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Brief Report **Oncology**

The immune system has a powerful ability to recognize and kill cancer cells, but its function is often suppressed within tumors, preventing clearance of disease. Functionally diverse innate and adaptive cellular lineages either drive or constrain immune reactions within tumors. The transcription factor (TF) BACH2 regulates the differentiation of multiple innate and adaptive cellular lineages, but its role in controlling tumor immunity has not been elucidated. Here, we demonstrate that BACH2 is required to establish immunosuppression within tumors. Tumor growth was markedly impaired in *Bach2*-deficient mice and coincided with intratumoral activation of both innate and adaptive immunity. However, augmented tumor clearance in the absence of *Bach2* was dependent upon the adaptive immune system. Analysis of tumor-infiltrating lymphocytes from *Bach2*-deficient mice revealed high frequencies of rapidly proliferating effector $CD4^+$ and $CD8^+$ T cells that expressed the inflammatory cytokine IFN-γ. Effector T cell activation coincided with a reduction in the frequency of intratumoral $Foxp3^+$ Tregs. Mechanistically, BACH2 promoted tumor immunosuppression through Treg-mediated inhibition of intratumoral $CD8^+$ T cells and IFN-γ. These findings demonstrate that BACH2 is a key component of the molecular program of tumor immunosuppression and identify therapeutic targets for the reversal of immunosuppression in cancer.

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The transcription factor BACH2 promotes tumor immunosuppression

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The immune system has a powerful ability to recognize and kill cancer cells, but its function is often suppressed within tumors, preventing clearance of disease. Functionally diverse innate and adaptive cellular lineages either drive or constrain immune reactions within tumors. The transcription factor (TF) BACH2 regulates the differentiation of multiple innate and adaptive cellular lineages, but its role in controlling tumor immunity has not been elucidated. Here, we demonstrate that BACH2 is required to establish immunosuppression within tumors. Tumor growth was markedly impaired in *Bach2*-deficient mice and coincided with intratumoral activation of both innate and adaptive immunity. However, augmented tumor clearance in the absence of *Bach2* was dependent upon the adaptive immune system. Analysis of tumor-infiltrating lymphocytes from *Bach2*-deficient mice revealed high frequencies of rapidly proliferating effector CD4$^+$ and CD8$^+$ T cells that expressed the inflammatory cytokine IFN-γ. Effector T cell activation coincided with a reduction in the frequency of intratumoral Foxp3$^+$ Tregs. Mechanistically, BACH2 promoted tumor immunosuppression through Treg-mediated inhibition of intratumoral CD8$^+$ T cells and IFN-γ. These findings demonstrate that BACH2 is a key component of the molecular program of tumor immunosuppression and identify therapeutic targets for the reversal of immunosuppression in cancer.

Introduction

While the immune system has a powerful ability to recognize and kill cancer cells, its function is often suppressed, preventing clearance of disease. A variety of innate and adaptive immune lineages causes immunosuppression within tumors, including immature DCs, plasmacytoid DCs, myeloid-derived suppressor cells, CD4$^+$ Foxp3$^+$ Tregs, IL-10–secreting type-I Tregs, and CD1d-restricted natural killer T cells (1–5). Thus, a relatively well-characterized network of innate and adaptive immunosuppressive cell types drives immunosuppression, but molecular mechanisms required for the development of immunosuppressive responses within tumors are poorly elucidated. TFs play key roles in cellular differentiation and bind to regulatory DNA to control gene expression. BACH2 is a 92-kDa transcription factor (TF) of the basic leucine zipper family and functions within multiple innate and adaptive lineages to control immune function. In B cells, BACH2 is critical for somatic hypermutation and class-switch recombination, and its absence leads to impaired formation of class-switched antibody responses (6, 7). In CD4$^+$ T cells, BACH2 promotes the development of Foxp3$^+$ Tregs by suppressing effector cell transcriptional programs (8, 9). BACH2 is also required for normal development and function of alveolar macrophages (10). Consistent with its role in regulating the development and function of diverse immune cell types, the *BACH2* gene in humans is a prominent susceptibility locus for multiple autoimmune and allergic diseases (11–14). Thus, BACH2 functions in a variety of cellular lineages that can either promote or suppress immune responses against tumors. However, its function in controlling tumor immunity has not been elucidated.

