Immune evasion and the suppression of antitumor responses during cancer progression are considered hallmarks of cancer and are typically attributed to tumor-derived factors. Although the molecular basis for the crosstalk between tumor and immune cells is an area of active investigation, whether host-specific germline variants can dictate immunosuppressive mechanisms has remained a challenge to address. A commonly occurring germline mutation (c.1162G>A/rs351855 G/A) in the FGFR4 (CD334) gene enhances signal transducer and activator of transcription 3 (STAT3) signaling and is associated with poor prognosis and accelerated progression of multiple cancer types. Here, using rs351855 SNP–knockin transgenic mice and Fgfr4-knockout mice, we reveal the genotype-specific gain of immunological function of suppressing the CD8/CD4+FOXP3+CD25+ regulatory T cell ratio in vivo. Furthermore, using knockin transgenic mouse models for lung and breast cancers, we establish the host-specific, tumor-extrinsic functions of STAT3-enhancing germline variants in impeding the tumor infiltration of CD8 T cells. Thus, STAT3-enhancing germline receptor variants contribute to immune evasion through their pleiotropic functions in immune cells.

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STAT3-enhancing germline mutations contribute to tumor-extrinsic immune evasion

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Immune evasion and the suppression of antitumor responses during cancer progression are considered hallmarks of cancer and are typically attributed to tumor-derived factors. Although the molecular basis for the crosstalk between tumor and immune cells is an area of active investigation, whether host-specific germline variants can dictate immunosuppressive mechanisms has remained a challenge to address. A commonly occurring germline mutation (c.1162G>A/rs351855 G/A) in the FGFR4 (CD334) gene enhances signal transducer and activator of transcription 3 (STAT3) signaling and is associated with poor prognosis and accelerated progression of multiple cancer types. Here, using rs351855 SNP–knockin transgenic mice and Fgfr4−/−knockout mice, we reveal the genotype-specific gain of immunological function of suppressing the CD8/CD4+FOXP3+CD25+ regulatory T cell ratio in vivo. Furthermore, using knockin transgenic mouse models for lung and breast cancers, we establish the host-specific, tumor-extrinsic functions of STAT3-enhancing germline variants in impeding the tumor infiltration of CD8 T cells. Thus, STAT3-enhancing germline receptor variants contribute to immune evasion through their pleiotropic functions in immune cells.

Introduction
Immune evasion is considered the hallmark of cancers (1). Strategies that restore the capacity of the immune system to recognize and eliminate malignant cells have produced clinical benefits. However, due to a dearth of predictive biomarkers for patient stratification, only one-third of all patients are responsive to treatment (2). Immune evasion by tumor tissues has been the major bottleneck in the development of therapeutically effective anticancer strategies. The prominent mechanisms by which tumors evade immune attack include the evolution of tumor cell variants that are resistant to immune effectors and the progressive formation of an immune suppressive microenvironment within the tumor that impedes the infiltration of antitumor effector cells. Great emphasis has been placed on understanding the function of tumor-intrinsic somatic heterogeneity or the tumor-induced microenvironment in evading immune surveillance, but the role played by tumor-extrinsic, host-specific genetic heterogeneity in modulating the antitumor immune response is poorly understood and remains challenging to address.

An attribute common to all cancers is the presence of numerous cell types, including bone marrow–derived inflammatory cells, lymphocytes, fibroblastic cells, and the extracellular matrix composed of collagen and proteoglycans. Importantly, of diverse assemblages of tumor cell infiltrates, cytotoxic and regulatory T lymphocytes within the tumors are often the crucial factors that determine the outcome of anticancer therapy (3, 4). For example, the increased T cell numbers, particularly an increased ratio of CD8/FOXP3+ regulatory T cells (Tregs) within the tumor microenvironment (TME), predict a favorable therapeutic response, whereas severe lymphopenia negatively impacts the chemotherapeutic and immunotherapy response (5). It is well recognized that while the host immune system can recognize and reject cancerous cells, it can also mold the somatic heterogeneity of tumors by assisting in the generation of immune-resistant tumor variants (6). Various mechanisms, such as immunoediting, exist whereby primary tumor rejection is rendered compromised and ineffective by the inhibition of cytotoxic CD8 T cell infiltration or viability in the TME. An imminent question thus arises: can individual-specific heritable genetic variants regulate immune homeostasis such that immune surveillance is impaired in a host-dependent manner irrespective of the nature of oncogenic onslaught? Here, we attempted to address this question by dissecting the tumor-extrinsic immunological function of signal transducer and activator of transcription 3–enhancing (STAT3-enhancing) germline receptor variants in shaping the TME. Many of the genes that have been studied to modulate immune responses have variants that occur in frequencies ranging from rare (<1%) to common (>10%) in the general population. There are approximately 907.3 million SNPs catalogued in the Single Nucleotide Polymorphism Database (dbSNP) build 150 (Feb 3, 2017), and it is practically impossible to systemically evaluate all polymorphic SNPs in the human genome through association studies alone. Here, we demonstrate for the first time that by examining the cancer-associated germline receptor variants that enhance the amplitude of STAT3 signaling...
in a genotype-dependent manner (7), potential individual-specific
modulators of cancer immune surveillance can be systematically
evaluated. Amplified STAT3 signaling is promitotic in cancer
cells, whereas studies using targeted ablation of STAT3 signal-
ing in immune cells, such as DCs, CD8 T cells, regulatory T cells,
NK cells (8), and macrophages (9–11), establish the immunosup-
pressive properties of constitutively activated STAT3. We hypoth-
esized that STAT3-enhancing germline variants are potentially
the tumor-extrinsic germline-encoded determinants of immune
evasion in the TME. Our work provides valuable insights into the
predictive value of host-specific STAT3-enhancing germline vari-
ants in impeding the immune cell infiltration of tumors.

