Antibodies and Flow Cytometry

Fluorochrome antibodies were obtained from Thermo Fischer Scientific or BioLegend. We used the following antibodies: anti-H-2K^{b}(AF6-88.5.5.3), anti-CD4 (RM4-5/GK1.5), anti-CD8 (53-6.7), anti-CD25 (PC61.5), anti-Thy1.1 (HIS51), anti-CD45.1 (A20), anti–IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22), anti–CXCR3 (CXCR3-173), anti-CCR9 (CW-1.2), anti-α4β7 (DATK-32), anti-IFN-γ (XMG1.2), anti-IL-17 (eBio17B7), and anti- IL-4 (11B11). Live/Dead fixable viability dye (Thermo Fischer Scientific) was used for live/dead discrimination. For intracellular cytokine staining experiment, cells were stimulated with cell stimulation cocktail (plus protein transport inhibitors) (Thermo Fischer Scientific) for 5 h at 37°C. Cells were then stained for surface antigens, fixed, permeabilized using a Foxp3/Transcription factor staining buffer set (Thermo Fischer Scientific) and labeled with appropriate intracellular antibodies to detect intracellular antigens. Tregs were detected by staining cells for surface antigens, followed by fixation, permeabilization using a Foxp3/Transcription factor staining buffer Set (Thermo Fischer Scientific) and labeled with anti-Foxp3 (FJK-16s). BD LSRFortessa (BD Biosciences) was used to acquire cells and analyses were performed using FlowJo software.

Treg isolation and suppression assay

Tregs were isolated from CD45.2+ B6 lymph nodes and spleens by using negative selection as described in the methodology, but with the addition of anti-CD8 Ab (53–6.7, STEMCELL Technologies) and removal of anti-CD25 to enrich CD4+ T cells. CD4+ T cells were incubated with phycoerythrin-labeled (PE-labeled) anti-CD25 (PC61.5, eBioscience), followed by anti-PE beads (Miltenyi Biotec). CD25+ cells were selected

with magnetic columns (Miltenyi Biotec). Isolated Tregs were activated overnight with plate bound anti-CD3 (10 μg/ml, 145-2C11, Thermo Fisher Scientific), anti-CD28–coated (10 μg/ml, Thermo Fisher Scientific), 1,000 IU/ml recombinant human IL-2 (rhIL-2, Novartis) + IRX4204 or vehicle. After overnight activation, Tregs were washed twice to remove the drug, co-cultured with purified cell trace violet (CTV) labeled CD45.1+ CD25- T cells at different ratios (Tregs: Teffs; 0:1, 1:1, 1:3 and 1:12) in the presence of soluble anti-CD3 mAb (0.25 μg/ml, 145-2C11, Thermo Fisher Scientific) and T cell–depleted CD45.1+ mouse splenocytes. CTV dilution was analyzed after 72 hours by flow cytometry.

In vitro CTV proliferation study

Purified B6 CD44- T cells were labeled with 2.5 μM CTV (Life Technologies, Thermo Fisher Scientific) for 15 minutes at 37°C. Cells were stimulated with anti-CD3 mAb– and anti-CD28 mAb–coated beads (Dynabeads, Thermo Fisher Scientific) at a 4:1 cell/beads ratio in the presence of either IRX4204 (100nM) or vehicle DMSO. CTV dilution was analyzed after 72 hours by flow cytometry.

CFSE proliferation study

In selected experiments CFSE (2.5μM) labeled) purified T cells were infused into lethally irradiated recipients on day 0. Spleens were harvested from the recipients on day 6 and cells were analyzed for CFSE dilution and apoptosis using annexin V staining kit (Thermo Fischer Scientific). Dead cells were excluded from analysis. Responder frequency and proliferation capacity of donor CD4 and CD8 T-cells were calculated.[1]

Mixed lymphocyte reactions (human)

