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ORIGINAL RESEARCH

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Interferon regulatory factor 4 plays a pivotal role in the development of aGVHD-associated colitis

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ABSTRACT

Interferon regulatory factor 4 (IRF4) is a master transcription factor that regulates T helper cell (Th) differentiation. It interacts with the Basic leucine zipper transcription factor, ATF-like (BATF), depletion of which in $CD4^+$ T cells abrogates acute graft-versus-host disease (aGVHD)-induced colitis. Here, we investigated the immune-regulatory role of *Irf4* in a mouse model of MHC-mismatched bone marrow transplantation. We found that recipients of allogenic *Irf4*$^{-/-}$ $CD4^+$ T cells developed less GVHD-related symptoms. Transcriptome analysis of re-isolated donor *Irf4*$^{-/-}$ $CD4^+$ T helper (Th) cells, revealed gene expression profiles consistent with loss of effector T helper cell signatures and enrichment of a regulatory T cell (Treg) gene expression signature. In line with these findings, we observed a high expression of the transcription factor BTB and CNC homolog 2; (BACH2) in *Irf4*$^{-/-}$ T cells, which is associated with the formation of Treg cells and suppression of Th subset differentiation. We also found an association between BACH2 expression and Treg differentiation in patients with intestinal GVHD. Finally, our results indicate that IRF4 and BACH2 act as counterparts in Th cell polarization and immune homeostasis during GVHD. In conclusion, targeting the BACH2/IRF4-axis could help to develop novel therapeutic approaches against GVHD.

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Acute GVHD; BACH2; IRF4; T helper cells

Introduction

Graft-versus-host disease (GVHD) is a frequent complication after hematopoietic stem cell transplantation (HSCT) and still associated with high morbidity and mortality.[1–5] Acute GVHD (aGVHD) emerges due to differences in minor and major histocompatibility antigens (HLA) between donor and recipients leading to activation of allogenic T cells and induction of the disease.[6]

To date, the role of the distinct T helper cell (Th)-subsets in GVHD pathogenesis is not completely understood. Th-1 cells and regulatory T cells (Treg) induce and suppress GVHD, respectively.[7–15] Whereas numerous publications have shown that Th-2 cells are more suppressive,[11,15–18] the Th-17 cells are thought to be responsible for the development of severe aGVHD.[7,15,19–21]

Interestingly, these subsets share the transcription factor Interferon Regulatory Factor 4 (IRF4) which regulates T cell fate decisions.[22] IRF4 is not only essential for the formation of Th-2, Th-9, Th-17 and T-follicular helper cells (Tfh), but also acts as a central regulator for Th-1 cells.[23–33] Additionally, IRF4 plays an important role in Th-driven autoimmune diseases, transplant rejection and inflammatory bowel disease.[34–36]Furthermore, IRF4 was found to regulate the differentiation of different Treg subsets.[36–38]

Here, we investigated the immune regulatory role of IRF4 in aGVHD. In an allogenic bone marrow transplantation (BMT) mouse model, recipients of *Irf4*$^{-/-}$ $CD4^+$ T cells developed reduced clinical GVHD symptoms and GVHD-associated colitis compared to controls. We found lower expression of effector T helper cell signature genes while

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transcripts associated with Treg cell development, including the transcription factor BTB and CNC homolog 2 (BACH2) were more abundant in the absence of *Irf4*. Remarkably, we observed a similar impact of *BACH2* on Treg differentiation and of *IRF4* on inflammatory processes in patients with intestinal GVHD.

In summary, our results indicate that IRF4 and BACH2 act as counterparts in Th cell subset polarization and immune homeostasis in the development of GVHD.

Material and methods

Study approval

All mouse experiments were approved by the government committee (Regierungspräsidium Darmstadt, Germany) and conducted in accordance with the requirements of the German Animal Welfare Act. The patient biopsy studies and scientific analysis were approved by the local ethical review board (approval no 02/220 and 09/059) and were performed in accordance with the Declaration of Helsinki. All patients consented for biopsy.

Bone marrow transplantation and GVHD mouse model

A full MHC-mismatch C57BL/6 → BALB/c model was used for aGVHD studies *in vivo*. Briefly, BALB/c recipient mice were lethally irradiated with 8 Gy at day 0. Bone marrow cells were isolated from C57BL/6 donors, depleted for CD90.2 positive T-cells (MACS mouse CD90.2 microbeads, Miltenyi) and transplanted with a dose of 5×10^6 bone marrow cells per recipient at day 1. $CD4^+$ T cells were isolated from spleens of F2- and F3-generation C57BL/6-IRF4$^{-/-}$-CD45.1-Luc$^+$ (*Irf4*$^{-/-}$) or C57BL/6-IRF4$^{\pm}$-CD45.1-Luc$^+$ (*Irf4*$^{+/-}$) mice or wild type C57BL/6-CD45.1-Luc+ (WT) mice via MACS mouse $CD4^+$ T cell negative selection (Miltenyi) at day 2 and transplanted with a dose of 1×10^6 cells per BALB/c recipient. Control BALB/c mice did not receive $CD4^+$ T cells (bone marrow transplantation (BMT) control). GVHD development was tracked over 14–30 days by regular control of the GVHD score.[39] Mice from the WT and *Irf4*$^{+/-}$ group were euthanized latest 14–16 days after irradiation, whereas a fraction of mice from the *Irf4*$^{-/-}$ and BMT control group was analyzed for a longer period. Of note, mice did not receive any GvHD prophylaxis or treatment during the entire observation time.

In vivo bioluminescence imaging (BLI)

Luciferase positive $CD4^+$ T cells derived from T cell donors were tracked *in vivo* using the IVIS Lumina II device (PerkinElmer). 100 µL luciferin (15 mg/mL, Promega) were injected subcutaneously 15 minutes before measurement start. Bioluminescence was measured and analyzed with Living Image Software (PerkinElmer). *In vivo* tracking of the cells was performed until day 13 with an interval of 2–3 days. Target organ specific migration of the Luc$^+$ cells was measured at day 14 or 15 in the respective isolated organs.

Flow cytometry and cell sorting

For T cell phenotyping, cells were stained with the following antibodies: anti-CD122 (TM-beta1), anti-CD127 (A7R34), anti-CD25 (PC61), anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD44 (REA664 or IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD69 (H1-2F3), anti-CD8 (53–6.7). Intracellular staining was performed with the transcription factor buffer set (BD Pharmingen) and the following antibodies: anti-Foxp3 (REA788), anti-Helios (22F6), anti-IFN-γ (XMG1.2), anti-IL-17A (TC11-18H10.1), anti-IL-4 (11B11). For live/dead discrimination cells were stained with 7AAD (BD Pharmingen) or LIVE/DEAD Fixable Near-IR stain (Invitrogen).

For sorting of CD45.1$^+$ $CD4^+$ cells the following staining antibodies were used: anti-Nkp46 (29A1.4), anti-CD8 (53–6.7), anti-CD19 (6D5), anti-CD3 (145-2C11), anti-CD45.1 (A20), antiCD4 (GK1.5), 7AAD staining (BD). All antibodies were purchased from BD, Biolegend, eBioscience or Miltenyi.

