverse 5′-GGGAAAGAATGGTGCTGAAA-3′); *Nrm1* proximal promoter (forward 5′-GTGACTGATTTTCATCCCAGTG-3′, reverse 5′-ACCAGGACTCCCCGTCTC-3′); *Bcl2l1* core promoter (forward 5′-TCCACTTGGATTACACCAC-3′, reverse 5′-CAGACATTGGGTGGACGAG-3′); *Crabp2* proximal promoter (forward 5′-CTTGCCTTCTGACGCTTCTC-3′, reverse 5′-GGGTTCTCCAAGAGCCAAG-3′). Primers specific for *GJA5* were used as controls (forward 5′-ACCATGGAGGTGGCCTTCA-3′, reverse 5′-CATGCAGGGTATCCAGGAAGA-3′).

2.7. qRT-PCR

The primers and probes were purchased from IDT, Roche, and Applied Biosystems. qRT-PCR used primers and probes specific for *CCL1* (forward 5′-TCACCATGAAACCCACTGC-3′, reverse 5′-AGCAGCAGCTATTGGAGACC-3′, CTGGCTGC); *Crtam* (forward 5′-AGATCCAACAACGAGGAGACA-3′, reverse 5′-TCATGCAACGCTTAGACTGG-3′, CTGGCTGC); *Sema7A* (forward 5′-TCAATCGGCTGCAAGATGT-3′, reverse 5′-CGCAGACAGCTGAGTAGTTCC-3′, GAGCAGGA); *Lag3* (forward 5′-TGCTTTGGGAAGCTCCAGT-3′, reverse 5′-GCTGCAGGGAAGATGGAC-3′, CCAGGAGG); *4-1BB* (forward 5′-GAACGGTACTGGCGTCTGTC-3′, reverse 5′-CCGGTCTTAAGCACAGACCT-3′, CTGCTCTC); *Nrgn* (forward 5′-AACACCGGCAATGGACTG-3′, reverse 5′-AAACTCGCCTGGATTTTGG-3′, GCTGGATG); *Nrm* (forward 5′-TCCTCGCGGTGCAAATAG-3′, reverse 5′-GCCCTTAAAGACTGCATCACA-3′, CTGCTCTC); *Bcl2l1* (forward 5′-GGAGACGAGTTCAACGAAACTT-3′, reverse 5′-AACAGTTGTAAGATAACCATTTGAGG-3′, GGCTGAAG); *Crabp2* (forward 5′-AAATGGTGTGCGAGCAGAG-3′, reverse 5′-AACGTCATCTGCTGTCATTGTC-3′, CCAGGAGG). Relative RNA abundance was determined based on control 18S RNA (Hs99999901_s1, Applied Biosystems).

2.8. Immunoblot Analysis

Equal numbers of T cells were resuspended in ice-cold lysis buffer containing 50 mM Tris (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.5% Triton x-100, 1mM PMSF, 10mM NaF, 1mM Na₃VO₄, and 1x protease inhibitor mixture (Roche). After 30-minute incubation on ice, the cells were spun for 10 minutes at top speed at 4°C, and the supernatant was collected. The cellular lysate was loaded into 10% Tris-HCL gels (Bio-Rad Laboratories), separated by SDS-PAGE, and transferred to PVDF membranes (Millipore). Proteins were detected using primary antibodies against Sema7A (1:1000, AF1835, R&D System) and Crabp2 (1:1000, MAB5488, Millipore), a secondary HRP-linked anti-mouse IgG antibody (1:3000, GE Healthcare), an ECL Detection Kit (GE Healthcare).

2.9. Flow Cytometry

To stain for Crtam, cells were incubated with an anti-Crtam mouse IgG2a (30 ng/mL, 10 µl per 1 x 10⁶ cells; a gift from Dr. Andrew C. Chan, Genentech, CA) at 4°C for 20 minutes, and then an Alexa Fluor 647 goat anti-mouse IgG2a (1:100; A21241, Invitrogen) at 4°C for another 20 minutes.

