Transcriptional repressor DREAM regulates T-lymphocyte proliferation and cytokine gene expression

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Downstream Regulatory Element Antagonist Modulator (DREAM) is a Ca²⁺-dependent transcriptional repressor expressed in the brain, thyroid gland and thymus. Here, we analyzed the function of DREAM and the related protein KChIP-2 in the immune system using transgenic (tg) mice expressing a cross-dominant active mutant (EFmDREAM) for DREAM and KChIPs Ca²⁺-dependent transcriptional derepression. EFmDREAM tg mice showed reduced T-cell proliferation. Tg T cells exhibited decreased interleukin (IL)-2, -4 and interferon (IFN)γ production after polyclonal activation and following antigen-specific response. Chromatin immunoprecipitation and transfection assays showed that DREAM binds to and represses transcription from these cytokine promoters. Importantly, specific transient knockdown of DREAM or KChIP-2 induced basal expression of IL-2 and IFNγ in wild-type splenocytes. These data propose DREAM and KChIP-2 as Ca²⁺-dependent repressors of the immune response.

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Introduction

The transcriptional repressor Downstream Regulatory Element Antagonist Modulator (DREAM) is a Ca²⁺-binding protein that binds specifically to DRE sequences in DNA (Carrion *et al*, 1999). Binding of DREAM to DREs is regulated by the level of nuclear Ca²⁺, by the interaction with other nucleoproteins like αCREM and CREB and by the PI3 kinase pathway (Carrion *et al*, 1999; Ledo *et al*, 2000a, 2002; Sanz *et al*, 2001). Mutation of two key amino acids within any of the functional EF hands of DREAM (EFmDREAM) results in a protein insensitive to Ca²⁺ that remains bound to DNA

during Ca²⁺ stimulation (Carrion *et al*, 1999). Since DREAM binds to DRE sites as a tetramer, EFmDREAM was proposed to function as a dominant active mutant in a background of wild-type (wt) DREAM (Ledo *et al*, 2000b). DREAM, also known as KChIP-3, belongs to a group of structurally and functionally related Ca²⁺-binding proteins (KChIP-1 to -4) that interact with Kv4 potassium channels (An *et al*, 2000) and presenilins (Lilliehook *et al*, 2002) to regulate channel gating and Ca²⁺ release from the endoplasmic reticulum, respectively.

Engagement of the TCR/CD3 complex triggers a signal transduction cascade that involves early increases in intracellular Ca²⁺ and diacylglycerol (Cantrell, 1996), the activation of various kinases and phosphatases, and changes in gene expression (Feske *et al*, 2001). Activation of Ca²⁺-dependent pathways is required to induce expression of the cytokine genes interleukin (IL)-2, -4 and interferon (IFN)γ (Rao *et al*, 1997; Macian *et al*, 2001). The first and best characterized Ca²⁺-dependent event in the transcriptional control of cytokine genes is the dephosphorylation of NFAT by the Ca²⁺/calmodulin-dependent protein phosphatase calcineurin (Rao *et al*, 1997). This modification allows NFAT to translocate from the cytoplasm to the nucleus and activate transcription (Clipstone and Crabtree, 1992; Kubo *et al*, 1994; Campbell *et al*, 1996; Rincon and Flavell, 1997). Expression of IL-2, -4 and IFNγ is the signature for different types of effector T helper (Th) cells after differentiation. In response to antigen stimulation, naive Th cells differentiate into different types of effector cells that are classified based on their distinct cytokine profiles and immune-regulatory functions (Mosmann *et al*, 1986; Paul and Seder, 1994). Th1 cells produce IL-2 and IFNγ, and play an important role in cell-mediated immune response against intracellular pathogens. Th2 cells produce IL-4, -5 and -10, and are involved in humoral immunity and in the allergic response.

DREAM is highly expressed in many brain areas and in some peripheral organs, including the thyroid gland, testis and the thymus. While specific target genes for DREAM repression have been described in the brain (Carrion *et al*, 1999) and in the thyroid gland (Rivas *et al*, 2004), the role of DREAM in the immune response has not been previously investigated. Here we show that DREAM and the related protein KChIP-2 are expressed in all subpopulations of T cells and that DREAM expression is downregulated after TCR engagement. Expression of the Ca²⁺-insensitive DREAM mutant, EFmDREAM, in transgenic (tg) lymphocytes reduced proliferation and decreased IL-2, -4 and IFNγ production following TCR engagement and during primary antigen-specific T-cell responses. Importantly, DREAM binds to and represses the transcriptional activity of these cytokine promoters, suggesting a direct repression. Furthermore, specific transient knockdown of DREAM or KChIP-2 induced basal expression of IL-2 and IFNγ mRNAs in wt splenocytes.

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Results

DREAM is downregulated upon TCR stimulation

DREAM mRNA is abundantly expressed in the thymus (Carrion *et al*, 1999). To understand its function in the immune system, we first wanted to know the expression pattern of DREAM and related KChIP genes in the various lymphoid compartments. Qualitative reverse transcriptase (RT)-PCR showed, in addition to DREAM, expression of KChIP-2 in thymus and spleen, while KChIP-1 was barely detectable and KChIP-4 was not present (data not shown). We then quantified DREAM and KChIP-2 expression in different T-cell populations by real-time RT-PCR. DREAM and, to a lower extent, KChIP-2 were present in thymocytes, splenocytes and in purified T, CD4⁺ and CD8⁺ cells from spleen (Figure 1A).

