CD28-inducible transcription factor DEC1 is required for efficient autoreactive CD4+ T cell response

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During the initial hours after activation, CD4+ T cells experience profound changes in gene expression. Co-stimulation via the CD28 receptor is required for efficient activation of naive T cells. However, the transcriptional consequences of CD28 co-stimulation are not completely understood. We performed expression microarray analysis to elucidate the effects of CD28 signals on the transcriptome of activated T cells. We show that the transcription factor DEC1 is highly induced in a CD28-dependent manner upon T cell activation, is involved in essential CD4+ effector T cell functions, and participates in the transcriptional regulation of several T cell activation pathways, including a large group of CD28-regulated genes. Antigen-specific, DEC1-deficient CD4+ T cells have cell-intrinsic defects in survival and proliferation. Furthermore, we found that DEC1 is required for the development of experimental autoimmune encephalomyelitis because of its critical role in the production of the proinflammatory cytokines GM-CSF, IFN-γ, and IL-2. Thus, we identify DEC1 as a critical transcriptional mediator in the activation of naive CD4+ T cells that is required for the development of a T cell–mediated autoimmune disease.

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Abbreviations used: BSB, basiliximab; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; PCA, principal component analysis.

For productive activation, naive T cells require two signals: an antigen-specific signal through the TCR and a second signal via the CD28 co-stimulatory receptor (Lenschow et al., 1996; Bour-Jordan et al., 2011). The delivery of the combined signals rapidly promotes a complex pattern of transcriptional changes that leads to efficient T cell proliferation and differentiation (Diehn et al., 2002; Riley et al., 2002). Many studies have focused on the membrane-proximal events involved in CD28 signals and their immediate effects on nuclear translocation of transcription factors, including AP-1, NFAT, and NF-κB family members (Jain et al., 1993; Köntgen et al., 1995; Kempiak et al., 1999; Rao et al., 2003; Marinari et al., 2004; Sánchez-Valdepeñas et al., 2006). Based on the central role of the CD28/B7 signaling pathway in immune responses, autoimmune diseases, and allograft rejection, two drugs that block this pathway, abatacept and belatacept, have been developed and FDA approved (Linsley and Nadler, 2009). However, in spite of the functional and clinical progress in developing co-stimulation antagonists for clinical purposes, there are few studies on the transcriptional program initiated after CD28/B7 engagement, and

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few specific transcription factors have been directly associated with CD28 signals. Some studies have suggested that there may be no unique transcriptional program after CD28 engagement that cannot be recapitulated by stronger TCR signals (Diehn et al., 2002; Riley et al., 2002). However, these studies may have underestimated the qualitative effects of CD28 co-stimulation as they have, in general, used mixed T populations without taking into account the relative differences in CD28 dependency between distinct T cell subsets (Whitney et al., 2003; Radich et al., 2004; Amyes et al., 2005) or the temporal changes in the gene transcription during the initial hours after T cell activation (Ellisen et al., 2001; Acuto and Michel, 2003). Thus, in this study, we examined the consequences of CD28-dependent signals in a highly co-stimulation–dependent T cell subset, naive $CD4^+$ T cells. We performed gene expression microarrays of human and mouse naive $CD4^+$ T cells to identify genes uniquely regulated by CD28 signaling that may play a role in the global transcriptional changes required for T cell activation and differentiation.

Among the many genes identified in the CD28 co-stimulation screen, one transcription factor, *DEC1* (also referred to as *BHLHE40*, *Stra13*, *Sharp2*, or *Bhlhb2*), stood out as a factor important for the maximal expression or repression of many early transcripts during T cell activation. Many of these transcripts were also dependent on CD28 co-stimulation. DEC1 is a basic helix-loop-helix transcription factor that can directly repress or cooperatively activate transcription in a wide variety of cells. *DEC1* gene expression has been implicated in repression of neurotrophic factor production in neurons (Jiang et al., 2008), regulation of circadian rhythms (Honma et al., 2002; Kon et al., 2008; Rossner et al., 2008), lipid metabolism homeostasis (Iizuka and Horikawa, 2008), and control of cellular responses to a variety of other stimuli such as exposure to cytokines and hypoxia (Boudjelal et al., 1997; Honma et al., 2002; Miyazaki et al., 2002). An initial study on DEC1-deficient mice showed that they have defective T cell–mediated recall responses and they develop spontaneous autoimmune disease caused by defects in activation-induced cell death (Sun et al., 2001). However, other groups have found DEC1-deficient mice do not develop spontaneous autoimmune disease (Jiang et al., 2008) or they develop disease with a very low penetrance (Miyazaki et al., 2010). Thus, we set out to better understand the role of DEC1 in $CD4^+$ conventional T cells (T conv cells) during in vivo immune responses, especially in CD28-dependent settings.

To investigate the function of DEC1 in autoreactive $CD4^+$ T conv cell responses, we took advantage of a DEC1-deficient mouse strain and the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis. EAE is a well-defined $CD4^+$ T cell–driven autoimmune disease initiated by self-antigen peptide immunization. Importantly, it is highly dependent on efficient CD28 signaling (Perrin et al., 1995, 1999; Oliveira-dos-Santos et al., 1999). Microarray expression analysis of DEC1-deficient cells further demonstrated that DEC1 controls a subset of CD28-depedendent genes, and the expression of these genes is required for optimal $CD4^+$ T conv cell function in a mouse model of autoimmune disease.

RESULTS

Identification of potential regulators of the T cell early activation transcriptome

Effective T cell function requires profound transcriptional reprogramming upon stimulation. To characterize transcriptional changes during the first hours after T cell activation, primary human $CD4^+CD45RA^+$ T cells were stimulated in vitro with antibodies to the CD3 component of the TCR and antibodies to the CD28 co-stimulatory receptor. CD69 and CD25 expression were analyzed to ensure similar activation among the seven donors (not depicted). Expression microarray analysis was performed on RNA samples from each donor at 4 and 24 h after stimulation. Approximately 2,500 genes were either up- or down-regulated more than twofold at both 4 and 24 h after stimulation with anti-CD3 and anti-CD28 (not depicted). However, a total of ~5,000 genes were more than twofold up-regulated by 24 h after activation. Thus, the transcriptional profile of the activated T cells at 24 h both preserves aspects of the immediate early transcriptional profile (4 h) and shows significant temporal changes.

We analyzed the transcriptome of the activated T cells using GeneGo (Thomson Reuters) to identify transcription factors whose activity might underlie the observed expression patterns. The transcription factor candidates were ranked based on their connectivity ratio, which is calculated using the number of known target genes differentially expressed in the T cell activation signature compared with the total number of genes regulated by the transcription factor (Fig. 1 A). Transcription factors with higher connectivity ratios have a larger overlap between their known targets and the genes that are differentially regulated in our dataset. Using this approach, we confirmed regulatory networks known to be essential for efficient T cell activation, such as NFAT, c-Myc, and the NF-κB family (Jain et al., 1993; Sánchez-Valdepeñas et al., 2006; Wang et al., 2011). However, the transcription factor with the highest connectivity ratio was DEC1, a basic-helix-loop-helix family member.

We next asked whether the transcription factors identified above are themselves transcriptionally induced by TCR and CD28 signaling. Interestingly, several transcription factors predicted to be involved in the transcriptional regulation of T cell activation in our analysis above were highly up-regulated at both 4 and 24 h after initial activation compared with unstimulated cells. These factors include *DEC1*, *NFKB1*, *MYC*, and *NFATC2* (Fig. 1 B). Thus, the increased expression of specific transcription factors during the initial hours after T cell activation correlates with their predicted role in transcriptional regulation.

CD28 co-stimulation stabilizes the TCR transcriptome independently of IL-2

Because CD28 signaling is critical for productive activation of naive $CD4^+$ T cells (Lenschow et al., 1996; Bour-Jordan et al.,

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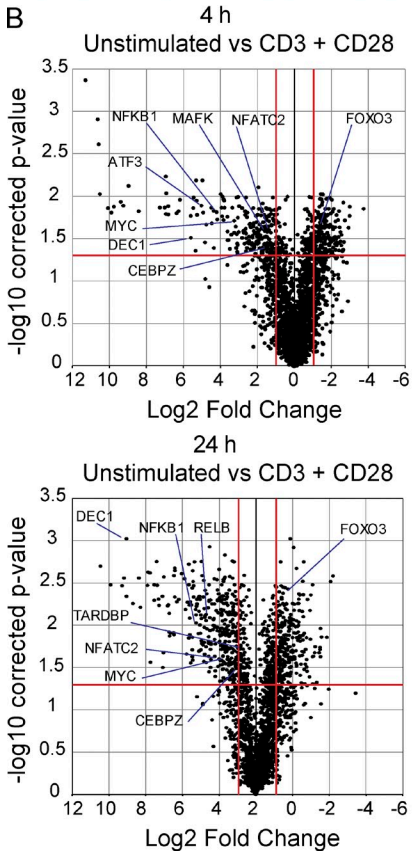
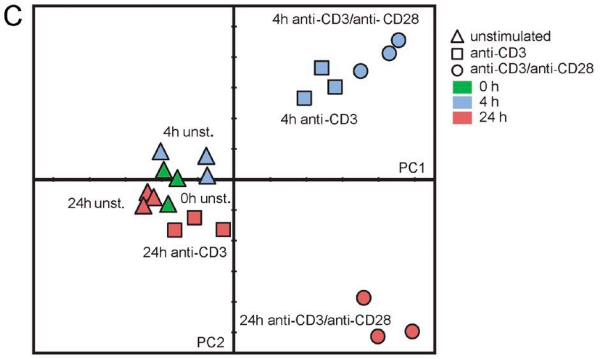
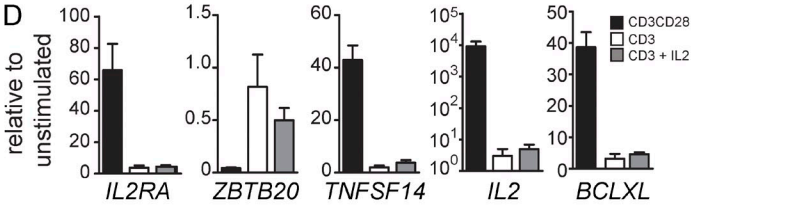
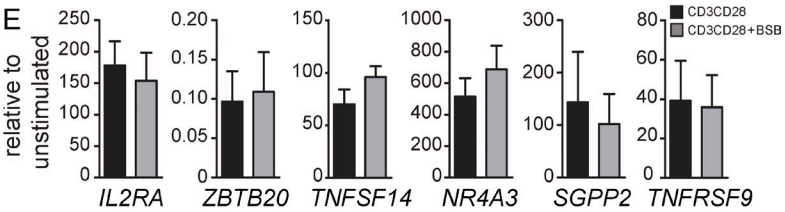
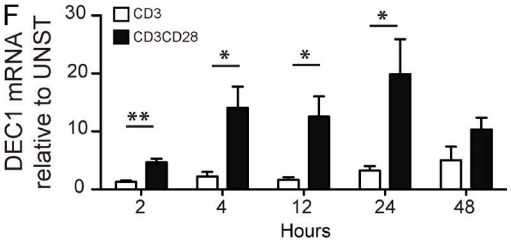
Transcription Factor	Connectivity Ratio	p-value
DEC1	2.41	6.40E-10
ATF3	2.32	1.05E-10
STAT5B	2.25	1.45E-10
NFKB1	2.25	1.11E-12
FOXO3	2.23	1.37E-15
CEBPZ	2.21	3.14E-11
RELB	2.20	4.09E-09
MAFK	2.20	3.28E-09
NFATC2	2.19	1.84E-10
BCL6	2.13	8.60E-17
TARDBP	2.06	4.12E-15
MYC	2.04	1.05E-101