In this study, we have found that BACH2 is required to establish immunosuppression within tumors. We found that growth of B16 melanoma and EL-4 lymphoma tumors was markedly impaired in *Bach2*-deficient mice and coincided with intratumoral activation of both innate and adaptive immunity. However, augmented tumor clearance in the absence of *Bach2* was dependent upon adaptive immunity. Analysis of tumor-infiltrating lymphocytes in *Bach2*-deficient mice revealed high frequencies of rapidly proliferating CD4$^+$ and CD8$^+$ effector cells expressing the inflammatory cytokine IFN-γ. Lymphocyte activation coincided with reduction in the frequency of intratumoral CD4$^+$ Foxp3$^+$ Tregs. Treg-dependent inhibition of intratumoral CD8$^+$ T cells and IFN-γ was required for BACH2-mediated tumor immunosuppression. These findings identify BACH2 as a key component of the molecular program of tumor immunosuppression and identify a target for therapies aimed at reversing immunosuppression in cancer.

Results and Discussion

BACH2 promotes tumor immunosuppression. To determine the function of BACH2 in regulating tumor immunity, we implanted syngeneic B16 melanoma cells subcutaneously into littermate

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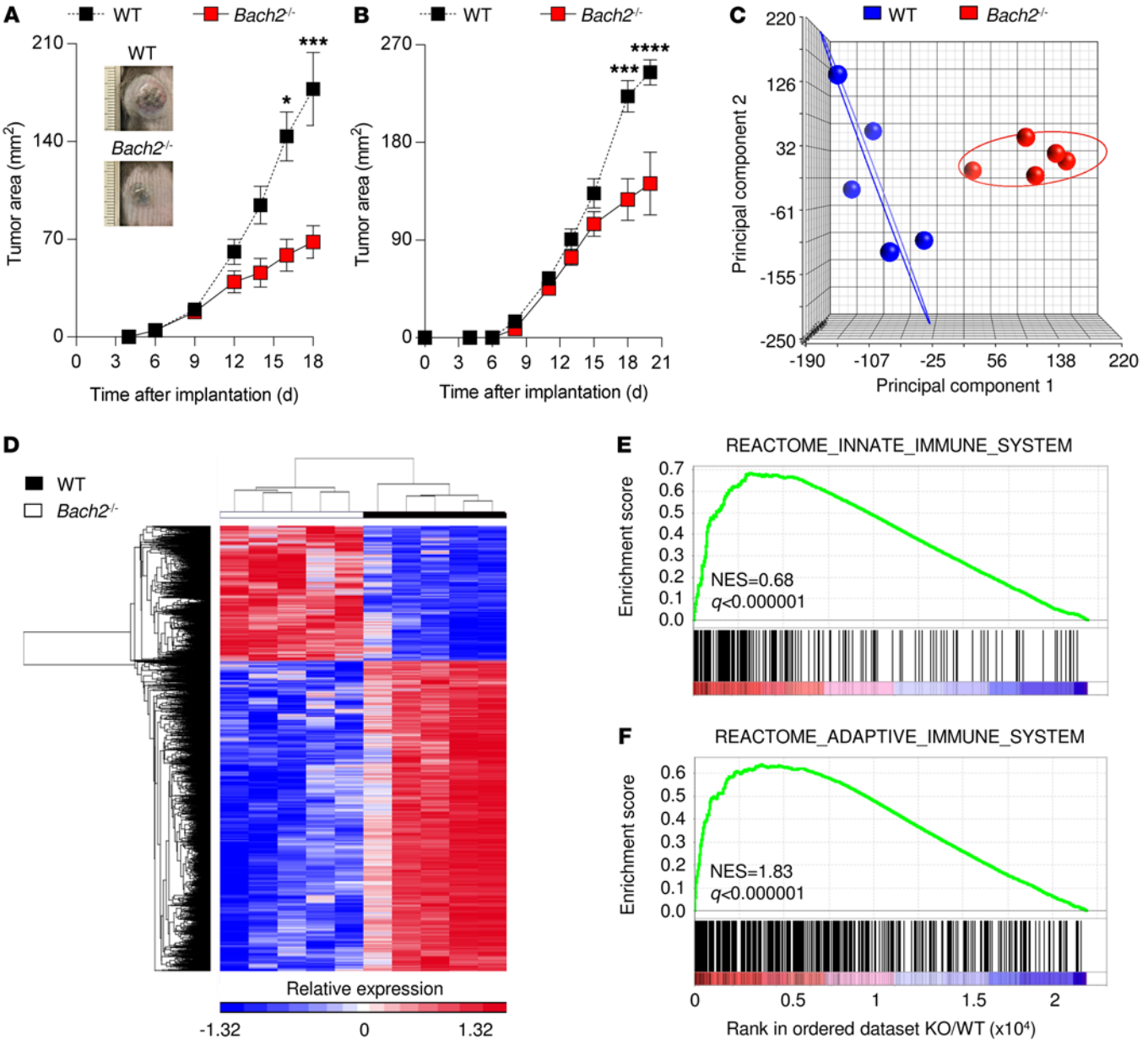