To investigate a potential role for DREAM and KChIP-2 in the immune response, we analyzed changes in both transcripts upon TCR activation. Primary cultured thymocytes

and splenocytes were stimulated for different times with plate-bound α CD3. The levels of DREAM mRNA were decreased as early as 30 min following TCR engagement and the decrease was still noticeable 24 h later (Figure 1B), while KChIP-2 expression was unaffected (data not shown). The downregulation of DREAM after TCR engagement indicates a potential role for DREAM in T-cell function.

EFmDREAM tg mice, a tool to study DREAM function *in vivo*

Since genetic ablation of DREAM did not result in any noticeable phenotype in the immune system (Cheng *et al*, 2002), we aimed to analyze the function of DREAM and KChIP-2 in T cells by developing a tg mouse model using a DREAM mutant insensitive to Ca^{2+} , EFmDREAM, that could block the Ca^{2+} -dependent derepression function of DREAM and other KChIP proteins endogenously expressed. Conceptually, the strategy was based on the known property of DREAM to bind DNA and repress transcription when forming a tetramer (Carrion *et al*, 1999; Osawa *et al*, 2001). Since the other KChIP proteins are structurally and functionally related to DREAM, we analyzed whether DREAM and KChIP-2 are able to physically interact and form functional heterotetramers. We performed yeast two-hybrid assays using EF-mutated proteins, since oligomerization has been shown to be regulated by Ca^{2+} (Osawa *et al*, 2005). The DREAM-DREAM interaction was confirmed and also a specific interaction between DREAM and KChIP-2 could be detected by this assay (Figure 2A). In addition, we performed coimmunoprecipitation experiments after transient cotransfection of Flag-DREAM and Myc-KChIP-2 expression vectors in HeLa cells. An anti-Flag antibody successfully immunoprecipitated Myc-KChIP-2 (Figure 2B), confirming the DREAM/KChIP-2 interaction.

To ascertain the formation of functional heterotetramers and, more importantly, to investigate whether the EFmDREAM protein could act as a cross-dominant mutant for the DREAM transcriptional function, we performed transient transfections with a DRE-reporter (pHD3CAT) and DREAM or KChIP-2 expression vectors, alone or in combination with EFmDREAM in HEK293 cells. As previously reported (Carrion *et al*, 1999; Link *et al*, 2004), DREAM, EFmDREAM and KChIP-2 repressed basal transcription from the pHD3CAT reporter (Figure 2C). Stimulation by caffeine relieved DREAM- and KChIP-2-induced repression, transactivating five- to six-fold the expression from the reporter (Figure 2C) by a mechanism that involves Ca^{2+} release from intracellular stores (Hernandez-Cruz *et al*, 1990) and increased CREB-dependent transcription (Carrion *et al*, 1999; Ledo *et al*, 2002). However, repression by the EFmDREAM was only slightly reversed by caffeine through an EF-independent mechanism not yet clarified (Figure 2C). Cotransfection of EFmDREAM with DREAM or KChIP-2 in a ratio 1 to 3 repressed basal transcription to a similar extent as each expression vector alone; however, the presence of EFmDREAM blocked the five- to six-fold derepression following caffeine stimulation, indicating that heterotetramers between EFmDREAM and DREAM or KChIP-2 remained bound to DNA in the presence of high Ca^{2+} concentrations (Figure 2C). Block of the DRE-dependent caffeine-induced derepression by EFmDREAM has been previously shown to be still noticeable at ratios of up to 1:6 of EFmDREAM with DREAM or KChIP-2 (Ledo *et al*, 2000b). Thus, since

Figure 1 DREAM expression is downregulated after stimulation in lymphoid cells. (A) Quantitative real-time RT-PCR analysis of DREAM and KChIP-2 mRNA levels in thymocytes, splenocytes and purified T, CD4⁺ and CD8⁺ cells. Results are representative of two experiments. (B) DREAM mRNA levels in thymocytes or splenocytes at different times after stimulation with α CD3 (4 μ g/ml). Results are expressed as fold induction compared to non-stimulated cells and are representative of four experiments.

EFmDREAM blocks the functional response of DREAM and KChIP-2 to $[Ca^{2+}]_i$ increase, preventing derepression, hereafter, we will refer to EFmDREAM as a cross-dominant active mutant for the Ca^{2+} -mediated derepression function, though EFmDREAM is still a DRE-dependent repressor.

Figure 2 EFmDREAM interacts with DREAM and KChIP-2 and blocks Ca^{2+} -mediated derepression. (A) Yeast two-hybrid assay showing the specific interactions between EFmDREAM and DREAM or KChIP-2. pAS2-1 is the empty vector used as control. Use of low (DDO) or high (QDO) selective medium is indicated. (B) Coimmunoprecipitation analysis of Flag-DREAM and Myc-KChIP-2 after transient transfection in HeLa cells. (C) Repression of the DRE-containing pH3CAT reporter by DREAM, KChIP-2, EFmDREAM or the combinations of EFmDREAM with DREAM or KChIP-2 (in a 1:3 ratio) after transient cotransfection in HEK293 cells. Treatment with caffeine was started 6 h before harvesting the cells. Results are the mean ± s.d. of triplicates and are representative of three independent experiments.

