The transcription factor DREAM represses the deubiquitinase A20 and mediates inflammation

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Here we found that the transcription repressor DREAM bound to the promoter of the gene encoding A20 to repress expression of this deubiquitinase that suppresses inflammatory NF-κB signaling. DREAM-deficient mice displayed persistent and unchecked A20 expression in response to endotoxin. DREAM functioned by transcriptionally repressing A20 through binding to downstream regulatory elements (DREs). In contrast, binding of the transcription factor USF1 to the DRE-associated E-box domain in the gene encoding A20 activated its expression in response to inflammatory stimuli. Our studies define the critical opposing functions of DREAM and USF1 in inhibiting and inducing A20 expression, respectively, and thereby the strength of NF-κB signaling. Targeting of DREAM to induce USF1-mediated A20 expression is therefore a potential anti-inflammatory strategy for the treatment of diseases associated with unconstrained NF-κB activity, such as acute lung injury.

The transcriptional repressor DREAM is a member of a Ca2+-binding family that contains four Ca2+-binding motifs (EF hands) that interact as a tetramer with downstream regulatory elements (DREs) to inhibit transcription1. Ca2+ signaling has been linked to the activation of DREAM because a decrease in the intracellular concentration of Ca2+ increases the affinity with which DREAM binds to DREs and thereby transcriptionally represses the target genes1. The binding of DREAM to DREs is reversed by activation of protein kinase A by cAMP through phosphorylation of the DREAM-interacting protein α-CREM, which blocks the binding of DREAM to DREs2,3. DREAM is involved in sensing pain4,5, a hallmark of inflammation. DREAM is expressed in pain-sensing areas of the spinal cord in association with κ-opiate receptors4,5, but it is also present in cells of the immune system, such as T lymphocytes and B lymphocytes6,7, in which its function is not understood. Mice with transgenic expression of a dominant-active DREAM mutant show markedly reduced production of the cytokines interleukin 2 (IL-2), IL-4 and interferon-γ, increased B cell numbers and decreased production of immunoglobulin G8,9. As activation of the transcription factor NF-κB may regulate some of those responses, we surmised that DREAM might be involved in the mechanism of inflammation through its ability to control NF-κB signaling. In addition to the transcription-repressive function of DREAM, the transcription factor USF1, which binds to the E-box domain associated with DREs on the promoter of the gene encoding the deubiquitinase A20, is also involved in initiating transcription of that gene8. The question arises, therefore, of whether DREAM and USF1 function cooperatively to coordinate transcription of the gene encoding A20 and thus the magnitude of proinflammatory NF-κB signaling.

NF-κB is composed of dimers of five proteins (p50, p52, p65 (RelA), RelB and c-Rel) that exist in an inactive form in the cytoplasm bound to three inhibitory proteins (IκBα, IκBβ and IκBε)9–11. Activation of NF-κB in the classical pathway requires activation of the IκB kinase (IKK) complex, which contains the kinases IKKα and IKKβ and the regulatory protein IKKγ. Activated IKK complexes phosphorylate IκBα and IκBβ, which leads to their proteolytic degradation and frees NF-κB dimers to translocate to the nucleus to induce the expression of various target genes. Signaling via Toll-like receptors, the receptor for IL-1, the receptor for tumor-necrosis factor (TNF) and G protein–coupled receptors induces the activation of IKK complexes, which results in NF-κB activity9–12. The identification of feedback checks on NF-κB activation has been of great interest as possible drug targets. A key downregulator of NF-κB is A20 (ref. 13), first identified as an antipoptotic protein in human umbilical vein endothelial cells14. NF-κB induces A20 expression within hours of being activated by TNF or lipopolysaccharide (LPS)15–17. A20 in turn inhibits the functions of the transcription factors TRAF2, TRAF5 and TRAF6, the adaptor RIP1, the effector RIP2, and IKKγ upstream of IKK complexes by editing ubiquitin chains on those proteins essential for IKK activation18–21. The ovarian tumor domain of A20 mediates the deubiquitination of Lys63 (K63)-linked polyubiquitinated proteins, and the carboxy (C)-terminal zinc finger domain of A20 has ubiquitin ligase activity that mediates K48-linked polyubiquitination of target proteins to induce their proteasomal degradation and terminate NF-κB signaling19,20. A20-deficient mice develop spontaneous inflammation and cachexia and die prematurely22. Targeted cardiac overexpression of A20 improves outcome in a mouse model of myocardial infarction.
by suppressing inflammation. A20 overexpression is also protective in a mouse model of atherosclerosis, whereas haploinsufficiency in A20 results in severe atherosclerosis. Such studies underscore the importance of A20 in restricting inflammation. However, the transcriptional mechanisms of A20 expression are poorly understood. Therefore, to gain insight into these mechanisms, we analyzed the promoter of the gene encoding A20 and detected DREAM-binding DREs both upstream and downstream of the transcription start site (TSS) in intron 1 of human and mouse genes encoding A20. In addition, we found that the E-box domain was an integral component of DREs. Further, we observed that both basal and endotoxin-induced A20 expression in endothelial cells and macrophages was markedly augmented in mice deficient in DREAM (Knip3+/−; called ‘Dream−/−’ here), which in turn prevented the activation of NF-kB signaling. Production of the inflammatory cytokines IL-6, MCP-1 and TNF, the sequestration of polymorphonuclear neutrophils (PMNs) in the lungs and expression of the integrin ligand ICAM-1 in the lungs were suppressed following intraperitoneal injection of LPS (10 mg per kg body weight). LPS induced severe lung injury and sequestration of PMNs in the lungs and increased expression of ICAM-1 protein in a time-dependent manner in wild-type mice, whereas those responses were significantly reduced in Dream−/− mice (Fig. 1a–c). To quantify changes in lung vascular permeability (an index of inflammatory injury), we measured the pulmonary microvessel filtration coefficient. LPS significantly increased this value in wild-type lungs, whereas deletion of DREAM abrogated this response (Fig. 1d). We also observed considerably fewer PMNs and less MPO activity in bronchoalveolar lavage fluid (BALF) from Dream−/− mice than in that from wild-type mice (Fig. 1e). In addition, in response to LPS, concentrations of the proinflammatory mediators IL-6, MCP-1 and TNF in BALF from Dream−/− mice were lower than those in BALF from wild-type mice (Fig. 1f). In survival studies, 90% of wild-type mice died within 6 d of LPS administration, whereas only 50% of Dream−/− mice died during the same period, and thereafter there were no further deaths (Fig. 1g). To confirm the findings reported above in a severe model of sepsis, we used cecal ligation and puncture (CLP) to induce polymicrobial sepsis in age-, sex- and weight-matched wild-type and Dream−/− mice. In these studies, we noted 100% mortality in wild-type mice within 36 h of CLP, whereas only 20% of Dream−/− mice died in the same period, and thereafter there were no further deaths (Fig. 1h). To confirm the findings reported above in a severe model of sepsis, we used cecal ligation and puncture (CLP) to induce polymicrobial sepsis in age-, sex- and weight-matched wild-type and Dream−/− mice. In these studies, we noted 100% mortality in wild-type mice within 36 h of CLP, whereas only 20% of Dream−/− mice died in the same period, and thereafter there were no further deaths (Fig. 1h).

