

Supplemental Information

Interferon Regulatory Factor 4 plays a pivotal role in the development of aGVHD-associated colitis

Jochen T. Frueh*, Julia Campe*, Daniele Yumi Sunaga-Franze, Nikita A. Verheyden, Sakhila Ghimire, Elisabeth Meedt, Denise Haslinger, Sabine Harenkamp, Daniel Staudenraus, Sascha Sauer, Andreas Kreft, Ralf Schubert, Michael Lohoff, Andreas Krueger, Halvard Bonig, Andreas G. Chiocchetti, Robert Zeiser, Ernst Holler & Evelyn Ullrich

* These authors contributed equally to the work

Content:

- **Supplemental material & methods**
- **References to supplemental material & methods**
- **Supplemental figures and figure legends**

Mice

Female BALB/c (H-2^d, CD45.2) and C57BL/6 (H-2^b, CD45.2) mice (BL6), 10-15 weeks of age were purchased from Janvier Laboratories (France). C57BL/6 *Irf4*^{-/-} mice⁵ were crossed with C57BL/6-CD5.1-Luc⁺ (H-2^b) mice, which were kindly provided by Andreas Beilhack (Julius-Maximilians-University Würzburg, Germany).

Mouse colonoscopy

For macroscopic assessment of colon integrity colonoscopy was performed with the Mini-Endoscope (Karl Storz). Colitis severity was assessed at day 14 according to a modified murine endoscopic score (MEICS) (1)

Histology

Colon, lung and liver were collected from the mice at day +15/31 after TBI, fixed in 3% paraformaldehyde and embedded in paraffin. Serial histological sections were stained with Hematoxylin/Eosin. Histologic evaluation was performed blinded. Colon evaluation included quantification of apoptotic crypt epithelia cells, granulocytes and the crypt destruction using optical fields of 0.25 mm² (2). The livers were evaluated for portal and periportal inflammation, bile duct damage, hematopoietic islands, prominent granulocytic infiltrates, and inflammatory infiltrate around the central veins. In the lungs the evaluation included peribronchial- and perivascular inflammatory infiltrate, endothelialitis and prominent alveolar macrophages.

Cytokine Analysis

Serum cytokines were analyzed using BD CBA Enhanced Sensitivity Flex Sets (BD Biosciences-Pharmingen). Briefly, standards (0.274 – 200 pg/mL) and samples were incubated with capture bead mixture, mouse detection reagent and enhanced sensitivity detection reagent in 96-well plates at room temperature in the dark for 1h. After washing, samples were measured with a BD FACS Verse™ cytometer (BD Bioscience-Pharmingen, USA). FCAP Array Software (BD Bioscience-Pharmingen, USA) was used to analyze the data.

RNA sequencing analysis

Transcripts with less than 5 read counts per sample were filtered and p-values were corrected for multiple testing using the Benjamini-Hochberg (BH) procedure. A gene was considered differentially expressed if BH < 0.05. The functional enrichment analysis (GO term) was performed with ClusterProfiler R package. STRING analysis was performed with STRING database v.11 and Cytoscape v.3.2.1.

Mixed lymphocyte reaction and Immunoblot

MACS (Miltenyi) isolated CD4⁺ T cells from *Irf4*^{-/-} or WT mice splenocytes were co-incubated with allogenic BALB/c BM-derived dendritic cells (DCs) in a DC/T cell ratio of 1:2. For protein isolation, cells were harvested before and at day 10 of co-culture. At day 10 living CD4⁺ T cells were enriched via flow-cytometry based sorting, using anti-CD4 BV-605 (Biolegend) antibody and 7-AAD. Cells were lysed in RIPA-buffer and yielded protein was separated in a 12% SDS-Gel and blotted on a PVDF membrane. Immunodetection of IRF4 and BACH2 was performed with the following primary and horseradish peroxidase (HRP)- linked secondary IgG antibodies: rat-anti-IRF4 (clone 3E4, Santa Cruz, sc-130921, 1:200); rat-anti-BACH2 (clone 7A4, Abcam, 1:10000); rabbit-anti-β-Actin (clone D6A8, Cell Signaling, 1:5000); goat-anti-rat HRP (polyclonal, 31470, Invitrogen, 1:10000) or goat-anti-rabbit IgG-HRP (Cell Signaling, #7074, 1:10000).

Biopsy characteristics

Biopsies were obtained either during screening study in asymptomatic patients or because of clinical symptoms indicative of de novo onset or persistence/recurrence of acute intestinal GVHD. Biopsies were obtained by upper GI or lower GI endoscopy or sigmoidoscopy. Overall, 4 biopsies were obtained from duodenum, 11 from Ileum or upper caecum, 19 from colon and 75 from sigmoid colon. For analysis, biopsies from different intestinal compartments were used since homogenous expression of IRF4 and BACH2 was proven throughout the different locations (Supplemental Figure 6).

Donor lymphocyte isolation and activation

For *in vitro* studies CD4⁺ T cells were isolated from buffy coats by Rosette Sep negative CD4⁺ T cell isolation kit (Stemcell). Plates were pre-coated with anti-human CD3 (OKT-3, InVivoMab, 10µg/mL) and CD4⁺ T cells were cultivated with 2 mg/mL anti-human CD28 (CD28.2, Biolegend), 100 IU/ml human IL-2 (Novartis) and either 0.25 mM Methylprednisolone (URBASON® soluble forte, Sanofi), 2.4 µM Cyclosporine A (Sandimmun®, Novartis), a combination of both, or none of them for three days. For unstimulated controls, no anti-CD28 was added. RNA was extracted by peqGold Total RNA Kit (Pepqlab).