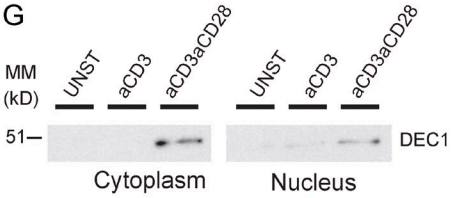


Figure 1. Early transcriptional regulation of activated naive T cells. (A) List of transcription factors highly correlated ($P < 1 \times 10^{-9}$) with the transcriptome of in vitro anti-CD3/anti-CD28–activated primary human T cells, ranked in descending order of connectivity ratio. This ratio indicates the correlation between known transcriptional targets and transcript expression levels. The input gene list is composed of transcripts with a fold change of more than two by 4 h after T cell activation. (B) Volcano plot of transcripts involved in transcriptional control as annotated by GO Biological Processes. Microarray data were obtained by activating primary human T cells with anti-CD3/anti-CD28, with harvest at the times indicated. Transcription factors from the table in A with fold change greater than two and $P < 0.05$ are labeled in the figure. (C) Two-dimensional representation of global gene expression by PCA. 16,279 transcripts for each sample are included in the analysis. Each dot represents a sample, which is colored and shaped according to activation parameters. The combination of PC1 and PC2 summarizes 64% of the original dataset variation. (D and E) Gene expression analysis of human naive T cells by quantitative real-time PCR after 24 h of activation as indicated with or without 100 U/ml of recombinant human IL-2 and 25 µg/ml BSB. (F) DEC1 mRNA expression in human naive CD4+ T cells at different time points after activation, indexed to expression in unstimulated cells. Data are represented as mean ± SEM. P-values were calculated with an unpaired Student's *t* test: *, $P < 0.05$; **, $P < 0.01$. (G) DEC1 protein expression by Western blot from 5×10^6 human naive CD4+ T cells 24 h after activation. Results are from at least three independent experiments. MM, molecular mass.

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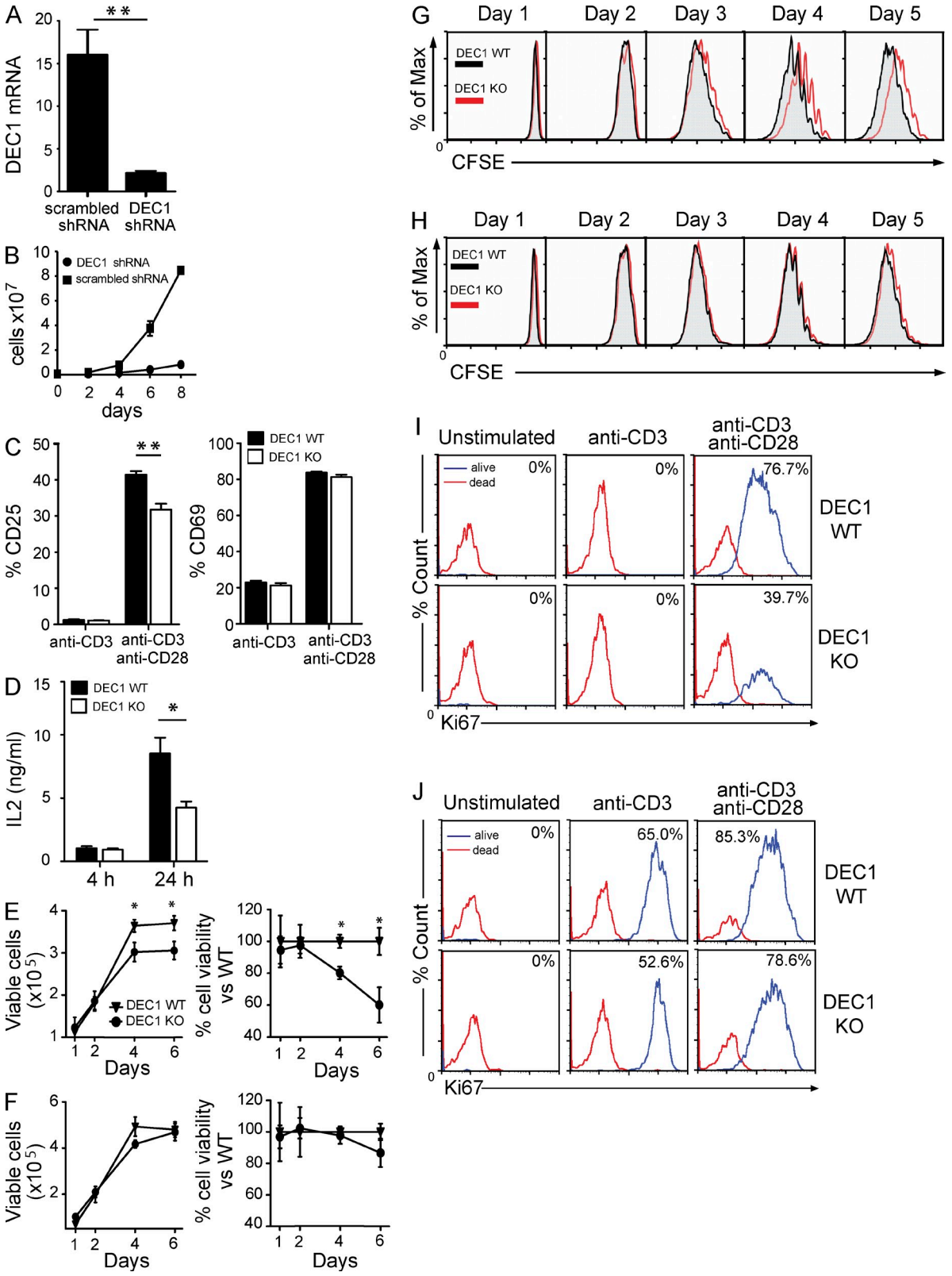

Figure 2. DEC1 is necessary for efficient in vitro T cell function. (A) Jurkat T cells were infected with lentivirus containing pSICO-R vectors with shRNA complementary to human *DEC1* transcript or a scrambled shRNA. RNA was harvested, and expression of *DEC1* transcript was measured by quantitative real-time PCR. (B) 10^6 Jurkat cells previously infected with shRNA expression virus were seeded on day 0. The total number of cells in culture was followed for 8 d. (C) Percentage of CD69$^+$ and CD25$^+$ naive CD4$^+$ T cells from DEC1^{WT} and DEC1^{KO} mice after activation and 24 h of culture. (D) IL-2

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2011), we analyzed the gene expression differences between cells activated with or without anti-CD28 co-stimulation, at 4 and 24 h after stimulation (not depicted). The transcriptional effect of CD28 was greater at 24 h than at 4 h, with 4.5 times the number of CD28-regulated genes at the later time point (not depicted). Principal component analysis (PCA), a technique which reduces the dimensionality of the microarray data, showed that the overall transcriptional effects of CD28 co-stimulation were initially modest at 4 h, becoming more pronounced by 24 h after T cell activation (Fig. 1 C). Moreover, by 24 h after activation, the cells that received anti-CD3 stimulation alone had an overall transcriptional profile similar to cells that had never been stimulated at all. Collectively, these results suggest that one important characteristic of CD28 co-stimulation is the stabilization of the transcriptome induced by TCR ligation (anti-CD3).

IL-2 has been implicated in initiating STAT5-dependent signals important for the production and maintenance of T conv cells, regulatory T cells (T reg cells), and memory T cells (Dooms and Abbas, 2006; Lohr et al., 2006). Thus, we examined whether early induction of IL-2 could substitute for CD28 co-stimulation in naive T cells to generate a similar gene expression signature. Gene expression of five transcripts highly up- or down-regulated by CD28 co-stimulation was analyzed in human $CD4^+CD45RA^+$ T cells activated with anti-CD3 with or without exogenous recombinant human IL-2 (Fig. 1 D). In no instance did exogenous IL-2 stimulation substitute for CD28 co-stimulation in modulating the level of expression of these genes. The biological activity of IL-2 used in these experiments was confirmed using NKL cells (not depicted), a strictly IL-2–dependent cell line (Robertson et al., 1996). Consistent with this observation, antibody-mediated CD25 blockade did not reduce the expression of a panel of CD28-dependent transcripts 24 h after T cell activation with CD28 co-stimulation (Fig. 1 E). Before activation, an mAb (basiliximab [BSB]) was added to the cultures to efficiently block IL-2 binding to the high-affinity α chain of the IL-2 receptor (CD25; not depicted; Binder et al., 2007). These results show the limited effect of IL-2 signals on genes controlled by CD28 signals in the first 24 h after T cell activation.

CD28 co-stimulation enhances *DEC1* expression upon T cell activation

We set out to identify transcription factors that could be responsible for the temporal effects of CD28 co-stimulation identified in the aforementioned microarray experiments. Among the transcripts highly dependent on CD28 co-stimulation at both 4 and 24 h, we identified the transcription factor DEC1 (not depicted). The effect of CD28 signaling on *DEC1* transcription was validated by real-time PCR in human naive $CD4^+$ T cells at various times after activation (Fig. 1 F). *DEC1* expression rapidly increased upon stimulation in a CD28-dependent manner, and the level of transcript increased over the initial 24 h after activation. Similar results were observed in mouse naive T cells (not depicted). To assess DEC1 protein production and potential functional activity, cytoplasmic and nuclear lysates were analyzed by Western blot at 24 h after naive T cell activation (Fig. 1 G). Although cytoplasmic DEC1 was equally undetectable in nonactivated and CD3-stimulated cells, large amounts of DEC1 were observed upon CD3 plus CD28 co-stimulation. The DEC1 production triggered by efficient T cell activation also correlates with a higher concentration of the transcription factor in the nucleus. Collectively, these observations suggest that DEC1 may be an important early transcription factor during T cell activation.

DEC1 is necessary for efficient T cell activation in vitro

To elucidate the relevance of DEC1 in T cell activation, Jurkat T cells were infected with shRNA-expressing lentiviruses targeting DEC1. The DEC1 shRNA, but not the control (scrambled) shRNA, significantly attenuated *DEC1* mRNA expression (>15-fold; Fig. 2 A) and reduced DEC1 protein levels by >40% (not depicted). In side by side culture, Jurkat cells infected with the shRNA against DEC1 expanded more slowly than the cells infected with control virus (Fig. 2 B). This attenuated increase in the cell number was, in part, caused by greater cell death in cells with diminished *DEC1* expression (not depicted).

