CREB coactivators CRTC2 and CRTC3 modulate bone marrow hematopoiesis

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Populations of circulating immune cells are maintained in equilibrium through signals that enhance the retention or egress of hematopoietic stem cells (HSCs) from bone marrow (BM). Prostaglandin E2 (PGE2) stimulates HSC renewal and engraftment through, for example, induction of the cAMP pathway. Triggering of PGE2 receptors increases HSC survival in part via the PKA-mediated induction of the cAMP response element-binding protein (CREB) signaling pathway. PKA stimulates cellular gene expression by phosphorylating CREB at Ser133 and by promoting the dephosphorylation of the cAMP- responsive transcriptional coactivators (CRTCs). We show here that disruption of both CRTC2 and CRTC3 causes embryonic lethality, and that a single allele of either CRTC2 or CRTC3 is sufficient for viability. CRTC2 knockout mice that express one CRTC3 allele (CRTC2/3m mice) develop neutrophilia and splenomegaly in adulthood due to the up-regulation of granulocyte-colony stimulating factor (G-CSF); these effects are reversed following administration of neutralizing anti–G-CSF antiserum. Adoptive transfer of CRTC2/3m BM conferred the splenomegaly/neutrophilia phenotype in WT recipients. Targeted disruption of both CRTC2 and CRTC3 in stromal cells with a mesenchymal Prx1-Cre transgene also promoted this phenotype. Depletion of CRTC2/3 was found to decrease the expression of Suppressor of Cytokine Signaling 3 (SOCS3), leading to increases in STAT3 phosphorylation and to the induction of CEBPβ, a key regulator of the G-CSF gene. As small molecule inhibition of JAK activity disrupted CEBPβ induction and reduced G-CSF expression in CRTC2/3m stromal cells, our results demonstrate how cross-coupling between the CREB/CRTC and JAK/STAT pathways contributes to BM homeostasis.

cAMP | CREB | CRTC | C/EBPβ | G-CSF

Triggering of the TLR4 signaling pathway in response to bacterial infection stimulates the NFkB- and C/EBPβ-mediated induction of cytokine genes by innate immune cells (1, 2). Circulating monocytes and tissue-resident macrophages recruit neutrophils to sites of infection in part by releasing granulocyte colony-stimulating factor (G-CSF) into the circulation, thereby promoting the proliferation and mobilization of neutrophils from bone marrow (BM). This response is circumscribed by the parallel production and release of prostaglandin E2 (PGE2), which attenuates cytokine signaling by activating the cAMP/PKA pathway (3).

In addition to its role in immunosuppression, cAMP also has been found to up-regulate hematopoietic stem and progenitor cell (HSPC) numbers during development (4–6). Activation of the PKA-cAMP response element-binding protein (CREB) pathway, either through triggering of adenosine A2b receptors or in response to shear stress, stimulates the PKA-mediated expression of CXCL8, which in turn appears to promote HSPC specification.

cAMP stimulates cellular gene expression through the PKA-mediated phosphorylation of CREB and the dephosphorylation of the CRTC family of CREB coactivators (7). CRTC1 is largely confined to the CNS, whereas CRTC2 and CRTC3 are expressed at comparable levels in peripheral tissues. Consistent with their overlapping effects on CREB target gene expression, single knockouts of either CRTC2 or CRTC3 have relatively modest phenotypes at birth (8, 9). In the process of characterizing mice with a knockout of both CRTC2 and CRTC3, we uncovered an unexpected role for these coactivators in BM homeostasis. The results may provide new therapeutic targets for treatment of BM transplant recipients.

Results

In studies to characterize mice with a knockout of both CRTC2 and CRTC3, we intercrossed $CRTC2^{+/-}$;$CRTC3^{+/-}$ compound heterozygotes; however, we recovered no double-knockout animals from multiple litters totaling 447 pups (*SI Appendix*, Fig. S1*A*). In contrast, mice expressing only one allele of either CRTC3 ($CRTC2^{-/-}$;$CRTC3^{+/-}$) or CRTC2 ($CRTC2^{+/-}$;$CRTC3^{-/-}$) were viable. Protein amounts for CRTC2 were undetectable in BM stromal cells from $CRTC2^{-/-}$;$CRTC3^{+/-}$ mice, and CRTC3 levels were reduced by one-half (*SI Appendix*, Fig. S1*B*).

Although they appeared comparable to wild-type (WT) controls at birth, the $CRTC2^{-/-}$;$CRTC3^{+/-}$ mice (hereinafter referred to as CRTC2/3m mice) developed splenomegaly by 15–20 wk of age (Fig. 1*A*). Spleen/body weight ratios were increased fivefold on average in the CRTC2/3m mice relative to WT littermates (Fig. 1*B*). Microscopically, splenic architecture appeared disorganized, with a blurring of red and white pulp margins that is often indicative of extramedullary hematopoiesis (*SI Appendix*, Fig. S2*A*).