RESULTS

DREAM mediates inflammatory lung injury and mortality

We first assessed the expression of ICAM-1, pathological changes in lungs, and lung myeloperoxidase (MPO) activity (an indicator of PMN sequestration) in wild-type and Dream−/− mice at various times after intraperitoneal injection of LPS (10 mg per kg body weight). LPS induced severe lung injury and sequestration of PMNs in the lungs and increased expression of ICAM-1 protein in a time-dependent manner in wild-type mice, whereas those responses were significantly reduced in Dream−/− mice (Fig. 1a–c). To quantify changes in lung vascular permeability (an index of inflammatory injury), we measured the pulmonary microvessel filtration coefficient. LPS significantly increased this value in wild-type lungs, whereas deletion of DREAM abrogated this response (Fig. 1d). We also observed considerably fewer PMNs and less MPO activity in bronchoalveolar lavage fluid (BALF) from Dream−/− mice than in that from wild-type mice (Fig. 1e). In addition, in response to LPS, concentrations of the proinflammatory mediators IL-6, MCP-1 and TNF in BALF from Dream−/− mice were lower than those in BALF from wild-type mice (Fig. 1f). In survival studies, 90% of wild-type mice died within 6 d of LPS administration, whereas only 50% of Dream−/− mice died during the same period, and thereafter there were no further deaths (Fig. 1g). To confirm the findings reported above in a severe model of sepsis, we used cecal ligation and puncture (CLP) to induce polymicrobial sepsis in age-, sex- and weight-matched wild-type and Dream−/− mice. In these studies, we noted 100% mortality in wild-type mice within 36 h of CLP, whereas only 20% of Dream−/− mice died in the same period (Fig. 1h); 50% of Dream−/− mice were alive 3 d after CLP, and 40% remained alive more than 2 weeks after CLP (Fig. 1h).
To determine whether DREAM deficiency in hematopoietic cells, rather than its deficiency in nonhematopoietic cells such as endothelial cells (which constitute ~50% of the total lung cell population) and epithelial cells, was responsible for the attenuation of inflammation in Dream−/− mice, we transplanted wild-type mouse bone marrow (BM) cells into lethally irradiated Dream−/− mice. We used those chimeras (WT-BM→Dream−/−) for experiments 6 weeks after transplantation. Analysis of the male-specific Sry gene in DNA isolated from blood cells of recipient mice showed highly efficient reconstitution of wild-type bone marrow (Supplementary Fig. 1a).

After challenging wild-type, Dream−/− and WT-BM→Dream−/− mice similarly with LPS, we found that sequestration of PMNs in the lungs, the presence of chemokines and cytokines (MCP-1, IL-6 and TNF) in BALF and the expression of ICAM-1 in the lungs of WT-BM→Dream−/− mice were not substantially different from that of Dream−/− mice (Supplementary Fig. 1b–d).