To further investigate the functional consequences of DEC1 deficiency in primary $CD4^+$ T cell activation, naive $CD4^+CD62L^+CD25^-$ T cells from DEC1 WT ($DEC1^{WT}$) and DEC1-deficient ($DEC1^{KO}$) mice were harvested and activated in vitro with beads conjugated to anti-CD3 or anti-CD3 plus anti-CD28. The mean fluorescence intensity of CD25, a CD28 co-stimulation–dependent gene, was significantly decreased in DEC1-deficient T cells (Fig. 2 C). However, there was no difference between $DEC1^{WT}$ and $DEC1^{KO}$ cells in up-regulation of CD69, a TCR-dependent gene. This observation was consistent with previous data, in which transgenic overexpression of *Dec1* increased CD25 expression in T cells (Miyazaki et al., 2010). Differences in supernatant IL-2 protein level between $DEC1^{WT}$ and $DEC1^{KO}$ T cell cultures

cytokine production measured by ELISA in primary $CD4^+$ T cells from $DEC1^{WT}$ and $DEC1^{KO}$ animals at 4 and 25 h after activation. (E and F) Cultured primary $DEC1^{WT}$ and $DEC1^{KO}$ $CD4^+$ T cells were followed for 6 d after stimulation with anti-CD3 + anti-CD28 without (E) or with (F) 100 U/ml of recombinant mouse IL-2 at day 0. Viable cells were automatically counted by video imaging (Vi-CELL). Percentage of live $DEC1^{KO}$ $CD4^+$ T cells is expressed relative to the $DEC1^{WT}$ cells. (G and H) CFSE labeling analysis of viable $DEC1^{WT}$ and $DEC1^{KO}$ cells activated with anti-CD3 + anti-CD28 and cultured for the indicated time periods without (G) or with (H) the addition of 100 U/ml IL-2 at day 0. (I and J) Representative histogram plots of Ki67 expression after 6 d of culture with the conditions described in G and H. Cells were cultured without (I) or with (J) the addition of 100 U/ml IL-2 at day 0. Results are from at least three independent experiments. Data are represented as mean ± SEM. P-values were calculated with an unpaired Student's *t* test: *, $P < 0.05$; **, $P < 0.01$.

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were present at 24 h but not at 4 h after activation in vitro (Fig. 2 D). *IL2RA* and *IL2* mRNA expression was also diminished in DEC1 knocked down Jurkat cells (not depicted). Together with the decreased level of IL-2, we observed a significant reduction in the number of viable DEC1^{KO} T cells compared with WT T cells 4 d after activation in culture (Fig. 2 E). Similar to the observations in Jurkat cells, these results demonstrated that DEC1^{KO} cells display a survival defect, a proliferation defect, or both when compared with WT cells. To distinguish these defects, we analyzed CFSE-labeled DEC1^{KO} versus DEC1^{WT} T cells activated for 5 d (Fig. 2 G). Viable DEC1^{KO} T cells showed reduced cell division as compared with DEC1^{WT} cells starting 3 d after stimulation. This decreased cell division was accompanied, at day 6 after activation, by reduced viability of DEC1^{KO} cells (39.7% compared with 76.7% in WT cells; Fig. 2 I). Thus, DEC1^{KO} cells in culture have both decreased proliferation and decreased survival, consistent with the reported consequences of reduced IL-2.

Next, we tested whether the addition of exogenous IL-2 might reverse the observed survival and proliferation defects. DEC1^{KO} T cells expanded similarly to DEC1^{WT} T cells in the presence of 100 U/ml IL-2 in culture (Fig. 2 F). CFSE-labeled DEC1^{KO} cells cultured with exogenous IL-2 showed identical cell division compared with WT cells (Fig. 2 H). Additionally, the percentage of viable and dividing DEC1^{KO} T cells after 6 d of culture in the presence of exogenous IL-2 was nearly identical to WT cells (Fig. 2 J). Thus, DEC1 is necessary for optimal CD4$^+$ T cell proliferation and survival after activation, and this defect can be corrected in vitro with exogenous IL-2.

DEC1 deficiency protects mice from EAE

To interrogate whether DEC1 is important for efficient CD4$^+$ T cell activation in vivo in an autoimmune setting, we used the EAE model, a CD4$^+$ T cell–mediated and CD28-dependent inflammatory disease of the central nervous system (CNS), which serves as an experimental model of multiple sclerosis (Perrin et al., 1995, 1999; Oliveira-dos-Santos et al., 1999; Schwartz, 2003). DEC1^{WT} and DEC1^{KO} mice were immunized with MOG$_{35–55}$ peptide in CFA to induce EAE. DEC1^{KO} mice were completely protected from EAE (Fig. 3 A). Notably, DEC1 heterozygous mice were partially protected from EAE as compared with DEC1^{WT} controls (Fig. 3, A and B), consistent with the reduced *Dec1* mRNA expression observed in activated T cells from these animals (not depicted). Histological analysis by hematoxylin and eosin (H&E) staining of the spinal cords from DEC1^{KO} mice showed no evidence of mononuclear cell infiltration, as compared with the extensive infiltrates found in the spinal cords of WT mice during the peak of disease (Fig. 3 C). To further investigate the infiltration of CD4$^+$ T cells in the CNS, we analyzed leukocytes in spinal cord and cerebellum at day 15 after immunization. This analysis showed that DEC1^{KO} tissue had similar numbers of infiltrating CD45^{hi} cells as the unimmunized control (not depicted). Similarly, the total number of DEC1^{KO} CD4$^+$ T cells and their proportion among the CNS cells was similar to that in unimmunized mice and significantly reduced compared with DEC1^{WT} mice (Fig. 3 D). EAE disease incidence correlated with the activation level of the CNS microglia and myeloid cells as measured by expression of MHC class II (Fig. 3 E). In addition, the capacity of infiltrating cells to produce proinflammatory cytokines, including IFN-γ, IL-17, and GM-CSF, was reduced in DEC1^{KO} mice (Fig. 3 F). Overall, these results indicate that the absence of clinical EAE in DEC1-deficient mice is associated with the absence of CD4$^+$ T conv cell infiltration in the CNS.

We next probed whether the inability of DEC1^{KO} mice to develop EAE was the result of an intrinsic defect in CD4$^+$ T cells or whether DEC1 deficiency in non-T cells was affecting T cell activity indirectly. We transferred nonmanipulated DEC1^{WT} or DEC1^{KO} CD4$^+$ T cells into lymphocyte-deficient RAG2^{KO} mice and then immunized the recipient mice with MOG$_{35–55}$ peptide 7 d later. No differences in transferred T cell numbers were found between groups of animals before the immunization (not depicted). Mice that received DEC1^{WT} CD4$^+$ T cells developed clinical EAE, whereas those receiving DEC1^{KO} cells were protected (Fig. 3, G and H). Thus, DEC1 is required in a CD4$^+$ T cell–intrinsic manner for the development of EAE.

DEC1 is required during the priming phase of EAE

We analyzed CD4$^+$ T cells in the periphery during the priming phase (7 d after immunization) to better understand the role of DEC1 in EAE induction. At this time point, very few lymphocytes have trafficked to the CNS and the MOG-specific response is largely confined to the lymphoid organs. Although no significant differences were observed when comparing DEC1^{KO} and DEC1^{WT} nonimmunized mice (not depicted), the total cellularity and CD4$^+$ T cell count from pooled spleen and lymph nodes were reduced in immunized DEC1^{KO} mice as compared with control (Fig. 4, A and B). Peripheral CD4$^+$ T cells were stained with MOG$_{38–49}$-loaded tetramer to examine the antigen-specific population of pathogenic cells directly (Fig. 4 C). Although the percentages of activated (CD44$^+$) and nonactivated (CD44^{lo}) T cells were similar between the DEC1^{WT} and DEC1^{KO} mice, there was a significant reduction in the percentage of MOG-specific CD4$^+$CD44$^+$ T cells in DEC1^{KO} mice as compared with DEC1^{WT} controls (Fig. 4 D). The percentages of T reg and T conv cells were measured. There were decreased numbers of Foxp3$^+$CD44$^+$ T reg cells in DEC1^{KO} mice but no difference in T reg cell numbers among MOG-specific cells (not depicted), indicating that alterations in antigen-specific T reg cell numbers were not responsible for the abrogation of EAE induction in DEC1^{KO} mice.

CD25 expression was defective in DEC1-deficient T cells after in vitro stimulation (Fig. 2 C; Miyazaki et al., 2010). Thus, we evaluated whether changes in CD25 expression were associated with decreased in vivo T cell expansion after priming. The expression level of CD25 was examined in

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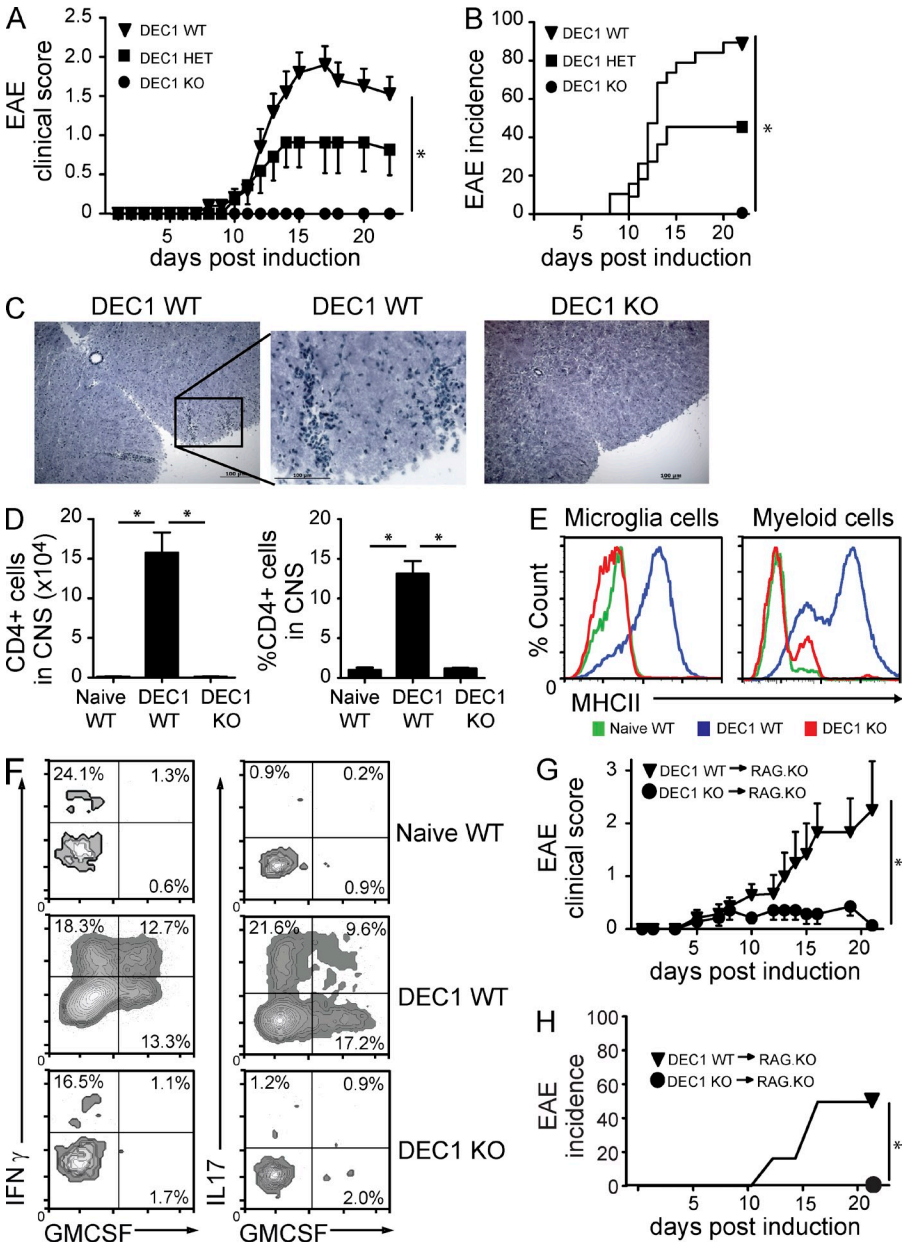