Significance

The second messenger cAMP mediates the salutary effects of prostaglandin E2 on hematopoietic stem cell (HSC) engraftment in bone marrow (BM). We found that disruption of the cAMP-inducible coactivators CRTC2 and CRTC3 in BM stromal cells promotes neutrophilia and HSC egress due to up-regulation of granulocyte colony-stimulating factor (G-CSF). CRTC2/3 were found to reduce G-CSF expression by inhibiting signaling through the transcription factor STAT3. These results demonstrate how cross-talk between cAMP and cytokine signaling pathways contributes to BM homeostasis.

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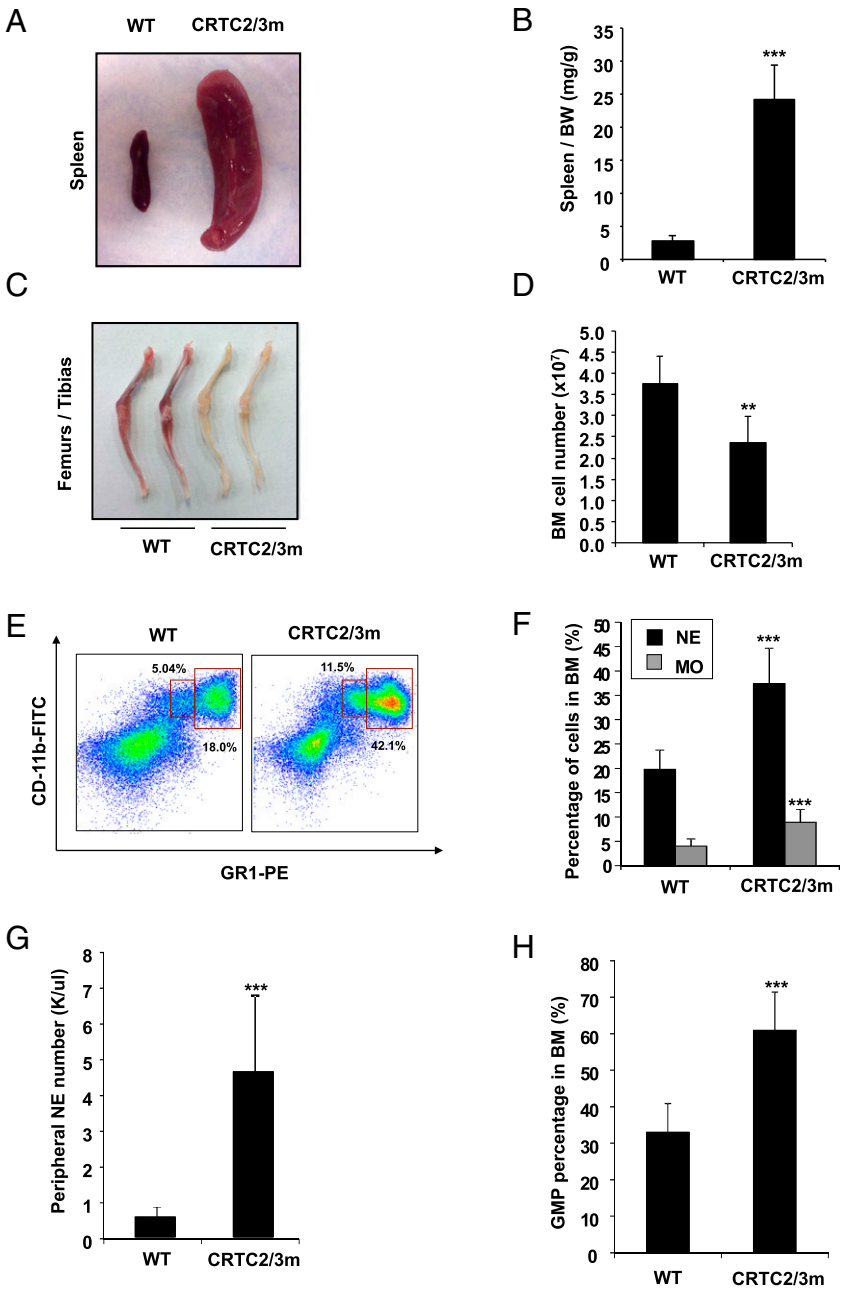