Figure 3 The T-cell proliferative response is impaired in EFmDREAM tg mice. (A) Quantitative real-time RT-PCR analysis of the expression of EFmDREAM in the thymus and spleen of transgenic mice from lines 1 and 33. Results are the mean ± s.d. of 4–8 mice. (B) Flow-cytometric analysis of cells from thymus and spleen. Single-cell suspensions were stained with the indicated antibody combination. Positive cells (%) within a quadrant are indicated. The results are representative of six wt and six tg mice. (C) Cell proliferation was quantified in wt and tg thymocytes stimulated with the indicated amount of plate-bound αCD3 alone or αCD3 (6 μg/ml) in combination with αCD28 for 48 h. Results are the mean ± s.d. of four mice per group and are representative of three independent experiments. (D) Cell proliferation was analyzed in wt and tg thymocytes 48 h after stimulation with plate-bound αCD3 (6 μg/ml) in the presence or absence of recombinant murine IL-2 (20 ng/ml). Results are the mean ± s.d. of four mice per group and are representative of two independent experiments. Asterisks represent the statistical significance versus the appropriate control in each case. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Normal development but impaired proliferation in EFmDREAM tg T-cells

In order to characterize the role of DREAM and KChIP-2 in the immune function *in vivo*, we used tg mice that ubiquitously express the EFmDREAM mutant. Two lines of tg mice that showed different levels of transgene expression in lymphoid organs were used. As quantified by real-time RT-PCR (Figure 3A), expression of the transgene in the thymus and spleen of line 33 was seven- and six-fold, respectively, higher

than in line 1, in which the levels of EFmDREAM and endogenous DREAM mRNAs were at ratios 1:1 and 1:5 in thymus and spleen, respectively. Importantly, in spite of the different expression of the EFmDREAM transgene in the two lines, the results reported hereafter correspond to experiments performed with line 1 and were completely comparable to data from line 33, indicating that a low level of expression of EFmDREAM, in the same range as endogenous DREAM or lower, is sufficient to disturb DREAM and KChIP-2 function in T cells.

Analysis of 6–8-weeks-old tg mice showed that expression of EFmDREAM in lymphoid compartments did neither affect total cellularity nor the distribution of single- and double-positive CD4 and CD8T lymphocytes compared to wt thymus or peripheral lymphoid organs (Figure 3B). Furthermore, evaluation of several lymphoid markers in the thymus, spleen and lymph nodes of tg mice did not reveal major abnormalities in the development of the immune system (data not shown). Thus, overexpression of EFmDREAM does not affect the normal development of T lymphocytes.

Since TCR activation results in T-cell proliferation and sustained downregulation of DREAM mRNA levels, we wanted to check whether DREAM could function as a negative regulator of lymphocyte proliferation. Proliferation of thymocytes stimulated with α CD3 alone or in combination with α CD28 was significantly lower in EFmDREAM expressing T cells than in wt cells (Figure 3C), without change in apoptosis (Supplementary Figure S1). The reduced proliferative response after polyclonal stimulation was still noticeable even when the experiment was performed in the presence of saturating concentrations of recombinant IL-2, though in these conditions both wt and tg T cells showed an increased response (Figure 3D).

Given that the proliferative response in tg cells was not restored in the presence of IL-2, we analyzed the expression of IL-2R α chain (CD25) and CD69, well-characterized markers of the early phase of T-cell activation. Interestingly, the induction of CD25 and CD69 was smaller in both tg CD4+CD8– and CD4–CD8+ T cells as compared to the corresponding wt cells (Figure 4A and B). Thus, EFmDREAM is able to function as a negative regulator of TCR-induced lymphocyte proliferation.

Reduced cytokine expression in tg lymphoid cells

T-cell proliferation is dependent on cytokine gene expression, which has been shown to be Ca²⁺-dependent (Rao *et al*, 1997); hence, we analyzed the cytokine response in tg T cells. Importantly, induction of both IL-2 and IFN γ in tg thymocytes (Figure 5A and B) or purified CD4+ tg T cells from the spleen (Figure 5C and D) was weaker than in wt cells. The reduction was observed following α CD3 (Figure 5A–D) or α CD3 plus α CD28 (data not shown) stimulation and irrespectively of the antibody concentrations used, and remained significantly diminished up to 72 h after the stimulation (data not shown). Furthermore, the polarized effector T-cell tg populations Th1 and Th2 showed a significant reduction in IFN γ and IL-4 production, respectively (Figure 5E and F). Thus, in addition to IL-2 and IFN γ , expression of the Th2-specific cytokine IL-4 is as well repressed in EFmDREAM tg mice.

Moreover, we examined the effect of EFmDREAM on the primary antigen-specific T CD4+ cell response. Mice were immunized with ovalbumin (OVA)–complete Freund's

Figure 4 Reduced expression of activation markers in tg T cells. (A, B) Thymocytes were stimulated with plate-bound α CD3 (6 μ g/ml) and analyzed by cytometry for CD25, CD69, CD4 and CD8 expression after 24 h. (A) shows a representative example and (B) shows the % $\pm$ s.d. of CD25+ and CD69+ cells of the gated CD4+CD8– (left panel) or of the gated CD4–CD8+ (right panel) after stimulation of four mice per group. Asterisks represent statistical significance versus the appropriate control in each case. * P <0.05 and *** P <0.001.

adjuvant (CFA) and the polarization of the antigen-specific T-cell response in the draining lymph nodes was tested 9 days later. The number and the repartition of CD4+ and CD8+ cells in the lymph node were not different between wt and tg mice (data not shown). Thus, EFmDREAM does not modify lymphocyte recruitment in lymph nodes. Exposure to OVA induced IL-2 and IFN γ production (Figure 6A and B) and T-cell proliferation (Figure 6C) that were significantly reduced in EFmDREAM as compared to wt lymphocytes. These data indicate that expression of EFmDREAM downregulates cytokine production both after polyclonal activation and during antigen-specific T-cell response.