Figure 3. DEC1 deficiency protects mice from EAE. EAE was induced by CFA and MOG_{35-55} peptide. (A and B) Clinical disease score (A) and incidence (B). Number of animals per group: WT $n = 20$, het $n = 11$, and KO $n = 18$. (C) Spinal cord tissue sections were stained with H&E from $DEC1^{WT}$ and $DEC1^{KO}$ mice at day 15 after MOG_{35-55} immunization (peak disease for WT mice). Bars, 100 µm. (D) Single cell suspensions from cerebellum and spinal cord of $DEC1^{WT}$ and $DEC1^{KO}$ mice at day 15 after immunization were used for quantification of $CD4^+$ T cells among CNS cells. (E) Flow cytometric analysis of MHC class II expression in CNS-infiltrating myeloid cells and microglia cells from unprimed mice and $DEC1^{WT}$ or $DEC1^{KO}$ mice at day 15 after MOG_{35-55} immunization. (F) Production of IFN-γ, GM-CSF, and IL-17 by CNS-infiltrating cells of unprimed, $DEC1^{WT}$, and $DEC1^{KO}$ mice at day 15 after priming (gated on $CD45^{hi}CD11b^-CD4^+$). (G and H) RAG^{KO} mice were transferred with 2×10^6 $CD4^+$ T cells isolated from $DEC1^{WT}$ or $DEC1^{KO}$ mice. 7 d after the transfer, EAE was induced by MOG_{35-55} immunization. EAE clinical score was determined every other day. Data are representative of at least three different independent experiments. Number of animals per group: WT $n = 7$ and KO $n = 8$. Data are represented as mean ± SEM. P-values were calculated with an unpaired Student's *t* test: *, $P < 0.05$.

T reg and T conv cells 7 d after MOG immunization (Fig. 4 E). Although antigen nonspecific $CD44^+$ T reg $DEC1^{KO}$ cells showed a modest reduction of CD25 mean fluorescence intensity, both MOG antigen–specific and antigen-nonspecific activated $CD4^+CD44^+Foxp3^-$ T conv $DEC1^{KO}$ cells expressed significantly lower CD25. Proliferation of activated T conv cells, as measured by Ki67 nuclear protein staining, was decreased in $DEC1^{KO}$ as compared with control mice (Fig. 4 F). However, T reg cell proliferation was intact in $DEC1^{KO}$ mice. To verify the effects on the antigen-specific

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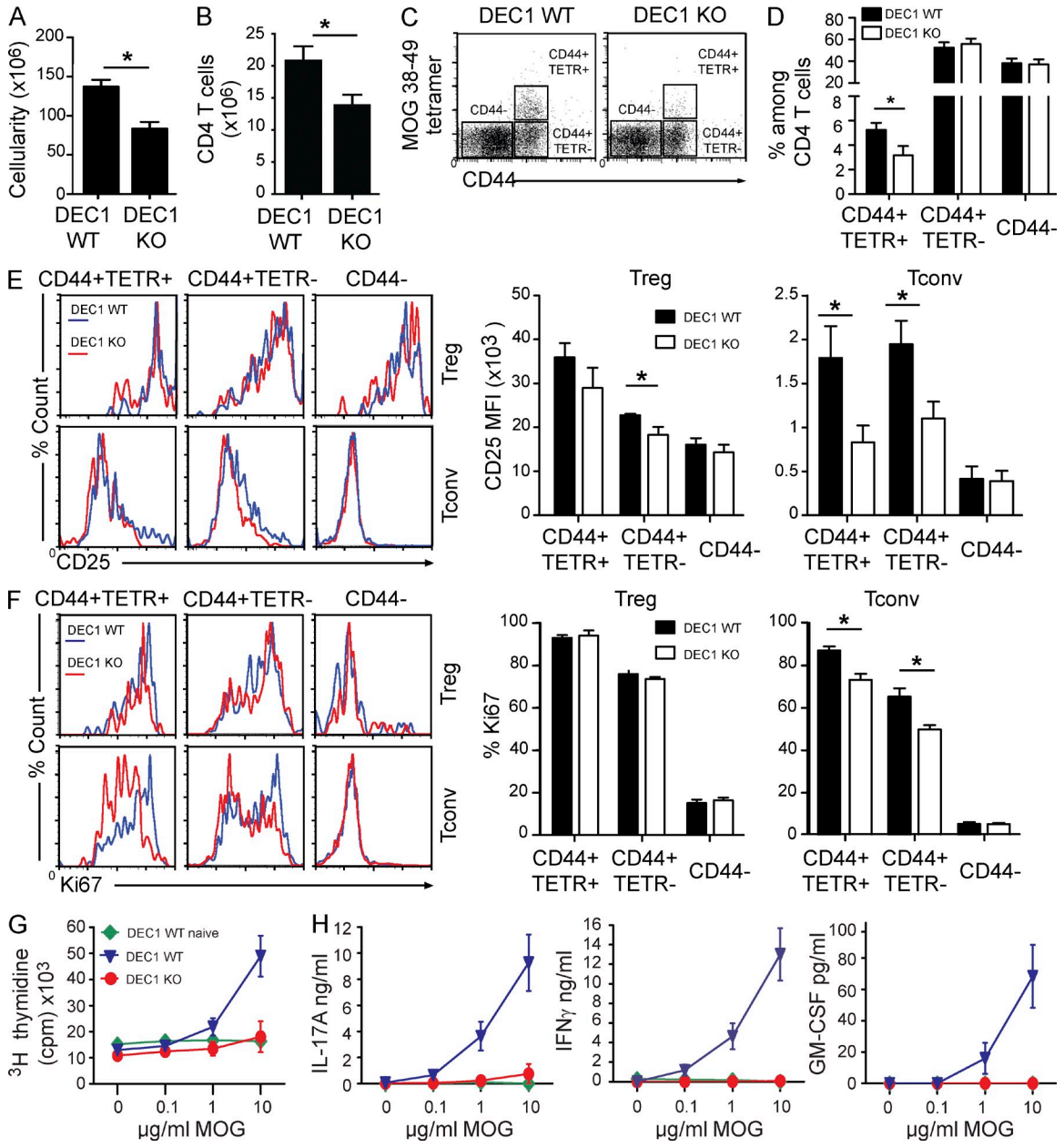

Figure 4. DEC1 is required for efficient T cell priming during EAE. (A and B) Total cellularity (A) and CD4+ T cell quantification (B) from pooled spleen and lymph nodes of DEC1^{WT} or DEC1^{KO} mice at day 7 after MOG$_{35-55}$ immunization. (C and D) Flow cytometric analysis (C) and quantification (D) of MOG$_{38-49}$ tetramer staining of CD4+ T cells subpopulations from spleen and lymph nodes of DEC1^{WT} or DEC1^{KO} mice at day 7 after MOG$_{35-55}$ immunization. Data are representative of at least six independent experiments. (E) Flow cytometric analysis (left) and CD25 quantification (right) of MOG$_{38-49}$ tetramer–stained CD4+ T cell subpopulations. MFI, mean fluorescence intensity. (F) Flow cytometric analysis (left) and Ki67 quantification (right) of MOG$_{38-49}$ tetramer–stained CD4+ T cell subpopulations. (G and H) Spleen and lymph node cells collected from immunized DEC1^{WT} or DEC1^{KO} mice or from unimmunized control mice were cultured in the presence of the indicated concentrations of MOG$_{35-55}$ peptide for 3 d. Tritiated thymidine was added to restimulated cultures for 14 h before harvest. Cytokine production was measured by ELISA after 48 h of culture. Results are from at least three independent experiments. Data are represented as mean ± SEM. P-values were calculated with an unpaired Student's *t* test: *, $P < 0.05$.

effector responses of the CD4+ T cells during the EAE priming phase, peripheral lymphocytes harvested at day 7 after immunization were restimulated in vitro with MOG$_{35-55}$ peptide. T cells from previously immunized DEC1^{KO} mice failed to proliferate or to produce IFN-γ, IL-17, and GM-CSF, cytokines shown to be necessary for EAE pathogenesis (Fig. 4, G and H). Similar results were observed 15 or 25 d after immunization (not depicted), suggesting that the lack of T cell cytokine production in DEC1^{KO} mice was not caused by delayed response kinetics. Together, the data suggest that

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DEC1 expression is required for optimal proliferation and cytokine production of T conv cells in the priming phase of EAE.

Exogenous survival signals reverse in vivo proliferation defect of DEC1-deficient T cells

Previous studies have shown that the addition of IL-2 can restore $DEC1^{KO}$ proliferation and survival defect in vitro. To examine whether in vivo administration of IL-2 could improve T cell expansion after $MOG_{35–55}$ immunization in DEC1-deficient mice, we treated the animals with IL-2–anti–IL-2 mAb complexes to enhance IL-2 signaling in $CD4^+$ T cells during the priming phase of EAE (Macián et al., 2002; Acuto et al., 2003; Boyman et al., 2006; Webster et al., 2009). IL-2 complex treatment at days 1, 3, and 5 after MOG immunization increased total lymphocyte numbers in the periphery of $DEC1^{KO}$ mice at day 7 to the same levels as $DEC1^{WT}$ mice (Fig. 5 A). Moreover, the percentages of $MOG_{38–49}$ antigen–specific T cells equalized with IL-2 complex therapy between $DEC1^{KO}$ and $DEC1^{WT}$ mice (Fig. 5 B). We also followed the mice to examine whether enhancement of IL-2 signaling could induce EAE in DEC1-deficient mice (Fig. 5 C). $DEC1^{KO}$ mice primed with $MOG_{35–55}$ peptide did not develop EAE after IL-2 complex treatment during the priming phase (days 1, 3, and 5) or just before onset (days 6–8). To further evaluate the function of cells treated with IL-2 during the priming phase of EAE, we restimulated T cells harvested at day 7 from IL-2 complex–treated animals with $MOG_{35–55}$ peptide (Fig. 5 D). $DEC1^{KO}$ cells did not proliferate in response to increasing concentrations of antigen after in vivo IL-2 treatment. Thus, although exogenous IL-2 can restore the proliferation defect of DEC1-deficient T cells during the priming phase of EAE, the expanded antigen-specific T cells are unable to cause CNS inflammation. This result suggests that the protection from EAE in $DEC1^{KO}$ mice is caused by a T cell–intrinsic differentiation defect altering pathogenicity and not by an IL-2 deficit alone.

