Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo

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T cell receptor engagement in the absence of costimulation results in a hyporesponsive state termed anergy. Understanding the transcriptional regulation of other T cell differentiation states has provided critical information regarding the biology of T cell regulation in vivo. However, the transcriptional regulation of T cell anergy has been poorly understood. Using the key anergy target gene diacylglycerol kinase (DGK) α as a focal point, we identified early growth response gene 2 (Egr2) as a central transcription factor that regulates the anergic state. Conditional Egr2 deletion in peripheral T cells abolishes induced expression of DGK-α and other anergy genes and restores Ras/MAPK signaling, IL-2 production, and proliferation upon attempted anergy induction. Using superantigen- and tumor-induced anergy models, we found that Egr2 is necessary for anergy induction in vivo. Collectively, our results implicate Egr2 as an essential transcriptional regulator of the T cell anergy program.

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TCR engagement in a NFAT-dependent manner, and that overexpression of Egr2 inhibits T cell activation (Harris et al., 2004; Safford et al., 2005). However, the exact role of Egr2 in anergy is poorly understood. Here, we demonstrate that Egr2 is a major transcription factor that directly up-regulates DGK-α as well as other anergy-associated genes. Furthermore, Egr2 deletion prevents anergy induction in vitro and in vivo. Our data support the notion that Egr2 is an essential transcription factor of the anergy program, which drives functional alterations that characterize the anergic state.

RESULTS AND DISCUSSION

Egr2 directly regulates the expression of DGK-α upon anergy induction

In the search for transcription factors that regulate DGK-α expression during anergy induction, we identified an Egr binding site located between −909 and −901 bp of mouse DGK-α transcriptional start site, and a corresponding binding site was found in the homologous region of human sequence (Fig. 1 A). This was of interest because our previous gene expression profiling analyses on anergic T cells identified Egr2 as a highly up-regulated transcriptional factor in the anergic state (Zha et al., 2006). Kinetic analysis upon anergy induction revealed that Egr2 mRNA peaked as early as 1–3 h after anti-CD3 mAb stimulation (Fig. 1 B). In comparison, DGK-α transcription occurred later, coinciding with the reported onset of hyporesponsiveness (Gajewski et al., 1995). The expression of both Egr2 and DGK-α was substantially lower in T cells fully activated with anti-CD3 + anti-CD28 mAbs (unpublished data). These observations suggest that Egr2 could be involved in the transcription of DGK-α in the anergic state.

To determine whether Egr2 could induce the activity of DGK-α promoter, we cloned mouse DGK-α promoter region from −1054 to 192 bp, which contains the putative Egr binding site (−909 to −901 bp), into a luciferase reporter vector and cotransfected with an Egr2-expressing vector into Jurkat cells. A control reporter (−54 to 190 bp), lacking the putative Egr binding site, and an empty expression vector (empty vector [EV]) were included as controls. As seen in Fig. 1 C, the luciferase activity of the DGK-α reporter increased in the presence of Egr2, suggesting that Egr2 is capable of driving DGK-α transcription. Next, we performed a ChIP assay, and the particular DGK-α promoter region was coprecipitated with an anti-Egr2 Ab in anergic cells (Fig. 1 D). Thus, upon anergy induction Egr2 associates with the DGK-α promoter and can directly regulate its expression.

TG10 Th1 T cells were anergized with 1 µg/ml of immobilized anti-CD3, and Egr2 and DGK-α mRNA expression was assessed by qRT-PCR at the indicated time. (C) Jurkat cells were cotransfected with a control or a DGK-α reporter, and an EV or an Egr2-expressing vector, and luciferase activity was assessed 48 h later. (D) OVA-specific CAR Tg × Egr2flox/flox Th1 T cells were untreated (Control) or anergized with immobilized anti-CD3 mAb (Anergic) for 6–8 h and immunoprecipitated with an anti-Egr2 or a control IgG Ab, followed by qPCR using specific primers for the DGK-α promoter region. Data are presented as mean ± SD, and are representative of two to three independent experiments. *, P < 0.05; **, P < 0.01.