Suppression was assessed by using a 5-carboxyfluorescein-diacetatesuccinimide ester (CFSE) inhibition assay. Peripheral blood mononuclear cells (PBMCs) from two donors were ficolled from leukapheresis products (Memorial Blood Center, St. Paul, MN) were purified using magnetic CD3-labeled beads (Miltenyi Biotec) into groups of T cells (CD3+) and non-T cells (CD3-). CD3+ cells from each donor were labeled with CFSE (InVitrogen, Carlsbad, CA), and stimulated with allogeneic non-T cells (1:1, T cell: non-T cell) from the other donor in the absence or presence of IRX4204 (100nM) in 96-well round-bottomed plates. Assays were harvested on day 5 and stained with antibodies to CD4, CD8, CD25, and Foxp3, and then data were acquired by using FACScalibur or LSRII (both BD Biosciences, San Jose, CA). Data were analyzed by using the proliferation platform in FlowJo (Treestar, Ashland, OR), and suppression was determined from the division index.

iTreg generation

Naive ($CD44^{lo}CD62L^{+}$) $CD4^{+}$ T cells were negatively selected by using Easy sep (Stem Cell Technology) and further enriched by MACS microbeads (Miltenyi Biotec). Isolated CD4+ T cells were then cultured with irradiated T cell depleted splenocytes in the presence of 100 Units/ml of recombinant human IL-2 (rH IL-2, Novartis), 5ug/ml of anti-CD3 (145-2C11; Thermo Fischer Scientific) with (1-3ng/ml) or without recombinant human TGF-β (rhTGF-β; Peprotech) for 4 days.

FITC- dextran assay

The mucosal integrity was evaluated by using a FITC-dextran (Sigma) assay as described previously.[2] Plasma samples fluorescence intensity was analyzed on a plate reader with excitation/emission wavelength of 485/535 nm.

Histopathology

Colon, small intestine, liver, and spleen were harvested on day 14 after transplantation, embedded in Optimal Cutting Temperature (OCT) compound (Miles), snap frozen, and stored at -80ºC. Frozen blocks were cut in 5µm sections and mounted on microscope slides (Superfrost/Plus). Sections were fixed, stained with hematoxylin and eosin (Thermo-Fisher) per manufacturer's instructions.

Lamina propria lymphocyte (LPL) isolation

Intestinal LPL were isolated as described previously.[2,3] Briefly, intestines from BMT recipients were harvested and incubated at 37°C in a shaker with 10% FBS PBS containing EDTA before digesting the tissues at 37°C in a shaker with 10% FBS PBS containing either collagenase D alone 0.5mg-1mg/ml (Roche) or a mix of 1 mg/ml collagenase D (Roche, Indianapolis, IN), 0.15 IU/ml Dispase (Sigma-Aldrich), and 0.5 mg/ml DNase I (Sigma-Aldrich). LPLs were purified on a Percoll gradient (40%/80%).

Bioluminescence imaging (BLI) studies

A Xenogen IVIS imaging system was used for in vivo and ex vivo imaging. Mice were injected i.p. with firefly luciferin substrate (i.p.; 0.1 ml; 30 mg/ml; PerkinElmer) five minutes before imaging. Tissues from GVHD mice were incubated with 500µg/ml

luciferin substrate 5 minutes before ex-vivo imaging. Data were analyzed using Living Image Software (Xenogen) and presented as photon counts per area.

RNA sequencing

Lethally irradiated BALB/c recipients were infused with B6 CD45.2+ BM (10^7) + B6 CD45.1+ CD4+ T cells ($1.5x10^6$) and treated with either IRX4204 or vehicle. On day 18, donor CD4+ T cells were isolated from the small intestine LPLs of those treated recipients. RNA was extracted from sorted CD45.1+ CD4+ T cells using the RNeasy Mini Kit (Qiagen). According to manufacture's directions, purified mRNA were loaded onto a nanostring cartridge with a custom codeset of 365 genes previously determined to be related to T cell function.[4,5] Cartridge were then prepared on a nanostring platform and scanned by a digital analyzer. Data are normalized based on four house keeping genes and log-transformed prior to analysis. Raw count data and data transformation were processed using the DESeq2 package using default parameters.[6] Significance thresholds included a log fold change cutoff of 1 and a q value threshold of 0.05. A variance-stabilized transformation using of raw gene count values was used for both principal component analysis and heatmap generation. All analysis was performed in the R statistical environment.