Isolation of RNA, cDNA synthesis and RNA sequencing analysis

For RNA sequencing $CD4^+$ T cells were re-isolated from recipient mouse spleens using the $CD4^+$ T cell isolation Kit II (Miltenyi) and then sorted for CD45.1 with > 99% purity. RNA was isolated using the RNeasy Mini or Micro Kit (Qiagen) depending on the cell number. RNA libraries for sequencing were prepared with Paired-End TruSeq Cluster Kit and sequenced on an Illumina HiSeq 4000 instrument (2×76 bp) with an average read depth of 23 million reads. Adapter sequences were trimmed (Trimmomatic v0.36)[40] and low-quality reads were filtered out. Reads were mapped to Ensembl Gencode GRCm38 using STAR v2.5.3a.[41] RSEM v1.3.0[42] was used to quantify the expression of transcripts. DESeq2 R package v1.16.1[43] was used with default parameters to find the differentially expressed genes. The sequencing data generated in this study have been deposited in the EMBL BioStudies under accession number [S-BSST1167].

Patient characteristics

109 intestinal biopsies were obtained and analyzed from 76 adult patients receiving allogenic stem cell transplantation between January 2012 and March 2016. Patient characteristics are summarized in Supplemental Table S1.

Statistical analyses

Statistical analyzes were performed with the Graphpad Prism software (version 9). Non-parametric tests were applied as indicated in the respective figure legends. A p-value <0.05 was considered as statistically significant.

Results

Irf4$^{-/-}$ $CD4^+$ T cell do not induce acute GVHD in an allogenic mouse model

To determine whether *Irf4* expression in $CD4^+$ T cells impacts intestinal acute GVHD development, we

transplanted *Irf4*-sufficient (*Irf4*$^{+/-}$ and wild type (WT)) or *Irf4*-deficient (*Irf4*$^{-/-}$) CD4^{+} T cells from C57BL/6 mice to a C57/Bl6 (H2^{b}) → BALB/c (H2^{d}) model (Figure 1a). Recipients transplanted with either WT or *Irf4*$^{+/-}$ T cells developed GVHD to a similar degree and in a cell-dose dependent manner, suggesting that heterozygous loss of *Irf4* is not associated with a gene-dosage effect in this model system (Figure 1b, Supplemental Figure S1A). In contrast, *Irf4*$^{-/-}$ CD4^{+} T cells failed to induce GVHD above scores also observed in BMT control mice irrespective of CD4^{+} T cell numbers transplanted. GVHD-associated colitis was advanced in recipients of WT CD4^{+} T cells on day 14 after total body irradiation (TBI), whereas the colon of recipients of *Irf4*$^{-/-}$ CD4^{+} T cells appeared healthy also on day 30 (Figure 1c, Supplemental Figure S1B). This finding was confirmed by histopathology scoring of colon sections (Figure 1d, Supplemental Figure S1C).

Serum concentrations of IL-6, IL-2 and TNF and of the anti-inflammatory cytokine IL-10, but not IFN-γ, were elevated on day 15 post TBI in recipients of *Irf4*$^{\pm}$ and WT CD4^{+} T cells (Supplemental Figure S1D). In contrast, recipients of *Irf4*$^{-/-}$ CD4^{+} T cells showed similar serum cytokine concentrations as the BMT control group, indicating the absence of inflammatory processes.

Overall, these data indicate that *Irf4* expression in CD4^{+} T cells contributes to aGVHD-associated colitis, and systemic inflammation in our murine full MHC-mismatch model of aGVHD.