Fig. 1. Neutrophilia and splenomegaly in CRTC2/3 mutant mice. (*A* and *B*) Spleen appearance (*A*) and spleen/body weight (BW) ratios (*B*) of CRTC2$^{-/-}$;CRTC3$^{+/-}$ (CRTC2/3m) mice and WT littermates ($n = 12$). ***$P < 0.001$. (*C*) Appearance of femurs from WT and CRTC2/3m mice. (*D*) Relative cellularity of BM in femurs and tibias from WT and CRTC2/3m mice ($n = 6$). **$P < 0.01$. (*E*) FACS analysis of BM cells from WT and CRTC2/3m littermates stained with CD11b and Gr1 antibodies. (*F*) Relative percentage of neutrophil (NE) and monocyte (MO) populations in BM ($n = 9$). ***$P < 0.001$. (*G*) Peripheral neutrophil counts ($n = 11$). ***$P < 0.001$. (*H*) Granulocyte macrophage progenitor (GMP) percentage in BM ($n = 6$). ***$P < 0.001$.

In contrast to the reddish appearance of BM from WT littermates, CRTC2/3m BM was pale (Fig. 1*C*). Correspondingly, total BM cellularity was decreased in the CRTC2/3m mice (Fig. 1*D*), with resident erythrocyte and lymphocyte populations most severely affected. Flow cytometry profiling revealed substantial increases in CD11b^{+}Gr-1^{+} neutrophils as well as CD11b^{+}Gr-1^{lo} monocytes in CRTC2/3m BM (Fig. 1 *E* and *F*). The numbers of circulating neutrophils and other myeloid cells (monocytes, eosinophils, and basophils) were also up-regulated, whereas B and T lymphocyte populations were reduced (Fig. 1*G* and *SI Appendix*, Fig. S2*B*). Indeed, the numbers of granulocyte-monocyte progenitors (Lin^{-}IL7R^{-}Sca1^{-}c-kit^{+}CD34^{+}CD16/32^{+}) were increased in BM from the CRTC2/3m mice (Fig. 1*H*), indicating that disruption of the CRTC2 and CRTC3 genes leads to myeloproliferative changes in BM. Consistent with this idea, early myeloid activity was mild to moderate in WT marrow, while marrow from CRTC2/3m mice was significantly shifted to the left (earlier) stages (*SI Appendix*, Fig. S3*A*). In contrast, hematopoietic stem cell (HSC)-enriched populations of Lin^{-}Sca-1^{+}c-kit^{+}, short-term HSCs, and multipotential progenitor cells were decreased in CRTC2/3m BM (*SI Appendix*, Fig. S3*B*), suggesting that loss of CRTC2/3 promotes depletion or mobilization of HSPCs from BM.

Based on the capability of granulocyte-colony stimulating factor (G-CSF) to promote neutrophilia and splenomegaly as

well as HSC egress, we examined whether this cytokine is upregulated in CRTC2/3m mice (10, 11). Circulating concentrations of G-CSF were elevated fivefold in CRTC2/3 mutants compared with controls (Fig. 2*A*). Markers for G-CSF signaling in BM cells, most notably phospho-(Y705, S727) STAT3 (P-STAT3) amounts were also increased (Fig. 2*B*). Disrupting G-CSF action by administration of anti–G-CSF antiserum reduced peripheral neutrophil counts, indicating that G-CSF drives granulocyte mobilization in CRTC2/3m mice (Fig. 2 *C* and *D*).

Because G-CSF is expressed in a variety of cells (i.e., monocytes and endothelial, epidermal, and stromal cells), we sought to identify the cellular context in which CRTCs modulate the expression of this cytokine (12). Depletion of CRTC2 and CRTC3 increased G-CSF expression in BM-derived macrophages (BMDMs)—which arise from monocyte/macrophage precursors—and, to a greater extent, in stromal cells, but not in fibroblasts or endothelial cells (Fig. 2*E* and *SI Appendix*, Fig. S4). Exposure to IL-1β further enhanced G-CSF mRNA and protein amounts to a greater extent in CRTC2/3m stromal cells compared with WT stromal cells.