After LPS challenge, the concentrations of MCP-1, IL-6 and TNF in serum from WT-BM→Dream−/− mice were not substantially different from those in serum from wild-type mice (Supplementary Fig. 1e). However, the concentration of MCP-1 in serum from LPS-treated Dream−/− mice was substantially lower than that of their wild-type counterparts (Supplementary Fig. 1e), which suggested that unlike the changes in the serum concentrations of IL-6 and TNF, the main source of MCP-1 was hematopoietic cells; this finding was consistent with a study showing that MCP-1 is generated mainly by hematopoietic cells. The mortality of WT-BM→Dream−/− mice resembled that of Dream−/− mice (Supplementary Fig. 1f). These results together suggested that DREAM signaling in hematopoietic cells was not responsible for the full-blown inflammatory lung-injury response.

Figure 2. DREAM and USF1 coordinate TNFAIP3 transcription. (a) DREAM-binding DREs (DRE1–DRE4) and other transcription factor–binding sites in human TNFAIP3; DRE3 overlaps with the E-box sequence. Numbers indicate position relative to the TSS. (b–f) ChIP assay of the interaction of DREAM with TNFAIP3 DRE1 (b), DRE2 (c), DRE3 (d) or DRE4 (e) in human lung microvessel endothelial cells challenged for 0–180 min (horizontal axes) with LPS (1 μg/ml) or TNF (500 units/ml); results were normalized to those of input DNA and are presented relative to basal values. (d) ∗P < 0.05, 0 min versus 120 min, and **P < 0.001, 0 min versus 30, 60 or 90 min (unpaired two-tailed Student’s t-test). (e) ∗P < 0.01, 0 versus 90 min, and **P < 0.001, 0 min versus 30 or 60 min (unpaired two-tailed Student’s t-test). (f) ChIP assay of the interaction of USF1 with the DRE3 E-box in cells as in b–e; results are presented relative to basal values. *P < 0.001, 0 versus 60 min or 90 min (unpaired two-tailed Student’s t-test). (g) Chromoblot analysis of USF1 (top left) in human umbilical vein endothelial cells transfected with Scr-siRNA (left) or USF1-specific siRNA (right), and of A20 (bottom left) in human umbilical vein endothelial cells left untransfected (UT control), left or transfected with Scr-siRNA (middle) or USF1-specific siRNA (right) and then not treated with TNF (−) or treated for 4 h with TNF (+). Right, quantification of results at bottom left (relative to β-actin); each symbol in bars represents an individual sample. ∗P < 0.001, untreated versus TNF, or Scr-siRNA versus USF1-specific siRNA (unpaired two-tailed Student’s t-test). Data are pooled from four experiments (b–f; mean and s.d.) or are representative of three experiments (g; mean ± s.e.m.).
within 90 min in response to LPS or TNF (an increase opposite to the diminished binding of DREAM during this period) and, as with the binding of DREAM, the binding of USF1 returned to baseline within 120 min of stimulation (Fig. 2f). To investigate whether USF1 was essential for regulating TNFAIP3 transcription, we next silenced USF1 expression in human umbilical vein endothelial cells and measured A20 expression. Knockdown of USF1 prevented TNF-induced A20 expression (Fig. 2f). These results showed that DREAM functioned basally to repress transcription of TNFAIP3, but in response to inflammatory stimuli, DREAM dissociated from the DREs, and USF1 bound to the DRE3 E-box to signal TNFAIP3 transcription.

To address whether DREAM also represses the mouse gene encoding A20 (Tnfaip3), we analyzed the Tnfaip3 promoter sequence and observed that this promoter had a DRE site downstream of the TSS in intron 1 and two additional DRE sites upstream of the TSS (Fig. 3a). Similar to DRE3 of TNFAIP3, DRE2 of Tnfaip3 had an overlapping E-box sequence (Fig. 3a). We observed that in basal conditions, DREAM bound mainly to the DRE3 domain of the Tnfaip3 promoter and, to a lesser extent, to DRE2 (Fig. 3b–d). As expected, we did not find DREAM binding in DREAM−/− cells (Fig. 3b–d). The binding of DREAM to DRE2 and DRE3 decreased after LPS challenge in a time-dependent manner in wild-type macrophages, as noted for the human cells above, and returned to baseline by 90 min (Fig. 3c,d). We also observed a positive correlation between the amount of DREAM protein in the nucleus and the binding of DREAM to the DREs (Supplementary Fig. 2a).
Since DRE2 in the Tnfaip3 promoter overlapped with the E-box, we assessed the interaction between the DRE2 E-box and USF1. The binding of USF1 to the DRE2 E-box increased maximally within 90 min of challenge with LPS (Fig. 3e), similar to the results obtained above for human cells showing temporal binding of USF1 to the DRE3 E-box of Tnfaip3 (Fig. 2f). USF1 bound to the DRE2 E-box of Tnfaip3 in basal conditions in Dream−/− macrophages (Fig. 3e), which indicated a role for such binding in mediating the persistent Tnfaip3 transcription in the absence of the repressive effect of DREAM. The binding of USF1 to the DRE E-box increased in Dream−/− macrophages until it peaked, at 90 min after the LPS challenge, at the same level as in wild-type cells (Fig. 3e); this indicated that USF1 continued to bind to the Tnfaip3 promoter in the absence of DREAM binding. These findings collectively demonstrated that similar to the results obtained for Tnfaip3, DREAM repressed Tnfaip3 transcription by binding to DREs, whereas the binding of USF1 to the DRE-associated E-box domain in response to inflammatory stimuli promoted Tnfaip3 transcription. Thus, these results analyzing the promoter of the gene encoding A20 suggested a model for the regulation of transcription of this gene by the coordinated actions of DREAM and USF1 (Supplementary Fig. 2b).