Quantitative real-time PCR (qPCR)

Human and murine intestinal tissues were stored at –80°C and RNA was extracted using RNeasy Mini Kit (QIAGEN). For murine tissue, 5µg of RNA was reverse transcribed to cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific)- qPCR was performed with the StepOnePlus device using the 2x blue qPCR SYBR Green Master Mix with ROX (Steinbrenner). For human tissues 1 µg of RNA was reverse transcribed to cDNA using moloney murine leukemia virus reverse transcriptase (Promega). qPCR was performed on a LightCycler 480 (Roche) using QuantiFast SYBR Green PCR Kit (QIAGEN). The specificity of PCR reactions was controlled by monitoring melting curves. 18S ribosomal RNA was used as human reference gene. For murine samples, RPL13A and Beta-Actin were used as reference genes. Gene of interest was normalized to the reference gene (dCT). In case of murine samples additional normalization to the BMT group was performed (ddCT). Primer sequences are displayed in Supplemental Table 2.

Immunohistochemistry

For human tissue, 2-3µm thick slides sectioned from the formalin-fixed and paraffin-embedded (FFPE) biopsies were stained, treated with CC1 buffer, and detection of FoxP3 was performed with a monoclonal mouse antibody (1:120, eBioscience 14-4777, clone 236A/E7) and OptiView DAB IHC Detection Kit (Ventana). The mean number of FoxP3-positive stromal cells was determined microscopically per high power field (HPF) with a Zeiss Axioskop 40 microscope (Zeiss), counting 3-12 HPF exhibiting the highest histological GVHD damage.

For murine tissue, 4µm thick slides sectioned from formalin-fixed, paraffin-embedded organs were deparaffinized and rehydrated, followed by Heat Induced Epitope Retrieval (HIER) with EDTA buffer pH 8,0 and a blocking step. A primary polyclonal rabbit anti-mouse/human CD3e antibody (#TA327165 1:100, Origene) and a secondary goat anti-rabbit IgG (H+L) conjugated to Alexa Fluor 488 (#A-11008 1:250, Invitrogen/Thermo Fisher Scientific) were used to observe immune cell infiltration in colons. Additionally, a monoclonal rat anti-mouse FoxP3 (#14-5773-82 1:50, eBioscience/Thermo Fisher Scientific) and a polyclonal rabbit anti-mouse/human T-bet antibody (#13700-1-AP 1:50, Proteintech) were added. Secondary chicken anti-rat IgG (H+L) and donkey anti-rabbit IgG (H+L), both conjugated to Alexa Fluor 647 (#A-21472 and #A-31573 1:250, Invitrogen/Thermo Fisher Scientific) antibodies were used. Nuclei were stained with 4', 6-Diamidin-2-phenylindol (DAPI) (#D9542, Sigma-Aldrich). Pictures were taken with a BZ-X810 All-in-One Fluorescence Microscope (Keyence, Osaka, Japan) with basic lenses (CFI PlanApo λ 10x 0.45/4.00mm, CFI PlanApo λ 20x 0.75/1.00mm) and filter cubes Ex: 360/40 DM: 400 BA: 460/50 (DAPI), Ex: 470/40 DM: 495 BA: 525/50 (Alexa Fluor 488), Ex: 620/60 DM: 660 BA: 700/75 (Alexa Fluor 647).

References

1. Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. *Nature protocols*. 2006;1(6):2900-2904. doi:10.1038/nprot.2006.446.
2. Kaplan DH, Anderson BE, McNiff JM, Jain D, Shlomchik MJ, Shlomchik WD. Target antigens determine graft-versus-host disease phenotype. *Journal of immunology (Baltimore, Md. : 1950)*. 2004;173(9):5467-5475. doi:10.4049/jimmunol.173.9.5467.