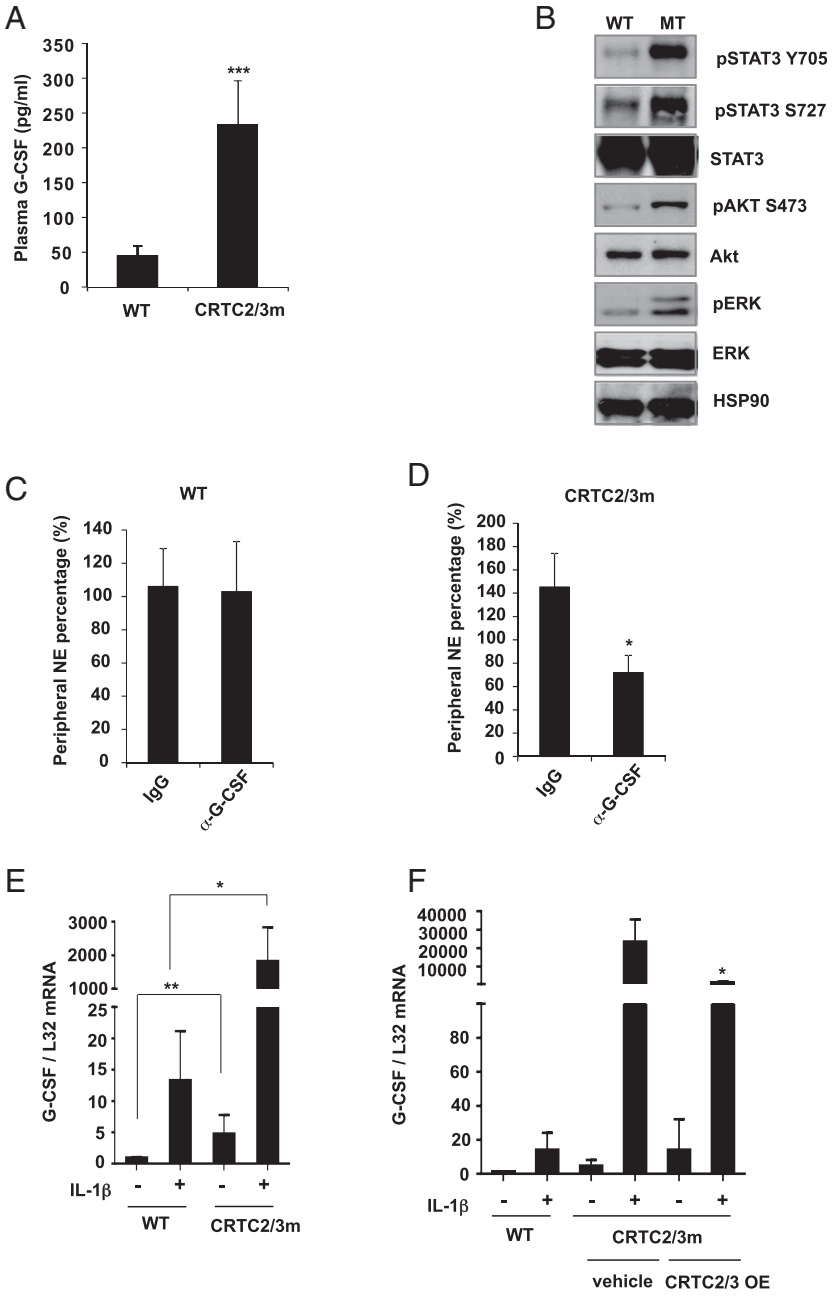

Fig. 2. Increased G-CSF expression in stromal cells from CRTC2/3 mutant mice. (*A*) Plasma G-CSF concentrations in WT and CRTC2/3m mice ($n = 6$ mice/group) measured by ELISA. ***$P < 0.001$. (*B*) Immunoblot analysis of P-STAT3, P-Akt, and P-ERK levels in BM from CRTC2/3 mutant (MT) and WT littermates at age 20 wk. (*C* and *D*) Effect of anti–G-CSF or control (IgG) antibody (administered i.p. for 7 d) on peripheral neutrophil (NE) counts in WT (*C*) and CRTC2/3m (*D*) mice. Data are shown as the ratio of (% NE after injection)/(% NE before injection) × 100 ($n = 3$). *$P < 0.05$. (*E*) G-CSF mRNA amounts in BM-derived stromal cells under basal conditions and following exposure to IL-1β ($n = 5$). *$P < 0.05$; **$P < 0.01$. (*F*) Effect of CRTC2 and CRTC3 reexpression on G-CSF mRNA amounts in CRTC2/3m stromal cells ($n = 3$). *$P < 0.05$.

We considered the possibility that CRTC2 and CRTC3 could exert developmental effects that alter the abundance of certain G-CSF–expressing cell populations rather than affecting the level of G-CSF transcription per se. Arguing against this scenario, however, acute depletion of CRTC2 and CRTC3 by RNAi-mediated knockdown increased mRNA amounts for G-CSF in WT stromal cells (*SI Appendix*, Fig. S5). Conversely, acute overexpression of CRTC2 and CRTC3 in CRTC2/3m cells reduced G-CSF expression (Fig. 2*F*).