DREAM modulates A20 expression during inflammation

We observed that DREAM protein was expressed in variety of cells involved in inflammation, including lung endothelial cells (LEC), PMNs and bone marrow–derived macrophages (BMDMs) of mice (Fig. 4a–c). There was approximately threefold higher expression of A20 protein in LECs, PMNs and BMDMs from Dream−/− mice than in their wild-type counterparts (Fig. 4a–c) consistent with the role of DREAM in suppressing Tnfaip3 transcription in these cells (as reported above). As LPS-induced acute lung injury was much less in Dream−/− mice than in their wild-type counterparts (Fig. 1), we next investigated the possibility that augmented A20 expression in these mice was responsible for the diminished lung-injury response. Challenge with LPS induced three- to fourfold higher A20 expression in Dream−/− lungs than in wild-type lungs (Fig. 4d). DREAM represses expression of the transcription factor c-Fos by binding to DREs in the Fos promoter. As a positive control, we assessed LPS-induced c-Fos expression in wild-type and Dream−/− mice. We observed that c-Fos expression was higher in the lungs of LPS-treated Dream−/− mice than in their wild-type counterparts (Supplementary Fig. 3), which indicated that DREAM deficiency augmented the expression of genes that are targets of DREAM.
**Figure 6** DREAM deletion attenuates TNF-induced Jnk and p38 MAPK activation in LECs. (a,b) Immunoblot analysis (top) of total and phosphorylated Jnk (a) or p38 (b) in Dream⁺/⁺ or Dream⁻/⁻ LECs treated for 0–30 min (above lanes) with TNF. Below, quantification of results above, presented as the ratio of phosphorylated Jnk to total Jnk (a) or phosphorylated p38 to total p38 (b). * P < 0.001 (unpaired two-tailed Student’s t-test). (c) Immunoblot analysis of A20 (top) or total and phosphorylated p38 (below) in Dream⁺/⁺ or Dream⁻/⁻ LECs left untransfected (UT) or transfected for 48 h with Scr-siRNA or A20-specific siRNA, and then assessed without further treatment (top blots) or treated for 0–30 min (above lanes) with TNF (bottom blots). Numbers below lanes (bottom blots), quantification of band intensity, presented as the ratio of phosphorylated p38 to total p38. (d) Quantitative RT-PCR analysis of MCP-1 mRNA, ICAM-1 mRNA and A20 mRNA in Dream⁺/⁺ or Dream⁻/⁻ LECs treated for 0–6 h (horizontal axes) with TNF (1,000 U/ml), presented relative to that in untreated Dream⁺/⁺ cells, set as 1. * P < 0.001 (unpaired two-tailed Student’s t-test). (e) Immunoblot analysis (left) of A20 in Dream⁺/⁺ or Dream⁻/⁻ BMDMs cultured for 0–6 h (above lanes) with LPS (100 ng/ml). Right, quantification of results at left, presented as the ratio of A20 to β-actin. * P < 0.05, ** P < 0.01 and *** P < 0.001 (unpaired two-tailed Student’s t-test). Data are representative of three experiments (a,b,c,e) or four experiments (d); mean ± s.d. (a,b) or mean and s.e.m. (e).}

**Knockdown of A20 restores inflammation in Dream⁻/⁻ mice**

To address the causal role of the augmented A20 expression noted in Dream⁻/⁻ mice (Fig. 4a–d) in mediating their considerably diminished inflammatory lung-injury response (Fig. 1), we silenced A20 expression in lung vascular endothelial cells in vivo through the use of small interfering RNA (siRNA) by liposome-mediated delivery, which targets LECs28,29. Here we studied DREAM signaling in endothelial cells, as we found the role of DREAM in these cells was essential for inflammatory lung injury (Supplementary Fig. 1). At 48 h after siRNA delivery, we observed >80% less A20 protein in Dream⁻/⁻ mice

**Figure 7** DREAM regulates the expression of NF-xB signaling components and target genes differently. (a) Immunoblot analysis (top) of TRAF2 and TRAF6 (left) and RIP1, RIP2 and IxBα (right) in lung tissue (LT) from Dream⁺/⁺ or Dream⁻/⁻ mice (n = 5 per group). Below, quantification of results above, presented relative to β-actin. (b,c) Immunoblot analysis (left) of IKKα, IKKβ and IKKγ (b) or the NF-xB components p65 (RelA), NF-xB1 (p50), NF-xB2 (p52), RelB and c-Rel (c) in lung tissue from Dream⁺/⁺ or Dream⁻/⁻ mice (n = 6 per group). Right, quantification of results at left, presented relative to β-actin. (d) Immunoblot analysis of NF-xB signaling components (as in a–c) in Dream⁺/⁺ and Dream⁻/⁻ LECs. * P < 0.001 (unpaired two-tailed Student’s t-test). Data are representative of three experiments (mean ± s.d. in a–c).
given injection of A20-specific siRNA than in Dream−/− mice given injection of either saline or nontargeting siRNA with a scrambled sequence (Scr-siRNA) (Fig. 4e). ICAM-1 expression induced by LPS in Dream−/− mice given injection of A20-specific siRNA and treated with LPS was significantly greater than that of their Dream−/− counterparts given injection of either saline or Scr-siRNA (Fig. 4f,g). Also, PMN sequestration (assessed by MPO activity) was greater in the lungs of Dream−/− mice given injection of A20-specific siRNA than in their Dream−/− counterparts given injection of either saline or Scr-siRNA (Fig. 4h). Thus, the upregulated A20 expression in LECs seen in Dream−/− mice was required for the mitigation of inflammatory lung injury.