To further characterize the functional defects and rule out a cell-extrinsic effect of DEC1 deficiency, we generated $DEC1^{WT}/DEC^{KO}$ mixed bone marrow chimeras in T cell–deficient mice. Genetically marked $DEC1^{WT}$ and $DEC1^{KO}$ T cells showed similar proliferative capacity and cell numbers both before and after the $MOG_{35–55}$ peptide immunization (not depicted). Peripheral $DEC1^{WT}$ and $DEC1^{KO}$ $CD4^+$ T cells were analyzed for $MOG_{38–49}$ tetramer staining at peak clinical disease (Fig. 5 E). These data show that the relative proportion of activated $CD44^+$ and nonactivated $CD44^-$ T cells was equal between $DEC1^{WT}$ and $DEC1^{KO}$ cells. However, there were a reduced percentage of DEC1-deficient antigen-specific cells (Fig. 5 F). This reduction correlated with reduced CD25 expression in $DEC1^{KO}$ T conv and T reg cells (Fig. 5 G). However, there was no difference in Ki67 staining between the DEC1 KO and WT cells (Fig. 5 H). Collectively, these results suggest that in vivo, the presence of $DEC1^{WT}$ cells can drive normal proliferation of $DEC1^{KO}$ cells but cannot correct an intrinsic cell survival defect in antigen-specific $DEC1^{KO}$ $CD4^+$ T cells.

DEC1 is necessary for proinflammatory cytokine production in the effector phase of EAE

We sought to further investigate the intrinsic defects in DEC1-deficient T cells. Chimeric mice had similar total numbers of $DEC1^{WT}$ and $DEC1^{KO}$ $CD4^+$ T cells infiltrating the CNS during EAE (Fig. 6 A), demonstrating that DEC1 was not required for efficient T cell trafficking to the CNS when the survival signals are provided by WT cells. However, the proportion of MOG-specific T cells was reduced among the $DEC1^{KO}$ T cells in the CNS (Fig. 6 B). Next, we isolated mononuclear cells from the CNS of chimeric animals during peak of disease and analyzed cytokine production (Fig. 6 C). IL-2 production was decreased in $DEC1^{KO}$ cells compared with $DEC1^{WT}$, supporting a role for DEC1 in directly controlling IL-2 production. The proinflammatory cytokines GM-CSF and IFN-γ were also reduced in $DEC1^{KO}$ T cells. In contrast, IL-17 production was increased in $DEC1^{KO}$ cells, indicating that these cells did not have a global cytokine production defect. Thus, DEC1 is necessary in a T cell–intrinsic manner for efficient cellular pathogenesis in EAE by controlling the production of IL-2, GM-CSF, and IFN-γ.

DEC1 regulates a subset of CD28-dependent genes after T cell activation

To characterize the transcriptional effects of DEC1 deficiency, we used expression microarrays to generate transcriptional profiles of activated $DEC1^{WT}$ and $DEC1^{KO}$ T cells. Naive $CD4^+$ T cells from $DEC1^{WT}$ and $DEC1^{KO}$ mice were sorted and activated with beads coated with anti-CD3 and anti-CD28. Expression microarray analysis was performed 24 h after activation. $DEC1^{WT}$ cells showed a statistically significant increase in the expression of 158 genes and a reduction in 436 genes as compared with activated $DEC1^{KO}$ T cells. The GO functional annotations of the genes differentially expressed between $DEC1^{WT}$ and $DEC1^{KO}$ included multiple genes and pathways associated with processes that are highly regulated during T cell activation (not depicted). To further link the expression data with the function of DEC1-dependent genes, we performed analysis of the functional annotations (Ingenuity pathways analysis [IPA]) of transcripts showing >1.4-fold change between $DEC1^{WT}$ and $DEC1^{KO}$ cells during T cell activation (Fig. 7 A). Among the most statistically significant DEC1-dependent cellular functions that emerged from this analysis, several are directly related to T cell activation, proliferation, survival, and differentiation.

We next sought to assess the potential role of DEC1 in mediating key CD28 signals by comparing the newly defined DEC1-dependent transcripts with CD28 co-stimulation–dependent transcripts. We performed expression microarray analysis of $DEC1^{WT}$ naive $CD4^+$ T cells activated for 24 h with anti-CD3 or with anti-CD3 plus anti-CD28 antibodies (Fig. 7 B, top). There were 6,842 transcripts differentially regulated in this analysis (Fig. 7 B, bottom), which was consistent with the CD28-dependent human signature (Fig. 1 D and not depicted). The correlation of the DEC1-dependent signature was highly significant with the CD28-regulated gene

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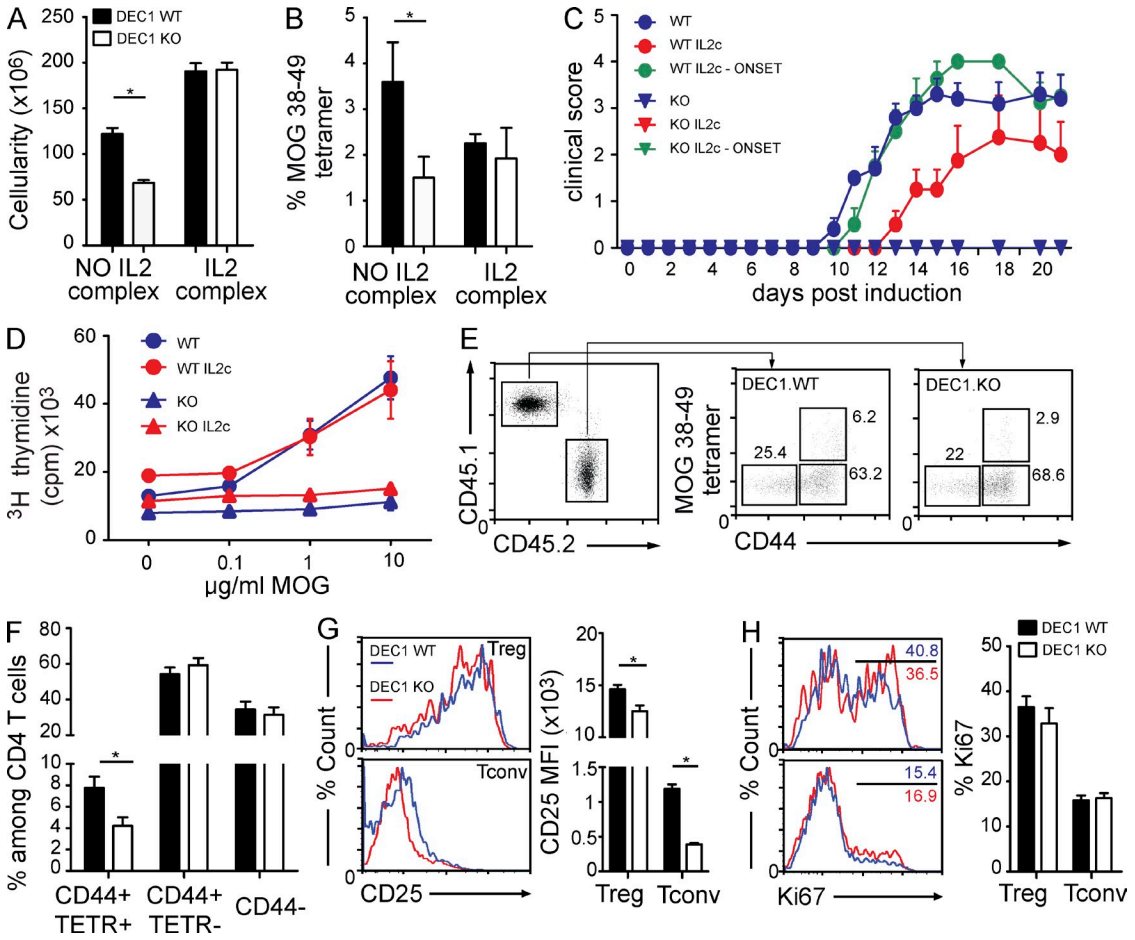

Figure 5. Exogenous survival signals can restore DEC1-deficient T cell expansion during EAE priming phase. (A) Total cellularity from pooled spleen and lymph nodes of DEC1^{WT} or DEC1^{KO} mice at day 7 after MOG$_{35-55}$ immunization with or without IL-2 complex treatment (days 1, 3, and 5). (B) Quantification of MOG$_{38-49}$ tetramer-positive CD4$^+$ T cells of the DEC1^{WT} or DEC1^{KO} cells described in A. (C) Development of EAE clinical disease in DEC1^{WT} or DEC1^{KO} mice treated with IL-2 complex during priming phase (treated on days 1, 3, and 5) or during EAE onset (labeled "ONSET," treated on days 6–8). (D) Spleen and lymph nodes cells collected at day 7 after immunization from DEC1^{WT} or DEC1^{KO} mice treated with IL-2 complex or untreated were cultured in the presence of the indicated concentrations of MOG$_{35-55}$ peptide for 3 d. Tritiated thymidine was added to restimulated cultures for 14 h before harvest. (E and F) Bone marrow cells from DEC1^{WT} and DEC1^{KO} mice were mixed and transferred to mice lacking endogenous T cells. Mixed bone marrow chimeras were immunized with MOG$_{35-55}$ peptide at least 7 wk after cell transfer. Splenocytes and CNS-infiltrating lymphocytes were harvested and analyzed at day 15 after immunization (peak of disease). Flow cytometry (E) was used for quantification (F) of MOG$_{38-49}$ tetramer staining of CD4$^+$ T cells subpopulations from chimera splenocytes. (G) CD25 staining and quantification of T reg and T conv cells from mixed bone marrow chimera splenocytes. MFI, mean fluorescence intensity. (H) Ki67 staining and quantification of T reg and T conv cells from mixed bone marrow chimera splenocytes. Results are from at least three independent experiments. Data are represented as mean ± SEM. P-values were calculated with an unpaired Student's *t* test: *, $P < 0.05$.

list ($P < 0.0001$). 72% of the genes up-regulated by DEC1 were also up-regulated by CD28 co-stimulation, whereas 83% of the DEC1 down-regulated genes were again down-regulated by CD28 signaling (Fig. 7, C and D, top). Only a small set of significantly regulated genes (7%) showed expression changes in opposite directions between DEC1 and CD28 signatures (Fig. 7, C and D, bottom). To provide further support to the DEC1–CD28 association, we performed a PCA using the defined DEC1-dependent genes. This analysis demonstrates that DEC1-regulated genes are highly differentially regulated in T cells activated with or without CD28 co-stimulation (Fig. 7 E). As expected, the DEC1 transcriptional signature efficiently segregated the samples based on DEC1 expression (Fig. 7 E, blue and red). Interestingly, a set of DEC1-dependent genes differentiated the samples activated through TCR alone versus TCR plus CD28 engagement (Fig. 7 E, squares and circles, respectively). Collectively, these observations suggest that the transcription factor DEC1 regulates a subset of the CD28-dependent genes.