We performed BM transplantation studies to determine whether the effects of CRTC2/3 are cell-autonomous. Supporting this idea, adoptive transfer of BM from CRTC2/3m mice to WT recipients promoted neutrophilia and splenomegaly, as well as increases in circulating concentrations of G-CSF (Fig. 3 *A–D* and *SI Appendix*, Fig. S6). We further tested the role of BM stromal cells using mice with conditional alleles for both CRTC2 and CRTC3. We crossed double-floxed (CRTC2/CRTC3 fl/fl) mice (13) with mice carrying a Prx1-Cre transgene, which is expressed in mesenchymal cells of the developing limb bud (14). Similar to whole-body knockouts, mice depleted of both CRTC2 and CRTC3 with the Prx1-Cre transgene (CRTC2/3$^{\Delta STR}$) also exhibited increases in circulating neutrophils and G-CSF plasma concentrations (Fig. 3 *E* and *F* and *SI Appendix*, Figs. S7 and S8). Despite the capability of CRTC2/3m BM to promote splenomegaly and neutrophilia, stromal cells account for only a small fraction of cells recovered from BM during transplantation, and they do not engraft to a significant extent. The presence of splenomegaly and neutrophilia in CRTC2/3$^{\Delta STR}$ mice supports the involvement of both cell-autonomous and non–cell-autonomous processes.

Based on the importance of NFκB and C/EBPβ in promoting G-CSF gene expression (15, 16), we compared the relative levels of each factor in WT and CRTC2/3m cells. Notably, both C/EBPβ mRNA and protein amounts were elevated by threefold to fourfold in CRTC2/3m cells relative to controls (Fig. 4*A*). Correspondingly, C/EBPβ occupancy over the G-CSF promoter was selectively increased in CRTC2/3m stromal cells on chromatin immunoprecipitation (ChIP) assays, whereas NF-κB amounts were unchanged (Fig. 4*B*).

Realizing that STAT3 mediates the induction of C/EBPβ in response to cytokine signaling (17), we evaluated the phosphorylation state of this factor in CRTC2/3m cells. Amounts of (Y705) phosphorylated STAT3 were increased in CRTC2/3m cells relative to control cells (Fig. 4*C*). We performed transcriptomic studies to identify CREB target genes that modulate G-CSF expression,