**DREAM promotes kinase TAK1−mediated NF-κB activation**

We next addressed the mechanisms by which the DREAM-induced inhibition of A20 expression mediated inflammatory lung injury. As A20 cleaves K63-linked polyubiquitin chains in TRAF2 and TRAF6 to prevent TAK1 kinase activity15−21 and the subsequent activation of NF-κB15−21, we focused on TNF-induced activation of both TAK1 and downstream IKK complexes in LECs obtained from wild-type and Dream−/− mice. We observed time-dependent TNF-induced phosphorylation of IKKβ in wild-type LECs, but this effect was suppressed in Dream−/− LECs (Fig. 5a). We next investigated the role of DREAM in mediating the expression of IkBα on the basis of the proposal that NF-κB signaling is required for IkBα expression and that IκBα, in a negative feedback manner, inhibits the activation of NF-κB9,10. We observed that basal expression of IκBα was significantly lower in LECs from Dream−/− mice than in their wild-type counterparts (Fig. 5b). TNF challenge elicited time-dependent increases in IκBα transcripts (Fig. 5c) and IκBα protein (Fig. 5b) in wild-type LECs. Those responses were abrogated in Dream−/− LECs (Fig. 5b,c). To address whether activation of TAK1 was also suppressed in Dream−/− LECs, we treated LECs from wild-type and Dream−/− mice with TNF and measured the phosphorylation of TAK1 (ref. 30). Here we observed a time-dependent increase in the phosphorylation of TAK1 in wild-type LECs but not in Dream−/− LECs in response to TNF (Fig. 5d). To determine whether the suppressed activation of IKK in Dream−/− LECs was the result of enhanced A20 expression itself, we did a ‘rescue’ experiment in which we expressed wild-type DREAM or mutant DREAM unable to bind DNA in Dream−/− LECs. We observed that wild-type DREAM interacted with TNFAIP3 DRE sequence (Fig. 5e). TNF-induced phosphorylation of those kinases was the result of the higher A20 expression noted in Dream−/− mice, silenced A20 and found that TNF-induced p38 phosphorylation was restored in in Dream−/− LECs in which A20 was knocked down (Fig. 6c). In further support of those findings, we observed much lower expression of transcripts encoding MCP-1 and ICAM-1 in Dream−/− LECs in response to TNF challenge than in their wild-type counterparts (Fig. 6d); in contrast, A20 expression was augmented in Dream−/− LECs (Fig. 6e).

Next we studied the role of DREAM in regulating endotoxin-induced NF-κB signaling. A20 protein expression was increased to a greater extent in BMDMs from Dream−/− mice than in their wild-type counterparts (Fig. 4c). To address the functional relevance of the enhanced A20 expression in the endotoxin response, we studied the activation of TAK1 and IKK in macrophages from Dream−/− and wild-type mice following LPS challenge. As in the studies reported above, the LPS-induced activation of TAK1 and IKK was much lower and delayed in Dream−/− cells relative to that in wild-type cells (Supplementary Fig. 4a,b). These results supported the proposal that augmented A20 expression restricted TAK1-mediated activation of IKK and MAPKs in Dream−/− cells.