To confirm the observed microarray findings, we measured mRNA expression of several genes in DEC1^{WT} and DEC1^{KO} mouse naive T cells (Fig. 7 F). We found a significant decrease in the expression of *Bclxl* in DEC1^{KO} cells. BCL-XL is a key regulator of T cell survival after activation

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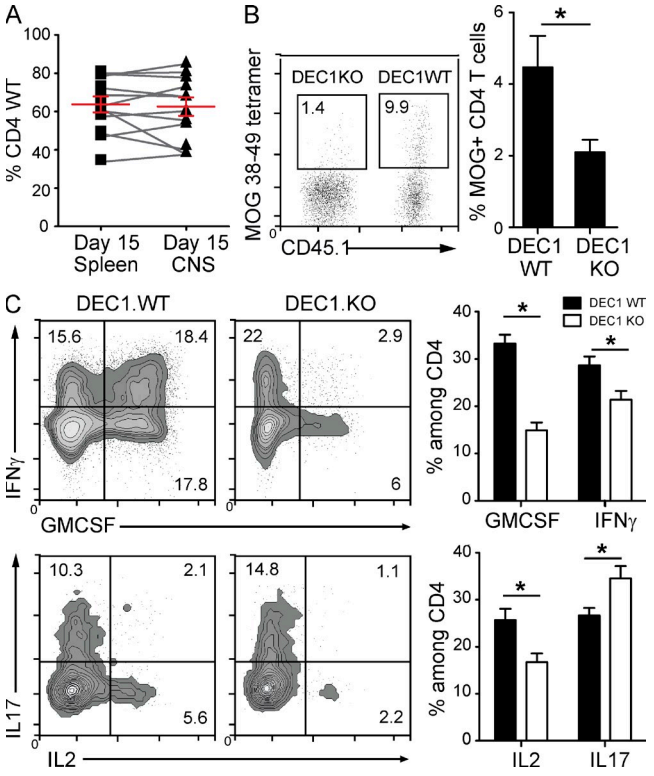

Figure 6. DEC1 is required for proinflammatory cytokine production in a T cell–intrinsic manner. (A) Percentage of DEC1^{WT} CD4^{+} T cells during EAE peak disease in spleen and CNS of DEC1$^{WT/KO}$ mixed bone marrow chimeras compared with DEC1^{KO} CD4^{+} T cells. (B) Flow cytometry and analysis of CD4^{+} MOG$_{38-49}$ tetramer T cells from CNS-infiltrating cells. (C) Production of IFN-γ, GM-CSF, IL17, and IL2 by CNS-infiltrating DEC1^{WT} and DEC1^{KO} cells (gated on CD45^{hi}CD11b^{-}CD4^{+}). Results are from at least three independent experiments. Data are represented as mean ± SEM. P-values were calculated with an unpaired Student's *t* test: *, $P < 0.05$.

(Boise et al., 1995; Noel et al., 1996; Wang et al., 2012). DEC1-deficient cells show defects in *Il2* and *Il2ra* gene transcription, which correlate with the lack of IL-2 production and CD25 expression reported here and by others (Sun et al., 2001; Miyazaki et al., 2010). We also found that DEC1^{KO} cells were defective in the transcription of cytokines involved in the pathogenesis of EAE, namely *Ifng* (IFN-γ) and *Csf2* (GM-CSF). In addition, we confirmed that expression of these genes depends on both DEC1 and CD28 during T cell activation. IL-2 signaling has a well-characterized role in the transcriptional regulation in T cells (Jin et al., 2006). Because IL-2 production is decreased in DEC1-deficient T cells, we next sought to investigate the effect of IL-2 signals on the DEC1-dependent transcription. Similar to Fig. 1 F, DEC1^{WT} and DEC1^{KO} mouse naive T cells were activated for 24 h with anti-CD3 and anti-CD28 in the presence of exogenous IL-2 or anti-CD25 antibodies (Fig. 7 G). Efficient blockade of CD25 was confirmed by phospho-STAT5 staining (not depicted). Although exogenous IL-2 reversed proliferation and survival defects in DEC1-deficient T cells (Fig. 2 J), the expression of a subset of DEC1-dependent genes was not affected by maximizing or repressing IL-2 signaling. Thus, these results support an intrinsic role for DEC1 in the transcriptional control of genes independent of IL-2 signaling.

DISCUSSION

T cell co-stimulation via CD28 plays an essential role in the development and maintenance of T cell responses (Lenschow et al., 1996; Bour-Jordan et al., 2011). However, few studies have used global transcriptional analyses to link the CD28 co-stimulation effects to specific transcription factors. In contrast to previous expression microarray studies (Diehn et al., 2002; Riley et al., 2002), we analyzed highly purified human and mouse naive T cells to avoid interference caused by the heterogeneous responses of various T cell subsets to CD28 co-stimulation. We defined a conserved CD28-dependent expression pattern, which we contrast with the anergy-like transcriptional profile observed 24 h after maximal TCR signaling alone. We confirmed that CD28 co-stimulation has profound qualitative, and not only quantitative, effects on the gene expression of activated T cells and contributes to the stabilization and amplification of the TCR transcriptional effects before cell division.

The distinct transcriptional signature found in T cells after CD28 co-stimulation suggests that specific transcription factors regulated by early CD28 signaling may be responsible for the temporal effect observed in the gene expression pattern. Using in silico analysis, we identified transcription factors already known to be involved in T cell activation such as NFAT, c-myc, and NF-κB, which are linked to CD28 signaling through PDK1 and PKC-θ activation (Takeda et al., 2008; Park et al., 2009). Among the predicted transcription factors related to the CD28 signature, we identified DEC1, which is a highly CD28-inducible transcript. DEC1 is thought to promote T reg cell survival in vivo by cooperating with Runx1 to induce expression of CD25, which is critical for T reg cell survival and function (Miyazaki et al., 2010). The connection between DEC1 and CD28 is also supported by the observation that CD28 is essential in the development and survival of T reg cells (Salomon et al., 2000; Bour-Jordan et al., 2004).

DEC1 is rapidly expressed after T cell activation, and its transcription is maintained over the initial 48 h in a CD28-dependent manner. Maximal *DEC1* transcription is observed at 24 h after stimulation, suggesting that its regulatory function is predominantly in the initial stages of activation. Although TCR signaling can trigger modest levels of *DEC1* expression at late stages of activation, the anergy-like transcriptional status of the cells 24 h after anti-CD3 stimulation may restrict DEC1 functions. Furthermore, we observed undetectable DEC1 signal in the cytoplasmic fraction in the absence of CD28 co-stimulation, which suggests posttranscriptional regulation of DEC1 through CD28 signaling, a phenomenon previously observed for other transcripts (Ledbetter et al., 1990; Sanchez-Lockhart et al., 2004; Butte et al., 2012). DEC1 expression can be induced by a variety of stimuli in different cell types (Boudjelal et al., 1997;

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A

Functions Annotatioin	Category	p-value
Maturation of leukocytes	Cellular Development	1.09E-06
Cell death of lymphocytes	Cell Death	2.26E-05
Arrest in G2/M phase transition of T lymphocytes	Cell Cycle	6.23E-05
Arrest in S phase of T lymphocytes	Cell Cycle	6.23E-05
Stimulation of lymphocytes	Cellular Growth and Proliferation	7.99E-05
Exposure of phospholipid	Lipid Metabolism	8.54E-05
T cell development	Cellular Development	3.38E-04
Apoptosis of T lymphocytes	Cell Death	6.59E-04
Proliferation of Th1 cells	Cellular Growth and Proliferation	8.71E-04
Proliferation of T lymphocytes	Cellular Growth and Proliferation	9.50E-04
Differentiation of lymphocytes	Cellular Development	1.81E-03
Binding of STAT binding site	Gene Expression	2.04E-03
Activation-induced cell death of T lymphocytes	Cell Death	3.10E-03
Stimulation of carbohydrates	Carbohydrates Metabolism	3.88E-03
Development process of Th1 cells	Cellular Development	4.27E-03
Development process of regulatory T lymphocytes	Cellular Development	9.92E-03

