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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.

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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) *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) (Olenchock 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 h 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

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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. Materials 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 × $Egr2^{flox/flox}$ Th1 clones, CAR Tg × $Egr2^{flox/flox}$ 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, 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 min, transferred to DMEM with 10% FBS, and cultured for another 16 h 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 (32–52 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 and 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 μL 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 μL 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 μg/mL (PRB-236P,

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