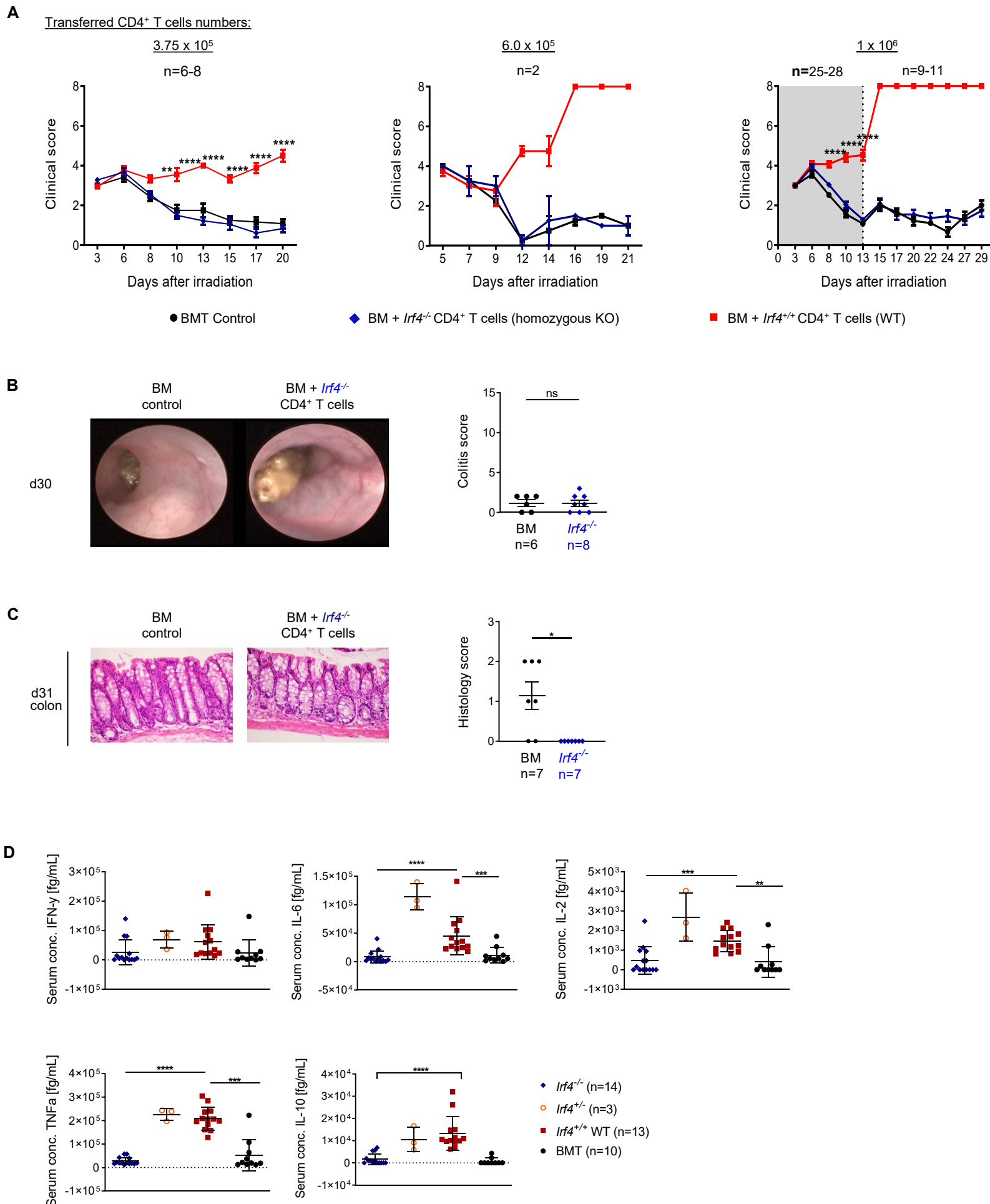


Figure S1: *lrf4*^{-/-} CD4⁺ T cell induce less aGVHD and aGVHD associated colitis to later time points in a dose independent manner.

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 different concentrations of CD4⁺ CD45.1⁺ Luciferase⁺ T cells from WT, *lrf4*^{-/-} or *lrf4*^{+/-} donors with BL/6 background at day two were monitored every 2-3 days for clinical GVHD score (A). The colons of BM and *lrf4*^{-/-} CD4⁺ T cell recipients were analyzed at day 30/31 post irradiation by colonoscopy (B) and by histopathological scoring (C). One representative picture is shown per condition. (D) Inflammatory responses in the recipients at day 15 post irradiation were monitored by CBA-based measurement of pro- and anti-inflammatory cytokines in the serum. Data represent the mean \pm standard deviation combined from 1-4 independent experiments (BM =4, *lrf4*^{-/-} =2, *lrf4*^{+/-} =1, WT =4 experiments). Data represent the mean + standard deviation combined from independent experiments. If applicable, all data points each representing an individual are shown. **P < .01, ***P < .005, **** P < .0001.