**DREAM regulates A20 targets that mediate NF-κB signaling**

We then set out to determine the consequence of DREAM-induced downregulation of A20 in mediating the activation of NF-κB (Fig. 1). For this, we evaluated the expression of A20 targets in lungs of Dream−/− and wild-type mice. The expression of TRAF2 and TRAF6 (Fig. 7a), RIP1 and RIP2 (Fig. 7a), IκBα (Fig. 7a) and IκKY (Fig. 7b) was suppressed in Dream−/− mouse lungs relative to the expression in wild-type mouse lungs. However, expression of IκKα and IKKB was unaffected (Fig. 7b). Next we assessed the expression of NF-κB proteins in the lungs of Dream−/− and wild-type mice. The expression of p65-RelA was not different in Dream−/− mouse lungs versus wild-type mouse lungs (Fig. 7c), whereas the expression of NF-κB1, NF-κB2, RelB and c-Rel was suppressed in Dream−/− mouse lungs (Fig. 7c). Also, the expression of TRAF2 and TRAF6, RIP1 and RIP2, IKKY, IκBα and NF-κB proteins (NF-κB1, NF-κB2, RelB, and c-Rel) was lower in Dream−/− LECs than in wild-type LECs (Fig. 7d). Next we measured the expression of mRNA encoding those NF-κB signaling components by quantitative RT-PCR. Expression of mRNA encoding RIP2 and TRAF2 was much lower in the lungs of Dream−/− mice than in their wild-type counterparts (Supplementary Fig. 5), whereas the expression of mRNA encoding RIP1, TRAF6, NEMO, NF-κB1, NF-κB2, RelB and c-Rel was not altered in lungs from Dream−/− mice relative to that in lungs from wild-type mice (Supplementary Fig. 5). To address whether the lower expression of NF-κB signaling components noted in Dream−/− mice was affected by A20, we did ‘rescue’ experiments in which we ectopically expressed wild-type DREAM or the DNA-binding–defective mutant DREAM in LECs from Dream−/− mice. In this study, expression of wild-type DREAM suppressed the expression of A20 as well as that of c-Fos (another DREAM-regulated protein1) in Dream−/− LECs, but expression of the mutant DREAM did not (Fig. 8). Expression of wild-type DREAM restored the expression of A20 targets and NF-κB signaling components (except RIP2) in LECs from Dream−/− mice; however, expression of the mutant...
DREAM did not produce this result (Fig. 8). These findings together demonstrated that DREAM-mediated suppression of A20 expression was responsible for activating NF-kB signaling and the NF-kB target genes responsible for inflammatory lung injury. On the basis of our results, we propose a model for the mechanism whereby DREAM regulates A20 expression and thereby regulates the inflammatory NF-kB signaling pathways (Supplementary Fig. 6).

**DISCUSSION**

Our results here have demonstrated a proinflammatory function of the transcription repressor DREAM and its interaction with the transcription factor USF1 in the mechanism of the expression of the deubiquitinase A20 and the subsequent ‘tuning’ of NF-kB activity. DREAM has been shown to be important in the spinal cord in mediating the sensation of pain4,5. Mice lacking DREAM (Dream−/−) have increased prodynorphin mRNA and dynorphin A peptides in the spinal cord and diminished pain sensation6. Here we found that Dream−/− mice failed to develop inflammatory lung injury in response to sepsis as a result of USF1-mediated expression of A20, and thereby the downstream inhibition of TAK1-mediated activity and signaling by NF-kB.

DREAM bound constitutively to DRE3 and DRE4 in the human TNFAIP3 promoter. DREAM binding decreased for 90 min after exposure to LPS or TNF but returned to baseline within 180 min. The cyclic nature of the DREAM-binding response was mirrored by binding of the A20-transcription activator USF1 to the DRE3 E-box; that is, binding of USF1 functioned to induce transcription of TNFAIP3. A similar pattern emerged for the mouse Tnfaip3 promoter, which suggested a well-conserved mechanism of coordinated regulation of transcription of the gene encoding A20 by DREAM-USF1. The reciprocal function of DREAM and USF1 in regulating such expression is consistent with the proposal that binding of USF1 to the E-box sequence on the promoter of the gene encoding A20 is important for mediating initiation of the transcription of that gene6.

We observed that in Dream−/− mice, in which binding of USF1 to the Tnfaip3 promoter remained intact, treatment with endotoxin resulted in lower ICAM-1 expression and sequestration of PMNs in lungs and normal lung vascular barrier function, compared with that of wild-type mice. The Dream−/− mice also showed much less generation of the NF-kB-transcribed proinflammatory mediators IL-6, MCP-1 and TNF and displayed enhanced survival in a CLP model of severe polymicrobial sepsis, results consistent with augmented A20 expression and decreased NF-kB activation in these mice. Therefore, inactivation of DREAM signaling had an indispensable anti-inflammatory function.

As DREAM expressed in hematopoietic cells6,7 as well as in endothelial cells may be essential for the mechanism of inflammatory lung injury, we addressed whether DREAM deficiency was responsible for the proinflammatory role of DREAM identified in Dream−/− mice. We observed that the sequestration of PMNs in lungs, lung production of MCP-1, IL-6, and TNF and lung vascular ICAM-1 expression in chimeras generated by the transplantation of wild-type-mice bone marrow cells into Dream−/− mice was similar to that of Dream−/− mice. Those findings ruled out the possibility of a chief role for DREAM expression in hematopoietic cells in the mechanism of the inflammatory lung injury response. However, they were consistent with published results showing that selective expression of the degradation-resistant form of NF-kB in the vascular endothelium prevents inflammation in mice31. Our results suggested that the proinflammatory role of DREAM identified in Dream−/− mice was probably the result of DREAM expressed by endothelial cells.

Since inflammatory lung injury was considerably diminished in Dream−/− mice, we investigated whether augmented A20 expression was responsible for mediating the response. We silenced A20 expression in lung vascular endothelial cells by liposome-mediated delivery of A20-specific siRNA28,29. We found that ICAM-1 expression and sequestration of PMNs in the lungs induced by LPS challenge of Dream−/− mice treated with A20-specific siRNA were significantly greater than that of Dream−/− mice treated with Scr-siRNA. Thus, A20 expression in the LECs of Dream−/− mice was required and sufficient to reestablish the inflammatory lung-injury response in these mice.