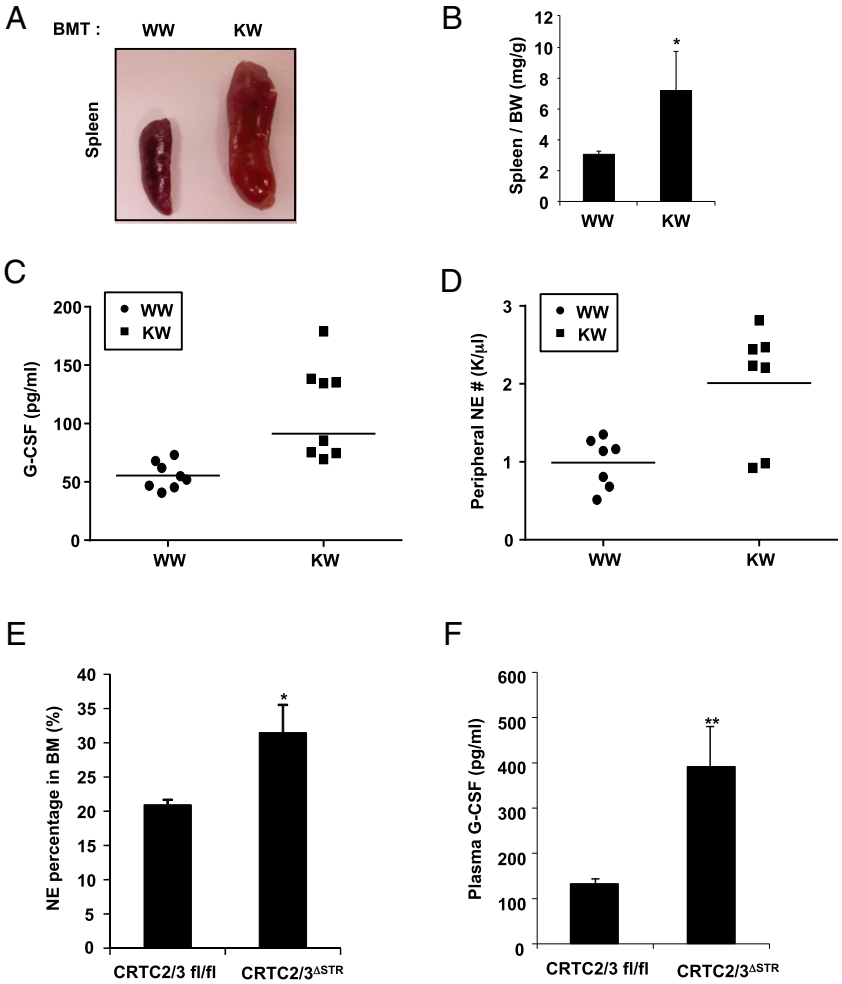

Fig. 3. BM stromal cells mediate the effects of CRTC2/3 depletion on neutrophilia and splenomegaly. (*A* and *B*) Effect of BM transplantation (BMT) with WT (WW) or CRTC2/3m (KW) BM ($n = 4$ mice/group) on spleen size (*A*) and spleen/body weight ratios (*B*) in irradiated recipient WT mice. (*C* and *D*) Circulating G-CSF protein concentrations (*C*) and peripheral neutrophil numbers (*D*) in reconstituted mice. Plasma G-CSF was measured by ELISA ($n = 10$). *$P < 0.05$. (*E* and *F*) Effect of CRTC2/3 gene disruption in BM stromal cells on leukocytosis and G-CSF expression. CRTC2/CRTC3 double-floxed mice (CRTC2/3 fl/fl) were crossed with Prx1-Cre transgenic mice. Shown are the percentage of NEs in BM (BM CD11b$^+$/Gr1) (*E*) and plasma G-CSF concentrations (*F*) in CRTC2/3$^{\Delta Str}$ mutant and double-floxed (CRTC2/3 fl/fl) controls. *$P < 0.05$; **$P < 0.01$.