Results and Discussion
To identify all SNP variants that create membrane-proximal
tyrosine-based STAT3 docking motifs, we performed a compre-
hensive computational analysis of all publicly available human
genotyping data sets, namely, the 1,000 Genomes, Phase 3 (12),
the Catalogue of Somatic Mutations in Cancer (COSMIC) (13),
the Cancer Cell Line Encyclopedia (CCLE) (14), the dbSNP
(15), the Exome Aggregation Consortium (ExAC.r0.3) (16), the
International HapMap Project (HAPMAP) (17), the United States
National Cancer Institute (NCI) 60 human tumor cell line (NCI-
60) exomes (18), the Cancer Genome Atlas (TCGA) (19), and
the whole genome sequences of healthy elderly people (Well-
derly) (20), using our new python-based algorithm called the
Transmembrane Protein Sequence Variant Identifier (TraPS-
Varl) (21). We reviewed the approximately one billion human
variants analyzed, and identified SNPs in the human variome
(Supplemental Table 1; supplemental material available online
with this article; https://doi.org/10.1172/JCI96708DS1) that cre-
ate membrane-proximal STAT3 binding sites in juxtamembrane
segments. Interestingly, a large majority of these rare SNPs in
type I membrane proteins exhibited expression patterns restric-
ed to either professional antigen-presenting cells (namely,
CD11b+Ly6c+MHCIi+ monocytes, CD11b+F4/80+CD11c+MHCIi–
SiglecF– macrophages, or CD45+CD11b+CD11c+MHCIi+ DCs)
or immunosuppressive CD3+CD4+CD25+FOXP3+ regulatory T
cells (Supplemental Table 1 and Supplemental Figure 1, A–C;
see complete unedited blots in the supplemental material). The
SNP allele rs351855-A encoding the fibroblast growth factor
receptor 4 variant (FGFR4 p.Gly388Arg) is the only commonly
occurring STAT3-enhancing receptor variant with a minor allele
frequency of 0.3 in the general population. Among the immune
cells profiled, FGFR4 (alias CD334) was highly expressed in
CD4+CD25+FOXP3+ Tregs (Figure 1A; see complete unedited
blots in the supplemental material). Using FOXP3-GFP−knockin
reporter mice (22), we found that protein levels of FGFR4 in
CD4+CD25+FOXP3+ or CD4+GITR+FOXP3+ Treg populations
are elevated when Tregs are resident in the lymph nodes (Figure
1B and Supplemental Figure 2). Here, using transgenic rs351855
SNP–knockin mice (homozygous for minor allele rs351855-A
denoted hereafter by Fgfr4 rs351855−A/A) and their WT littermates
(homozygous denoted by Fgfr4 rs351855−G/G), we asked whether
STAT3-enhancing germline variants can shape the TMEs pleio-
tropically independent of the cancer types. We first ascertained
that the minor allele variant rs351855-A was functional in Tregs
as indicated by elevated levels of 705-tyrosine phosphorylated
STAT3 (pY705) (Supplemental Figure 3, A–D; see complete
unedited blots in the supplemental material) in Fgfr4rs351855−A/A
mice. No obvious differences were detected when we monitored
Fgfr4rs351855−A/A− and Fgfr4rs351855−G/G−knockin mice at 5 to 6 months
of age by assessing the proportions of monocytes/macrophages and
B and T lymphocytes in lymphoid compartments, including bone
marrow, thymus, blood, lymph nodes, and spleen (Supplemen-
tal Figure 4, A–C). Likewise, analyses for the proportions of NK
cells and TCRγδ+ T cells in the thymus, spleen, and lymph nodes
of Fgfr4rs351855−G/G and Fgfr4rs351855−A/A mice (Supplemental Figure 5,
A and B) showed no significant differences between the 2 genotypes. However, the numbers of CD8+ T cells were significantly decreased in the thymus, blood, lymph nodes, and spleen of Fgfr4 rs351855–A/A mice compared with those of the WT Fgfr4 rs351855–G/G littermates (Figure 2A and Supplemental Figure 6, A and B). The suppressed levels of CD8+ T cells in Fgfr4 rs351855–A/A genotypes appeared as a systemic trait, since lower levels were also found in the nonlymphoid organs analyzed, including parenchymal tissues such as lung and mammary tissue pads (Figure 2B). On the other hand, the quantification of FOXP3+CD25+ Tregs revealed an apparent increase in Tregs under unchallenged homeostasis conditions in healthy adult mice (Figure 2C and Supplemental Figure 6C). Concordantly, the levels of Foxp3 and Il10 transcripts were significantly elevated, whereas the levels of Cd8 mRNA transcripts were decreased in the spleens of Fgfr4 rs351855–A/A mice (Supplemental Figure 7, A–C), further supporting a general decrease in the CD8/Treg ratio in vivo. Interestingly, immunophenotyping analyses of WT (Fgfr4+/+) and Fgfr4-deficient (Fgfr4−/−) mice showed no significant alterations in the CD8/Treg ratio (Figure 2D and E) or other immune cells analyzed, including NK cells, TCRγδ T cells, B cells, and macrophages (Supplemental Figure 8, A–C) either in lymphoid or in parenchymal organs (data not shown).