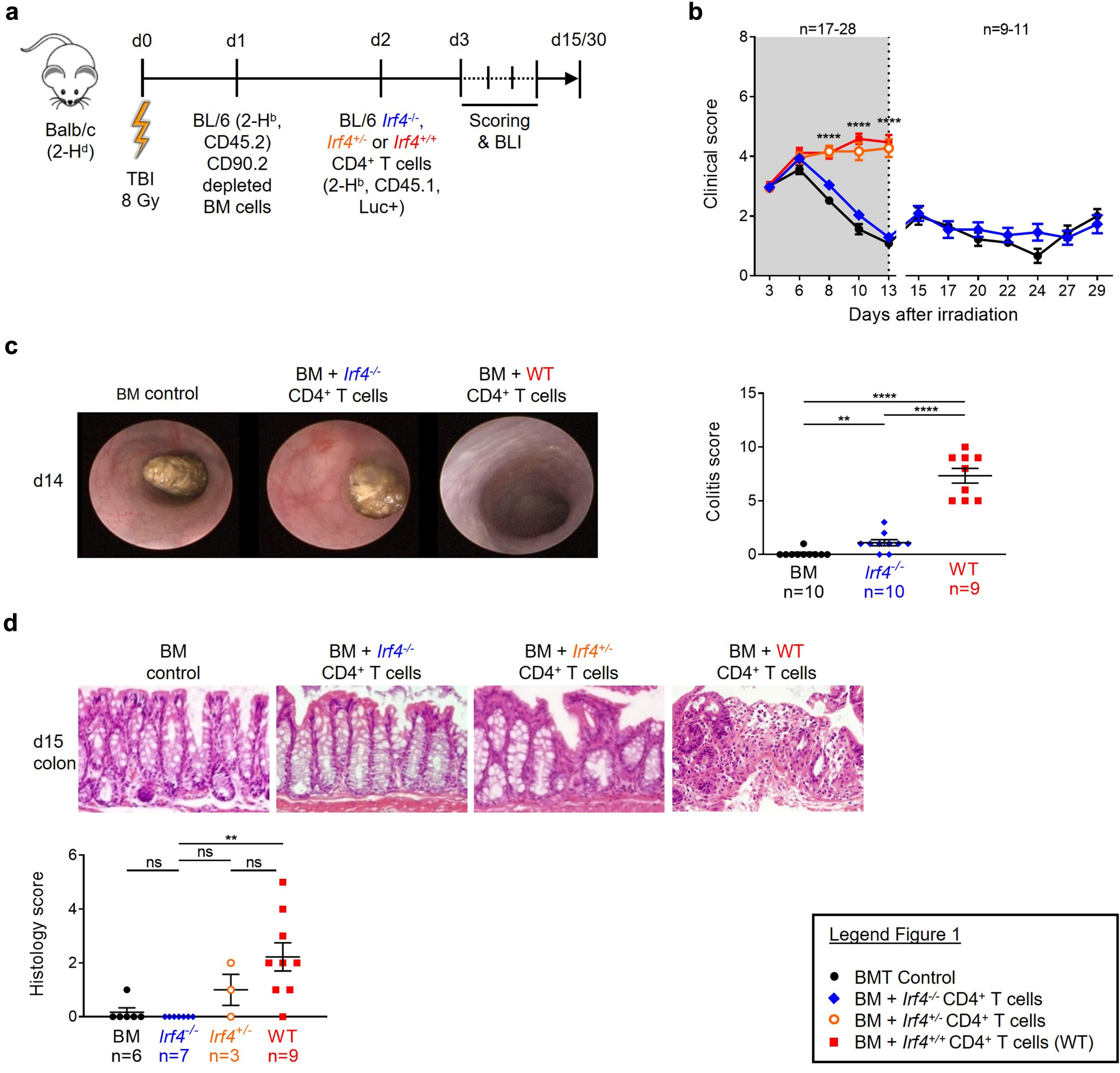

Figure 1. *Irf4*$^{-/-}$ CD4^{+} T cell induce less aGVHD and GVHD associated colitis. Lethally irradiated BALB/c recipients that received 5×10^{6} CD45.2^{+} WT BL/6 BM depleted for CD90.2^{+} cells at day one and 1×10^{6} CD4^{+} CD45.1^{+} Luciferase^{+} T cells from WT, *Irf4*$^{-/-}$ or *Irf4*$^{\pm}$ donors with BL/6 background at day two (a) were monitored every 2–3 days for clinical GVHD score (b) and for colitis development at the end of the experiment (c). Data represent the mean ± standard deviation combined from 3 to 8 independent experiments (a-b: BM = 8, *Irf4*$^{-/-}$ = 7, *Irf4*$^{\pm}$ = 4, WT = 5 experiments; c: all groups = 3 experiments). (d) The colons of BM and CD4^{+} T cell recipients were analyzed at day 14 post irradiation. Histological sections were stained with Hematoxylin/Eosin (HE) and scored for histopathological changes. One representative picture is shown per condition. Data represent the mean ± standard deviation combined from 1 to 3 independent experiments (BM = 2, *Irf4*$^{-/-}$ = 2, *Irf4*$^{\pm}$ = 1, WT = 3 experiments). If applicable, all data points each representing an individual are shown. ***P < .01, ***P < .005, **** P < .0001*. Analyses were performed with the two tailed Mann-Whitney test.

Irf4$^{-/-}$ *CD4*$^{+}$ *T cells migrate to GVHD target organs with impaired proliferation capacity*

Next, we assessed whether the difference in aGVHD development between recipients of *Irf4*$^{-/-}$, *Irf4*$^{+/-}$ and WT CD4^{+} T cells can be explained by alterations in migration to GVHD target organs or defective T cell expansion, as we had also observed *in vitro* (Supplemental Figure S2A). We observed lower frequencies of donor CD45.1^{+} CD4^{+} T cells in spleens from the *Irf4*$^{-/-}$ group in comparison to WT and *Irf4*$^{\pm}$ group (Figure 2a). Moreover, we traced the spatial and temporal distribution of the transplanted Luciferase-positive (Luc^{+}) CD4^{+} T cells by bioluminescence signal. Bioluminescence intensity revealed substantially reduced expansion of donor *Irf4*$^{-/-}$ CD4^{+} T cells in comparison to the WT group (Figure 2b,c). Bioluminescence imaging (BLI) also showed lower amounts of Luc^{+} CD4^{+} T cells in the GVHD target organs lung, liver, and colon, on day 15 (Figure 2d,e, Supplemental Figure S2B and C). However, we did not detect qualitative differences in tissue distribution dependent on donor genotype, suggesting that loss of *Irf4* does not affect homing of T cells to GVHD target organs. Furthermore, we noticed that transplanted *Irf4*$^{-/-}$ CD4^{+} T cell showed delayed expansion to the same GVHD target organs as WT and *Irf4*$^{+/-}$ CD4^{+} T cells without eliciting disease at these later time points. (Figure 2b; Figure 1b; Supplemental Figure S1B and C; Supplemental Figure S2b – e). This finding implies that impaired expansion of *Irf4*$^{-/-}$ CD4^{+} T cells early after transplantation