Figure 1. BACH2 promotes immunosuppression within tumors. (**A** and **B**) Growth of subcutaneous (**A**) B16 and (**B**) EL-4 tumors in littermate *Bach2*$^{+/+}$ (WT) and *Bach2*$^{-/-}$ mice at indicated time points following implantation. (**A**) Representative photographs of tumors at day 18 after implantation (inset). Rulers show millimeters. Error bars represent mean ± SEM. *$P < 0.05$; ***$P < 0.005$; ****$P < 0.001$, 2-tailed Student's *t* tests. (**C**) Principal component analysis of transcriptional profiles from tumors of *Bach2*$^{+/+}$ (blue) and *Bach2*$^{-/-}$ (red) mice at day 18 following implantation of B16 tumor cells. Principal component 3 is indicated by the axis perpendicular to the *x* and *y* axes. (**D**) Global differences in transcriptional profiles of tumors from *Bach2*-deficient and WT mice. 3,623 differentially expressed genes identified in the analysis (adjusted $P < 0.05$; fold change > 2, 2-tailed Student's *t* tests) are shown. (**E** and **F**) Gene set enrichment analysis of global transcriptional differences between tumors from *Bach2*-deficient and WT mice. Positions of genes from indicated gene sets within a list of gene expression differences rank ordered by fold change are indicated. Kolmogorov-Smirnov statistic was used to calculate statistical significance. All data are representative of ≥2 independent experiments. NES, normalized enrichment score.

WT or *Bach2*-deficient mice and measured tumor size at serial time points following implantation. Strikingly, we noted reduced growth of B16 tumors in *Bach2*-deficient animals (Figure 1A). Similar observations were made using the EL-4 lymphoma model (Figure 1B). To determine the basis for impaired tumor growth in *Bach2*-deficient animals, we performed whole-tumor transcriptional profiling of B16 tumors from WT and *Bach2*-deficient animals. Whole-transcriptome profiles of tumors from *Bach2*-deficient animals were uniformly distinct from those of WT animals (Figure 1C). Analysis of transcriptional profiles for global differences in gene expression identified 3,623 differentially expressed transcripts (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI82884DS1), which enabled tumors to be distinguished based upon hierarchical cluster analysis (Figure 1D). Gene set enrichment analysis (15) indicated induction of transcriptional signatures of both innate and