Figure 1. Egr2 is associated with DGK-α promoter upon anergy induction and can regulate DGK-α expression. (A) The homologous region of human and mouse DGK-α promoter sequences. The boxed site is a putative Egr2 binding site. The arrows represent the predicted transcriptional start sites of human and mouse DGK-α according to NCBI reference sequences. (B) TG10 Th1 cells were anergized with 1 µg/ml of immobilized anti-CD3, and Egr2 and DGK-α mRNA expression was assayed by qRT-PCR at the indicated time. (C) Jurkat cells were cotransfected with a control or a DGK-α reporter, and an EV or an Egr2-expressing vector, and luciferase activity was assayed 48 h later. (D) OVA-specific CAR Tg × Egr2flox/flox Th1 T cells were untreated (Control) or anergized with immobilized anti-CD3 mAb (Anergic) for 6–8 h and immunoprecipitated with an anti-Egr2 or a control IgG Ab, followed by qPCR using specific primers for the DGK-α promoter region. Data are presented as mean ± SD, and are representative of two to three independent experiments. *, P < 0.05; **, P < 0.01.
Egr2 is necessary for the up-regulation of DGK-α upon anergy induction

To determine the necessity of Egr2 in the expression of DGK-α upon anergy induction, we developed a system to be able to delete Egr2 conditionally from Th1 clones, to be able to use a monoclonal population of T cells, and to use the Th1 cellular context, which is the best characterized model for the anergy process (Schwartz, 2003). To achieve targeted gene deletion, we used a mouse that expresses Coxackie/adenovirus receptor as a transgene (CAR Tg) in T cell lineage, along with a Cre-expressing adenovirus (Wan et al., 2000). Egr2<sup>flox/flox</sup> mice were interbred with CAR Tg mice to generate CAR Tg × Egr2<sup>flox/flox</sup> mice, from which OVA-specific Th1 cell clones were generated (Fitch et al., 2006). To delete Egr2, the Th1 T cell clones were transduced with the Cre adenovirus. As assessed by qRT-PCR and immunoblotting, the Egr2 expression normally induced during anergy induction by anti-CD3 mAb was reduced to minimal levels (Fig. 2, A and B).

Cre adenovirus-transduced T cell clones were anergized by immobilized anti-CD3 mAb. As shown in Fig. 2 (C and D), deletion of Egr2 abrogated detectable binding to DGK-α promoter, which, as expected, was associated with markedly reduced levels of DGK-α transcripts. Therefore, Egr2 is necessary for DGK-α up-regulation under anergizing conditions.

Egr2-deleted T cells are resistant to anergy induction in vitro

To determine whether Egr2 was essential for the functional characteristics of T cell anergy, CAR Tg × Egr2<sup>flox/flox</sup> T cell clones were transduced with the EV or Cre adenovirus and left untreated or anergized with immobilized anti-CD3 mAb. Upon rechallenge with anti-CD3 + anti-CD28 mAbs, the EV-transduced cells showed diminished IL-2 production and proliferation consistent with the hyporesponsive state of anergy (Fig. 3, A and B). However, with Egr2 deletion, the T cells remained active with nearly normal IL-2 secretion and proliferation. Of note, although the nonanergic control Cre-transduced cells produced a modestly increased amount of IL-2 upon anti-CD3 + anti-CD28 mAb stimulation compared with the control EV-transduced cells, the difference between the anergic Cre- and EV-transduced cells was more than sevenfold. Thus the restored IL-2 production found in the anergic Cre-transduced cells was not simply a result of hyperactivity; rather, Egr2 deletion rendered the cells less susceptible to anergy induction. Similar functional results were observed with additional independently derived CAR Tg × Egr2<sup>flox/flox</sup> Th1 clones (unpublished data). In agreement with these functional results, the blunted ERK phosphorylation normally observed in anergic cells was restored with Egr2 deletion (Fig. 3 C). Quantitative densitometry averaged over three independent experiments confirmed the statistical significance of this difference (P < 0.05).