With studies of LECs from Dream−/− mice, we demonstrated that the persistent A20 expression in these mice interfered with phosphorylation of TAK1 and thus downstream activation of IKKβ and NF-kB. To assess the functional relevance of this finding, we did a crucial ‘rescue’ experiment in which we expressed wild-type DREAM or mutant DREAM that was unable to bind to DREs in Dream−/− LECs. In this study, expression of wild-type DREAM in Dream−/− cells restored IKK activation in response to TNF, but expression of the mutant DREAM did not. These studies demonstrated a key role for DREAM and its relationship with USF1 described above in regulating the activation of TAK1-mediated NF-kB signaling.

Because DREAM and USF1 function through modulating A20 expression, we also assessed the expression of various constituents of the NF-kB signaling pathway that as a consequence might be altered by A20 expression. We observed that expression of p65-RelA, IKKα and IKKβ was similar in wild-type and Dream−/− mice. A likely explanation of this finding is that those factors are not transcriptionally regulated by NF-kB32. However, we found that the expression of other NF-kB signaling components, including TRAF2, TRAF6, RIP1, RIP2, IκBα, IκBβ, IκBγ, NF-kB1, NF-kB2, RelB and c-Rel, was downregulated in Dream−/− mice. The expression of mRNA encoding TRAF2 and RIP2 was substantially lower in Dream−/− mice than in wild-type mice. Until now, the transcription mechanisms of TRAF2 expression have not been identified, to our knowledge. Our promoter analysis revealed the presence of multiple binding sites for the transcription factor AP-1 in both human and mouse genes encoding TRAF2 (data not shown). It is known that NF-kB signaling mediates the transcription of both the human and mouse genes encoding RIP2 (ref. 33). A20 restricts the activation of IKK and MAPK (MAPK signaling is essential for AP-1 activation34) by blocking TAK1 activation35-37; therefore, the enhanced A20 expression seen in Dream−/− cells probably prevented the transcription of genes encoding TRAF2 and RIP2 by this mechanism. A20 activity also decreased the expression of NF-kB signaling components through proteosomal pathway, which involves A20-mediated deubiquitination of K63-linked ubiquitin chains followed by ubiquitination of K48-linked ubiquitin chains on the target molecules38. Thus, it is possible that lower expression of NF-kB signaling components such as TRAF6 and RIP1 may be the result of constitutive A20-mediated proteosomal degradation of such molecules in Dream−/− cells.

Our findings collectively support the proposal of a key role for USF1-mediated enhanced A20 expression in Dream−/− mice in inhibiting TAK1-mediated signaling. Our findings suggest that the reciprocal relationship between DREAM and USF1 functions as a ‘rheostat’ that regulates A20 expression and thus enables ‘fine tuning’ of NF-kB signaling. The anti-inflammatory result of the deletion of DREAM described here would suggest that targeting DREAM might be a potentially useful therapeutic strategy in inflammatory diseases such as acute lung injury.

**METHODS**

Methods and any associated references are available in the online version of the paper.
Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
We thank J.M. Penninger (Institute of Molecular Biotechnology of the Austrian Academy of Sciences) for DREAM-deficient mice, and Y.B. Wu for help with the isolation and culture of LECs. Supported by the US National Institutes of Health (P01 HL077806).

AUTHOR CONTRIBUTIONS
C.T., D.S., I.W.C., A.M. and A.B.M. designed the research; C.T., D.S., D.-M.W., J.X., V.S., P.B.T., R.P.C., R.K.M., A.D., Z.Q., K.B., Y.-Y.Z. and S.M.V. did the experiments; C.T. analyzed data; and C.T. and A.B.M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Antibodies and other reagents. The following polyclonal antibodies were from Santa Cruz Biotechnology: anti-DREAM (sc-9142), anti-ICAM-1 (sc-1511), anti-USF1 (sc-229), anti-TAK1 (sc-876), anti-TRAF6 (sc-7220), anti-NF-kB1 (sc-114), anti-NF-kB2 (sc-848), anti-ReLB (sc-226), anti-c-Rel (sc-70) and anti-IkBα (sc-1643). Mouse monoclonal antibody (mAb) to A2O (59A426) was from Calbiochem. The following antibodies were from Cell Signaling: polyclonal antibody (pAb) to c-Fos (4384), pAb to TAK1 phosphorylated at Thr184 and Thr185 (4531), rabbit mAb to TAK1 (D94D7), mAb to p38 phosphorylated at Thr180 and Tyr182 (28810), pAb to p38 (9212), rabbit mAb to Jnk phosphorylated at Thr183 and Tyr185 (81E11), rabbit mAb to Jnk (56G8), pAb to Ikkα/β phosphorylated at Ser176 and Ser180 (2687), pAb to IKKβ (2684), rabbit mAb to IκBα phosphorylated at Ser32 (14D4), pAb to RIP1 (4926) and pAb to RIP2 (4982). mAb to IKKγ (72C67) and mAb to DREAM (40A5) were from Upstate. pAb to p65-RelA (AB1604) was from Chemicon. Control siRNA was from Qiagen, and siRNA specific for human USF1 (SMARTpool; L003617) was from Dharmacon. The siRNA-transfection reagent was from Santa Cruz Biotechnology. Lipids (dimethyldioctadecylammonium bromide and cholesterol) for liposome preparation and mAb to β-actin (AC-15) were from Sigma. PCR primers were custom-synthesized from Integrated DNA Technologies.