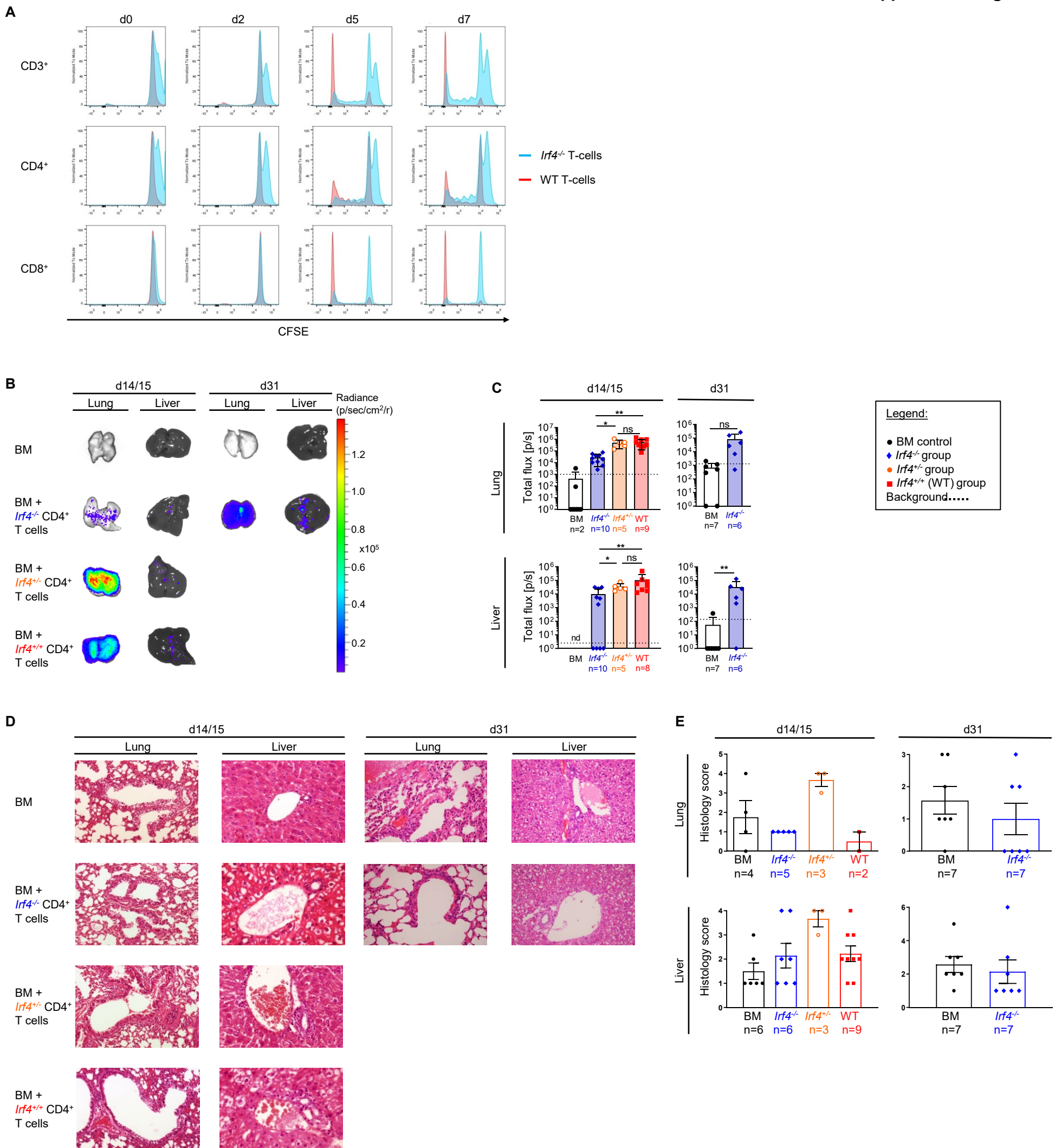
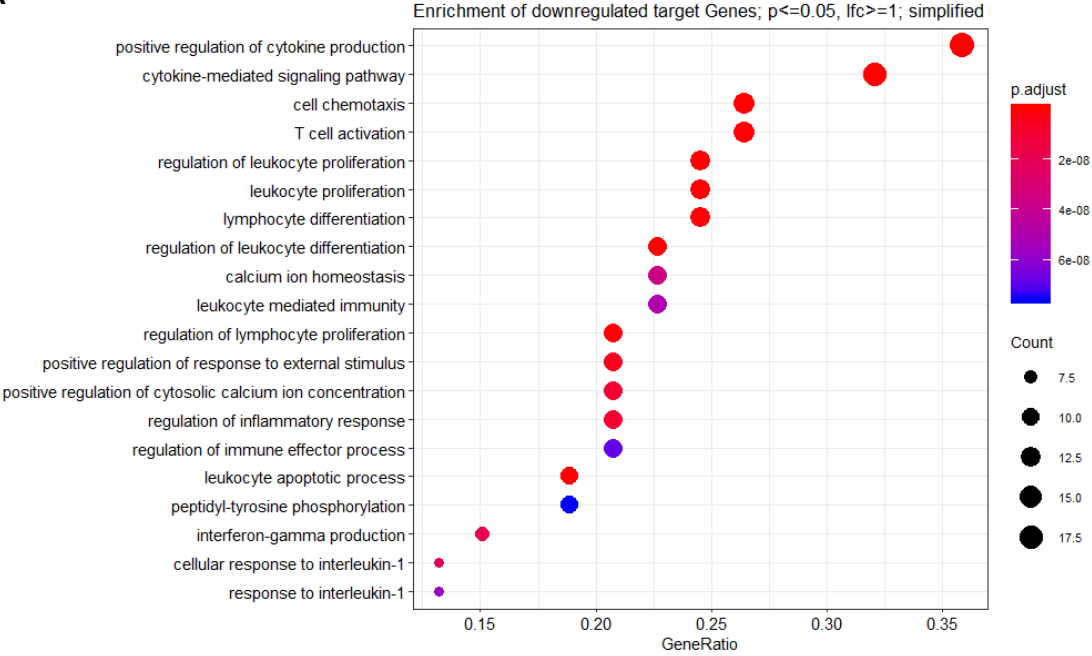


Figure S2: *lrf4*^{-/-} CD4⁺ T cells show decelerated proliferation but migrate to the same aGVHD target organs like WT and *lrf4*^{+/-} CD4⁺ T cells.