DREAM binds to and represses the IL-2, -4 and IFN γ promoters

Since DREAM and KChIP-2 proteins display different functions in different cellular compartments (Carrion *et al*, 1999; An *et al*, 2000; Ledo *et al*, 2002; Lilliehook *et al*, 2002), we first excluded that expression of EFmDREAM could modify the early and late signaling pathways that activate cytokine gene expression (Supplementary Figure S2) to then explore a direct repressor effect by DREAM and KChIP-2 at the transcriptional level. We searched for potential DRE sites (Carrion *et al*, 1998; Ledo *et al*, 2000b) in the promoter regions of IL-2, -4 and IFN γ genes. One direct DRE site (GTCA) and one inverted DRE (TGAC) are present in the mouse IL-2 and IL-4 genes at positions +74 and +39 downstream from the transcription initiation site, respectively, while the mouse IFN γ gene contains a DRE site at position +20 (Figure 7A). EMSA using thymocyte extracts from wt mice and oligo-

Figure 5 Decreased IL-2 and IFN γ protein in tg T cells. Thymocytes (A, B) or CD4+ T (C, D) from wt and tg mice were stimulated for 48 h with different concentrations of α CD3. Levels of IL-2 (A, C) or IFN γ (B, D) were quantified by ELISA. In (A, B), results are the mean $\pm$ s.d. of five mice per group and are representative of four independent experiments. In (C, D), results are the mean $\pm$ s.d. of triplicates and are representative of two experiments. (E, F) CD4+ T cells from wt and EFmDREAM mice were cultured under Th1 or Th2 differentiating conditions for 2 weeks and cytokine levels analyzed. Results are representative of three experiments. Asterisks represent statistical significance versus the appropriate control in each case * P <0.05, ** P <0.01 and *** P <0.001.

Figure 6 EFmDREAM decreases cytokine production following antigen-specific stimulation. Wt and tg mice were immunized s.c. with 50 μ g of OVA in CFA. Draining lymph node cells were harvested 9 days later and cultured in the presence of the indicated concentration of OVA. (A) IL-2, (B) IFN γ secretion and (C) antigen-induced proliferation were quantified after 48 h. Data are expressed as mean $\pm$ s.d. of five mice per group. Results are representative of two experiments. Asterisks represent statistical significance versus the appropriate control in each case. * P <0.05, ** P <0.01 and *** P <0.001.

nucleotide probes encompassing these DRE sites revealed specific probes retarded bands. As shown for DRE_{IL-2}, protein-DNA complexes could be competed by an excess of cold DRE_{IL-2} and DRE_{Dyn}, but were unaffected by competition with a cold unrelated oligonucleotide (Sp1). Incubation with a specific α DREAM antibody substantially reduced the protein-DNA complex of the DRE_{IL-2}, indicating that endogenous DREAM is participating in the retarded bands (Figure 7B). Similar results were observed with oligonucleotide probes encompassing DRE_{IFN γ} and DRE_{IL-4} sites (data not shown). These results indicate that the DRE sites identified in the promoters of *IL-2*, *IL-4* and *IFN γ* genes can bind DREAM *in vitro*. To show the dynamic binding of DREAM and KChIP-2 to DRE sites, we performed EMSA using extracts from HEK293 cells transiently transfected with DREAM or KChIP-2 and prepared in the presence of EGTA or CaCl₂. Binding of DREAM or KChIP-2 to the IL-2 probe was reduced in the presence of Ca²⁺ (Figure 7C). These data show that DREAM and KChIP-2 are able to bind DRE sites in a Ca²⁺-dependent manner.

To investigate whether the binding of DREAM and KChIP-2 to the DRE sites in cytokine promoters is functional and affects their basal transcription, we performed transient transfection experiments in HEK293 cells. Expression of DREAM or KChIP-2 significantly repressed basal transcription from the pIL2CAT, pIFN γ CAT or pIL4Luc reporters (Figure 7D and Supplementary Figure S3), but failed to repress transcription from reporter plasmids pIL2mDRECAT, pIFN γ mDRECAT and pIL4mDRELuc, in which the DRE sites are mutated (Figure 7D and Supplementary Figure S3). To test whether

the binding of EFmDREAM on cytokine promoters also occurs *in vivo*, we performed chromatin immunoprecipitation using a specific α DREAM antibody and genomic DNA from acutely dissociated tg thymocytes. α DREAM specifically immunoprecipitated chromatin fragments containing promoter regions for the three cytokine genes (Figure 7E), while an irrelevant antibody failed to do so (Figure 7E). Furthermore,

the α DREAM antibody did not immunoprecipitate chromatin containing the β -actin promoter that was used as a negative control (Figure 7E). Moreover, using genomic DNA from Jurkat T cells, the binding of DREAM to the IL-2 promoter was reduced by 65% after 2 h of Ca^{2+} stimulation (Figure 7F). Real-time quantitative PCR revealed reduced levels of IL-2 and $\text{IFN}\gamma$ both in tg thymocytes and splenocytes in basal conditions and at different times after stimulation, supporting a transcriptional effect of DREAM on cytokine gene expression (Supplementary Figure S4). Taken together, the results from EMSA, transient transfections and ChIP assays support a Ca^{2+} -dependent binding of DREAM and KChIP-2 to cytokine promoters.

Specific transient knockdown of endogenous DREAM or KChIP-2 upregulates IL-2 and $\text{IFN}\gamma$ expression