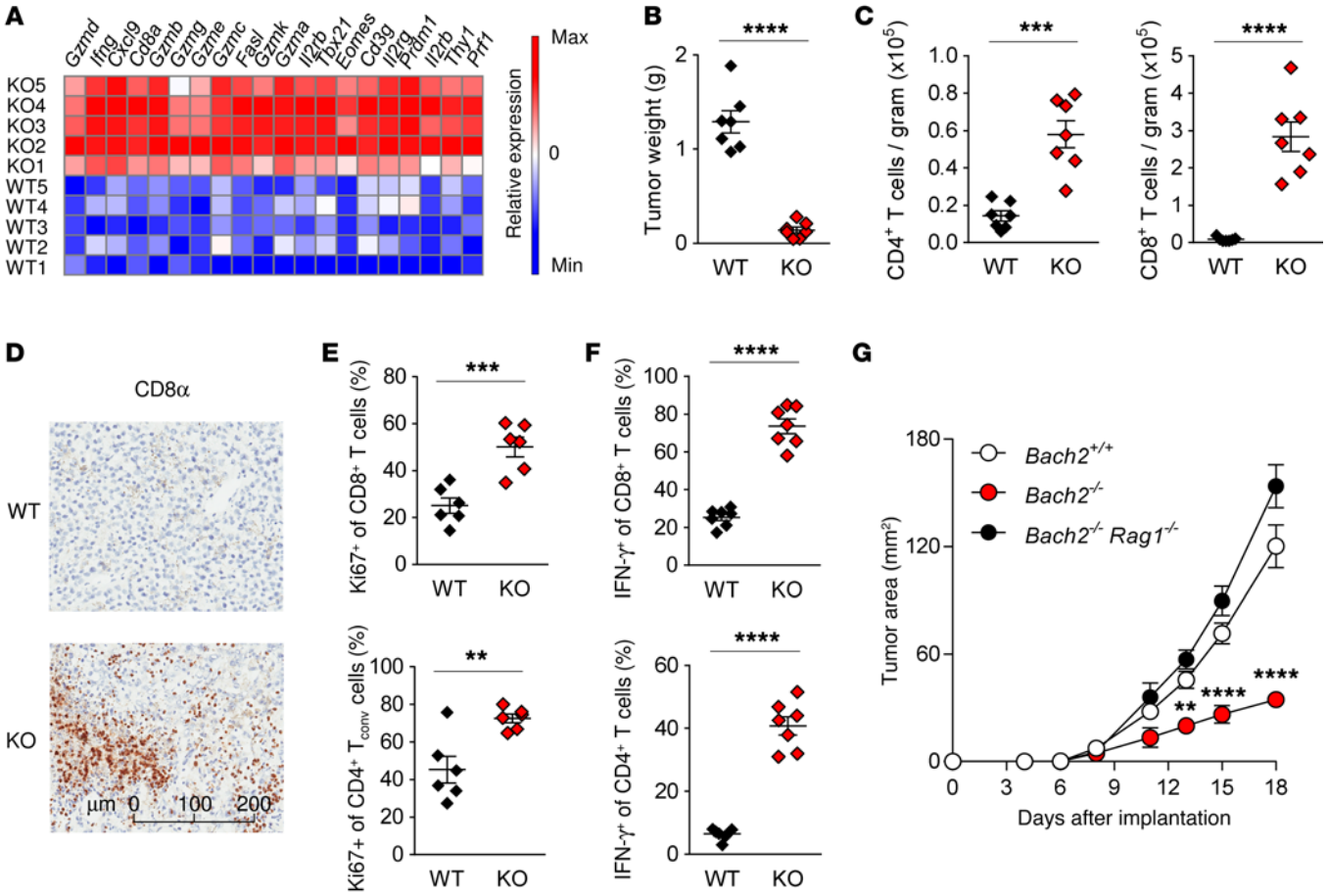


Figure 2. BACH2 constrains T cell–mediated antitumor immunity. (**A**) Heatmap indicating relative expression of indicated genes within global transcriptional profiles of tumors from *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ (KO) animals. (**B**) Wet weight of tumors dissected from WT and *Bach2*$^{-/-}$ animals at day 18 following implantation. (**C**) Density of CD4$^+$ and CD8$^+$ T cells normalized by tumor mass. (**D**) Immunohistochemical analysis of CD8$^+$ T cells in sections of B16 tumors from mice of indicated genotypes at day 18 after implantation. Scale bar: 200 μm. (**E**) Frequency of Ki67$^+$ cells among CD8$^+$ T cells and CD4$^+$ Foxp3$^-$ conventional T (Tconv) cells at day 18 after implantation. (**F**) IFN-γ expression among intratumoral CD4$^+$ and CD8$^+$ T cells at day 18 after implantation. (**G**) Growth of subcutaneous B16 tumors in littermate mice of the indicated genotypes at selected time points following implantation. Significant differences from growth in *Bach2*$^{+/+}$ mice are indicated. Error bars represent mean ± SEM. Results are representative of ≥2 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$, 2-tailed Student's *t* tests.

adaptive immune activation in tumors from *Bach2*-deficient mice (Figure 1, E and F, and Supplemental Tables 2–4). Collectively, these data indicate that BACH2 promotes tumor growth in a tumor cell–extrinsic fashion and suppresses induction of transcriptional profiles indicative of innate and adaptive immune.

BACH2 suppresses adaptive immunity to promote immunosuppression within tumors. CD4$^+$ and CD8$^+$ T cells form a key component of adaptive immune responses against tumors. Whole-tumor transcriptional profiles indicated striking induction of genes associated with CD4$^+$ and CD8$^+$ effector T cells