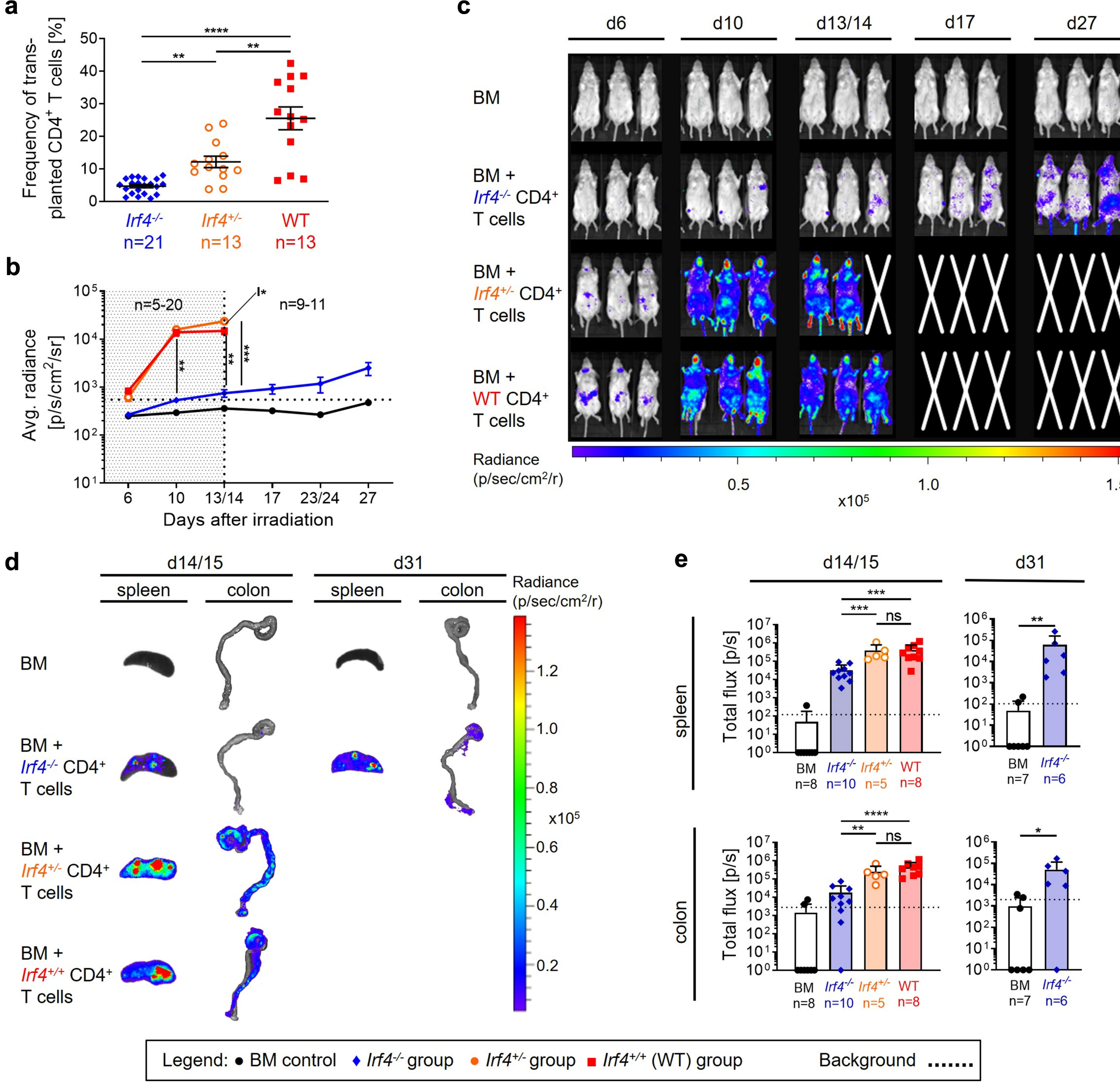

Figure 2. *Irf4*$^{-/-}$ CD4^{+} T cells show decelerated proliferation but migrate to the same aGVHD target organs like WT and *Irf4*$^{\pm}$ CD4^{+} T cells. The migration of 1×10^{6} CD4^{+} CD45.1^{+} Luciferase^{+} T cells from WT (red), *Irf4*$^{-/-}$ (blue) or *Irf4*$^{+/-}$ (orange) donors with BL/6 background in the lethally irradiated WT BL/6 CD0.2-depleted BM transplanted recipients was traced by measuring the frequency of CD45.1^{+} CD4^{+} T cells in the spleen at day 15 after irradiation via flow cytometry (a) and by bioluminescent imaging of the Luc^{+} CD4^{+} T cells considering the total bioluminescent signal (b) and the signal distribution throughout the body (c) at different time points. At day 14/15 (d, e) and day 31 (d, e) bioluminescent signal was additionally measured in the spleen and colon after dissection. Representative BLI pictures for the respective groups are shown. Data represent the mean ± standard deviation combined from independent experiments. If applicable, all data points each representing an individual are shown. *$^{*}P < .05$, $^{**}P < .01$, $^{***}P < .005$, $^{****}P < .0001$*. The legend of Figure 2 applies for all subfigures. Analyses were performed with the two tailed Mann-Whitney test.

cannot fully explain attenuated intestinal aGVHD in *Irf4*$^{-/-}$ CD4^{+} T cell recipients. Therefore, we hypothesize that loss of *Irf4* is associated with altered functional features of donor T cells.

Irf4$^{-/-}$ CD4^{+} T cells depict an altered Th differentiation profile on a transcriptomic level

To gain more mechanistic insight into *Irf4*-dependent T cell differentiation during GVHD, we performed transcriptome analysis of re-isolated *Irf4*$^{-/-}$ from five recipients and *Irf4*$^{\pm}$ donor CD4^{+} T cells from eight recipients on day 14–16 after GVHD induction (Figure 3a). Genes linked to immune cell cytokines, chemotaxis, activation proliferation and effector function were less abundant in*Irf4*$^{-/-}$ CD4^{+} T cells compared to *Irf4*$^{+/-}$ cells (Supplemental Figure S3A).

At protein-protein interaction level, the interactors of IRF4 (Supplemental Figure S3B) were associated with the KEGG pathways *Th differentiation*, *Cytokine-cytokine receptor interaction* and the *JAK-STAT-signaling pathway*. Mapping of the differentially expressed genes (p-value, fold-change) onto the IRF4 STRING network predicted a downregulation of Th-differentiation associated genes except for FoxP3 and STAT-Proteins (Supplemental Figure S3B, Supplemental Table S3).

Indeed, comparing the expression levels of genes involved in Th lineage differentiation revealed an overall lower expression of genes associated to Th-1, Th-2 and Th-17 lineages and a higher expression of Th-9, Th-22, Tfh and Treg cell associated genes in the absence of *Irf4* (Figure 3b). Analysis of expression profiles of Th lineage defining transcription factors substantiated this finding (Figure 3c). In addition, genes coding for chemokine receptors, cytokines and cytokine receptors specific for Th-1, Th-2 and Th-17 were also expressed at lower levels in *Irf4*$^{-/-}$ donor CD4^{+} T cells (Supplemental Figure S3C).

Taken together, transcriptome analysis supports the notion that IRF4 promotes pathologic effector T cell differentiation in GVHD at the expense of differentiation of T cells with protective function, such as Treg cells.