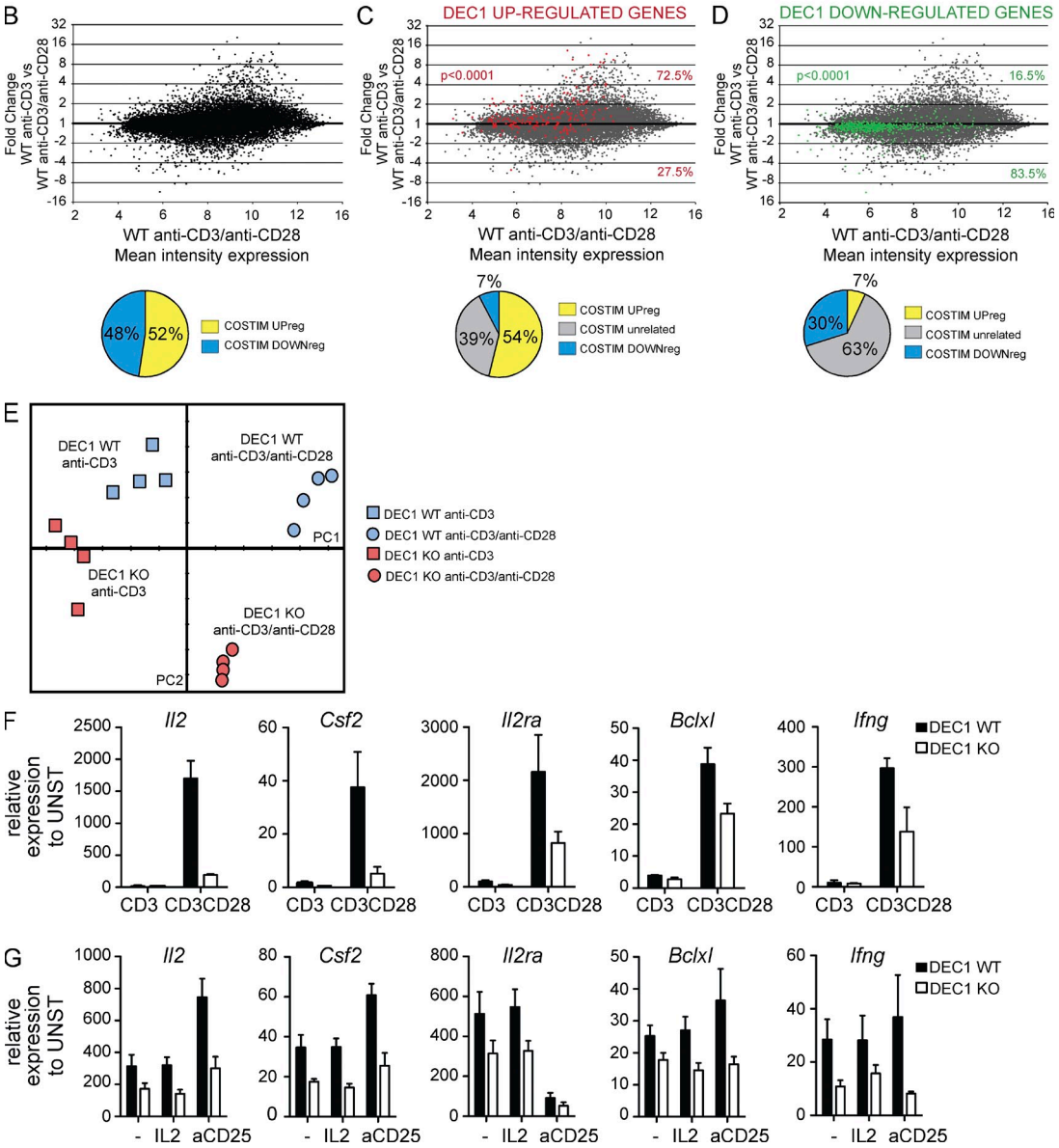

Figure 7. Overlap in CD28- and DEC1-dependent transcriptional profiles after T cell activation. (A) Functional analysis performed by IPA based on genes 1.4-fold differentially expressed between DEC1^{WT} and DEC1^{KO} naive T cells activated with anti-CD3 plus anti-CD28 for 24 h. The significance of the association of the gene expression pattern with a biological function is calculated using right-tailed Fisher's exact test. (B, top) CD28 co-stimulation–dependent signature described as a scatter plot summarizing the gene expression differences between DEC1^{WT} naive T cells activated with anti-CD3 versus anti-CD3 plus anti-CD28. (bottom) Quantification of genes significantly up- and down-regulated by the effect of CD28 signaling ($P < 0.05$). (C, top) Red dots represent significantly up-regulated genes between DEC1^{WT} naive T cells activated with anti-CD3 plus anti-CD28 and DEC1^{KO} naive

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Honma et al., 2002; Miyazaki et al., 2002). Microarray analysis performed in human glioblastoma cells described transcriptional regulation of *DEC1* through PI3K and MEK/ERK (Tullai et al., 2004), suggesting these signaling pathways as potential candidates linking CD28 activation and DEC1 expression in T cells. Nevertheless, other members of the B7 co-stimulation family could participate in the transcriptional regulation of DEC1 during T cell activation (Sharpe and Freeman, 2002). Microarray analyses performed to compare T cell activation with different co-stimulatory signals showed that ICOS and BTLA can slightly increase the expression of *DEC1* at late stages of activation (Riley et al., 2002; Wakamatsu et al., 2013). Because ICOS and BTLA expression is restricted to activated cells, their stimulation may complement CD28 signaling in inducing DEC1 expression in some T cell subsets.

We performed gene expression analysis of $DEC1^{WT}$ and $DEC1^{KO}$ naive $CD4^+$ T cells 24 h after activation. Importantly, at this time point, there were no measurable differences between proliferation and survival of the DEC1-null cells and control cells, which allowed us to capture the primary effects of DEC1 deficiency rather than the indirect effects. The significant correlation observed between the DEC1 and CD28 transcriptional pattern suggests that DEC1 is a central transcription factor during T cell activation that regulates part of the specific CD28-dependent transcriptome. To evaluate the magnitude of DEC1 contribution to the CD28 signature, we tested whether *DEC1* overexpression could substitute for CD28 co-stimulation in primary T cells (not depicted). Although we observed an increase in the expression of CD28/DEC1-dependent genes and a slight improvement in cell proliferation compared with control, the overexpression of *DEC1* alone did not recapitulate CD28 signaling function. Complementary with this data, loss of DEC1 does not completely block the transcriptional response initiated by CD28 co-stimulation. These results imply that there may be some partial compensation by other transcription factors involved in the CD28 transcriptional signature. Thus, DEC1 is necessary but not sufficient for the transcriptional changes induced by CD28 signaling in naive T cells. Interestingly, DEC1 has a closely related homologue, DEC2 (BHLHE41), which has been shown to be necessary for Th2 differentiation, IL-2 production, and CD25 expression in primary mouse T cells (Liu et al., 2009; Yang et al., 2009). Thus, it is possible that DEC1 and DEC2 synergize to drive IL-2 production. Further work is now underway to determine whether DEC2 collaborates to direct transcription after $CD4^+$ T cell activation.

Microarray analysis and *DEC1* expression kinetics during T cell activation suggest that the transcriptional function of DEC1 is maximized by CD28 co-stimulation. However, because we observed weak induction of *DEC1* expression after TCR signaling, we have not ruled out a potential function for DEC1 in our experimental settings after T cell activation without CD28 signaling. In fact, we identify a different transcriptional pattern between $DEC1^{WT}$ and $DEC1^{KO}$ naive T cells after anti-CD3 stimulation (Fig. 7 E and not depicted). However, these differences may have limited consequences during in vivo T cell activation as a result of the usual presence of CD28 ligands on antigen-presenting cells.

The proliferation and survival defects observed in DEC1-deficient T cells could have been caused by direct or indirect effects of DEC1 transcriptional targets. We identified many DEC1-dependent genes involved in cell cycle control. Interestingly, several studies have shown abundant expression of *DEC1* in tumor tissue from colon, breast, stomach, lung, and kidney cancers (Li et al., 2003; Chakrabarti et al., 2004; Ivanova et al., 2005; Zheng et al., 2009). DEC1 has also been described to promote cell survival through both reducing caspase activation and increasing the transcription of antiapoptotic genes (Li et al., 2002, 2006; Wang et al., 2010). Thus, DEC1 may play a role in cell proliferation and survival in many cell types, including T cells. We observed an improvement in T cell expansion in vivo and in vitro after supplying survival signals to DEC1-deficient cells. Therefore, DEC1 may also support T cell proliferation and survival by enhancing IL-2 signaling.

We showed that IL-2 cannot substitute for CD28 co-stimulation under our in vitro activation conditions. Similarly, we also demonstrated that the reduced IL-2 signaling in DEC1-deficient cells did not interfere with DEC1-dependent transcriptional regulation. Furthermore, DEC1 functioned in a cell-intrinsic manner to promote $CD4^+$ T cell effector function and clinical disease in the highly CD28-dependent EAE model (Perrin et al., 1995, 1999; Oliveira-dos-Santos et al., 1999). The analysis of CNS-infiltrating T cells revealed decreases in EAE-mediated cytokine production by $DEC1^{KO}$ T cells, in particular IL-2, IFN-γ, and GM-CSF but not IL-17 (Petermann and Korn, 2011). Interestingly, although deficiency of GM-CSF has been shown to induce complete resistance to the induction of EAE, the expression of only

T cells activated with anti-CD3 plus anti-CD28. (bottom) Pie graph indicates the percentage of a set of DEC1 up-regulated genes whose expression was regulated by CD28 co-stimulation, in the direction indicated. (D, top) Green dots represent significantly down-regulated genes between $DEC1^{WT}$ naive T cells activated with anti-CD3 plus anti-CD28 and $DEC1^{KO}$ naive T cells activated with anti-CD3 plus anti-CD28. (bottom) Pie graph indicates the percentage of a set of DEC1 down-regulated genes whose expression was regulated by CD28 co-stimulation, in the direction indicated. P-values were calculated by χ^2 test. (E) PCA of DEC1-dependent genes in $DEC1^{WT}$ and $DEC1^{KO}$ naive T cells activated with anti-CD3 alone or anti-CD3 plus anti-CD28 at 24 h. The DEC1-regulated genes include genes differentially expressed between CD28–co-stimulated $DEC1^{WT}$ and $DEC1^{KO}$ T cells (594 genes; $P < 0.05$). Each dot represents a sample, which is colored and shaped according to activation parameters. The combination of PC1 and PC2 summarizes 84% of the dataset variation. (F) mRNA expression of the indicated genes at 24 h after activation of $DEC1^{WT}$ and $DEC1^{KO}$ naive T cells with anti-CD3 alone or anti-CD3 plus anti-CD28. (G) Gene expression analysis as in F with or without 100 U/ml of recombinant IL-2 or 25 μg/ml anti-CD25. Results are from at least three independent experiments. Data are represented as mean ± SEM.

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IFN-γ or IL-17 by T cells is not enough to induce CNS infiltration and disease (Chu et al., 2000; McQualter et al., 2001; Haak et al., 2009). The inability of DEC1^{KO} T cells to produce GM-CSF is likely responsible for their inability to activate CNS-resident innate immune cells required for EAE pathogenesis (Ponomarev et al., 2007; Codarri et al., 2011). Thus, DEC1 is necessary for efficient autoreactive T cell responses by supporting T cell proliferation after antigen encounter and promoting proinflammatory cytokine production in a cell-intrinsic manner.

MATERIALS AND METHODS

Human samples. Informed written consent from healthy blood donors was obtained in accordance with the reviewed and approved policies and procedures at the University of California, San Francisco (UCSF). Ethics approval was granted by the UCSF Institutional Review Board (approval number H7023-22712-08). Whole blood was obtained via venipuncture from healthy adult volunteers (males, age range 19–29 yr). Blood was collected in sodium heparin–containing Vacutainer tubes (BD). Peripheral blood mononuclear cells were isolated by using Ficoll-Paque PLUS (GE Healthcare) as previously described (Putnam et al., 2009).

Mice. *DEC1^{KO}* mice on a pure C57BL/6 background (from the Jackson Laboratory) were all derived from the mouse line described in Jiang et al. (2008). Mice were bred and housed in a specific pathogen–free barrier facility at the UCSF or at the National Institute on Aging at the Johns Hopkins Bayview Campus in Baltimore, MD. All animal experiments were approved by the UCSF Animal Care and Use Committee (approval number AN082188-01). All of the experiments were conducted comparing DEC1^{WT} and DEC1^{KO} littermate mice with rare exceptions, in which we used DEC1^{WT} controls from the Jackson Laboratory. No differences were observed intra-experiment between control mice bred in our facility and mice from outside sources. RAG^{KO} and TCRA^{KO} mice on pure C57BL/6 background were obtained from the Jackson Laboratory.