in tumors from *Bach2*-deficient mice. In particular, we noted elevated expression of genes that are expressed by T cells (including *Cd3g*, *Il2rg*, *Thy1*, and *Cd8a*) (16) or associated with effector differentiation (including *Tbx21* and *Prdm1*) (17, 18) and function (including *Ifng*, *Gzmb*, and *Fasl*) (ref. 16 and Figure 2A). We therefore measured infiltration of CD4$^+$ and CD8$^+$ T cells within tumors using flow cytometry. Normalized for differences in tumor mass (Figure 2B), the density of both CD4$^+$ and CD8$^+$ T cells was higher in tumors from *Bach2*-deficient animals (Figure 2C). Increased density of CD4$^+$ and CD8$^+$ T cells was also apparent from immunohistochemical analysis of tumor sections (Figure 2D and Supplemental Figure 1A). Consistent with their increased density, we observed elevated frequencies of proliferating Ki67$^+$ cells among both CD8$^+$ and CD4$^+$ Foxp3$^-$ effector T cells within tumors from *Bach2*-deficient mice (Figure 2E). Moreover, intracellular cytokine staining following brief restimulation of tumor-infiltrating cells ex vivo showed strikingly elevated frequencies of CD4$^+$ and CD8$^+$ T cells expressing the effector cytokine IFN-γ within tumors from *Bach2*-deficient mice (Figure 2F and Supplemental Figure 1B). Thus, BACH2 limits the proliferation of CD8$^+$ and CD4$^+$ effector T cells and their expression of effector cytokines within tumors.

The protein encoded by recombination-activating gene 1 (*Rag1*) is required for rearrangement of T and B cell receptor loci, and its loss results in a complete defect in generation of mature T and B lymphocytes (19). To test whether decreased tumor growth in the absence of BACH2 was dependent upon lymphocytes, we measured growth kinetics of subcutaneously implanted B16 melanoma cells in *Bach2 Rag1* double-deficient animals (Figure 2G).