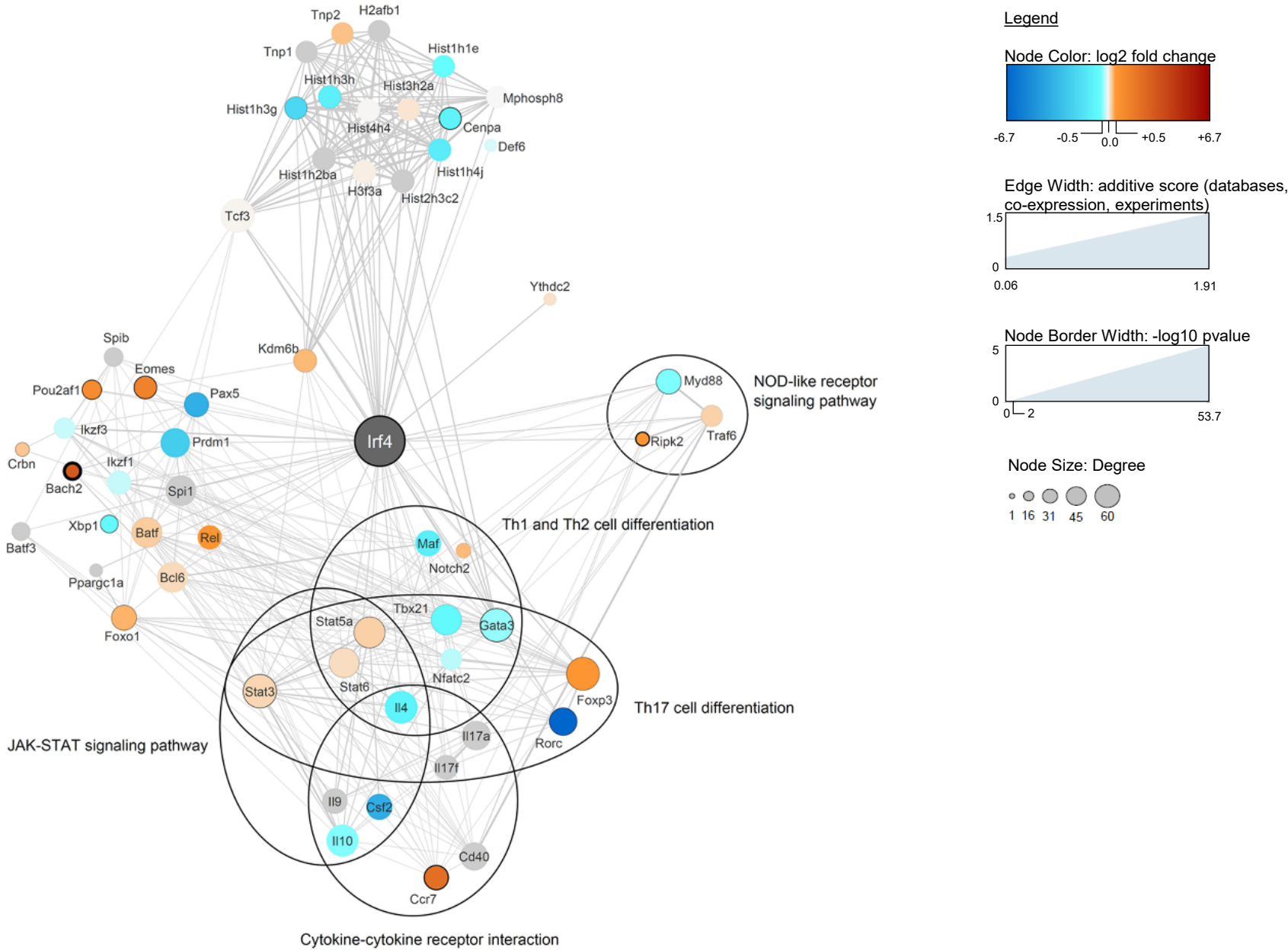
(A) Proliferation of *lrf4*^{-/-} T cells versus WT T cells was monitored via CFSE decrease over time, by stimulation of isolated T cells (BL/6 background derived) with dendritic cells (Balb/c background derived) in a mixed lymphocyte reaction. The migration of 1×10^6 CD4⁺ CD45.1⁺ Luciferase⁺ T cells from WT, *lrf4*^{-/-} or *lrf4*^{+/-} donors with BL/6 background in the lethally irradiated WT BL/6 CD0.2-depleted BM transplanted recipients was traced by imaging of the Luc⁺ CD4⁺ T cells in the in the GVHD target organs lung and liver after dissection at day 14/15 and day 31 after irradiation. Representative BLI pictures (A) and measured BLI signals (B) for the respective groups are shown. Histological sections of lungs and livers were additionally stained with Haematoxylin/Eosin (HE) and scored for histopathological changes (D-E).

Data represent the mean \pm standard deviation combined from independent experiments. If applicable, all data points each representing an individual are shown. * $P < .05$, ** $P < .01$. Analyses were performed with the two tailed Mann-Whitney test.