Mice. DREAM-deficient (DREAM−/−) mice4 generated on the C57BL/6 background were from the laboratory of J. Penninger. DREAM−/− mice were back-crossed onto the C57BL/6j background for eight generations. Age-matched Dream−/− and Dream+/− littermates were used for all experiments. All mice were housed in the University of Illinois Animal Care Facility in accordance with institutional guidelines and guidelines of the US National Institute of Health. Veterinary care of these animals and related animal experiments was approved by the University of Illinois Animal Resources Center.

Generation of bone marrow chimeras. DREAM−/− mice were lethally irradiated as described. At 3 h after irradiation, mice were given transplantation of 1 × 10⁷ isolated DREAM−/− bone marrow cells through injection of the tail vein. The bone marrow reconstitution was assessed at 3 weeks after transplantation by presence of the male-specific Sry gene in recipient mice blood cells by quantitative PCR. Mice were used for experiments 6 weeks after bone marrow transplantation.

Lung injury in mice. Age- and weight-matched Dream−/− and Dream+/− mice received a single intraperitoneal dose of LPS (10 mg per kg body weight; ultrapure Escherichia coli strain 0111:B4; InvivoGen). For histology, paraffin-embedded sections 5 μm in thickness prepared from the lungs were stained with hematoxylin and eosin. For MPO assay, lungs were perfused with PBS or hematoxylin. For MPO assay, lungs were perfused with PBS or 1 M NaCl, 1 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS and protease-inhibitor mixture. The homogenate was centrifuged (14,000 g for 4 °C for 10 min), and cleared supernatant was used for immunoblot analysis. BMDMs stimulated with LPS or endothelial cells stimulated with TNF were lysed with lysis buffer containing a phosphatase-inhibitor mixture4. Lysate were centrifuged and clear supernatants were used for immunoblot analysis.

Promoter analysis. Consensus binding sites for transcription factors and repressor elements in 5′ regulatory region and intron 1 of the human and mouse A2O-encoding genes were analyzed with Genomatix Software (Germany).

ChIP assay. ChIP assays were done as described. The primers used for PCR after ChIP were as follows: TNFαIP3 DRE1 forward, 5′-GGATTCAATGATTACCTTGGC-3′, and reverse, 5′-GGTGTCGTGCCTGAAAACTACAT-3′; TNFαIP3 DRE2 forward, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-TGCAAGGCCTGGTGCCTCAT-3′; and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-AACCAGGGTATGGGAACCT-3′; TnpaIP3 DRE3 forward, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-AACCAGGGTATGGGAACCT-3′; TnpaIP3 DRE4 forward, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-AACCAGGGTATGGGAACCT-3′; TnpaIP3 DRE5 forward, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-AACCAGGGTATGGGAACCT-3′; and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-AACCAGGGTATGGGAACCT-3′; and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′, and reverse, 5′-GTCCTGGTGTTTGAAGCTTGG-3′. The DNA-protein interaction was calculated with the following formula: 2ΔCtΔCt = 2ΔCtb2, where ΔCt = the cycling threshold of input DNA – the cycling threshold of sample DNA, and ΔCtb = the cycling threshold of input DNA – the cycling threshold of control antibody.

Quantitative real-time PCR. Total RNA was isolated from lung tissue or LECs and was reverse-transcribed with oligo(dT) primers and SuperScript reverse transcriptase (Invitrogen). The cDNA obtained was mixed with DNA, and ∆Ct = the cycling threshold of input DNA – the cycling threshold of sample DNA, and ∆Ctb = the cycling threshold of input DNA – the cycling threshold of control antibody.
GTCGAGG-3′, and reverse, 5′-AAAGTACGTGGAGGTGCT-3′; and Tnfaip3 forward, 5′-CAGTGGAAGGGACACAATC-3′, and reverse, 5′-GCAGTGCAAGAAAATCTC-3′.

**Preparation and expression of DREAM constructs.** Plasmids encoding human wild-type-DREAM and a DNA-binding–defective DREAM mutant were custom prepared by GenScript. The wild-type or mutant DREAM construct was cloned into pCMV-SPORT6 vector for experiments. In the mutant DREAM, alanine was substituted for arginine at position 98, for lysine at position 101, for lysine at position 115, for lysine at position 166, for lysine at position 168, for lysine at position 178, for lysine at position 184, for lysine at position 221 and for lysine at position 224 (R98A-L101A-L115A-L166A-L168A-L178A-L184A-L221A-L224A). Mouse LECs grown to ~70% confluence were transfected with the wild-type or mutant DREAM construct 12,41. Cells were transfected with plasmid DNA (1 µg/ml) through the use of SuperFect transfection reagent (Qiagen). At 48 h after transfection, the cells were used for experiments. Wild-type or mutant DREAM was ectopically expressed in HEK293 human embryonic kidney cells. Nuclear extracts prepared from cells expressing wild-type or mutant DREAM were used for electrophoretic mobility-shift assays to assess the binding of DREAM to the TNFAIP3 DRE4 sequence.

**Statistical analysis.** Data were analyzed by an unpaired two-tailed Student’s t-test and log-rank test. Difference in mean values were considered significant at a P value of ≥0.05.

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