To functionally consolidate our findings, we determined that an increase in pSTAT3 at Y705 in Tregs resulted in enhanced proliferation and suppressive functions of Tregs. Isolated Tregs from CD4+CD25+FOXP3+ cells in the thymus and spleen of 6- to 8-week-old Fgfr4 rs351855–A/A and Fgfr4 rs351855–G/G mice measured by flow cytometry. Data represent the percentages of total single-cell suspensions (mean ± SEM, n = 5–8, **P < 0.01, ***P < 0.001). Infiltrating cells were measured by preparing single-cell suspensions. (C) Analysis of CD4+CD25+FOXP3+ T cell numbers by flow cytometry of live splenocytes from Fgfr4 rs351855–A/A and Fgfr4 rs351855–G/G mice. Data represent the percentages of total single-cell suspensions (mean ± SEM, n = 5–8, **P < 0.05, ***P < 0.001). Statistical comparisons of groups were performed using 2-way ANOVA and Tukey’s t test with multiple comparisons. (D) Quantitative analysis of CD4+ and CD8+ cells in the thymus and spleen of 6- to 8-week-old Fgfr4 rs351855−/− and Fgfr4−/− mice and (E) CD4+CD25+FOXP3+ cells in the thymus and spleen of 6- to 8-week-old Fgfr4 rs351855−/− and Fgfr4−/− mice measured by flow cytometry. Data represent the percentages of total single-cell suspensions (mean ± SEM, n = 5–8, NS = not significant). Statistical comparisons of groups were performed using 2-way ANOVA and Tukey’s t test with multiple comparisons. All flow cytometry measurements on WT and mutant cohorts of mice were performed on the same day.
models (GEKIMM) for breast and lung cancers (see Methods).