Transient transfection and receptor activation analysis

Cos_7 cells were plated in 96-well plates and then grown until ~90 % confluency (overnight). Next, those cells were transfected with effectors plasmid pCMX-GAL4-mNurr1, pCMX-L-RXRα, reporter plasmid tk-(MH100) x4-luciferase and transfection control pCMX-βgal followed by incubation for 24 hours.[7] Cells were then treated with

either a graded dose series of IRX4204 or 0.05% DMSO vehicle. After 24 hours, cells were lysed and assayed for luciferase and β-galactosidase activity as described.[7] All transfections were performed in triplicate and repeated in multiple experiments. Data are reported as fold induction over vehicle (0.1% DMSO) controls ± S.E.M.

pTreg identification

Lethally irradiated BALB/c mice were transplanted with WT B6 BM and FACS sorted (FACSAria II) donor T cells containing Treg depleted T conventional cells (GFP -) from B6 Foxp3 GFP KI reporter mice. Recipients were treated with either vehicle or IRX4204 for two weeks. pTregs were identified by GFP expression in donor T cells in recipient's spleen, mesenteric lymph nodes and small intestines using a BD LSR Fortessa (BD Biosciences).

Bone marrow chimera

Lethally irradiated CD45.1 B6 mice were transplanted with equal numbers (2x 10^6) of B6 CD45.1+ NTCD BM and B6 *Scurfy* CD45.2+ NTCD BM to generate mixed bone marrow chimera. After three months of reconstitution, *Scurfy* CD45.2+ $CD4^+CD25^-$ cells from chimeric mice were MACS sorted to inject into secondary lethally irradiated BALB/c recipients. The purity of the sorted cells were >94%.

Tamoxifen administration

B6 (Rosa-RFP × $Foxp3^{eGFP\text{-}Cre\text{-}ERT2}$) reporter mice were given tamoxifen i.p injections (Sigma-Aldrich) at a dose of 1.5 mg/mouse, daily for consecutive 4 days in corn oil to

induce RFP+ on eGFP+ Foxp3 T cells as described previously.[8] RFP expression was detected in 40-50% of eGFP+ Foxp3 T cells after 2 weeks of injections.

Serum cytokine analysis

Serum was collected from BMT recipients on day 7 and assayed for IFN-γ and TNF-α using commercially available ELISA kits (Thermo Fischer Scientific).

Statistical analysis

For survival studies, Kaplan-Meier product-limit method was used. Differences between groups were determined using log-rank test. Student's *t*-test was used to analyze differences between groups in other experiments. A P value of .05 or less was considered significant in all tests.

1. Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J Clin Invest*. 1997;100(12):3173-3183.
2. Aoyama K, Saha A, Tolar J, et al. Inhibiting retinoic acid signaling ameliorates graft-versus-host disease by modifying T-cell differentiation and intestinal migration. *Blood*. 2013;122(12):2125-2134.
3. Thangavelu G, Lee YC, Loschi M, et al. Dendritic Cell Expression of Retinal Aldehyde Dehydrogenase-2 Controls Graft-versus-Host Disease Lethality. *J Immunol*. 2019;202(9):2795-2805.
4. Gaublomme JT, Yosef N, Lee Y, et al. Single-Cell Genomics Unveils Critical Regulators of Th17 Cell Pathogenicity. *Cell*. 2015;163(6):1400-1412.
5. Chihara N, Madi A, Kondo T, et al. Induction and transcriptional regulation of the co-inhibitory gene module in T cells. *Nature*. 2018;558(7710):454-459.
6. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.

7. Janesick AS, Dimastrogiovanni G, Vanek L, et al. On the Utility of ToxCast and ToxPi as Methods for Identifying New Obesogens. *Environ Health Perspect*. 2016;124(8):1214-1226.
8. Fan MY, Low JS, Tanimine N, et al. Differential Roles of IL-2 Signaling in Developing versus Mature Tregs. *Cell Rep*. 2018;25(5):1204-1213 e1204.