3. Results

3.1. Identification of *Egr2* transcriptome using microarray-based gene expression profiling and ChIP-Seq analyses

Th1 T cell clones anergized by immobilized anti-CD3 were used as our T cell anergy model. This model has been well-characterized and can provide sufficient cellular material for microarray and ChIP-Seq analyses (Schwartz, 2003). T cell-specific *Egr2* deletion was mediated by use of a Cre-expressing adenovirus and a CAR Tg x *Egr2*^{flox/flox} mouse in which CAR is expressed exclusively in the T cell compartment from a Lck promoter/CD2 enhancer cassette. This system allows for peripheral deletion of *Egr2* without affecting T cell development in the thymus, as we recently described (Zha et al., 2008). Briefly, OVA-specific Th1 cell clones were generated from CAR Tg x *Egr2*^{flox/flox} mice (Fitch et al., 2006; Zha et al., 2008). *Egr2* deletion was then achieved by transduction of the CAR Tg x *Egr2*^{flox/flox} Th1 T cell clones with the Cre adenovirus. This system was proven to be very efficient, and qRT-PCR and immunoblot analyses confirmed that the induction of *Egr2* mRNA and protein normally seen under anergy conditions was decreased to minimal levels following Cre adenovirus-mediated *Egr2* gene deletion (Zheng et al., 2012).

In order to map the complete *Egr2* transcriptome of anergic T cells, we conducted two sets of genome-wide screens. The first was a microarray-based gene expression profiling analysis in which anergized Th1 cells were compared with or without prior *Egr2* deletion. Specifically, CAR Tg x *Egr2*^{flox/flox} Th1 T cells were infected with an empty (EV) or a Cre-expressing adenovirus. Upon confirmation of *Egr2* deletion by immunoblot, the T cells were anergized by immobilized anti-CD3 for 16 hours, and microarray was conducted after 1 day of rest in culture medium. This analysis helped to identify the set of genes upregulated (Supplementary Table 1) and down-regulated (Supplementary Table 2) upon anergy induction in an *Egr2*-dependent manner. Gene array analysis revealed that 938 out of the total 45101 probes demonstrated at least a 2-fold increase upon anergy induction. Among those, 90 probes met our defined criteria for *Egr2*-dependence, in that the elevated gene expression seen in anergy was reduced by more than 1.5-fold in *Egr2*-deleted cells.

To identify the direct transcriptional targets of *Egr2*, we also performed a ChIP-Seq analysis. CAR Tg x *Egr2*^{flox/flox} Th1 T cells were untreated or anergized by immobilized anti-CD3, and nuclear extracts were immunoprecipitated with an anti-*Egr2* Ab. ChIP-Seq analysis was carried out on the DNA fragments bound to *Egr2*. The result of one of the two ChIP-Seq

analyses is shown here in Figure 1A. Egr2 was found to be associated with a variety of regulatory regions in the genome of anergic T cells, including core promoters (50 bp upstream of transcription start site (TSS)), proximal promoters (500 bp upstream of TSS), 5'-untranscription regions (UTRs), 3'-UTRs, introns, and intergenic regions. A similar pattern was identified from a second independently performed ChIP-Seq analysis as well (data not shown). These binding interactions appeared to be specific, since the consensus sequence of Egr2 binding site derived from ChIP-Seq was highly similar to that published on TRANSFAC, a comprehensive transcription factor database (Figure 1B).

When the gene array results were merged with the ChIP-Seq data, 62 of the 90 probes that showed Egr2-dependency for their expression were found to be directly bound by Egr2. Because of some duplication, those 62 probes represented 49 genes (Figure 1C). In summary, 2.08% (938/45101) of the gene array probes demonstrated upregulation upon anergy induction, among which 9.59% (90/938) were dependent on Egr2, and 6.61% (62/938) were directly regulated by Egr2.