As expected, although the tumor incidence rates in both the disease models were not dramatically altered, the tumor burden and progression were significantly elevated in animals expressing the minor allele variant rs351855-A (25). Flow cytometric analysis of the age- and sexual phenotype–matched cohorts of knockin transgenic mouse models for breast (26) (Fgfr4rs351855–G/G WAP-Tgfa and Fgfr4rs351855–A/A WAP-Tgfa) and lung (27) (Fgfr4 rs351855–G/G SPC-CrafBxB and Fgfr4rs351855–A/A SPC-CrafBxB) cancers revealed a significant increase in the proportions of CD4+CD25+FOXP3+ Tregs in the tumors extracted from Fgfr4rs351855–A/A–knockin mice (Figure 4, A and B). Furthermore, the significant increase in tumor-infiltrating Tregs correlated with increased Tregs in lymphoid organs (data not shown) and elevated serum levels of IL10 in Fgfr4rs351855–A/A WAP-Tgfa (Figure 4C) and Fgfr4rs351855–A/A SPC-CrafBxB (Figure 4D) mice. On the other hand, a marked reduction in the numbers of tumor-infiltrating CD8+ T cells was observed in the Fgfr4rs351855–A/A cohorts of GEKIMMs for both breast (Figure 4E) and lung (Figure 4F) cancers. Although STAT3 signaling is considered crucial for T helper cell differentiation during immune challenges, we did not observe any significant differences between the 2 genotypes in the transgenic disease models for breast or lung cancer. The proportions of differentiated CD4+ T cell subsets (namely, Th1, Th2, and Th17) in spleens and tumors of GEKIMM for lung cancer (Supplemental Figure 10) and GEKIMM for breast cancer (Supplemental Figure 11) were not altered. We propose that the in higher CD8/Treg ratios (32:1, 16:1), led to similar suppressive capacities by both genotypes (Supplemental Figure 9). IL10 signals primarily by inducing pSTAT3 at Y705 via the STAT3 docking sites in the cytoplasmic domains of IL10R (23). Therefore, we conclude that the synergistic action of the rs351855–A allele with IL10 signaling explains the enhanced suppressive functions of Tregs in Fgfr4rs351855–A/A–knockin mice. Thus, under healthy homeostatic conditions, a germline-encoded increase in basal pSTAT3 (Y705) levels in Tregs leads to a systemic decrease in the CD8/Treg ratio. This finding suggests that alterations in the CD8/Treg ratio in vivo are mechanistically linked to the presence of the minor allele rs351855-A and are not determined by the activity of Fgfr4. The STAT3-enhancing gain of function by the minor allele of rs351855 is independent of the extracellular or intracellular domains of FGFR4 and is mediated by the membrane-proximal STAT3 docking site in the juxtamembrane segment of the Fgfr4 p.Gly388Arg variant (24). We therefore attribute the genotype-dependent systemic suppression of the CD8/Treg ratio to the pleiotropic effect of STAT3-enhancing gain of function by the SNP rs351855-A. Disruption of the STAT3 membrane-recruitment event by the depletion of Fgfr4 in the Fgfr4−/− mice abolished the SNP-specific gain of the immunological phenotype (Figure 3C).

To determine whether STAT3-enhancing germline variants mediate a tumor-extrinsic immune evasive pleiotropic phenotype, we generated genetically engineered SNP-knockin mouse models (GEKIMM) for breast and lung cancers (see Methods). As expected, although the tumor incidence rates in both the disease models were not dramatically altered, the tumor burden and progression were significantly elevated in animals expressing the minor allele variant rs351855-A (25). Flow cytometric analysis of the age- and sexual phenotype–matched cohorts of knockin transgenic mouse models for breast (26) (Fgfr4rs351855–G/G WAP-Tgfa and Fgfr4rs351855–A/A WAP-Tgfa) and lung (27) (Fgfr4rs351855–G/G SPC-CrafBxB and Fgfr4rs351855–A/A SPC-CrafBxB) cancers revealed a significant increase in the proportions of CD4+CD25+FOXP3+ Tregs in the tumors extracted from Fgfr4rs351855–G/G–knockin mice (Figure 4, A and B). Furthermore, the significant increase in tumor-infiltrating Tregs correlated with increased Tregs in lymphoid organs (data not shown) and elevated serum levels of IL10 in Fgfr4rs351855–G/G WAP-Tgfa (Figure 4C) and Fgfr4rs351855–A/A SPC-CrafBxB (Figure 4D) mice. On the other hand, a marked reduction in the numbers of tumor-infiltrating CD8+ T cells was observed in the Fgfr4rs351855–A/A–knockin mice (Figure 4E and Figure 4F). Although STAT3 signaling is considered crucial for T helper cell differentiation during immune challenges, we did not observe any significant differences between the 2 genotypes in the transgenic disease models for breast or lung cancer. The proportions of differentiated CD4+ T cell subsets (namely, Th1, Th2, and Th17) in spleens and tumors of GEKIMM for lung cancer (Supplemental Figure 10) and GEKIMM for breast cancer (Supplemental Figure 11) were not altered. We propose that the
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Tor variants that enhance the amplitude of STAT3 signaling are potent modulators of tumor-intrinsic proliferative and tumor-extrinsic immune evasive functions. Overall, our work provides valuable insight into the prognostic value of STAT3-enhancing germline receptor variants in the immune-excluded and immunologically ignorant tumor phenotype. Given that the germline-encoded STAT3-enhancing SNPs are particularly prevalent in the coding regions of immune cell surface markers, further work is warranted to explore their significance as predictive biomarkers for immunotherapy responses.