To substantiate the role of endogenous DREAM and KChIP-2 proteins as Ca^{2+} -dependent repressors of the cytokine response, we performed specific transient knockdown of DREAM or KChIP-2 expression and analyzed the effect on basal levels of IL-2 and $\text{IFN}\gamma$ mRNA. In these experiments, we used an antisense DREAM expression vector (AS-DREAM) previously shown to dramatically reduce basal levels of endogenous DREAM protein in PC12 cells and to increase basal DRE-dependent transcription (Ledo *et al*, 2002), and a similar antisense KChIP-2 expression vector (AS-KChIP-2). Since DREAM and KChIP-2 show a high homology in their nucleotide sequence, we first characterized the specificity of each antisense vector using transient cotransfection of AS-DREAM or AS-KChIP-2 and HA-tagged DREAM or KChIP-2 expression vectors in HEK293 cells. Importantly, AS-DREAM substantially reduced HA-DREAM protein levels (40% decrease) without affecting the level of HA-KChIP-2 protein or Myc-KIAA1007, an unrelated corepressor protein used as a loading control (Figure 8A, upper panels). Conversely, AS-KChIP-2 reduced HA-KChIP-2 protein levels (32% decrease) without affecting the level of HA-DREAM or Myc-KIAA1007 (Figure 8A, lower panels). Given that TCR engagement downmodulates DREAM expression, we analyzed the effect of specific transient knockdown of DREAM or KChIP-2 in wt splenocytes. The specific transient knockdown of DREAM or KChIP-2 in wt splenocytes showed a significant increase in IL-2 mRNA, and a modest but reproducible increase in $\text{IFN}\gamma$ mRNA (Figure 8B). Interestingly,

Figure 7 DREAM regulates the transcriptional activity of IL-2, -4 and $\text{IFN}\gamma$ promoters *in vivo*. (A) Putative DRE sites present in IL-2, $\text{IFN}\gamma$ and IL-4 regulatory regions. Arrows indicate the orientation of the DRE sequence. (B) EMSA using extracts from wt thymocytes and the DRE_{IL-2} probe. Competitions with DRE_{IL-2}, DRE_{Dyn} and SP1 are shown. The arrow indicates the specific DRE retarded band eliminated by preincubation with α DREAM (Ab1014). The asterisks mark the appearance of nonspecific bands after serum incubation. (C) EMSA using extracts from HEK293 cells transiently transfected with DREAM or KChIP-2 and the DRE_{IL-2} probe. The arrow indicates the specific retarded band that is competed with Ca^{2+} . (D) DRE-dependent DREAM repression of IL-2, -4 and $\text{IFN}\gamma$ promoters. Plasmid pRL-CMV was used to correct for transfection efficiency. The transfection experiments were repeated twice in quadruplicate. Asterisks represent statistical significance versus the appropriate control in each case. * $P < 0.05$ and *** $P < 0.001$. (E) ChIP using tg thymocytes or (F) Jurkat cells nonstimulated cells (–) or cells after 2 h of PMA/Iono stimulation (+).

Figure 8 Specific transient knockdown of DREAM or KChIP-2 induces cytokine expression in splenocytes. **(A)** Western blot analysis of HEK293 cells transiently cotransfected with AS-DREAM or AS-KChIP-2 together with HA-DREAM or HA-KChIP-2 and Myc-KIAA1007 (loading control). The upper panel shows the specific knockdown of the HA-DREAM protein by AS-DREAM (arrowhead), and the lack of effect on HA-KChIP-2 (asterisk) or Myc-KIAA1007. The lower panel shows the specific knockdown of the HA-KChIP-2 protein by AS-KChIP-2 (asterisk) and the lack of effect on HA-DREAM (arrowhead) or Myc-KIAA1007. **(B)** Quantitative analysis of IL-2 and IFN γ mRNA expression by real-time quantitative RT-PCR in splenocytes from wt mice 48 h following electroporation with empty vector pcDNA3, AS-DREAM or AS-KChIP-2 or both. Results are expressed as fold induction compared to cells transfected with pcDNA3, and are the mean $\pm$ s.d. from two experiments in triplicates. Asterisks represent statistical significance versus the appropriate control in each case. * $P < 0.05$ and ** $P < 0.01$.

cotransfection with both antisense vectors resulted in a six- and two-fold increase in IL-2 and IFN γ mRNA, respectively (Figure 8B). This represents 10 and 28% of the maximum inducible level after 4 h of α CD3 stimulation for IL-2 and IFN γ , respectively. Thus, by only removing the DREAM- and/or KChIP-2-mediated repression, we observed a substantial induction of the basal expression without activating signaling pathways, that is, NFAT and MEF-2, known to participate in the up-regulation of cytokines. The level of IL-4 mRNA was below the detection limit both in vector- and in AS-DREAM-transfected cells (data not shown), which agrees with the Th1-prone cytokine response reported for B6 mice. In conclusion, the transcriptional repressors DREAM and KChIP-2 control basal expression of IL-2 and IFN γ genes.

Discussion

DREAM is a multifunctional protein able to specifically interact with DNA and/or other proteins to execute defined

functions in different cell compartments. In this study, we show that in T lymphocytes DREAM regulates the expression of three cytokine genes, IL-2, IL-4 and IFN γ . The proposed repressor mechanism involves recognition and binding to specific DREs located in their promoters. As previously shown for other Ca²⁺-activated genes like c-fos, ICER and AA-NAT (Carrion *et al*, 1999; Link *et al*, 2004), in IL-2 and IL-4 promoters DREAM binds to a doublet with one direct and one inverted DRE repeat located downstream from the transcription initiation site and downregulates their activity. In the case of the IFN γ gene, like for the prodynorphin and the fra-2 promoters, binding of DREAM to a single DRE site downstream from the TATA box is enough to repress transcription (Carrion *et al*, 1998, 1999; Link *et al*, 2004). Binding of DREAM or EFmDREAM to DRE sites downstream from the TATA box represses transcription of target genes by a mechanism sensitive to inhibitors of histone deacetylases (JR Naranjo, unpublished results), suggesting a role for DREAM as a Ca²⁺-regulated repressor in the transcription initiation complex. Whether the presence of several DRE core cassettes may contribute to the recruitment of a different repressor and to a tighter repressive control of target genes is presently unknown. Interestingly, a similar Ca²⁺-dependent derepression mechanism has been described for the IL-2 promoter and involves unbinding of cabin and the mSin3A repressor complex from transcriptional activators of the MEF2 class, which then can recruit CBP to activate transcription (Youn and Liu, 2000; Pan *et al*, 2004). Furthermore, expression of cytokine genes is also regulated by nuclear CREB/CBP complexes (Glimcher and Murphy, 2000). We have previously shown that the Ca²⁺-independent interaction between EFmDREAM and CREB/CREM proteins disturbs the recruitment of CBP by phosphoCREB and blocks CRE-dependent transcription (Ledo *et al*, 2002); hence, our data are also compatible with a CRE-dependent mechanism operating in EFmDREAM tg lymphocytes to decrease cytokine gene transcription. Future experiments using DREAM double mutants unable to respond to Ca²⁺ stimulation and to bind CREB will define more precisely the contribution of the CRE-dependent pathway in the regulation by DREAM of cytokine genes. The functional interaction between DREAM and NFAT or MEF2, transcription factors important for Ca²⁺-dependent regulation of cytokines, is presently under investigation.