To further confirm the functional relationship between Egr2 and DGK-α, we reintroduced DGK-α into the Egr2-deleted T cells by transduction with a DGK-α-expressing adenovirus (Fig. 3 D). In fact, directed expression of DGK-α dominated over Egr2 deletion, resulting in reinhibition of IL-2 production and proliferation (Fig. 3, E and F). Thus, DGK-α is operational downstream of Egr2 and is one of the critical anergy-associated genes that contribute to T cell dysfunction.

Egr2 deletion impairs anergy induction in vivo

We chose a well established model of superantigen-induced anergy to examine whether the Egr2 pathway was important for anergy induction in vivo (Kawabe and Ochi, 1990; Rellahan et al., 1990). Two methods were used. In the first, CD4-Cre × Egr2<sup>flox/flox</sup> mice were generated by interbreeding CD4-Cre Tg mice and Egr2<sup>flox/flox</sup> mice, and the lack of Egr2 expression in Egr2<sup>flox/flox</sup> Th1 clones was confirmed by immunoblot (Fig. 4 A). CD4-Cre × Egr2<sup>flox/flox</sup> mice and control mice were intraperitoneally injected with staphylococcal enterotoxin B (SEB) or PBS. 7 d later, splenic T cells were isolated, comparable numbers of Vβ8<sup>+</sup> T cells were restimulated with SEB ex vivo, and IL-2 secretion was assessed by ELISPOT (Fig. 4 B). The failure to detect anergy induction in Egr2-deleted T cells, as assessed by ELISPOT and qRT-PCR (Fig. 4 C), is consistent with these findings. In an independent study, we found that Egr2 deletion also impairs IL-2 production in vivo in CAR Tg × Egr2<sup>flox/flox</sup> T cells (unpublished data).

Figure 2. Egr2 deletion results in reduced DGK-α up-regulation upon anergy induction. (A and B) OVA-specific CAR Tg × Egr2<sup>flox/flox</sup> Th1 T cells were transduced with an EV or a Cre-expressing adenovirus (Cre) to delete Egr2. The cells were then untreated or anergized with immobilized anti-CD3 for 3–6 h, and deletion of Egr2 was confirmed by qRT-PCR (A) and immunoblotting (B). (C and D) Upon Egr2 deletion, the association of Egr2 with DGK-α was determined by ChIP Assay using primers specific for DGK-α and control primers for GJA5 (C). The up-regulation of DGK-α was assessed by qRT-PCR (D). Data are presented as mean ± SD and are representative of two to three independent experiments. ***, P < 0.01.
production was measured. Consistent with the superantigen-induced anergy model, T cells from SEB-treated control mice produced significantly less IL-2 than those obtained from PBS-treated counterparts (Fig. 4 B). In contrast, there was no reduction of IL-2 by T cells from conditionally Egr2-deficient mice treated with SEB, indicating that Egr2-deficient T cells were resistant to superantigen-induced anergy in vivo. This effect was not simply an artifact of Cre expression in T cells because CD4-Cre Tg mice remained susceptible to SEB-induced anergy induction (unpublished data).

Because of a concern that Egr2 deletion, even late in thymic development, using Cre driven by the CD4 promoter might yield an abnormal peripheral T cell compartment, a second method was used. T cells from CAR-Tg × Egr2flox/flox mice were purified, and Egr2 was deleted in vitro using the Cre-expressing adenovirus. After verification of Egr2 deletion by intracellular flow cytometry (Fig. 4 C), T cells were adoptively transferred into OT-1 TCR Tg × Rag2−/− mice. The rationale for using these recipients is to block homeostatic proliferation of the transferred T cells, which itself could interrupt or reverse anergy but without inducing immune-mediated regression of CAR-expressing T cells (Brown et al., 2006; Kline et al., 2008). In addition, the β chain of OT-1 TCR is Vβ5, which does not respond to SEB superantigen (Vβ8). 1 d after adoptive transfer, recipient mice were injected intraperitoneally with SEB, and 7 d later T cells were isolated and analyzed as described in the previous section. As with the CD4-Cre model, Cre-transduced T cells did not show reduced IL-2 production after SEB treatment in vivo (Fig. 4 D), confirming the importance of Egr2 for anergy induction in this model.