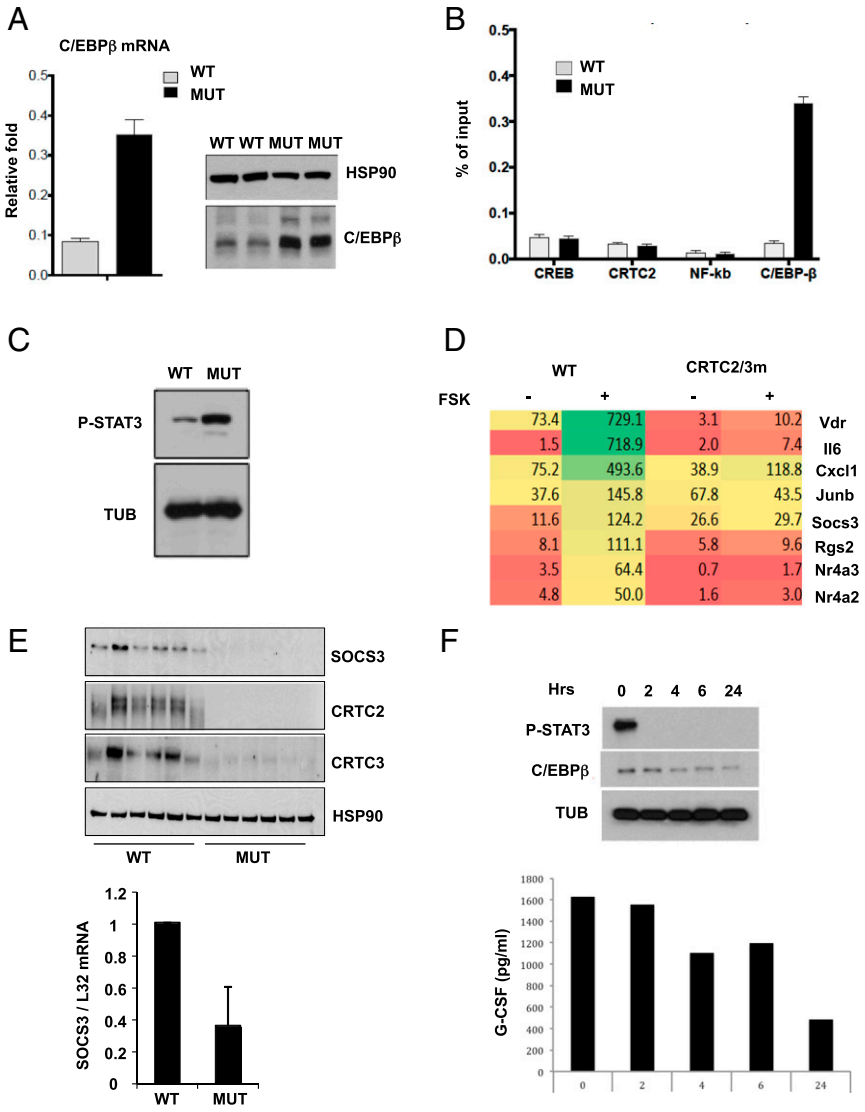

Fig. 4. Constitutive activation of STAT3 leads to G-CSF induction in CRTC mutant stromal cells. HSP90 is shown as a loading control for *A* and *E* and tubular (TUB) is shown as a loading control for *B* and *F*. (*A*) Relative C/EBPβ mRNA (*Left*) and protein amounts (*Right*) in WT and CRTC2/3m (MUT) cells. (*B*) Manual ChIP assay showing the effect of CRTC2/3 depletion on C/EBPβ and NF-κB levels over the G-CSF promoter in stromal cells. (*C*) Relative amounts of phospho (Y705) STAT3 in WT and CRTC2/3m stromal cells. (*D*) RNAseq data showing the effect of CRTC2/3 depletion on mRNA amounts for selected CREB target genes in CRTC2/3m and control cells exposed to forskolin (FSK). Data are reported in fragments per kilobase of exon per million. (*E*, *Top*) Immunoblot showing relative amounts of SOCS3 protein in BMDMs from WT and CRTC2/3m mice under basal conditions. (*E*, *Bottom*) Quantitative PCR analysis of basal SOCS3 mRNA amounts in WT and CRTC2/3m BMDMs. (*F*, *Top*) Immunoblot showing effect of exposure to the JAK2/3 inhibitor ruxolitinib for the indicated times on phospho (Y705) STAT3 and C/EBPβ protein amounts in CRTC2/3 stromal cells. (*F*, *Bottom*) ELISA showing the effect of ruxolitinib on G-CSF protein concentrations released into the medium after treatment of CRTC2/3m cells for the indicated time in hours (Hrs).

either directly or via their effects on STAT3 signaling. Exposure to forskolin (FSK) for 2 h increased the expression of 339 genes by twofold or greater in WT stromal cells (Fig. 4*D* and *SI Appendix*, Fig. S9); loss of CRTC2/3 abrogated the response to cAMP in two-thirds ($n = 223$) of these genes. Indeed, exposure to FSK increased the activity of a CRE-luc reporter by fourfold to fivefold in WT cells, but had no effect in CRTC2/3m cells (*SI Appendix*, Fig. S10). Collectively, these studies demonstrate the importance of the CRTC family in stimulating cAMP-dependent transcription.

Within the list of cAMP-responsive genes that are down-regulated in CRTC2/3m cells and that contain CREB-binding sites in their promoters, we identified the Suppressor of Cytokine Signaling 3 (SOCS3) gene. SOCS3 is a cAMP-regulated CREB target gene that inhibits cytokine signaling by disrupting JAK activity (18) (Fig. 4*D*). Similar to its effects on CREB reporter activity, exposure to cAMP agonist increased SOCS3 expression in WT stromal cells, but had no effect in CRTC2/3 mutant cells (Fig. 4*D*). Correspondingly, SOCS3 protein amounts were down-regulated in CRTC2/3m BMDMs and stromal cells (Fig. 4*E* and *SI Appendix*, Fig. S11), potentially explaining our observed increases in STAT3 phosphorylation.

We tested this idea using a small-molecule JAK1/2 inhibitor, ruxolitinib (19). Exposure to ruxolitinib sharply decreased phospho (Tyr705) STAT3 protein amounts in CRTC2/3m cells, and correspondingly reduced C/EBPβ protein amounts (Fig. 4*F*). Indeed, ruxolitinib treatment decreased G-CSF protein concentrations by fourfold in CRTC2/3m cells, demonstrating the importance of CRTC2/3 in regulating BM homeostasis, in part through the up-regulation of SOCS3 and inhibition of JAK/STAT signaling.

Discussion

Consistent with their ubiquitous pattern of expression, the CRTC coactivators appear to mediate transcriptional responses to cAMP in a variety of physiological settings. In keeping with these pleiotropic effects, knockout of both CRTC2 and CRTC3 causes embryonic lethality, but a single allele for either of these family members is sufficient for viability. We found that CRTC2 knockout mice harboring a single allele of CRTC3 develop splenomegaly with peripheral neutrophilia due to G-CSF overexpression in BM stromal cells and perhaps monocyte/macrophage precursors as well.

Within the BM, PGE2 has been found to promote the self-renewal and engraftment of HSCs in part through activation of the CREB pathway (20). Increases in extracellular PGE2 stimulate CRTC dephosphorylation, leading to increases in CREB target gene expression (13). Of the roughly 300 genes that are induced in stromal cells following exposure to cAMP agonist, more than 200 are down-regulated by depletion of CRTC2/3,

demonstrating the importance of both coactivators in mediating CREB-dependent transcription.