A



B



C

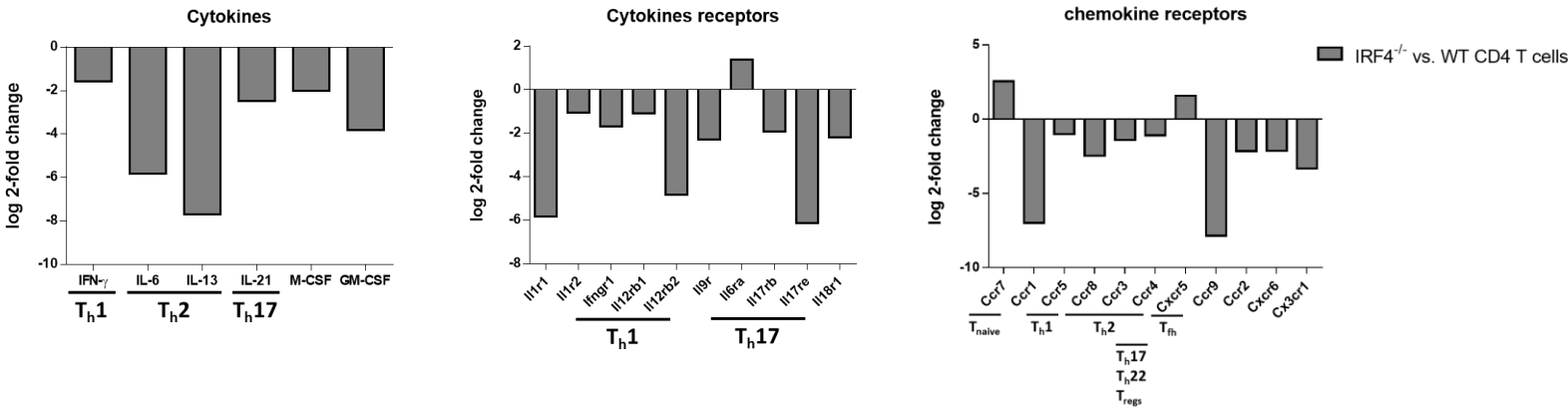


Figure S3: STRING analysis of interacting proteins with murine IRF4 impaired Th differentiation and *Irf4*^{-/-} CD4⁺ T cell according to GO term enrichment analysis and expression of Th specific cytokines, cytokine receptors and chemokine receptors.

Lethally irradiated BALB/c recipients received 5x10⁶ CD45.2⁺ WT C57BL/6 BM depleted for CD90.2⁺ cells at day one and 1x10⁶ 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) GO term enrichment analysis of downregulated genes between *Irf4*^{-/-} and *Irf4*^{+/-} recipients was performed with p-value<0.05. (B) The 60 most probable IRF4 protein-protein interactors were identified by STRING database analysis (all active interaction sources included, minimum required interaction score = medium confidence (0.400), max number of interactors=60). Results of differential gene expression analysis from RNA sequencing were plotted onto the STRING network using Cytoscape: Fold-change are depicted by node-colour, p-value by node-border width, node-degree (i.e. cumulative number of interactions) by node size and the interaction score based on the additive score of interaction scores for experiments, databases and co-expression sources as edge width. Predicted interactors with an additive interaction score to IRF4 of 0 which did not have any other interaction partner with an additive score >0 are not displayed in the network. KEGG pathways and the respective proteins involved are labeled by circles.

(C) Expression of genes coding for chemokine receptors, cytokines and cytokine receptors specific for Th-1, Th-2 and Th-17 between *Irf4*^{+/-} and *Irf4*^{-/-} CD4⁺ T cells was plotted by the mean of 2-fold change + standard deviation.