Among the 49 identified genes, there are transcriptional regulators: Egr2, four and a half LIM domains 2 (Fhl2), BTB and CNC homology 2 (Bach2), Kruppel-like factor 9 (Klf9), retinoic acid induced 14 (Rai14), JAZF zinc finger 1 (Jazf1); cytokines: CCL1, tumor necrosis factor (ligand) superfamily, member 11 (Tnfsf11); enzymes: carbonic anhydrase 12 (Car12), guanine nucleotide binding protein beta 5 (Gnb5), DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 11 (Ddx11), tetratricopeptide repeat domain 3 (Ttc3), ADP-ribosylation factor-like 3 (Arl3); kinase: protein kinase C and casein kinase substrate in neurons 1 (Pacsin1), pyruvate dehydrogenase kinase isoenzyme 2 (Pdk2), Dgk- ζ , calcium and integrin binding family member 2 (Cib2); peptidase: endothelin converting enzyme 1 (Ece1); membrane receptors: oxidized low density lipoprotein (lectin-like) receptor 1 (Olr1); transporters: pleckstrin homology, Sec7 and coiled-coil domains 3 (Pscd3), solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 (Slc17a6), solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (Slc13a3); ion channels: transient receptor potential cation channel, subfamily M, member 4 (Trpm4), ryanodine receptor 1, skeletal muscle (Ryr1).

3.2. Validation of novel targets of Egr2 in the anergic T cells

Previous mechanistic studies of T cell anergy have focused attention on identifying the key factors that are causal for T cell intrinsic dysfunction. However, a striking feature of the additional genes identified through the gene array/ChIP-Seq analyses is that several of the gene products are surface molecules or secreted factors. This observation suggests that anergic T cells may have additional functional properties oriented towards other cellular components of the immune system.

Nine of the newly identified Egr2-dependent targets were studied in more detail, including chemokine CCL1; five cell surface receptors (Semaphorin 7A (Sema7A), Class-I-MHC-restricted T cell associated molecule (Crtam), lymphocyte-activation gene 3 (Lag3), tumor necrosis factor receptor superfamily member 9 (Tnfrsf9, or 4-1BB), and Neuritin (Nrn1)); and three intracellular proteins (BCL2-like 11 (Bcl2l11 or Bim), Neurogranin (Nrgn), and cellular retinoic acid-binding protein 2 (Crabp2)). CCL1 is a member of the C-C motif chemokine family, and a recent publication indicated that CCL1 can recruit Foxp3⁺ regulatory T cells in the tumor context (Hoelzinger et al., 2010). Semaphorin 7A (Sema7A) belongs to membrane-bound Semaphorin family that associates with the plasma membrane via a GPI linker (Suzuki et al., 2008). Crtam is a type I transmembrane protein with V and C1-like Ig domains (Du Pasquier, 2004). Lag3, a CD4-related transmembrane protein, binds to MHC class II on APCs with higher affinity than does CD4 and has been reported to function as an inhibitory receptor (Baixeras et al., 1992; Grosso et al., 2007; Huang et al.,

2004; Triebel et al., 1990). 4-1BB is an inducible costimulation receptor on T cells, and belongs to the tumor necrosis factor receptor superfamily (Watts, 2005). *Nrn1* is a neural activity-regulated gene, encoding a small extracellular protein that serves as a neurotrophin to promote neuritogenesis, neuronal survival, and plasticity (Naeve et al., 1997; Nedivi et al., 1996; Nedivi et al., 1998). *Nrgn* is another protein that has been mainly studied in the central nervous system (Diez-Guerra, 2010). It interacts with calmodulin and regulates intracellular concentrations of calcium, and calcium-derived signaling in synapses. The functions of *Nrn1* and *Nrgn* in the immune system are yet to be clarified. *Bcl2l1* is a pro-apoptosis proteins in the intrinsic apoptosis pathway, and has been shown to regulate T cell deletion in both thymus and the peripheral (Bouillet and O'Reilly, 2009). Its interaction with pro-survival protein Bcl-2 releases BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer (BAK) to trigger mitochondria-mediated apoptosis. *Crabp2* is a retinoic acid (RA) binding protein, delivering retinoid acid from the cytoplasm to its selective receptors located in the nucleus (Hall et al., 2011).