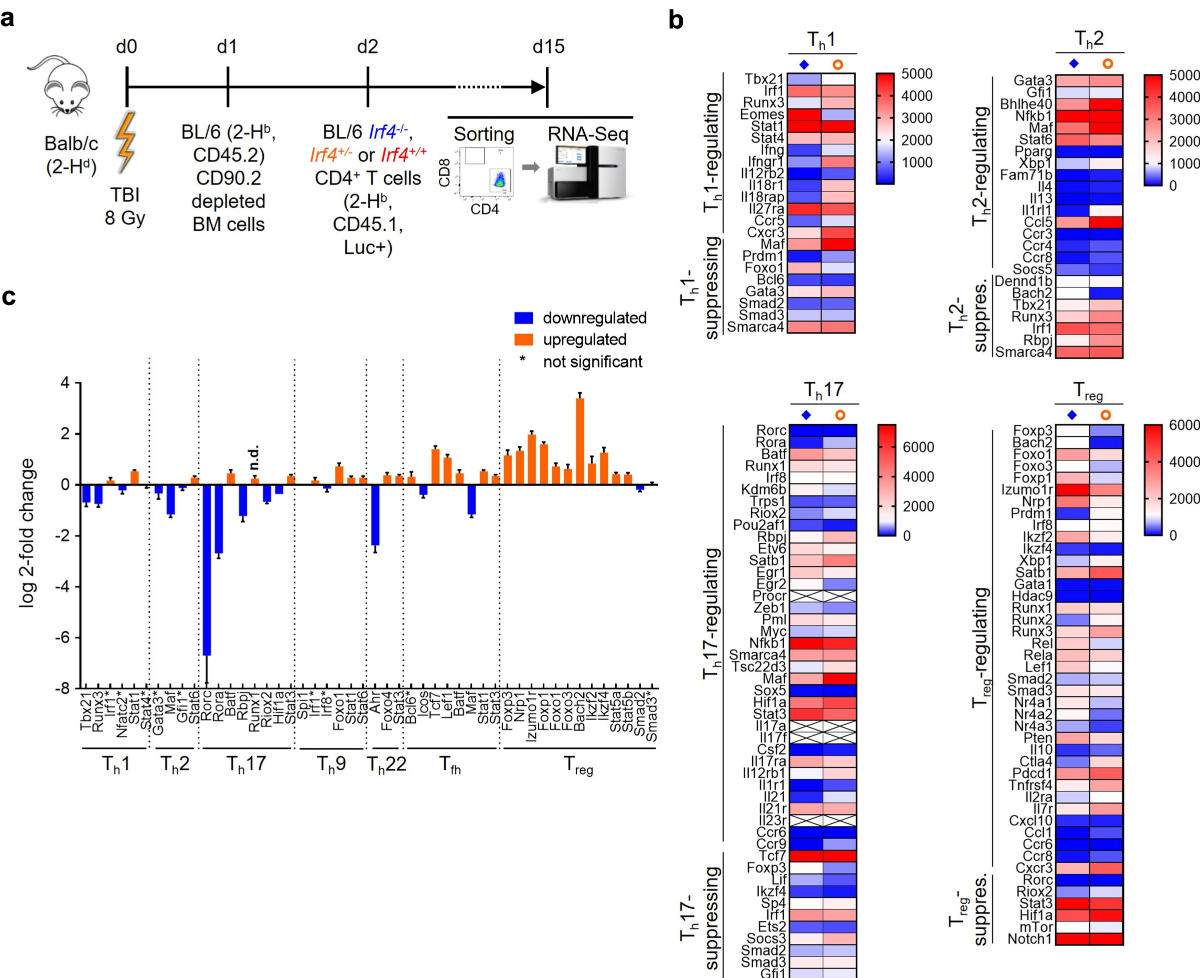

Figure 3. RNA sequencing analysis of re-isolated CD45.1^{+} CD4^{+} T cells shows differences in the transcriptomic landscape between transplanted *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ cells and reflects the observations described *in vivo*. Lethally irradiated BALB/c recipients received 5×10^{6} CD45.2^{+} WT C57BL/6 BM depleted for CD90.2^{+} cells at day one and 1×10^{6} CD4^{+} CD45.1^{+} Luciferase^{+} T cells from *Irf4*$^{+/-}$ or *Irf4*$^{-/-}$ donors with C57BL/6 background at day two. CD4^{+} T cells were re-isolated and sorted for CD45.1^{+} CD4^{+} T cells with high purity and RNA was extracted from these cells for RNA sequencing analysis (a). (b) T helper cell subset-regulating and -suppressing genes were plotted as mean of RNA sequencing reads between the two groups. (c) The up- and downregulation of Th-specific transcription factors between *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ CD4^{+} T cells was plotted by the mean of 2-fold change + standard deviation. Data represent the mean combined from two independent experiments from $n = 5$ *Irf4*$^{-/-}$ versus $n = 8$ *Irf4*$^{+/-}$ CD4^{+} T cell transplanted mice.

Irf4$^{-/-}$ *and WT CD4*$^{+}$ *T cells differ in effector/memory capacity and effector function*

To test this hypothesis, we analyzed subset composition, activation, and functional marker expression on the transplanted CD45.1^{+} CD4^{+} T cells isolated from spleens of mice from *Irf4*$^{-/-}$, *Irf4*$^{+/-}$ or WT groups on day 15. Whereas most WT and *Irf4*$^{+/-}$ donor cells displayed an effector memory phenotype (T_{EM}, CD62L^{low} CD44^{high}) with a smaller contribution of effector cells (T_{eff}, CD62L^{-} CD44^{low}), *Irf4*$^{-/-}$ donor CD4^{+} T cells had a more balanced subset composition with a majority of cells exhibiting a central memory phenotype (T_{CM}, CD62L^{high}, CD44^{high}) (Figure 4a).

In all groups, similar proportions of donor CD4^{+} T cells expressed the activation marker CD69 as well as the memory marker CD122. In contrast, surface expression of CD127 was virtually absent in *Irf4*$^{-/-}$ donor CD4^{+} T cells, but not in WT donor cells (Figure 4b).

Analysis of effector cytokine levels by intracellular flow cytometry revealed that *Irf4*$^{-/-}$ donor CD4^{+} T cells failed to express IFN-γ, suggesting defective Th1 polarization (Figure 4c). No substantial differences between the groups were detected for expression of the Th-17 and Th-2 specific cytokines IL-17A and IL-4, respectively (Figure 4c).

To test whether loss of *Irf4* in donor T cells expanded the Treg-cell compartment, we performed flow cytometric analyzes for the Treg-cell lineage defining transcription factor Forkhead-Box-Protein P3 (FoxP3) as well as Helios in CD45.1^{+} CD4^{+} T cells re-isolated on day 15 after irradiation. We found a higher proportion of Foxp3^{+} Treg cells within *Irf4*$^{-/-}$ donor CD4^{+} T cells compared to controls, consistent with the transcriptome analysis (Figure 4d). Frequencies of Helios^{+} CD4^{+} T cells were elevated in *Irf4*$^{-/-}$ donor CD4^{+} T cells to an even larger degree, suggesting that this marker is also induced in non-Treg cells (Figure 4d). In line with these findings, staining of colon tissue derived from the different groups at day 15 indicated a higher abundance of Foxp3^{+} CD3^{+} T cells in *Irf4*$^{-/-}$ CD4^{+} T cell transplanted mice, while T-bet expression was more abundant in the WT and *Irf4*$^{\pm}$ group (Supplemental Figure S4).

Taken together these findings indicate that loss of *Irf4* in CD4^{+} T cells alters their differentiation trajectory, resulting in predominant differentiation into central memory cells rather than effector memory cells as well as virtually complete abrogation of Th-1 polarization and increased differentiation to Tregs which leads to differential abundance of these cell types in GVHD target tissues.

BACH2 is strongly upregulated in *Irf4*$^{-/-}$ *CD4*$^{+}$ *T cells*

One of the most upregulated differentially expressed gene (DEG) identified in the RNA sequencing analysis between *Irf4*$^{-/-}$ and *Irf4*$^{+/-}$ CD4^{+} T cell transplanted mice was *Bach2* (log2fold change = 3.39; adjusted p-value = 5.59×10^{44}; Figure 5a), which acts as negative regulator of *Irf4* in Tregs and Tfh cells.[38,45] To validate differential expression of *Bach2* at protein level, we performed Western Blot analysis of *Irf4*$^{-/-}$ and WT CD4^{+} T cells, stimulated in a mixed lymphocyte reaction (MLR). The results confirmed the absence of IRF4 and the upregulation of BACH2 in *Irf4*$^{-/-}$ CD4^{+} T cells after allogenic stimulation. In comparison, WT CD4^{+} T cells exhibited lower amounts on BACH2 after stimulation (Figure 5b).

We hypothesize, that IRF4 and BACH2 have opposing but tightly connected roles in the immune regulation of CD4^{+} T cells during GVHD and the development of intestinal acute GVHD.

IRF4 *but not* BACH2 *expression are influenced by GVHD prophylactic therapy*

In mice, we found significantly decreased expression of *Bach2* and a trend toward higher expression of *Irf4* in colon tissue of the *Irf4*$^{-/-}$ WT and BM control groups on transcript level. In addition, the inflammatory marker NLRP3 was also found to be upregulated in the WT group, while the *Irf4*$^{-/-}$ group showed similar expression levels as the BMT control for all three target genes (Supplemental Figure S5).