To further examine the functional role for Egr2 in vivo, CD4-Cre × Egr2flox/flox mice and control mice matched for age and sex were injected subcutaneously with B16.SIY (SIY-expressing B16 melanoma cells). This is a tumor model system in which anergy appears to be one mechanism of immune escape in vivo (Kline et al., 2008). As shown in Fig. 4 E, tumor growth was significantly slowed in Egr2-deleted mice. Although the frequency of SIY-specific T cells in CD4-Cre × Egr2flox/flox and control mice were similar (Fig. 4 F), Egr2 deletion led to markedly augmented SIY-specific IFN-γ production ex vivo assessed by ELISPOT (Fig. 4 G). These results argue for increased functional capacity of the endogenously
activated anti-tumor T cells and indicate that T cell–intrinsic Egr2 expression can contribute to tumor escape from immune destruction in vivo. We did not observe any obvious evidence of spontaneous autoimmunity in CD4-Cre × Egr2flox/flox mice up to 12 mo of age (unpublished data).

In the present study, we demonstrate that Egr2 directly regulates the expression of DGK-α in anergic T cells. Furthermore, conditional deletion of Egr2 in T cells abolished anergy-induced DGK-α expression indicating the necessity of Egr2 in this process. In contrast, transduction of DGK-α back into the Egr2-deleted T cells resulted in decreased IL-2 production and T cell proliferation. These data suggest that DGK-α acts downstream of Egr2 and is one of the critical anergy-associated genes responsible for maintaining the anergic state. Besides DGK-α, multiple anergy-associated genes have been identified previously including DGK-ζ, Chl-b, Itch, GRAIL, Tob1, and Dtx1, which also contribute to T cell–intrinsic hyporesponsiveness and the regulation of peripheral tolerance. In fact, using ChIP assay, we found that Egr2 bound the regulatory regions of these additional anergy-associated genes (unpublished data), and conditional deletion of Egr2 prevented their up-regulation as determined by qRT-PCR (the fold increase upon anergy induction of these genes was compared in EV and Cre adenovirus-infected CAR Tg × Egr2flox/flox T cells, and the results are DGK-ζ: 5.64 ± 0.08 vs. 2.19 ± 0.01; Chl-b: 5.62 ± 0.05 vs. 1.65 ± 0.01; Itch: 6.54 ± 0.47 vs. 3.41 ± 0.18; Tob1: 2.56 ± 0.11 vs. 1.07 ± 0.01; Dtx1: 2.80 ± 1.16 vs. 0.73 ± 0.65; all P values <0.05). We have also performed gene expression profiling of control versus Egr2-deleted T cells and a genome-wide ChIP-SEQ analysis, which have identified 46 genes that represent the identifiable Egr2 transcriptome in T cell anergy (unpublished data). The functional importance of several of these new candidates is currently being characterized. Thus, Egr2 is a major transcriptional regulator of the full anergy program.

T cell anergy can be viewed as a differentiation state of activated T cells, and previous studies had suggested that anergic T cells are not inert but can have additional functional roles, such as the ability to function as suppressor cells (Chai et al., 1999). The identification of transcriptional regulators for other T cell differentiation states (e.g., Tbet for Th1, GATA-3 for Th17, etc.) has provided tremendous information about the biology of those subsets in immune models in vivo. The identification of Egr2 as a central factor for anergic T cells should similarly place studies of the anergic state on firmer footing for in vivo studies, and pursuit of additional gene targets of Egr2 might identify molecules that could be used as markers for anergic T cells for ex vivo identification.