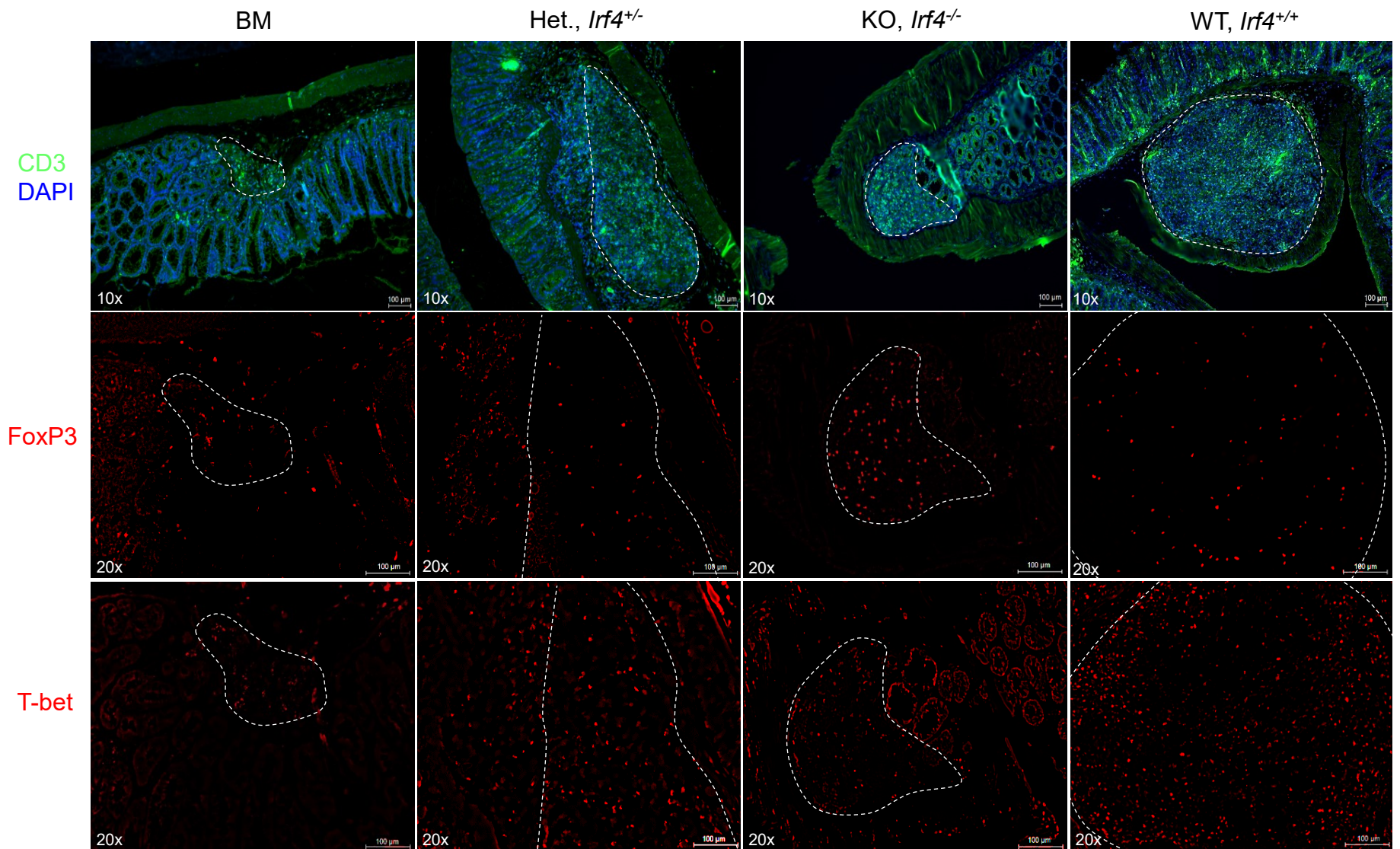


Figure S4: Distinct expression of T-bet *and* Foxp3 throughout murine colon biopsies.

Representative images gained by IHC-staining of colon biopsies with anti-CD3 and DAPI (upper row), anti-FoxP3 (middle row) as well as anti-T-bet (bottom row) in lymphocyte infiltrates (outlined by white line) present on colon tissue sections of mice 15 days after transplantation with BM only (BM, first column), BM + *Irf4*^{+/-} CD4⁺ T cells (Het., *Irf4*^{+/-}, second column), BM + *Irf4*^{-/-} CD4⁺ T cells (KO, *Irf4*^{-/-}, third column) or BM + *Irf4*^{+/+} CD4⁺ T cells (WT, *Irf4*^{+/+}, fourth column). The images are post-processed in terms of brightness and contrast.

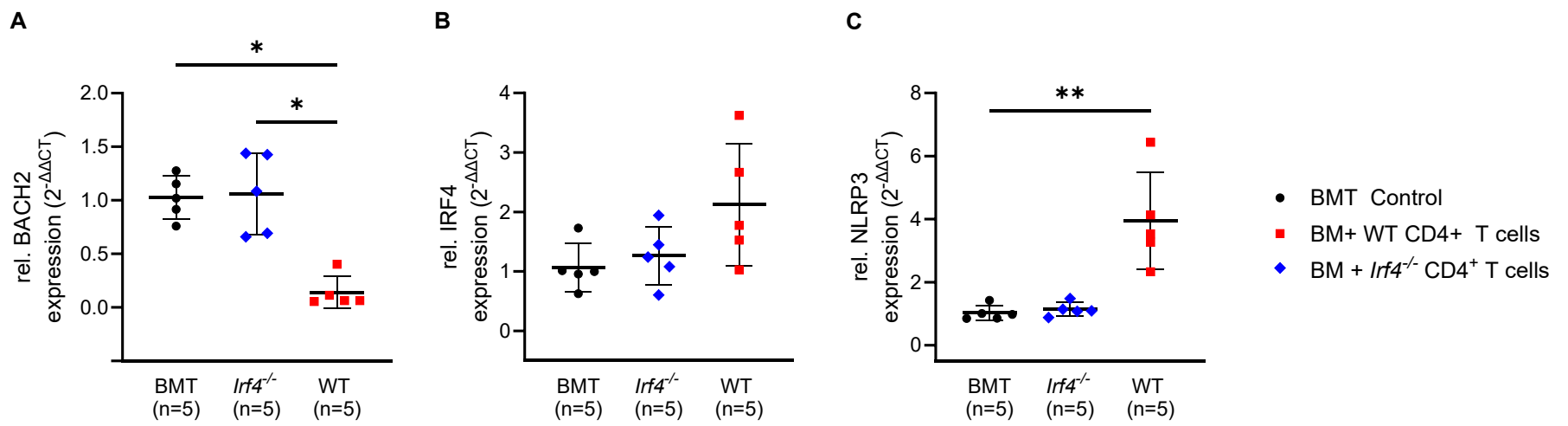


Figure S5: Distinct expression of *Irf4*, *Bach2* and *Nlrp3* throughout murine colon biopsies.

Relative expression of *Irf4* (A), *Bach2* (B) and *Nlrp3* (C) in colon biopsies of mice 15 days after transplantation of BM only (BMT), BM + *Irf4*^{-/-} CD4⁺ T cells and BM + WT CD4⁺ T cells quantified by qPCR. Bar plots indicate the mean, and standard deviation of the individual sample sizes. Transcript expression was normalized with the mean of the housekeeping genes RPL13A and β -Actin and with the mean of target gene expression by BMT reference samples *P < .05, **P < .01. Analyses were performed with Kruskal-Wallis test.