Isolation and activation of naive T cells. For the human microarray analysis, CD4$^+$CD45RA$^+$ T cells were isolated on a FACSAria II cell sorter (BD). Purity of the sorted population was >99%. These cells were then seeded into wells of a 12-well plate at a density of 10^6 cells per ml of RMPI-1640 supplemented with 5% human heat-inactivated pooled AB serum (Valley Biomedical). The cells were activated with latex beads conjugated to control antibody (clone MPC-11; BD) or antibodies specific for anti-CD3 (clone OKT3) or to both anti-CD3 and anti-CD28 (clone 9.3). For other in vitro experiments, human CD4$^+$CD45RA$^+$ T cells were isolated with a RoboSep (STEMCELL Technologies) and the human naive T cell enrichment kit (STEMCELL Technologies). This method consistently gave purities of >95% CD4$^+$CD45RA$^+$ cells. The cells were then activated on 12-well plates seeded the previous night with anti-CD3 or anti-CD3 and anti-CD28 at 1 μg/ml (anti-CD3) or 10 μg/ml (anti-CD28) in PBS. For isolation of mouse naive T cells, CD4$^+$CD25$^-$CD62L$^+$ cells were sorted according to the same methodology as human T cells. For in vitro activation experiments, cells were resuspended in DMEM supplemented with 10% FBS, glutamine, Hepes buffer, 0.1% β-mercaptoethanol, nonessential amino acids, and antibiotics. The cells were activated in plates precoated with 1 μg/ml anti-CD3 (clone 145-2C11) and 1 μg/ml anti-CD28 (clone PV1) or using a T Cell Activation/Expansion kit (Miltenyi Biotec). Dead cells were analyzed by staining with LIVE/DEAD Fixable Violet kit (Invitrogen) according to the manufacturer's instructions.

IL-2 signaling inhibition. For human T cell cultures, the concentration of BSB (Simulect; Novartis) was 25 μg/ml. Alternatively, recombinant human IL-2 (Proleukin; Chiron Therapeutics) was added at 100 U/ml. BSB was a gift from F. Vincenti (UCSF). Confirmation of BSB blocking of CD25 was performed with a competing mAb labeled "A" (clone 2A3; BD) and a noncompeting mAb labeled "B" (clone MA251; BD). For mouse IL-2RA blocking, anti-CD25 was added at 25 μg/ml (clone PC61), and efficiency was assessed by phospho-STAT5 staining.

RNA preparation and microarray analysis. RNA samples for microarray were prepared via TRIzol extraction, and the quantity, purity, and integrity of the samples were measured with a NanoDrop and a Bioanalyzer. The samples were then reverse transcribed and amplified before hybridization to Affymetrix human U133 Plus 2 microarrays or to Affymetrix mouse Gene 1.0 ST array. Hybridization and data collection were performed by the Genome Core of the J. David Gladstone Institutes. RNA samples for quantitative real-time PCR analysis were extracted using the RNeasy kit (QIAGEN) using the manufacturer's recommended protocol. All microarray data discussed in this publication have been deposited in NCBI's GEO database (GEO series accession no. GSE39596; human microarray accession no. GSE39594; mouse microarray accession no. GSE39595).

Microarray data analysis. Microarray image files were visually inspected by using the BioConductor package for R. Files with visible artifacts were excluded from the analysis. Data were normalized by using Genespring GX 10.0.2 software (Agilent Technologies) with GCRMA. Statistical significance of differential expression was calculated using ANOVA followed by Benjamini Hochberg FDR (false discovery rate), with a cut-off of $P < 0.05$. PCA was performed with MultiExperiment Viewer version 4.5 software to provide a global view of how the sample groups were related. The functional analysis was completed by loading the microarray datasets into IPA software (Ingenuity Systems). Transcription factor prediction analysis was performed using GeneGo (Thomson Reuters) with the software-predefined settings.

Quantitative real-time PCR. Reverse transcription of RNA samples and quantitative real-time PCR were performed as previously described (Esensten et al., 2009). TaqMan primer probes (Applied Biosystems) used for human experiments were IL-2 (Hs99999150_m1), BHLHE40 (Hs00186419_m1), CD25 (Hs00907777_m1), BCLXL (Hs99999146_m1), NR4A3 (Hs00545009_g1), SGPP2 (Hs00544786_m1), TNFRSF9 (Hs00155512_m1), TNFRSF14 (Hs00542476_g1), UHRF1 (Hs00273589_m1), CSF2 (Hs00929873_m1), and ZBTB20 (Hs00210321_m1); and for mouse experiments were Bhlhe40 (Mm00478593_m1), Cd25 (Mm00434261_m1), Nr4a3 (Mm01354011_m1), Il2 (Mm99999222_m1), Ifng (Mm00801778_m1), Zbtb20 (Mm00457765_m1), Bclxl (Mm00437783_m1), and csf2 (Mm01290062_m1).

DNA cloning and lentivirus production. Lentivirus encoding an shRNA specific for hBHLHE40 (pSICO-R-502) was produced by using the pSICO-R vector (provided by M.T. McManus, UCSF). Empty vectors were cut with XhoI and HpaI. The following primers were then annealed and ligated into the cut vector: forward, 5′-TGCATGTGAAAGCACTAACATTCAAGAGATGTTAGTGCTTTCACATGCTTTTTTC-3′; and reverse, 5′-TCGAGAAAAAAGCATGTGAAAGCACTAACATCTCTTGAATGTTAGTGCTTTCACATGCA-3′. For the control scrambled shRNA (pSICO-R-SCRM), the following primers were used: forward, 5′-TTCATCGAGTATTATTCCTATTCAAGAGATAGGAATAATACTCGATGATTTTTTC-3′; and reverse, 5′-TCGAGAAAAAATCATCGAGTATTATTCCTATCTCTTGAATAGGAATAATACTCGATGAA-3′. Lentivirus was produced in HEK 293 cells using psPAX2 and pCMV-VSV-G. Jurkat T cells were infected by mixing viral supernatant with Jurkat cells resuspended in RPMI-1640 plus 5% FCS at a concentration of 10^6 per ml.

Jurkat activation. Jurkat T cells (a gift from J. Roose, UCSF) were infected with pSICO-R lentivirus by mixing viral supernatant with Jurkat cells resuspended in RMPI-1640 plus 5% FBS at a 1:1 ratio. After 48 h, productive infection was confirmed by GFP expression. The Jurkat cells were activated with plate-bound anti-CD3 (clone OKT3) or anti-CD3 and anti-CD28 (clone 9.3). 1 μg/ml anti-CD3 with or without 10 μg/ml anti-CD28 in PBS was put into wells of 12- or 6-well plates. The next day, the wells were washed

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three times with PBS, and Jurkat cells were seeded at a density of 10^6 cells per ml of RPMI-1640 supplemented with 5% FCS, glutamine, penicillin, and streptomycin. Cell surface staining with fluorochrome-conjugated antibodies was performed as detailed above for primary human cells. Dead cells were excluded by staining with 7-aminoactinomycin D (BD) according to the manufacturer's instructions.

Western blot analysis. 5×10^6 human naive T cells were isolated and cultured for 24 h with different activation conditions. Total cell lysis and extraction of separate cytoplasmic and nuclear protein fractions were performed as described by the manufactures (NE-PER Nuclear Protein Extraction kit; Thermo Fisher Scientific). For each sample, the same number of cells was analyzed with an anti-DEC1 polyclonal antibody (A300-649A; Bethyl Laboratories, Inc.)

EAE/restimulation. EAE was induced by injecting 200 µg $MOG_{35–55}$ peptide (Genemed Synthesis) emulsified in CFA (Sigma-Aldrich) subcutaneously at three locations on the back of each mouse. Intraperitoneal injection of pertussis toxin (400 ng per dose; List Biological Laboratories) was administered to each mouse on the day of immunization and on day 2 after immunization. Clinical scores were assessed every 2 d, according to the following criteria: 1, weak tail or weak hind limb; 2, weak tail and weak hind limb; 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribund. For restimulation experiments, splenocytes and lymph node cells derived from mice immunized with MOG-CFA were harvested and stimulated in vitro with different concentrations of the $MOG_{35–55}$ peptide from 7, 15, or 25 d after initial immunization. Culture supernatants were collected 48 h later and evaluated for IFN-γ and IL-17A production by ELISA.

$MOG_{38–49}$ tetramer staining. Splenocytes and lymph node cells derived from mice immunized with MOG-CFA were harvested and stained with $MOG_{38–49}$-APC tetramer for 2 h at room temperature (National Institutes of Health Tetramer Facility). Before further antibody staining, tetramer-positive cells were enriched using anti-APC microbeads and MACS LS separation columns (Miltenyi Biotec) as previously described (Moon et al., 2009). The same protocol was used to stain CNS-infiltrating cells but without the microbead enrichment step.

CNS cytokine staining. CNS-infiltrating cells were stimulated for 2.5 h with phorbol 12-myristate 13-acetate and ionomycin in the presence on GolgiStop (BD). Then cells were stained for surface molecules, fixed, and made permeable with a Cytofix/Cytoperm kit (BD) and stained with the following antibodies: mouse GM-CSF (MP1-22E9; BD), mouse IL-17A (17B7; eBioscience), mouse IFN-γ (XMG1.2; BioLegend), and mouse IL-2 (JES6-5H4; eBioscience).

ELISA. For the IFN-γ ELISA, 96-well MaxiSorp Nunc-Immuno plates (Thermo Fisher Scientific) were coated with 2 µg/ml anti–IFN-γ antibody (clone R4-6A2; BD). Plates were blocked with PBS–2% BSA. Supernatant samples and standards were added, and cytokine was detected with 1 µg/ml biotinylated anti–IFN-γ (clone XMG1.2; BioLegend) and 1.25 µg/ml HRP-streptavidin (Invitrogen). Washing between all steps was performed with PBS 0.05% Tween-20. Plates were developed with TMB^+ Substrate-Chromogen (Dako), stopped with 1N HCl, and read at 450 nm, according to the manufacturer's instructions. For IL-17A and IL-2 ELISA, the same protocol was used, except plates were coated with 2 µg/ml anti-IL17A antibody (clone 17CK15A5; eBioscience) or 1 µg/ml anti–IL-2 antibody (clone JES6-1A12; eBioscience) and blocked with PBS–5% FCS, and cytokine was detected with 1 µg/ml biotinylated anti–IL-17A (clone 17B7; eBioscience) or 0.5 µg/ml biotinylated anti–IL-2 (clone JES6-5H4; eBioscience).

IL-2 complex. IL-2–anti–IL-2 mAb (JES6-1) complexes were prepared as previously described (Boyman et al., 2006; Webster et al., 2009). Mice immunized for EAE received an intraperitoneal injection of 5 µg of recombinant mouse IL-2 (eBioscience) complexed with 50 µg anti–IL-2 (clone JES6-1A12; eBioscience) at 37°C for 15 min on days 1, 3, and 5 or days 6–8.

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