Supplemental Figure 1. IRX4204 is more potent than FK506 in preventing and controlling acute GVHD

(A) Survival, weights, and clinical scores of lethally irradiated BALB/c recipients of 10^7 B6 NTCD BM cells with or without $5x10^6$ B6 splenocytes (n =5-8/group). Recipients were treated i.p with either vehicle or IRX4204 from days 0-56 (daily) or long term daily (0-100) post transplant. Vehicle vs. 0-56 $p<0.01$; vehicle vs. 0-100 $p<0.05$. **(B)** Survival, weights, and clinical scores of lethally irradiated BALB/c recipients of 10^7 B6 NTCD BM cells with or without $5x10^6$ B6 splenocytes. Recipients were treated i.p with either vehicle or IRX4204 from days 0-42 (daily) or intermittent (inter; 0-21, 25-28, 32-35, 39-42 days post transplantation). IRX4204 treated recipients survived longer ($p < 0.01$) than those of vehicle treated recipients. (n =5-8/group). **(C)** BALB/c mice were lethally irradiated and infused with 10^7 B6 NTCD BM cells with or without purified $1.5x10^6$ B6 T cells. Recipients were treated i.p with vehicle, FK506 or IRX4204 from either days -3 to 11 or 3 to 17 post transplant. FK506 or IRX4204 treatment from days -3 to 11 post transplant significantly prolonged the survival of GVHD mice vs. vehicle controls ($p < 0.01$; $p < 0.001$, respectively). IRX4204 vs. FK506 recipients $p < 0.01$. IRX4204 treatment from days 3 to 17 post transplant significantly prolonged the survival of GVHD mice vs. FK506 or vehicle controls ($p < 0.05$). No significant difference was observed between vehicle and FK506. (n =5-8/group). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Supplemental Figure 2. IRX4204 attenuates acute GVHD with impaired donor T cell proliferation

(A-D) Lethally irradiated BALB/c recipients were infused with 10^7 B6 NTCD BM cells and $5x10^6$ B6 luc spleen cells. Recipients were treated daily i.p with either vehicle or

IRX4204. **(A, C)** Bar graphs and bioluminescent imaging (BLI) picture of whole BLI of recipient mice on day 6 post transplantation. **(B, D)** Recipients were sacrificed on the same day and organs were harvested for ex-vivo organ BLI in the presence of luciferin. n=5/group. Data are shown as the mean ± SEM. $^{**}p < 0.01$.

Supplemental Figure 3. IRX4204 directly represses the proliferation of mouse and human T cells

(A) CTV labeled naïve B6 T cells were activated with anti-CD3/CD28 coated beads in the presence of either IRX4204 or vehicle. Cell proliferation was analyzed by flow cytometry after 72 hours of stimulation. Division index and representative histogram plots showing proliferation of CD4 + T-cells and CD8 + T-cells. Results are representative of three independent experiments with n=4/group. **(B)** Purified human T cells were labeled with CFSE and stimulated with allogeneic CD3- mononuclear cells in the presence of either IRX4204 or vehicle. Division index and representative histogram plots showing proliferation of CD4+ and CD8+ T cells on day 5 post stimulation. This result is a representative of two different donor T cells stimulated with allogeneic CD3- cells (n=3/group). **(C)** Lethally irradiated BALB/c recipients were infused with 10^7 B6 NTCD BM cells and 8.5 x 10^6 CFSE-labeled purified B6 T cells. Recipients were treated daily i.p with either vehicle or IRX4204. Mice were sacrificed on day 6 post transplant to determine Annexin-V expression of donor T cells in spleen. n=5/group. One experiment was performed. Data are shown as the mean ± SEM. $^{**}p < 0.01$.

Supplemental Figure 4. IRX4204 amelioration of acute GVHD is associated with reduced Th1 differentiation and serum cytokines

(A-C) BALB/c recipients were lethally irradiated and infused with 10^7 B6 NTCD BM cells with or without $5x10^6$ B6 splenocytes. Recipients were treated daily i.p with either vehicle or IRX4204. **(A)** Spleens were harvested two weeks post transplantation and restimulated in vitro to analyze donor CD4+ T cells expressing IFN-γ, TNF-α by flow cytometry. **(B-C)** Serum samples were harvested on day 7 post transplant and assayed for IFN-γ **(B)**, TNF-α **(C)**. n=4-5/group. **(D)** B6 NTCD BM (10^7) + purified T cells ($1x10^6$) from B6 Foxp3-GFP KI mice were infused into lethally irradiated BALB/c recipients. Recipients were treated daily i.p with either vehicle or IRX4204. On day 13, LI LPLs were isolated and restimulated in vitro to determine the frequency of donor T cells expressing IFN-γ, TNF-α by flow cytometry. n=3/group. Data are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Supplemental Figure 5. Donor T cells from IRX4204 treated recipients have upregulated gut homing receptors (α4β7, CCR9)