ChIP-Seq analyses identified *Egr2* binding sites in these nine genes (Supplementary Figure 1 and Table 3), which were subsequently confirmed by ChIP assay. As shown in Figure 2, *Egr2* was associated with variable regulatory regions of these genes upon immobilized anti-CD3-induced anergy. *Egr2*-dependent mRNA expression in anergic T cells was confirmed by qRT-PCR. As seen in Figure 3, TCR engagement alone highly upregulated these genes, and their expressions were reduced substantially with *Egr2* deletion. Similar results were also seen in two other CAR Tg x *Egr2*^{flox/flox} Th1 T cell clones (Supplementary Figure 2 and 3). To further validate the qRT-PCR results, we analyzed the expression of four gene products at protein level, as there were reagents available for this analysis. ELISA revealed that CCL1 was constitutively secreted by anergic cells in an *Egr2*-dependent manner (Figure 4A). The expression of *Sema7A* and *Crabp2* proteins were detected by immunoblot in anergic cells, which were partially reduced in the absence of *Egr2* (Figure 4B and 4D). Similarly, cell surface expression of *Crtam* was detected by flow cytometry on at least 14.60±1.88% anergic cells, and diminished to 3.41±0.2% when *Egr2* was deleted (Figure 4C represents one of six experiments). These results indicate that *Egr2* directly contributes to the expression of these nine genes in anergic cells.

4. Discussion

We have recently reported that *Egr2* is a critical transcription factor for the process of T cell anergy (Zheng et al., 2012). Deletion of *Egr2* enabled significant resistance to anergy induction both *in vivo* and *in vitro*. *Egr2* was proven to directly regulate the transcription of multiple established anergy-associated genes, including *DGK-α* and ζ , which contribute to the blunted TCR/CD28-induced signaling characteristic of the state of anergy. In the present report, we continued this focus on *Egr2*, and utilized gene expression profiling from T cells treated under anergy-promoting conditions that are competent or not for *Egr2* expression, along with ChIP-Seq analysis. Among the newly identified *Egr2* targets, we found a set of cell surface receptors and secreted molecules expressed in anergic T cells, suggesting that the anergic state might not be functionally inert but rather could generate a phenotype having an active functional role in the process of peripheral tolerance through interactions with other immune cells.

Several previously defined anergy-associated genes that were confirmed to be differentially expressed by qRT-PCR did not pass the threshold for positivity by gene array analysis. Specifically, microarray analysis failed to show the upregulated expressions of several defined genes in anergic cells, which, however, were detected by qRT-PCR using the same RNAs. These results speak to the lower sensitivity of the gene chip technique, and raise the possibility that our final list of genes defined to be upregulated in the anergic state may be

an underestimate. It has been estimated that the detection limit of current microarray technology can be a few copies of mRNA per cell (Draghici et al., 2006). In contrast, the sensitivity of qRT-PCR is generally between 10–20 copies of mRNA per reaction (Affymetrix), which in our experiments were derived from $1\text{--}5 \times 10^4$ cells. The results obtained by qRT-PCR have also been reported to be more quantitative, although qualitative expression results are reproducible using the microarray approach (Chuaqui et al., 2002). Therefore, alternative technologies may be required to fully capture the entire set of genes upregulated in the anergic state, such as the RNA-seq approach (Wang et al., 2009b).

The spectrum of new genes/gene products upregulated in an Egr2-dependent fashion raises new sets of hypotheses regarding the functional role for anergic T cells *in vivo*. We found that the chemokine CCL1 was constitutively produced by anergic T cells. Its receptor, CCR8, has been shown to be expressed on regulatory T cells (Treg) and Th2 cells (Soler et al., 2006). It has been recently reported that CCL1 can contribute to the recruitment as well as de novo conversion of Tregs in the tumor context *in vivo* (Hoelzinger et al., 2010). In that study, anti-CCL1 treatment promoted improved immune-mediated tumor control *in vivo*. In addition, CCL1 has been reported to be a potent pro-angiogenic factor. Its human homologue, I-309, has been shown to stimulate chemotaxis and invasion of endothelial cells (Bernardini et al., 2000). Therefore, it is possible that CCL1 production by anergic T cells in the tumor microenvironment might be driven by Egr2, and contribute both to Treg accumulation and to neoangiogenesis.