DREAM is highly expressed in different brain areas and in peripheral organs, including thyroid gland, testis and the thymus (Carrion *et al*, 1999). Here, we show that DREAM is constitutively expressed in unstimulated T lymphocytes and, more importantly, we report that DREAM is rapidly downregulated after stimulation. Activation of T cells has been associated with the downregulation of several transcription factors. They include LKLF, a nucleoprotein involved in the quiescence of T cells (Kuo *et al*, 1997; Buckley *et al*, 2001), Ets-2 that participates in the repression of the IL-2 gene (Argyropoulos *et al*, 2004) and Tob that acts as a negative regulator of activation in anergic and quiescent T cells (Tzachanis *et al*, 2001). The activation-dependent decrease of DREAM expression together with the profound effects of EFmDREAM on cytokine expression and T-cell proliferation indicates that endogenous DREAM, like these other proteins, might play an important role in regulating the function of resting T cells *in vivo*. Notably, in the case of DREAM, Ca²⁺-dependent T-cell activation simultaneously derepresses

target genes and downregulates the expression of the repressor itself. Thus, a dual mechanism operates to ensure the release of DREAM repression to allow T-cell activation.

DREAM is a member of the DREAM/KChIP family of neuronal Ca^{2+} sensors consisting of four independent genes, KChIP-1 to -4, being DREAM identical to KChIP-3 (An *et al*, 2000). All four DREAM/KChIP genes encode proteins structurally related that are able to bind DRE sites and block DRE-dependent transcription (Link *et al*, 2004). In addition to DREAM, KChIP-2 is expressed in different T-cell subpopulations and we have shown that after transient transfection KChIP-2 binds to DRE sites in a Ca^{2+} -dependent manner and represses cytokine promoters. Nevertheless, the role of KChIP-2 may not be strictly similar, since the basal KChIP-2 mRNA level is lower than that of DREAM and, more importantly, KChIP-2 expression is not downregulated following TCR engagement. The functional redundancy between DREAM and KChIP-2 and their coexpression in lymphocytes may account for the lack of phenotype in the immune system in DREAM $^{-/-}$ animals (Cheng *et al*, 2002). On the other hand, in our tg model, expression of cross-dominant active mutant EFmDREAM blocks the Ca^{2+} -dependent derepressor function of both DREAM and KChIP-2, which results in repression of cytokine genes.

At the plasma membrane, DREAM/KChIP proteins interact with Kv4 potassium channels, modulating type-A potassium currents (An *et al*, 2000). Expression of Kv4.2 channels has been reported in T cells (Koni *et al*, 2003), which opens up the possibility that EFmDREAM may also function by reducing normal potassium currents in T cells. However, several lines of evidence oppose a crucial role for Kv channels in murine lymphocyte physiology: (i) genetic ablation of the Kv1.3 mouse gene, encoding the most abundant voltage-dependent K^{+} channel in T cells, does not result in abnormalities in T-cell development or obvious defects in thymocyte apoptosis or T-cell proliferation (Koni *et al*, 2003), (ii) in Kv1.3 $^{-/-}$ mice, voltage-dependent potassium currents are not detectable (Koni *et al*, 2003) suggesting a minor functional significance of other Kv channels that are expressed in T murine cells, (iii) it has been proposed that Kv channels maintain a negative membrane potential to facilitate Ca^{2+} influx in T cells (Zweifach and Lewis, 1993); however, in our tg lymphocytes, expression of EFmDREAM does not affect the increase of $[\text{Ca}^{2+}]_i$ following TCR engagement and (iv) importantly, type-A potassium currents in wt and EFmDREAM expressing neurons are essentially identical, suggesting that the EFmDREAM is not acting as a dominant mutant for the channel function in the context of endogenous expression of DREAM and other KChIPs (JR Naranjo, unpublished observations). Taken together, these data indicate that the decrease of cytokine levels in EFmDREAM murine lymphocytes is not related to a change in potassium currents in tg T cells. A different scenario might occur in human T cells, where inhibitors of Kv1.3 K^{+} channels have profound effects on T-cell activation (Chandy *et al*, 2004).