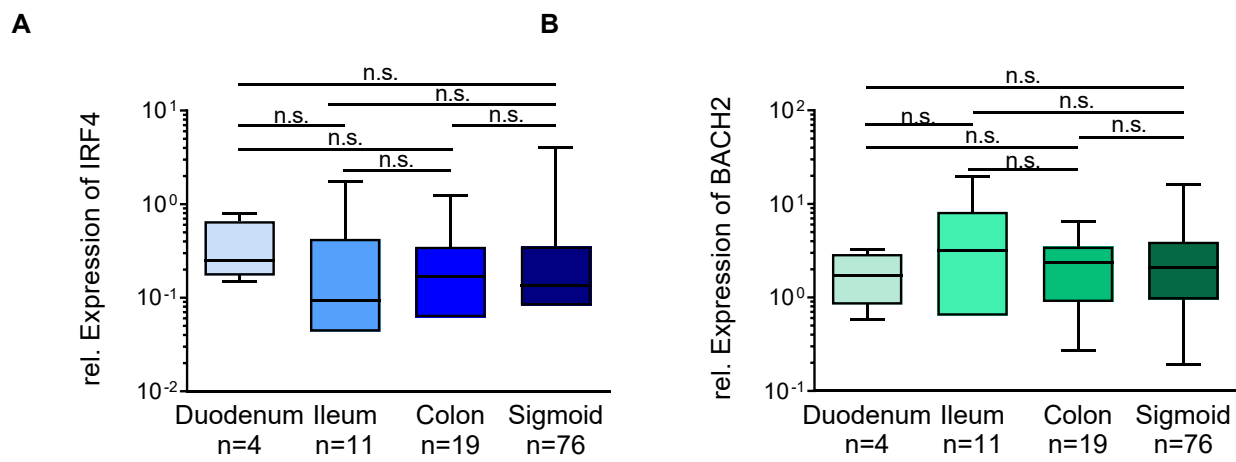


Figure S6: Homogenous expression of *IRF4* and *BACH2* throughout human biopsies from different intestinal compartments.

Relative expression of *IRF4* (A) and *BACH2* (B) in biopsies from GVHD patients from different intestinal compartments including duodenum, ileum, colon and sigma quantified by qPCR. Whisker-box plots indicate the mean, min and max value of individual sample sizes indicated in the respective plots. *P < .05, **P < .01, ***P < .001, ****P < .0001. Analyses were performed with the two tailed Mann-Whitney test.

Table S1: Summary of patients’ characteristics

Age in years		mean	(range)
	Patients (N=76)	53	(20-69)
Sex		n	(%)
	male	45	59.2
	female	31	40.8
Diagnosis			
	Acute leukemia	41	53.9
	Myelodysplastic syndrome	10	13.2
	Myeloproliferative syndrome	3	3.9
	Lymphoma	22	28.9
Stage of underlying disease			
	early	15	19.7
	intermediate	27	35.5
	advanced	34	44.7
Donor type			
	Unrelated donor	49	64.5
	Sibling	24	31.6
	Haploidentical donor	3	3.9
Stem cell source			
	PBSC	71	93.4
	BM	5	6.6
Conditioning regimen			
	Reduced intensity	68	89.5
	Standard	8	10.5
Conditioning regimen in detail			
	FBM	31	40.8
	FTM	6	7.9
	Thio/Treo/Flud	2	2.6
	Treo/Flud	12	15.8
	Thio/Flud	1	1.3
	Mel/Treo/Flud	5	6.6
	TBI/Flud	4	5.3
	TBI/Cy2	2	2.6
	FLAMSA-RIC/Treo	2	2.6
	FLAMSA-RIC	3	3.9
	TBI/Etoposid	1	1.3
	TBI/Mel/Flud	2	2.6
	Busulfan/Flud	1	1.3
	Treo/Cy2/Eto	1	1.3
	FBM+PostTxCy2	2	2.6
	Thiotepa+PostTxCy2	1	1.3
Immunosuppression			
	CyA/MTX	58	76.3
	CyA/MMF	13	17.1
	Everolimus	1	1.3
	Tacro/MMF	1	1.3
	PostTxCy/Tacro/MMF	3	3.9

Table S2: qPCR primer sequences for the respective human and murine transcripts

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
18S (human)	ACCGATTGGATGGTTTAGTGAG	CCTACGGAAACCTTGTTACGAC
IRF4 (human)	GACAACGCCTTACCCTTCG	GGGACATTGGTACGGGATT
BACH2 (human)	GCACAGGACCTGGAAAAATTA	TTCCGCTGGTCATTGAGG
NLRP3 (human)	GGACTGAAGCACCTGTTGTGC	TCCTGAGTCTCCCAAGGCATTC
IRF4 (murine)	TATGCCCCTGACCAGTCACA	AAAGGTTCTGTACCTGGC
BACH2 (murine)	CTTCTGGCAAGCACTGGTTG	TGGCAGTGTAGGCAAAGTGT
NLRP3 (murine)	GCTAAGAAGGACCAGCCAGA	CAGCAAACCCATCCACTCTT
Beta-Actin (murine)	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
RPL13A (murine)	TGAATACCAACCCCTCCCGA	CACCATCCGCTTTTTCTTGTCA