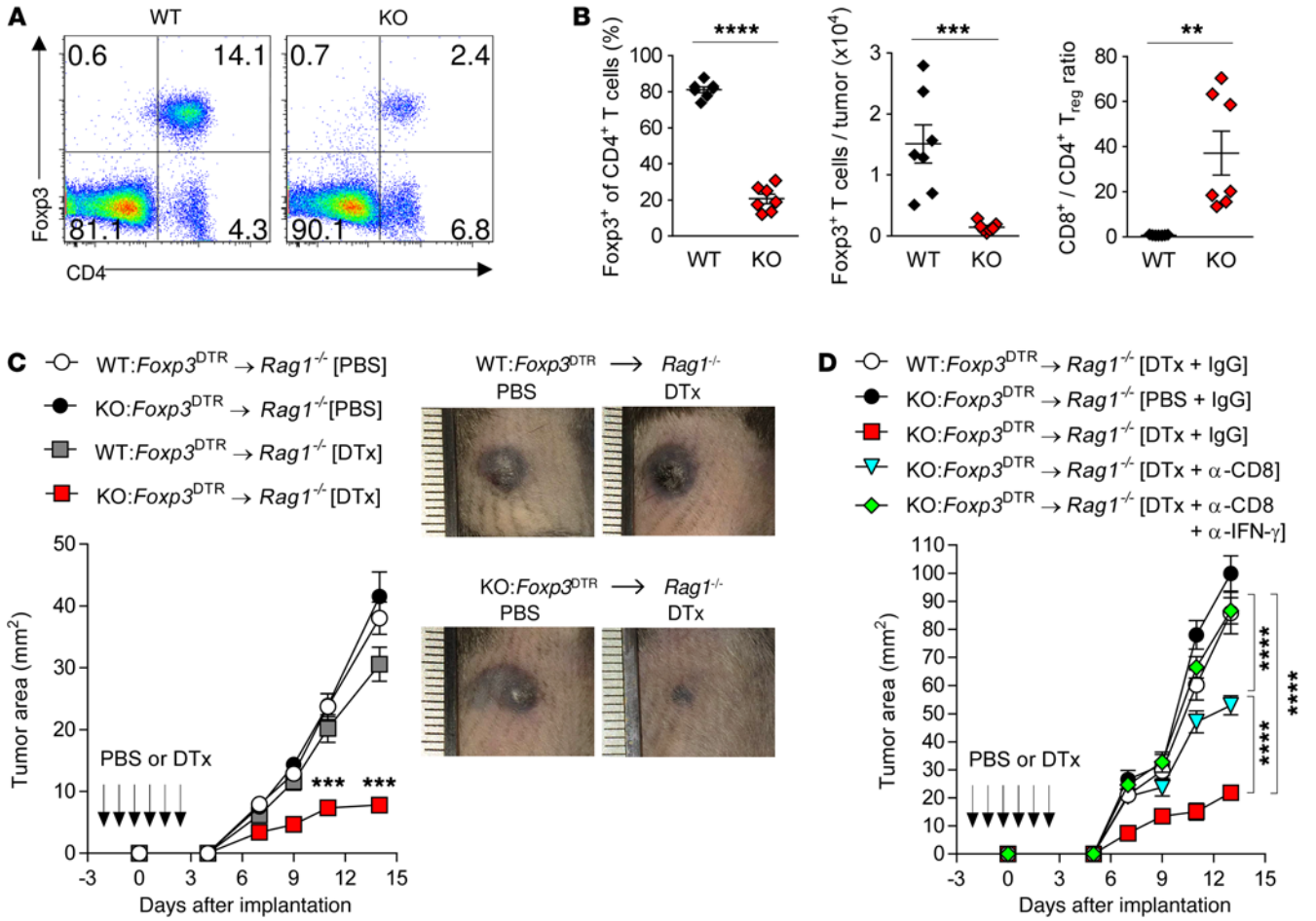


Figure 3. BACH2 promotes tumor growth through Treg-mediated suppression of intratumoral CD8⁺ T cells and IFN-γ. (**A**) Representative flow cytometry and (**B**) replicate measurements of CD4 and Foxp3 expression among T cells infiltrating B16 tumors at day 18 following implantation. Numbers in gates represent percentages of gated cells. (**C**) *Rag1*$^{-/-}$ mice were reconstituted with 1:1 mixtures of BM cells from *Foxp3*^{DTR} mice and either WT or *Bach2*-deficient (KO) mice and administered PBS or DTx at indicated time points relative to implantation. Tumor growth was measured after subcutaneous implantation of 1.25 × 10^5 B16 cells at indicated time points following implantation. Significant differences in tumor area compared with the WT:*Foxp3*^{DTR} →*Rag1*$^{-/-}$ (PBS) group are indicated. Representative photographs of tumors at day 18 after implantation are shown. Rulers show millimeters. (**D**) *Rag1*$^{-/-}$ mice were reconstituted with 1:1 mixtures of BM cells from *Bach2*-deficient and *Foxp3*^{DTR} mice and administered PBS or DTx in conjunction with α-CD8– and/or α–IFN-γ–depleting monoclonal antibodies or isotype control antibodies (IgG) at indicated time points relative to implantation. Tumor growth was measured at indicated time points after subcutaneous implantation of 2.5 × 10^5 B16 cells. All data are representative of ≥2 independent experiments. Error bars represent mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$, 2-tailed Student's *t* tests.

Consistent with our previous observations, tumor growth was markedly impaired in *Bach2*-deficient animals. However, this was dependent upon the activity of lymphocytes, since augmented tumor clearance was not seen in *Bach2 Rag1* double-deficient animals. Thus, decreased tumor growth in the absence of BACH2 requires the adaptive immune system.