In the cytoplasm, DREAM/KChIP-3, also named calsenilin (Buxbaum *et al*, 1998), has been associated with antiapoptotic or proapoptotic functions in different experimental systems (Jo *et al*, 2001; Sanz *et al*, 2001). In our work, spontaneous cell death or compromised T-cell viability following stimulation with various apoptotic stimuli was not different in tg lymphocytes compared to their wt counter-

parts. These results are in agreement with: (i) the normal TCR-induced Ca^{2+} signaling in EFmDREAM tg T cells and (ii) the IL-3-dependent antiapoptotic function of DREAM in hematopoietic cell lines through the control of *Hrk* gene expression (Sanz *et al*, 2001). On the other hand, these results do not support the proposed proapoptotic function for DREAM in Jurkat cells or human neuroblastoma HB4 cells (Jo *et al*, 2001; Lilliehook *et al*, 2002). In this regard, it is important to note that DREAM mRNA and protein levels are highly abundant in basal conditions in different adult human tissues like brain, heart, thymus, thyroid gland (Carrion *et al*, 1999) and reproductive organs, as well as in Jurkat cells and several human neuroblastoma cell lines (JR Naranjo, unpublished data). Thus, at the present state of knowledge, it is not feasible to explain the physiological meaning of the proposed proapoptotic role of DREAM/calsenilin.

The expression of EFmDREAM in tg lymphocytes results in a dramatic reduction in the proliferative response and a decreased expression of three key cytokines important for T-cell physiology. Since the repression of cytokine genes occurs in Th1 and Th2 differentiated lymphocytes, further studies in EFmDREAM tg mice will explore the possibility to prevent Th1- and Th2-mediated immune disorders. Supporting a central role for DREAM and KChIP-2 in the immune response, transient knockdown of endogenous DREAM and/or KChIP-2 in wt splenocytes is associated with a significant induction of the basal expression of IL-2 and IFN γ mRNAs. This result contradicts the lack of phenotype in DREAM $^{-/-}$ mice. However, as shown for retinoblastoma (Sage *et al*, 2003) and NFAT (Benedito *et al*, 2005) proteins, acute knockdown of DREAM in cultured splenocytes might reveal phenotypes that are masked in conventional knockout animals because of compensatory mechanisms that occur prior to the generation of the cells under study. Furthermore, it is important to notice that the increase in cytokine expression after transient knockdown of DREAM or KChIP-2 is reproducibly observed in spite of the low transfection efficiency, about 20%, with the electroporation method used for freshly prepared splenocytes. These data indicate that DREAM and KChIP-2, like NFAT, are central components of the Ca^{2+} -regulated transcriptional network that control expression of cytokine genes in lymphocytes. Further studies using DNA microarrays are in progress to uncover other potential target genes regulated by DREAM and/or KChIP-2 in immune cells. The relevant role of DREAM and KChIP-2 in cytokine expression and T-cell physiology identifies DREAM and KChIP-2 as potential new targets for immunosuppressant drugs.

Materials and methods

Generation of tg mice

The cDNA encoding human DREAM with two amino-acid substitutions at EF-hands 2, 3 and 4 (EFmDREAM) was inserted downstream of the human CaMK-II α promoter (Mayford *et al*, 1996) in a bicistronic expression vector containing an IRES and the LacZ reporter gene. The tg cassette was microinjected into the pronuclei of one-cell embryos (C57BL/6 $\times$ CBA F1) using standard techniques. Tg progeny were identified by Southern blot and qualitative PCR of tail DNA using specific primers: forward 5'-TTGCACTGCACGGCAGATACACTTGCTGA-3' and reverse 5'-CCACTGGTGTGGGCCATAATTCAATTCGC-3'. An amplified fragment of 326bp indicated the presence of the transgene. Founder males were backcrossed to C57BL/6 females to generate lines that were maintained as heterozygotes. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Cell cultures and flow cytometry analysis

Thymocytes, splenocytes and Jurkat cells were grown in RPMI with Glutamax containing 10% FCS (Invitrogen), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), penicillin (100 U/ml)/streptomycin (100 µg/ml) and 50 µM β-mercaptoethanol, and were maintained at 37°C in an atmosphere at 5% CO₂. For stimulation, 2 × 10⁶ thymocytes were cultured in 24-well plates, previously coated with different concentrations of αCD3 (Pharmingen) in 0.1 M Tris buffer (pH 8) overnight at 4°C. The antibody αCD28 (Pharmingen) was used in solution, added to the culture medium. Supernatants were collected between 24 and 72 h for cytokine analysis. For T-cell proliferation assays, 0.4 × 10⁶ thymocytes were cultured in 96-well plates and the cell cultures were pulsed for 16 h with 1 Ci [³H]thymidine (40 Ci/nmol, Amersham) before harvesting on glass fiber filters. Incorporation of [³H]dThd was measured by direct counting using an automatic β-plate counter (Wallac). IL-2, -4 and IFNγ production was quantified by a two-site sandwich ELISA protocol with pairs of monoclonal Abs (Pharmingen) (Guery *et al*, 1996). The detection limit in these assays was 15 pg/ml.

The subpopulations of T cells were isolated by negative selection by sequential incubation with a cocktail of antibodies and M-450 αrat IgG Dynabeads (Dyna), followed by magnetic cell separation as described (Foucras *et al*, 2000). The purity of the different subpopulations was above 95% assayed by flow cytometry. CD4 + T cells (0.2 × 10⁶) were stimulated in 96-well plates with αCD3 during 48 h before quantification of cytokines by ELISA. *In vitro* Th differentiation was performed as described in Supplementary data.

Single-cell populations of thymic and spleen cells were prepared and 1 × 10⁶ cells were stained in PBS/1% FCS, with the respective antibodies for 20 min at 4°C. Monoclonal antibodies were FITC-conjugated (CD4, CD3, B220, CD23, Pan-NK, TCRγδ), PE-conjugated (CD8, CD44, IgM) or biotin-conjugated (CD25, CD69, TCRβ, CD62L, IgD) (Pharmingen). Biotin-conjugated mAbs were developed using streptavidin Cy-Chrome. Analysis was performed on an Epics XL cytometer (Coulter).