Crtam was previously identified to be expressed mainly on subsets of T cells and NK cells. The interaction of Crtam and cell adhesion molecule 1 (Cadm1/ Nect2) on antigen presenting cells (APCs) has been reported to mediate T cell retention in lymph nodes (Takeuchi et al., 2009; Yeh et al., 2008). It is conceivable that Crtam expression on anergic cells limits their trafficking *in vivo*, or that adhesion between Crtam-expressing anergic T cells and APCs regulates immune responses.

Lag3 has been shown to be upregulated on activated Tregs, and serve to enhance their suppression activity (Huang et al., 2004). It has been described in some model systems that anergic cells can be suppressive as well, and their suppression activity is cell contact-dependent (Chai et al., 1999). Surface expression of Lag3 might therefore contribute to the immune suppressive activity of anergic T cells. In addition, a major subset of T cells from the tumor microenvironment has been reported to express Lag3 in both mouse and human systems, and these T cells have been shown to be dysfunctional in some models when analyzed *ex vivo* (Gandhi et al., 2006; Grosso et al., 2007). Interference with Lag3 binding, either alone or in concert with blocking PD-1 function, has been shown to improve immune-mediated tumor rejection *in vivo* (Woo et al., 2012). Future work will be necessary to examine whether Lag3, alone or in combination with other markers, might be useful for identifying anergic T cells *ex vivo*.

Semaphorins were initially identified as guidance factors of axon growth during neuron development. Accumulated evidence indicates several members of this family including Sema7A are also crucial in regulating immune responses. Sema7A-deficient T cells have been reported to be hyperproliferative in response to antigen challenge and in a lymphopenic environment *in vivo*. Sema7A-null mice have been shown to have exacerbated experimental autoimmune encephalomyelitis (EAE) and an augmented delayed type hypersensitivity response. Mechanistically, Sema7A-deficient T cells exhibit delayed TCR down-regulation, increased calcium influx, and defective focal adhesion kinase (FAK) phosphorylation. Therefore, it is possible that this molecule could contribute to T cell dysfunction in the anergic state (Czopik et al., 2006). On the other hand, regulation of APC function by T cells

via Sema7A- α 1 β 1 integrin interactions also has been demonstrated (Suzuki et al., 2008; Suzuki et al., 2007).

4-1BB is an inducible costimulation receptor on T cells, and binds to 4-1BB ligand on APCs. 4-1BB engagement can lead to augmented CD8⁺ T cell proliferation, cytokine production, and prolonged cell survival. An agonistic antibody against 4-1BB has been reported to enhance anti-tumor and anti-viral immune responses *in vivo* (Wang et al., 2009a). Therefore, 4-1BB ligation might provide a means by which to reverse T cell anergy under appropriate conditions *in vivo*. A recent publication in the tumor context supports this hypothesis (Curran et al., 2011).

Crabp2 functions to transport RA from the cytoplasm to its nuclear receptors retinoic acid receptors (RAR)/ retinoid X receptors (RXR) (Hall et al., 2011). RA is derived from Vitamin A and a critical regulator of immune tolerance. Enriched in CD103⁺ gastrointestinal tract and associated lymphoid tissue (GALT) DCs, RA serves as a cofactor of TGF- β in the generation of inducible Treg (iTreg). RA can also block Th17 differentiation by suppressing the expression of IL-6R and IL-23R. In addition, RA promotes T cell mucosal homing by upregulation of mucosal homing markers integrin α 4 β 7 and chemokine receptor CCR9. Of note, the effects of RA on T cell differentiation and trafficking are RAR α -dependent as shown by RAR α deficient mice and promoter studies. Therefore, it is possible that upregulation of Crabp2 in anergic cells might allow RA to direct the trafficking and promote/enhance the suppressive activity of the anergic cells. Examining the functional contributions of these molecules in T cell regulation both *in vitro* and *in vivo* will be attractive pursuits for future studies.