Therefore, we wondered whether the expression of *BACH2* or *IRF4* correlated with the development of aGVHD-associated colitis following HSCT in humans. We quantified *BACH2* and *IRF4* gene transcripts in intestinal biopsies from 76 HSCT patients, during different stages of GVHD development (Supplemental Table S1). Unexpectedly, *IRF4* expression was significantly reduced during GVHD progression, whereas *BACH2* showed no major alterations (Figure 5c). We hypothesized that immunosuppressive therapy interferes with *IRF4* and *BACH2* expression. Indeed, *IRF4* expression in CD4^{+} T cells was partially abrogated upon treatment with the most common prophylactic GVHD-therapeutics, Cyclosporine A (CyA) and Methylprednisolone (MP), whereas *BACH2* expression only slightly changed (Figure 5d). These *in vitro* results reflect the observed expression patterns of *IRF4* and *BACH2* in the intestinal biopsies and might explain the lower *IRF4* expression during GVHD-associated colitis in humans, as all included patients received GVHD prophylactic CyA therapy.

To test potential consequences of elevated or reduced expression of *IRF4* and *BACH2* in biopsies, we separated patient samples in groups according to the expression of *IRF4* or *BACH2* either above or below median. Patient samples with higher *IRF4* expression exhibited elevated *NLRP3* inflammasome levels (Figure 5e). Higher FoxP3 expression was found in samples with *BACH2* expression above median (Figure 5f). These data support the idea of a relationship between *BACH2* and Treg development in human aGVHD-associated colitis.

Taken together, combined analysis of patient samples and *in vitro* experiments support the notion, that IRF4 and BACH2 contribute to shaping the immune responses in human intestinal aGVHD.

Discussion

Donor CD4^{+} T cells are the key mediators of aGVHD following HSCT. The transcription factor IRF4 plays an essential role in CD4^{+} T cell differentiation and effector function via interaction with the transcription factor BATF and Jun-factor proteins.[46,47] As earlier studies showed, the depletion of BATF in CD4^{+} T cells abrogates aGVHD-associated colitis and the deficiency of IRF4 reduced colitis symptoms in an experimental murine colitis model.[48,49]

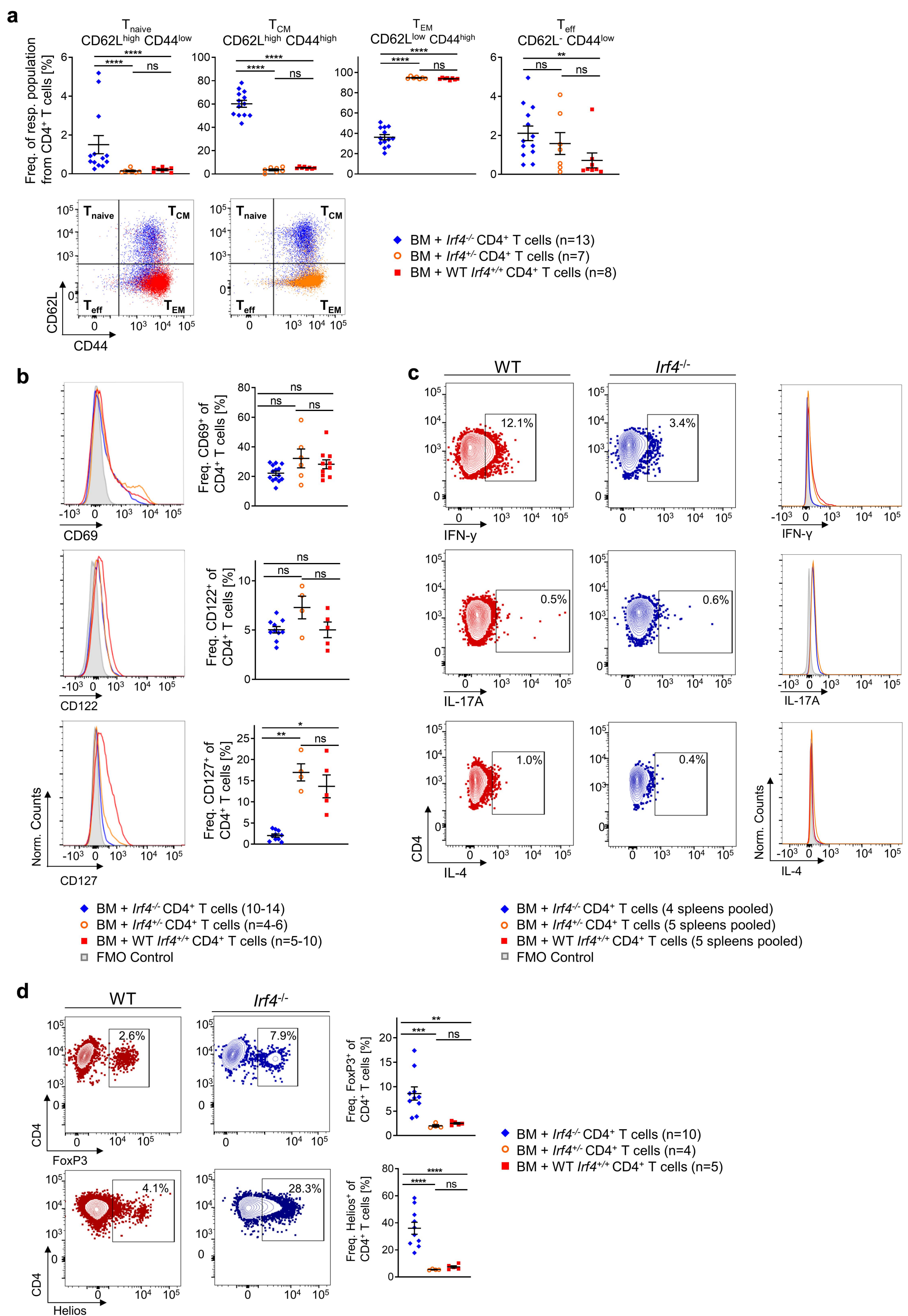

Figure 4. $Irf4^{-/-}$ CD4^{+} T cells exhibit an altered memory/effector and a compromised Th phenotype on comparison to $Irf4^{+/-}$ and WT CD4^{+} T cells. Lethally irradiated BALB/c recipients received 5×10^6 CD45.2^{+} WT BL/6 BM cells depleted for CD90.2^{+} cells at day one and 1×10^6 CD4^{+} CD45.1^{+} Luciferase^{+} T cells from WT, $Irf4^{-/-}$ or $Irf4^{+/-}$ donors with BL/6 background at day two. Re-isolated CD45.1^{+} CD4^{+} T cells were analyzed for CD62L and CD44 expression to determine effector/memory development (a) and for activation- (CD69), memory- (CD122) and maturation- (CD127) markers (b) at day 15 post irradiation by flow cytometry. Representative flow cytometry plots for the distribution of $Irf4^{+/-}$ (orange) and WT (red) versus $Irf4^{-/-}$ (blue) CD4^{+} T cells in the effector/memory compartment are shown. (c) The differentiation to Th-1, Th-17 and Th-2 CD4^{+} T cells was determined by intracellular staining for IFN-γ, IL-17A and IL-4 respectively and flow cytometry analysis upon PMA/Ionomycin stimulation of re-isolated CD45.1^{+} CD4^{+} T cells at day 15 post irradiation. (d) Re-isolated CD45.1^{+} CD4^{+} T cells at day 15 after irradiation were analyzed for the Treg specific transcription factor FoxP3 and Helios via flow cytometry. Data represent the mean combined independent experiments from $n = 4$-10 CD4^{+} T cell transplanted mice. Data represent the mean ± standard deviation combined from independent experiments. If applicable, all data points each representing an individual are shown. *$P < .05$, **$P < .01$, ****$P < .0001$. Analyses were performed with the two tailed Mann-Whitney test.