We found that the CREB/CRTC pathway modulates cytokine signaling in BM stromal cells in part by up-regulating SOCS3 and thereby reducing STAT3 phosphorylation. Similar to the effects of CRTC depletion, knockout of SOCS3 also promotes neutrophilia and mobilization of HSCs (21). Indeed, we found that exposure to a small-molecule JAK antagonist reduced phospho-STAT3 levels and down-regulated G-CSF expression in CRTC mutant cells.

In addition to SOCS3, we also identified a number of CRTC2/3-regulated CREB target genes that may contribute to the induction of G-CSF and the loss or egress of HSCs from BM. For example, knockout of the transcription factor JunB has been shown to promote G-CSF expression and splenomegaly, although these effects have been localized to skin epidermal cells (22). Mouse CXCL1, an ortholog of human CXCL8, appears to promote HSC engraftment and self-renewal (23). Our results suggest that the down-regulation of CXCL1 in CRTC mutant stromal cells may contribute to the loss of HSCs in this setting. Small molecules that stimulate CRTC activity may provide therapeutic benefit to individuals undergoing BM transplantation.

Materials and Methods

Mice. Crtc2 and Crtc3 KO mice have been described previously (8, 9). $CRTC2^{-/-}$; $CRTC3^{+/-}$ mice were obtained from intercrosses between Crtc2 and Crtc3 double-heterozygous mice. Stromal cell-specific KO of Crtc2/3 was obtained by a two-step cross of Crtc2/3 fl/fl mice (13) with Prx1-cre mice. All animal procedures were performed following an approved protocol from the Salk Institute for Biological Studies Animal Care and Use Committee.

Cells. BMDMs and BM-derived stromal cells were prepared from mouse bone marrow cells as described previously (24).

Reagents and Antibodies. Reagents and antibodies were obtained as follows: PGE2 and FSK were from Sigma-Aldrich; IL-1β was from Abcam; recombinant human G-CSF was from Invitrogen; pSTAT3(Y705,S727), STAT3, pAkt(S473), Akt, pERK(T202/Y204), ERK antibodies were from Cell Signaling; HSP90 was from Invitrogen; and α-G-CSF antibodies were from R&D Systems. All antibodies used for flow cytometry were purchased from e-bioscience. Ruxolitinib was obtained from LC Laboratories and used at 10 nM concentration.

BM Transplantation. Eight- to 10-wk-old recipient mice were lethally irradiated with 1,000 rad. The BM from donor mice was harvested from femurs and tibiae. After lysis of RBC, 2×10^6 cells were administered i.v. into recipient mice after lethal irradiation. After 12 wk, chimeric mice were harvested for analysis.

Peripheral Neutrophil Counts. Blood was collected into EDTA-coated tubes by retro-orbital bleeding. Complete blood count (CBC) was performed at the University of California San Diego's hematology and coagulation core laboratory.

Neutralizing G-CSF. Control IgG or anti–G-CSF antibodies were injected i.p. into WT and $CRTC2^{-/-}$;$CRTC3^{+/-}$ (CRTC2/3m) mice at days 0, 3, and 6, and mice were harvested at day 7. Peripheral and BM $CD11b^{+}GR\text{-}1^{+}$ cells were determined by CBC and flow cytometry.

FACS Analysis. Femora and tibiae were separated and flushed with 0.5% FBS HBSS. After lysis of RBCs, the remaining cells were counted and stained with indicated antibodies.

RNA Studies. Total RNA from cells was extracted using the RNeasy Kit (QIAGEN), and cDNA was generated using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science). cDNA was quantified on a Lightcycler 480 instrument (Roche Applied Science). Gene expression data were calculated relative to the expression of housekeeping gene L32. RNAseq libraries were prepared and analyzed as described previously (25). The GEO accession number for RNAseq studies is (GSE103999).

ELISA. Quantification of G-CSF and PGE_2 protein in plasma was performed using a commercially available ELISA kit (Abcam) according to the manufacturer's instructions.

Western Blot Analysis. BM cell and stromal cell lysates were prepared using lysis buffer (50 mM Tris·Cl pH 8.0, 1% Nonidet P-40, 150 mM NaCl, and protease inhibitor mixture). Proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes, and incubated with indicated antibodies.

Chromatin Immunoprecipitation. Stromal cells were plated in 150-mm plates and exposed to FSK (10 μM) for 2 h. ChIP assays were performed as described previously (26). RNA was isolated using a QIAGEN RNeasy Kit.

Statistics. All studies were performed on at least three different independent occasions. Results are presented as mean ± SEM. Statistical analysis was performed using the two-tailed unpaired Student's *t* test. Differences were considered statistically significant at $P < 0.05$. In the figures, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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