BACH2 causes tumor growth through Treg-mediated suppression of CD8⁺ T cells. While CD8⁺ T cells primarily function to promote tumor clearance, CD4⁺ T cells can either drive or constrain immune responses against tumors through the reciprocal function of effector and regulatory cell lineages, respectively (2, 3, 20). Within tumors, Tregs can powerfully inhibit the accumulation and function of CD4⁺ and CD8⁺ effector T cells (3). We have previously found that BACH2 promotes Foxp3⁺ Treg development through repression of helper cell transcriptional programs (8, 21). We wished to determine whether BACH2-mediated tumor immunosuppression was dependent upon its role in Treg development. In mice, Tregs can be identified by their expression of the TF Foxp3 (22–24). We observed substantially diminished Foxp3⁺ Treg populations in tumors from *Bach2*-deficient animals when expressed as either their frequency relative to total CD4⁺ T cells or CD8⁺ T cells within tumors or as their absolute number (Figure 3, A and B). The frequency of Foxp3⁺ Tregs was similarly reduced in both tumors and spleens of BACH2-deficient mice (Supplemental Figure 2), suggesting that defective Treg-mediated tumor immunosuppression in BACH2-deficient mice may correspond to the generalized Treg deficiency in these animals.

We wished to determine the relative contribution of Foxp3⁺ Tregs to BACH2-mediated tumor immunosuppression. Since Tregs suppress immune function in an immunodominant fashion, phenotypes resulting from their deficiency are amenable to rescue by provision of WT Tregs. Tregs from mice expressing