Supplementary Material

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Glossary

APCs	antigen presenting cells
Bcl2l1/Bim	BCL2-like 11
Crabp2	cellular retinoic acid-binding protein 2
Crtam	Class-I-MHC-restricted T cell associated molecule
DAG	diacylglycerol
DGK-α and DGK-ζ	diacylglycerol kinase- α and - ζ
Egr2	early growth response gene 2
Lag3	lymphocyte-activation gene 3
Nrgn	Neurogranin
Nrn1	Neuritin

OVA	chicken ovalbumin
SEB	staphylococcal enterotoxin B
Sema7A	Semaphorin 7A
Tregs	regulatory T cells
TSS	transcription start site
4-1BB/Tnfrsf9	tumor necrosis factor receptor superfamily member 9
5'UTR	5'-untranscription region

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Highlights

- We reported Egr2 as a central transcriptional regulator of T cell anergy induction.
- Microarray and ChIP-Seq analyses were conducted to investigate Egr2 transcriptome.
- 49 new targets were identified to be directly regulated by Egr2 in anergic cells.
- Egr2 regulates several cell surface and secreted molecules in anergic cells.

Figure 1. Identification of direct targets of Egr2 in the context of T cell anergy by ChIP-Seq and gene expression profiling analyses

CAR Tg x Egr2^{flx/flx} Th1 T cells were anergized with immobilized anti-CD3, and Egr2-associated genes were identified by ChIP-Seq analysis. (A) Distribution of the types of Egr2 binding sites in the genome from one of two ChIP-Seq analyses. (B) Consensus sequence of Egr2 binding sites derived from ChIP-Seq were highly similar to that published on TRANSFAC. EV- or Cre-transduced CAR Tg x Egr2^{flx/flx} Th1 T cells were left untreated or anergized by immobilized anti-CD3, and anergy-associated and Egr2-dependent genes were determined by gene chip analysis. The results from ChIP-Seq and gene chip analyses were merged to identify the direct targets of Egr2. (C) A list of direct Egr2 targets and their fold changes in expression upon anergy induction (Upregulation) and with Egr2 deletion

(Ratio EV vs. Cre). The results were summarized from two independent ChIP-Seq analyses and three independent gene expression profiling analyses.

Figure 2. Confirmatory ChIP assay on selected targets of Egr2

CAR Tg x Egr2^{flox/flox} Th1 T cells were left untreated (Control) or anergized with immobilized anti-CD3 (Anergic), the nuclear lysate were immunoprecipitated with anti-Egr2-coated beads or empty beads, and the association of Egr2 with the indicated genes was determined by ChIP Assay. Data are presented as mean $\pm$ SD, and are representative of two to three independent experiments, **p < 0.01.

Figure 3. Confirmatory qRT-PCR on selected targets of *Egr2*

CAR Tg x *Egr2*^{flox/flox} Th1 clones were infected with an EV- or a Cre- expressing adenovirus to delete *Egr2*. The cells were then left untreated (Control) or anergized with immobilized anti-CD3 (Anergic), and the expression of the indicated genes was examined by qRT-PCR. Data are presented as mean $\pm$ SD, and are representative of three independent experiments, * p < 0.05, **p < 0.01.

A CCL1 **B Sema7A** **C Crtam** **D Crabp2**

Figure 4. Confirmatory protein expression on selected targets of *Egr2*

CAR Tg x *Egr2*^{flox/flox} Th1 clones were infected with an EV- or a Cre- expressing adenovirus to delete *Egr2*. The cells were then left untreated (Control) or anergized with immobilized anti-CD3 (Anergic), and the expressions of CCL1, Sema7A, Crtam, and Crabp2 were examined by ELISA (A), Immunoblot (B and D), and flow cytometry (C) respectively. Data are presented as mean $\pm$ SD, and are representative of two to six independent experiments, ** $p < 0.01$.