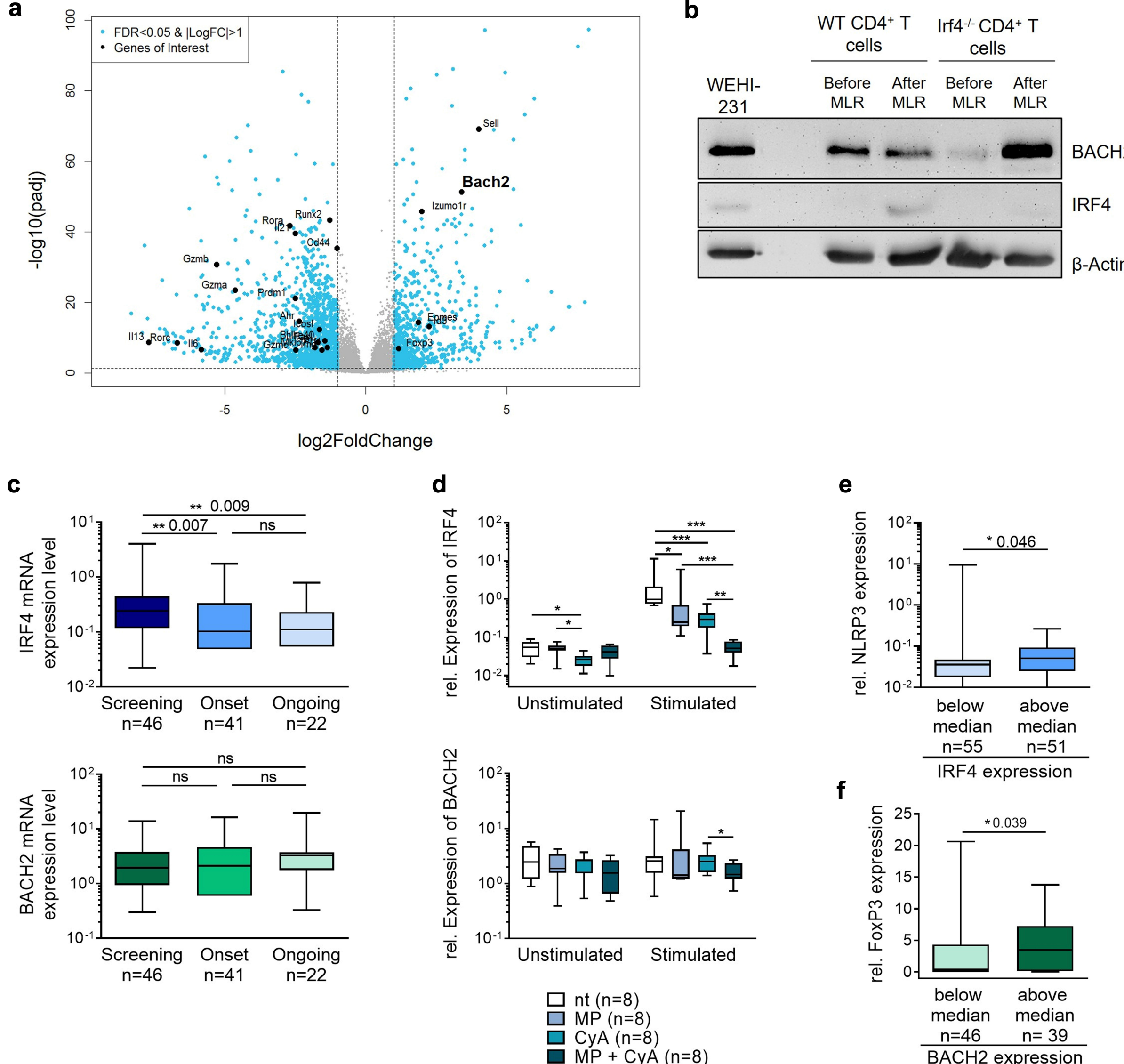

Figure 5. IRF4 but not BACH2 expression are altered by GVHD immunosuppressant therapy and influence inflammatory versus anti-inflammatory signatures in intestinal biopsies of GVHD patients. (a) Lethally irradiated BALB/c recipients received 5×10^6 CD45.2+ WT C57BL/6 BM depleted for CD90.2+ cells at day one and 1×10^6 CD4+ CD45.1+ Luciferase+ T cells from WT, *Irf4*-/- or *Irf4*+/- donors with C57BL/6 background at day two. Volcano plot mapping differentially expressed genes (DEGs) between *Irf4*-/- and *Irf4*± CD4+ T cells described in Figure 3 satisfying the criteria of log2 fold change > 1 or >-1 and $p < 0.05$. Significantly different expressed genes are labeled blue, genes of interest (GOI) are labeled black. (b) Western Blot detecting BACH2 and IRF4 protein expression of WT and *Irf4*-/- CD4+ T cells before and after 10 days of MLR. WEHI-231 cells were used as control for BACH2 and IRF4 detection. ß-Actin was used as loading control. (c) *IRF4* and BACH2 expression in intestinal biopsies of $n = 76$ patients who received HSCT during different stages of GVHD development analyzed by qPCR. (d) *IRF4* and *BACH2* expression were quantified in CD4+ T cells derived from healthy donors ($n = 8$) after 3 days under stimulated or unstimulated conditions and simultaneous treatment with Cyclosporine A (CyA), Methylprednisolone (MP), a combination of both or without treatment. Samples were analyzed with Kruskal-Wallis test. Intestinal biopsies of patients (n = 76) who received HSCT were divided into samples expressing *IRF4* (e) or *BACH2* (f) transcripts above and below median and plotted against *NLRP3* (e) or *FoxP3* (f) expression, quantified via qPCR (e) or immunohistochemistry (f) in these samples. Samples were analyzed by two-tailed Mann-Whitney-test. Whisker-box plots indicate the mean, min and max value of individual sample sizes indicated in the respective plots. Only significant statistical test results are indicated. **P < .05, **P < .01, ***P < .001, ****P < .0001.*

In this study, we identified IRF4 as an important regulator of Th-differentiation and associated inflammatory processes during GVHD in mice and human beings. The absence of *Irf4* in MHC-mismatched transplanted CD4+ cells abrogated GVHD development and GVHD-associated colitis in mice.