Egr2 is the first molecule in our hands which, when deleted from or blocked in peripheral T cells, results in anergy resistance in anti-CD3 induced in vitro anergy model as well as in the superantigen SEB-induced in vivo anergy models. Increased activities of protein tyrosine kinase Fyn, and Rap1 GTPase, which, driven by guanine nucleotide exchange factor C3G bound to adapter protein CrkL, have been described in anergic T cells (Gajewski et al., 1994; Boussiotis et al., 1997). However, Fyn– and
CrkL-deficient T cells remained subject to anergy induction, arguing that those pathways are dispensable (Fields et al., 1996a; Zha et al., 2006). Primary T cells from Cbl-b knockout and PTEN conditional knockout mice had been reported to be hyperresponsive and relatively anergy resistant (Bachmaier et al., 2000; Suzuki et al., 2001). However, introduction of a dominant-negative form of Cbl-b, or conditional deletion of PTEN directly in the peripheral T cells, led to hyperactivation but intact anergy susceptibility (Zha and Gajewski, 2007; unpublished data). Thus, not every genetic manipulation of peripheral T cells that can lead to increased activation results in anergy resistance, and these observations together argue more strongly for the particular importance of the Egr2 pathway.

In the anergy models using immobilized anti-CD3 or SEB, Egr2 deletion prevented anergy induction. However, Ramón et al. (2010) recently reported that CD4-Cre × Egr2fl/fl mice did not demonstrate increased immune responses against minor histocompatibility antigens, Toxoplasma gondii infection, or lymphocytic choriomeningitis virus. These data suggest that anergy is not a dominant mechanism of immune dysfunction in those models, and support the notion that Egr2 is not simply a global negative regulator of T cell activation. It will be of interest to examined the role of Egr2 and its target genes in other in vivo models in which anergy is thought to contribute to peripheral tolerance, such as costimulation blockade-induced immune suppression for solid organ transplantation (Alegre and Najafian, 2006).

**MATERIALS AND METHODS**

**Mice and cells.** Egr2fl/fl and CD4-Cre Tg mice were gifts from H. Singh and F. Gounari (University of Chicago, Chicago, IL), respectively. CAR Tg mice expressing the extradomal domain of CAR under control of a Lck promoter/CD2 enhancer were generated as previously described (Wan et al., 2000). All three mouse strains have been backcrossed with C57BL/6 mice for more than eight generations. C57BL/6 mice were purchased from Taconic. 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 × Egr2fl/fl-Th1 clones, CAR Tg × Egr2fl/fl-Th1 clones, CAR Tg × Egr2fl/fl-Th1 mice were immunized in the hind footpads with chicken oval (Sigma-Aldrich) emulsified in complete Freund’s adjuvant (Sigma-Aldrich). 7 d later, the draining lymph nodes were harvested, and CD4+ Th1 cell clones were derived and maintained as previously described (Gajewski and Fitch, 1990; Fitch et al., 2006).

**Luciferase reporter vector construction and analysis.** The mouse DGK-α promoter region from −1054 to 192 bp containing a putative Egr2 binding site (−909 to −901 bp) was amplified from genomic DNA by PCR using the following primers: forward, 5′-ATCACACTGAGTTCCAGCTGTCAAA-GCGTTCCCTTCT-3′; and reverse, 5′-CTAGTGTACCTCCTCTTCTGAC-CCTCTCTGACCC-3′. As control, the region from −54 to 190 bp was amplified using the following primers: forward, 5′-ATCACACTGAGTTCCAGCTGTCAAA-GCGTTCCCTTCT-3′; and reverse, 5′-CTAGTGTACCTCCTCTTCTGAC-CCTCTCTGACCC-3′. These primers were then cloned into a pGL4Luc2 vector (Promega) via KpnI and XhoI sites (underlined) to make DGK-α reporter vectors. A plasmid encoding Egr2 was a gift from DeWitt and Fitch, 1990; Fitch et al., 2006).