the human diphtheria toxin (DTx) receptor (DTR) under the control of the endogenous *Foxp3* locus (*Foxp3*^{DTR} mice) are sensitive to depletion following administration of DTx (25). To test whether BACH2-mediated immunosuppression is dependent upon the function of BACH2 in Treg development, we reconstituted irradiated *Rag1*$^{-/-}$ mice with 1:1 mixtures of bone marrow (BM) cells from *Foxp3*^{DTR} mice and either WT or *Bach2*-deficient mice, resulting in WT:*Foxp3*^{DTR} and KO:*Foxp3*^{DTR} mixed chimeric animals. This system allows experimental interrogation of Treg-dependent phenotypes attributable to *Bach2* deficiency, since *Bach2*-sufficient Tregs are provided by the *Foxp3*^{DTR} compartment, except in cases in which DTx is administered. Using this system, we observed equivalent chimerism of WT and *Bach2*-deficient CD4$^+$ and CD8$^+$ T cells within reconstituted WT:*Foxp3*^{DTR} and KO:*Foxp3*^{DTR} mixed chimeric animals, respectively (Supplemental Figure 3, A and B), and near-complete DTx -induced depletion of Foxp3$^+$ Tregs within *Foxp3*^{DTR} compartments (Supplemental Figure 3C). Strikingly, BACH2-mediated tumor immunosuppression was dependent upon Foxp3$^+$ Tregs, since impaired tumor growth and loss of immunosuppression was only observed when DTx was administered to KO:*Foxp3*^{DTR} mice (Figure 3C). Growth of subcutaneous tumors in reconstituted *Rag1*$^{-/-}$ radiation chimeras occurred with distinct kinetics compared with that in WT nonirradiated animals, as is consistent with our previous experience (our unpublished observations), and the duration of measurement in these experiments was limited by systemic effects of Treg depletion in mice reconstituted with mixtures of KO and *Foxp3*^{DTR} BM and administered DTx. Collectively, these findings suggest that BACH2-mediated tumor immunosuppression is dependent upon Foxp3$^+$ Tregs.

We observed increased frequencies of CD8$^+$ T cells and elevated production of IFN-γ within tumors of *Bach2*-deficient animals. This led us to ask whether Treg-dependent tumor immunosuppression mediated by BACH2 is caused by suppression of CD8$^+$ T cells or IFN-γ. Strikingly, augmented tumor immunity observed upon administration of DTx to KO:*Foxp3*^{DTR} mice was partially reversed upon depletion of CD8$^+$ T cells and fully reversed upon simultaneous depletion of CD8$^+$ T cells and blockade of IFN-γ (Figure 3D). Thus, we conclude that BACH2 causes tumor growth through Treg-mediated suppression of CD8$^+$ T cells and concomitant IFN-γ–dependent effector mechanisms.

In this study, we have found that the TF BACH2 is required for establishment of immunosuppression within tumors. Despite its activity in a diversity of innate and adaptive immune cell types, it predominantly exerts this function through its role in CD4$^+$ Tregs, thus potently suppressing the antitumor activity of CD8$^+$ T cells. These findings identify a molecular axis of tumor immunosuppression and provide targets for design of immune-based therapies aimed at reversing deleterious immunosuppression in cancer.

Methods

Additional details can be found in the Supplemental Methods.

Animals. C57BL/6J and *Rag1*$^{-/-}$ (B6.129S7-Rag1$^{tm1Mom/J}$) mice were purchased from The Jackson Laboratory. *Bach2*-deficient mice, which have been previously described (6), were backcrossed >16 times with C57BL/6 mice.

Data deposition. Data from whole-transcriptome analyses are deposited under GEO accession number GSE74653.

Statistics. Two-tailed Student's *t* tests were used to calculate statistical significance of the difference in sample means. *P* values of less than 0.05 were considered significant and are provided.

Study approval. Experiments were approved by the Institutional Animal Care and Use Committee of the National Cancer Institute and performed in accordance with NIH guidelines.

Author contributions

RR, RLE, DC, CAK, LG, and NPR conceived the study and wrote the manuscript. RR, RLE, DC, GM, ZY, HL, JHP, and PJ performed experiments. PJ and RR performed bioinformatic analyses. MS, FMG, ZY, YJ, DCP, AC, JGC, SJP, DS, EW, FMM, KO, and NPR edited the manuscript.

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