Phenotyping analysis revealed differences in the maturation and effector/memory state between transplanted *Irf4*-/- and WT CD4+ T cells in the GVHD mouse model. Similar to what has been found in *Irf4*-/- CD8+ T cells and for CD4+ T cells during acute infection,[33,50] we observed that *Irf4*-/- CD4+ T cells exhibited a biased differentiation to T_{CM} cells in the GVHD model. As IRF4 intrinsically translates TCR signaling strength, which is impaired in the absence of IRF4, the model that low TCR strength enforces T cell memory potential could explain this observation.[44,51,52] The expression of the memory markers CD127 and CD122 which were absent in *Irf4*-/-CD4+ T cells, was also shown to be directly regulated by IRF4 in CD8+ T cells.[53] By contrast, almost all IRF4 expressing cells exhibited an effector memory phenotype. In mice T_{EM} CD8+ T cells were found to be involved in GVHD-associated colitis development.[54] Hence, an altered development within the effector/memory compartment is likely to have contributed to the absence of GVHD development in *Irf4*-/- CD4+ T cell recipients.

Functional assays and RNA sequencing of re-isolated CD4$^+$ T cells indicated an impaired Th-1 differentiation and effector function in the absence of *Irf4*, which was expected since IRF4 regulates Th differentiation.[46] Wu et al. reported that *Irf4*$^{-/-}$ CD4$^+$ T cells failed to reject a heart allograft transplant.[35] Similar to our findings in aGVHD, *Irf4*$^{-/-}$ CD4$^+$ T cells were additionally shown to have compromised Th-1 and Th-17 differentiation capacity which led to abrogated EAE development and failure of infection control.[33,35] Hence, Th cell dysfunctionality could have contributed to the GVHD-abrogating effect in the absence of *Irf4*. Together with FoxP3 we also detected more Helios$^+$ CD4$^+$ T cells when *Irf4* was absent. Helios is expressed by more activated Treg cells but is also a marker for T cell exhaustion.[55–57] Wu et al. connected loss of *Irf4* to a higher expression of Helios and the exhaustion marker PD-1, which was reversible by anti-PD-1 antibody treatment.[35] Both Helios and PD-1 expression were also shown to be upregulated on activated CD4$^+$ T cells when *Irf4* was suppressed by the MAK1/2 inhibitor Trametinib.[35] In contrast, in CD8$^+$ T cells the expression of IRF4 sustained T cell exhaustion and reduction of IRF4 expression restored the functional properties of these cells.[58] Therefore, *Irf4*$^{-/-}$ associated abrogation could be mediated by both, inhibition of Th-1 differentiation or induction of exhaustion phenotypes in T cell subsets. Additionally, we cannot exclude the lack of tissue resident T cells, which has been associated with IRF4 deficiency, as a potent mechanism for attenuated GVHD in our model.[38,59]

Interestingly, we also found that the expression of Treg-associated transcription factors was increased in *Irf4*$^{-/-}$ CD4$^+$ T cells and a higher relative contribution of FoxP3$^+$ CD4$^+$ T cells was detected in murine recipients of *Irf4*$^{-/-}$ CD4$^+$ T cells. In line with that, others recently showed that more *Irf4*$^{-/-}$ CD4$^+$ T cells differentiated to FoxP3$^+$ Tregs under Treg-inducing conditions.[38] However, one should be aware that we do not know if the increase in frequency is connected to a numerical increase of Treg cells, especially taking into account that IRF4 is necessary for tissue resident Treg maintenance and eTreg differentiation.[36,37] In addition to FoxP3, we also found that the transcription factor BACH2, which is relevant for Treg differentiation,[60,61] was highly expressed in *Irf4*$^{-/-}$ CD4$^+$ T cells. Interestingly, recent publications showed that IRF4 and BACH2 directly compete for the binding of transcriptional motifs in murine Tfh and Treg cells and counter regulate T cell differentiation.[38,45] Additionally, a direct suppression of BACH2 transcription by IRF4 has been shown in B cells.[62] Therefore, we hypothesize that the downregulation of IRF4 leads to upregulation of BACH2 which enhances the differentiation of Tregs in the GVHD model.

To validate our hypothesis, we investigated IRF4 and BACH2 expression levels in human intestinal biopsies from patients during different stages of GVHD development. Analyzes were performed in a heterogeneous patient cohort at different times after HSCT and GvHD onset as well as different GvHD severity and treatment regimens and we cannot estimate how, and to which extent different immunotherapies might affect the expression of IRF4 and BACH2, thus limiting the interpretability of the data. However, we found that the immunosuppressive treatment of the patients with Cyclosporine A interfered with *IRF4* expression as reported before.[63] Additionally, we observed that corticosteroids had a similar effect on *IRF4* expression, whereas *BACH2* was not affected. The impressive effect of commonly used and potent prophylaxis and first-line treatment on *IRF4* expression underscores the contribution of IRF4 to the development and progression of GVHD. However, these standard therapies are associated to unspecific immune suppression and a more targeted IRF4 inhibition might be beneficial for GVHD patients. The MEK inhibitor Trametinib, which also inhibits IRF4,[35] for example, has been shown to suppress GVHD while sparing GVL-effectors in mice.[64] Consistent with our observations from the mouse model, high *BACH2* levels were associated with increased expression of FoxP3 in GVHD patients. On the contrary, higher IRF4 expression levels showed a correlation with an increased NLRP3 expression, an inflammasome factor known to contribute to aGVHD induction in intestinal tissues of mice.[65]

In summary, our study confirms the association of IRF4 expression with intestinal inflammatory responses as GVHD associated colitis by promoting pathologic effector T cell differentiation at the expense of differentiation of T cells with protective function, such as Treg cells. Additionally, our data support the idea that BACH2 expression is linked to Treg differentiation in mice and human. Finally, our results indicate that IRF4 and BACH2 act as counterparts in Th cell polarization and immune homeostasis during GVHD. On the aggregate strength of our data, we conclude that IRF4 could be a novel target for more targeted therapeutic approaches to treat GVHD patients in the future.

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Disclosure statement

The authors declare no conflict of interest related to this work. E.U. acts as medical advisor of Phialogics and received research funding from Gilead and Bristol-Myers Squibb. R.Z. received honorarium for participation in scientific meetings and advisory boards from Incyte, Mallinckrodt and Novartis.

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J.T.F., J.C., D.Y.S.F., S.G. and S.H. performed experiments, J.T.F., J.C., D. Y.S.F., N.V., S.G., E.M., S.H. and D.H. analyzed and interpreted data. E.H., S.G. and E.M. provided human colon RNA samples, performed analyses related to human colon biopsies and helped with the interpretation and critical discussion of the results. M.L., D.S., S.S., R.S., H.B. and A.G. C. provided critical reagents and mice. D.H., E.H. and R.Z. helped to design experiments and discussed the results, A.G.C. and A.K. gave critical advice and helped with the interpretation and critical discussion of the results.

E.U. designed and directed the study; J.C., J.T.F, and E.U. wrote the manuscript with contributions of all authors.

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