(A-C) HSCT was carried out in lethally irradiated BALB/c recipients with 10^7 B6 NTCD BM cells and $5x10^6$ B6 splenocytes (n=5/group). Recipients were treated daily i.p with either vehicle or IRX4204. After 7 days, donor T cells in recipient's spleens were analyzed by flow cytometry for the following homing markers: **(A)** α4β7, **(B)** CCR9 and **(C)** CXCR3. n=4-5/group. Data are shown as the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.001$.

Supplemental Figure 6. IRX4204 treated recipients have increased frequency of Tregs in their circulating lymphocytes

(A) Absolute numbers of Tregs from Figure 4B. (**B-G)** Lethally irradiated BALB/c recipients were infused with B6 NTCD BM (10^7) + B6 Foxp3-GFP KI mice purified T cells ($1x10^6$). Recipients were treated daily i.p with either vehicle or IRX4204. On day 6 post transplant, peripheral blood and spleens were collected from the recipients and analyzed for Treg and Teffector memory ($CD44^{hi}$ $CD62L^-$; T_{EM}) frequencies by flow cytometry. The percentages of Tregs in peripheral blood **(B-D)** and spleen (**C-E)** are shown. The percentages of T_{EM} in peripheral blood **(F)** and spleen are shown **(G)**. n=4/group. Data are shown as the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$.

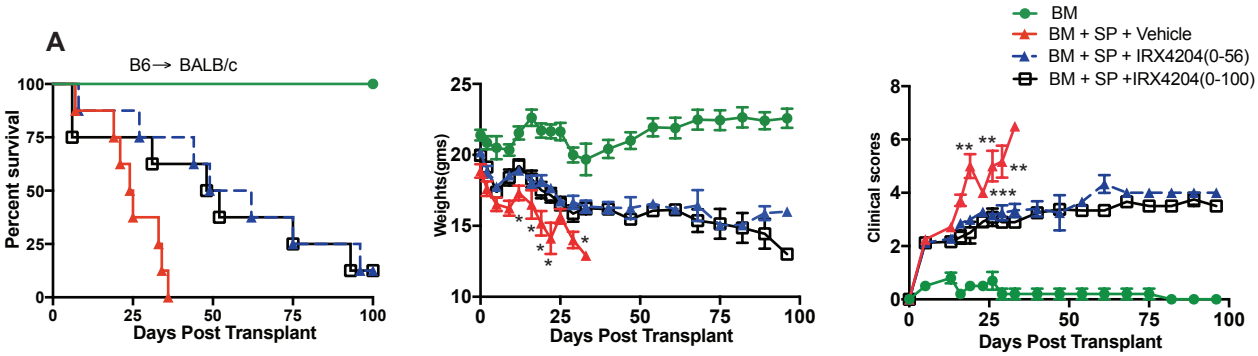
Supplemental Figure 7. IRX4204 can increase human Tregs in vitro and is also a potent activator of Nurr1 pathway