HEK293 and HeLa cells, two lines devoid of DREAM and KChIP-2 expression, were maintained in DMEM with Glutamax containing 10% FCS and penicillin (100 U/ml)/streptomycin (100 µg/ml) and were maintained at 37°C in an atmosphere at 5% CO₂. Jurkat cells were stimulated by phorbol myristate acetate (PMA, Sigma) (10 ng/ml) and ionomycin (Iono, Sigma) (1 µg/ml) for 2 h.

Immunization

The mice were immunized in the hind footpads with OVA in CFA and the polarization of CD4 + T-cell response was tested after *in vitro* restimulation with OVA as described (Maret *et al*, 2003).

Qualitative and quantitative real-time RT-PCR

RNA was isolated from whole tissues or cell suspensions using TRIzol (Invitrogen), treated with DNase (Ambion) and reverse transcribed using hexamer primers and Moloney murine leukemia virus RT. To confirm the absence of genomic DNA, each sample was processed in parallel without RT. Qualitative RT-PCR was performed as described (Link *et al*, 2004). Quantitative real-time PCR for endogenous DREAM and EFmDREAM was performed using a pair of primers able to amplify both: forward 5'-CACCTATGCACAC TTCCTCTTCA-3' and reverse 5'-ACCACAAAGTCCTCAA GTGGAT-3' and two TaqMan MGB probes; FAM-5'-TGCCTTCGATGCTGAT-3'-MGB and VIC-5'-CGCCTTTGCTGCGGC-3'-MGB, specific for DREAM and EFmDREAM, respectively. The results were normalized by quantification of HPRT mRNA using the specific primers forward 5'-TTGGATACAGGCCAGACTTTGTT-3' and reverse 5'-CTGAAGTACT CATTATAGTCAAGGGCATA-3', and the probe FAM-5'-TTGAAATCC AGACAAGTTT-3'-MGB.

Yeast two-hybrid screening

The yeast two-hybrid assay was performed as recommended (Clontech Matchmaker protocol handbook) using the yeast strain *Saccharomyces cerevisiae* AH109 (Clontech). The bait plasmid was constructed by inserting cDNA encoding EFmDREAM into the pAS2-1 vector. A library plasmid containing EFmDREAM or EFmKChIP-2, mutated at one of the functional EF-hands, were prepared by inserting respective cDNA into the pACT2 vector. Positive cotransformants were selected by sequential plating on plasmid selective media (without leucine and tryptophan: DDO), and on interaction selective media (without leucine, tryptophan, histidine and adenine: QDO). Cotransformation with EFmDREAM

or EFmKChIP-2 with empty pAS2-1 vector was used as negative control for the specificity of the interaction.

Electrophoretic mobility shift assays (EMSAs)

EMSA was performed as described (Ledo *et al*, 2002) using a double-stranded oligonucleotide probe encompassing the mouse IL-2 DRE double site (DRE_{IL-2}) 5'-ATCCTGTGTACATTGACACTT GTG-3'. For DNA competition, a two- to five-fold excess of the Dyn DRE (DRE_{Dyn}) site and the Sp1 site (Carrión *et al*, 1998, 1999) were used. Cells extracts from HEK293 cells transfected with DREAM or KChIP-2 were prepared as described (Ledo *et al*, 2002) in the presence of 10 µM of EGTA or 10 µM of CaCl₂.

Plasmid constructions

The vectors containing wt DREAM cloned in the sense or antisense orientation (AS-DREAM), the mutant EFmDREAM and the reporter plasmid pHD3CAT have been described previously (Carrión *et al*, 1999; Ledo *et al*, 2002). For details about reporter plasmids and expression vectors, see Supplementary data.

Coimmunoprecipitation

Whole-cell extracts were prepared in lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM KCl, 1 mM EDTA and 1% Triton X-100 and a protease inhibitor cocktail (Calbiochem). Coimmunoprecipitation was performed in lysis buffer containing 0.25% Triton X-100 overnight at 4°C using mouse αFlag agarose (Sigma). After five washes in lysis buffer containing 300 mM KCl and 0.25% Triton X-100, proteins were eluted in SDS sample buffer, resolved in SDS-PAGE and electroblotted onto Immobilon-P (Millipore). Blots were developed using a rabbit αMyc (Abcam).

Transient transfection and reporter assays

Transient transfections in HEK293 cells and reporter assays were carried out as described (Carrión *et al*, 1998; Rivas *et al*, 2004). In all cases, pRL-CMV (Renilla) (1 µg) was used to correct for transfection efficiency. Results are shown as relative activity compared to the controls in each experiment.

Knockdown of DREAM

Transfection in HEK293 cells was carried out by the Ca²⁺ phosphate method with AS-DREAM or AS-KChIP-2 together with HA-DREAM or HA-KChIP-2 and Myc-KIAA1007 that was used to control for equal loading. Western blot analysis was performed 24 h after transfection. The membranes were incubated sequentially with mouse αHA (Santa-Cruz) and with rabbit αMyc (Abcam). Freshly prepared splenocytes were electroporated with AS-DREAM and/or AS-KChIP-2 using the Nucleofector kit according to the manufacturer's protocol (Amaya Inc.) and cytokine mRNA levels were quantified 48 h later.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as described, with minor modifications (Takahashi *et al*, 2000). Chromatin from tg thymocytes or Jurkat T cells were subjected to immunoprecipitation with 15 µg of Ab 1014 (raised in rabbit using full-length recombinant DREAM and affinity-purified) or an irrelevant antibody (rabbit αGST, Santa Cruz, or αFlag, Sigma). Immunoprecipitated DNA was subjected to semiquantitative PCR with specific primers (Supplementary data). The PCR was performed within the linear range using trace amounts of [³²P]dCTP.

Statistical analysis

Statistical significance of the differences between experimental groups was analyzed using Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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