**qRT-PCR.** Total RNA was purified using an RNAeasy Mini kit (QIA-GEN). RNA was reverse transcribed by M-MMLV RT (Invitrogen). The primers and probes were purchased from IDT and Roche, and Applied Biosystems. qRT-PCR was used to determine expression of target genes by real-time qPCR using the primers specific for the following: 5′-CCCCGCCCCAACCACCGACTAATC-3′; reverse, 5′-TGCTCCTCTCACTCTTATTCCT-3′. Primers specific for GJA5 were used as controls (forward, 5′-ACCATGAGGTTGCGCTTCA-3′; reverse, 5′-CATTGGGAATGATCCAGGAAG-3′).

**Flow cytometry and intracellular staining.** Antibodies specific for CD3, CD4, CD8, and Vβ8 were purchased from BD, eBioscience, and BioLegend. 

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For Egr2 intracellular staining, cells were permeated for 1 h (Foxp3 Buffer Set; eBioscience), stained with the anti-Egr2 rabbit polyclonal Ab (1:100) for 1 h at 25°C, followed by a secondary antibody Alexa Fluor 647 goat anti-rabbit (1:100; Invitrogen) for 1 h at 4°C.

**Tumor implantation and IFN-γ ELISPOT.** B16. SIY tumor cells were washed three times with DPBS, and 2 × 10⁶ cells were injected subcutaneously in 100 μl DPBS at the flank. Tumor size was measured twice per week, and tumor area was calculated by multiplying the longest diameter and the shortest diameter of the tumor. 14 d later, the mice were sacrificed, and the percentage of SIY-specific CD8⁺ T cells was determined by SIY-K⁺ pentamer staining according to the manufacturer’s protocol (Proimmune). An irrelevant pentamer OVA-K⁺ was used as a negative control. To determine functional tumor antigen-specific immune responses by ELISPOT, splenocytes were plated at 1 × 10⁶ cells/well and stimulated overnight with medium alone or 80 nM SIY peptide. IFN-γ-producing cells were assessed using the manufacturer’s protocol (Mouse IFN-γ ELISPOT kit; BD).

**Statistical analysis.** Data from independent groups were analyzed using a Student’s t test. Western blot data were analyzed using ANOVA after digital denoentmetry.

**Online supplemental material.** Table S1 shows ChIP Assay primers used to confirm Egr2 association. Table S2 shows qRT-PCR primers and probes used to confirm dependency on Egr2 for expression in anergy.

We thank Dr. Harinder Singh and Dr. Fotini Gounari (University of Chicago, Chicago, IL) for providing Egr2hand and CD4-Cre Tg mice; Dr. Jonathan Powell (Johns Hopkins School of Medicine, Baltimore, MD) for providing an Egr2 plasmid; Fenge Gao, Mihir Vohra, and Mercedes B. Fuertes, and Robbert M. Spaapen for critical review of the manuscript. This work was supported by R01 Al008745 and R01 CA118153 from the National Institutes of Health. The authors have no competing financial interests.

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### Table S1. ChIP Assay primers used to confirm Egr2 association

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<tr>
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<td>Tob1</td>
<td>Forward, 5’-GGGAGGAGGCTATAACGAG-3’; Reverse, 5’-GGGATTTCCCTGGAGGTTTC-3’</td>
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<tr>
<td>Dtx1</td>
<td>Forward, 5’-CGGAGGAGGCTATAACGAG-3’; Reverse, 5’-GGGATTTCCCTGGAGGTTTC-3’</td>
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### Table S2. qRT-PCR primers and probes used to confirm dependency on Egr2 for expression in anergy

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<td>5’-AGCCAGAG-3’ (Roche)</td>
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