Figure 4. Rs351855 SNP–specific suppression of the CD69/Treg ratio in the TME. (A) Numbers of CD4+CD25+FOXP3+ T cells in tumor-bearing breast tissue of Fgf4δ4351855-G/G Wap-Tgfa, Fgf4δ4351855-G/A Wap-Tgfa, and Fgf4δ4351855-A/A Wap-Tgfa mice (mean ± SEM, n = 5, **P < 0.01, ***P < 0.001). (B) Numbers of CD4+CD25+FOXP3+ T cells in tumor-bearing lungs of Fgf4δ4351855-G/G SPC-CrafBxB, Fgf4δ4351855-G/A SPC-CrafBxB, and Fgf4δ4351855-A/A SPC-CrafBxB mice (mean ± SEM, n = 4–7, **P < 0.01, ***P < 0.001). (C) Quantification of IL10 in serum of tumor-bearing Fgf4δ4351855-G/G Wap-Tgfa and Fgf4δ4351855-A/A Wap-Tgfa breast cancer mice (mean ± SEM, n = 6–9, **P < 0.01) and (D) tumor-bearing Fgf4δ4351855-G/G SPC-CrafBxB and Fgf4δ4351855-A/A SPC-CrafBxB lung cancer mice (mean ± SEM, n = 8–12, ***P < 0.001) by ELISA. (E and F) Quantification of tumor-infiltrating CD8+ T cells in tumor nodules by immune staining for CD8 in breast tumor-bearing Fgf4δ4351855-G/G Wap-Tgfa and Fgf4δ4351855-A/A Wap-Tgfa and (F) lung tumor-bearing Fgf4δ4351855-G/G SPC-CrafBxB and Fgf4δ4351855-A/A SPC-CrafBxB mice (mean ± SEM, n = 19–26, ****P < 0.0001, **P < 0.01, 2-tailed unpaired t test with Welch’s correction). Insets: Representative images from immunofluorescence staining of tumor sections (×20 magnification) depicting tumor-infiltrating CD8+ T cells in lung and breast tumors (DAPI-blue, CD8-green). The red text in the figure denotes the minor allele of the SNP rs351855.

differences in these subsets may be notable in the mouse models for inflammation-induced cancers. Collectively, through data from knockin mice, knockout mice, and genetically engineered knockin mouse models for lung and breast cancers, our study illustrates a pleiotropic effect of cancer-associated STAT3-enhancing germline variants in shaping some aspects of the TME (Supplemental Figure 12). Hence, we conclude that the immune evasive phenotype of the TME can be determined by the pleiotropic functions of individual-specific germline variants in the immune cells. In this regard, cancer-associated germline receptor variants that enhance the amplitude of STAT3 signaling are potent modulators of tumor-intrinsic proliferative and tumor-extrinsic immune evasive functions. Overall, our work provides valuable insight into the prognostic value of STAT3-enhancing germline receptor variants in the immune-excluded and immunologically ignorant tumor phenotype. Given that the germline-encoded STAT3-enhancing SNPs are particularly prevalent in the coding regions of immune cell surface markers, further work is warranted to explore their significance as predictive biomarkers for immunotherapy responses.
Methods
A complete description of the methods and statistical analysis is provided in the Supplemental Materials.

Study approval. All of the experiments were performed under protocols approved and reviewed by the Institutional Animal Care and Use Committee at the Max Planck Institute of Biochemistry. Animal protocols and experimental procedures involving FGFR4-KO mice were approved by the Institutional Animal Care and Use Committee at the University of Miami Miller School of Medicine.

Author contributions
VKU conceived and designed the study. DK wrote the codes for the TraPS-VarI algorithm. VKU and DK performed the computation analyses. AG, CY, and CF performed the immune phenotyping analyses for the Fgfr4-knockout mice. VKU performed the experiments, analyzed the data, interpreted the results, and wrote the manuscript. The final version of the manuscript was reviewed by all the coauthors.

Acknowledgments
The authors thank Axel Ullrich (Max Planck Institute of Biochemistry) for reading the drafts of this manuscript and generously supporting this work, Kerstin Berer (Max Planck Institute of Neurobiology) and Gurumoorthy Krishnamoorthy (Max Planck Institute of Biochemistry) for advice and useful comments, and Susanne Wuchenerau and Bianca Sperl (Max Planck Institute of Biochemistry) for technical assistance. The authors acknowledge Heinz Brandstetter and Corrina Moerth (Max Planck Institute of Biochemistry) for their services with the animal housing facilities. AG and CF are supported in part by the American Heart Association. CF is supported by the American Diabetes Association (1-16-IBS-087) and by the NIH (R01HL128174).

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