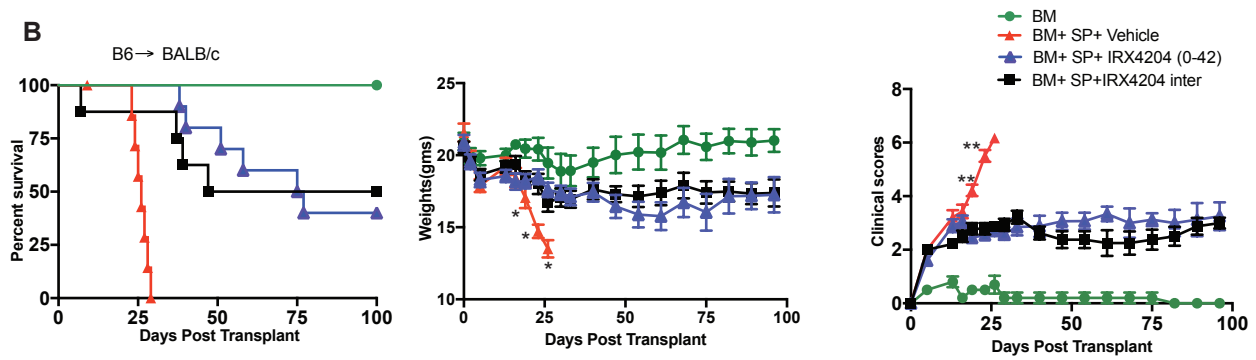
(A) Purified human T cells were stimulated with allogeneic CD3- mononuclear cells in the presence of either IRX4204 or vehicle. The frequency of CD4+ CD25+ Foxp3+ was determined on day 5 of culture. Bar graphs show the increased percentage of human Tregs in IRX4204 treated group. This result is a representative of two different donor T cells stimulated with allogeneic CD3- cells (n=3/group). **(B)** Cos cells were transfected with reporter tk-MH100x4-luciferase and plasmids pCMX-GAL-Nurr1 +/- pCMX-L-RXRα. After overnight incubation, transfected cells were treated with either IRX4204 or vehicle for about 24 hours. Cells were then lysed and assayed for luciferase and β-galactosidase activity. The data shown are representative of two experiments. **(C)** Absolute numbers of pTregs from Figure 5A-C. **(D)** CD45.2+ Tregs were activated with anti-CD3/CD28 overnight in the presence of either vehicle or IRX4204 and then co-

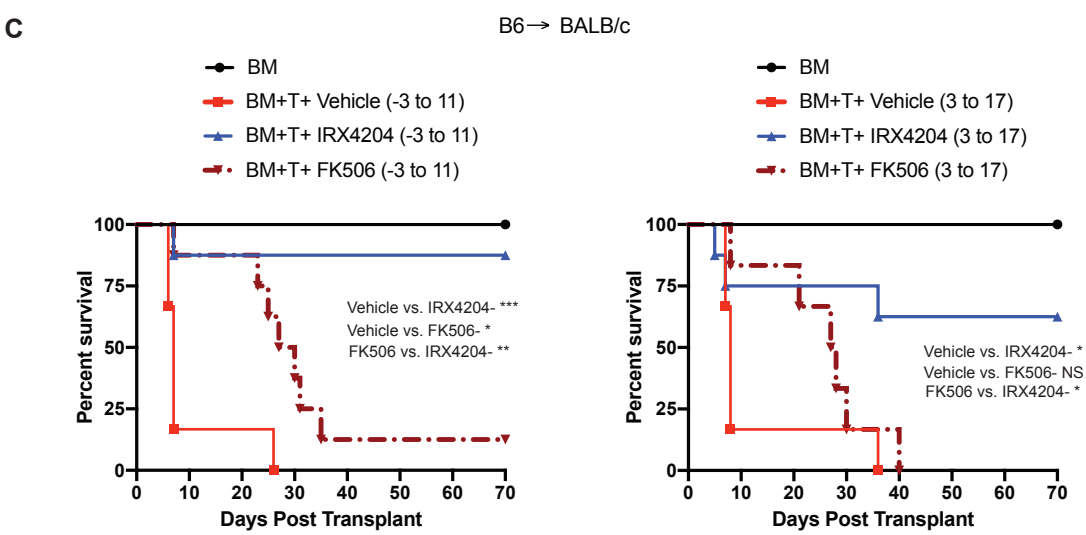
cultured with CTV labeled CD45.1+ CD25- T cells at different ratios. Suppression of CD45.1+ CD4+ and CD8+ T cells by Tregs was analyzed after 72 hours of co-culture. n=4/group. Data are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Supplemental Figure 8. IRX4204 retains GVL responses against a lymphoma cell line

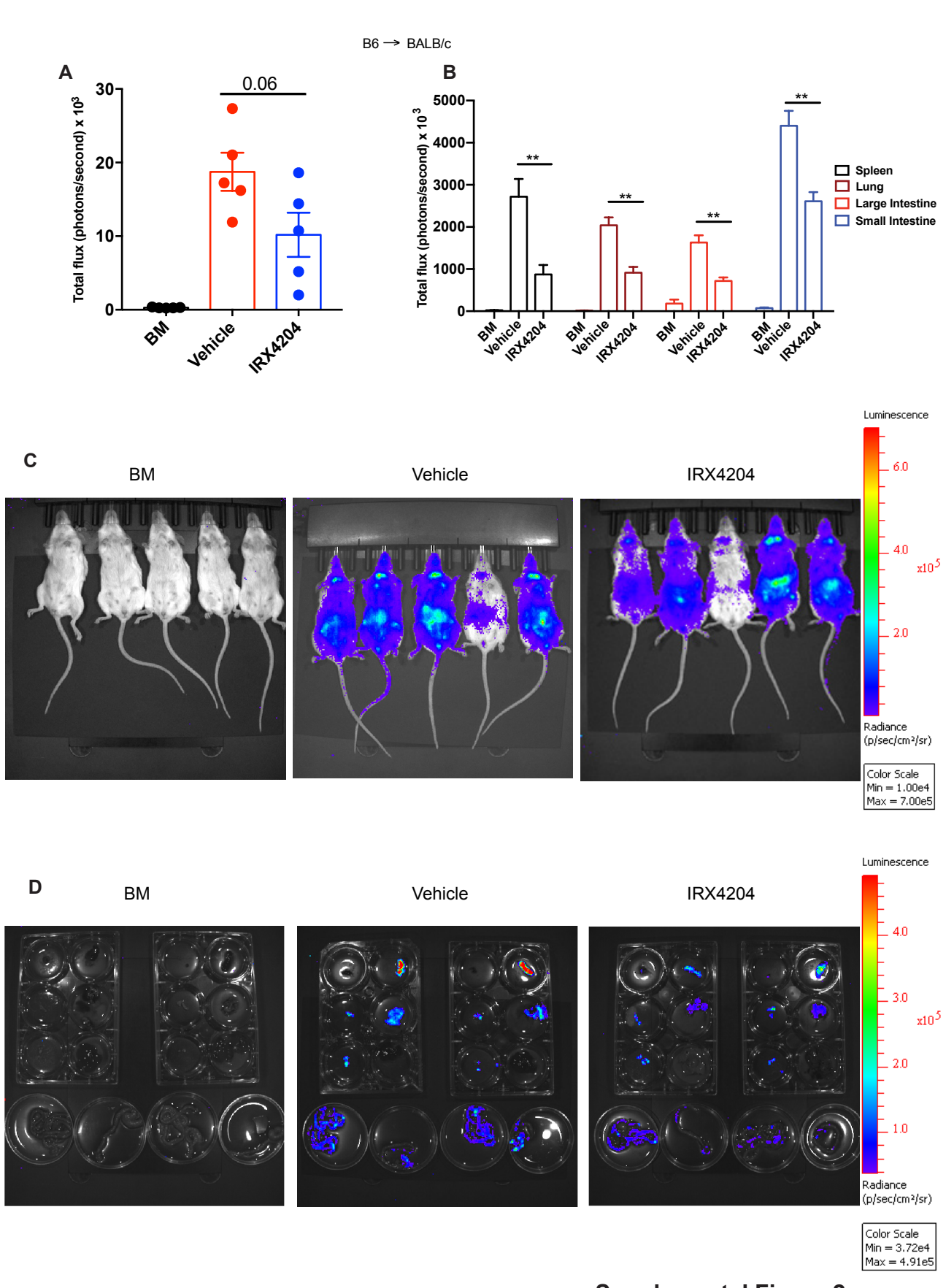
Survival, BLI curves and images (tumor growth signal) of lethally irradiated B6 mice receiving 10^7 BALB/c T cell depleted BM ± B6 TBL-12 luc (10^4) ± $1.5x10^6$ BALB/c CD25- T cells. Recipients were treated daily i.p with either vehicle or IRX4204. One experiment was performed. n=5-8/group. Data are shown as the mean ± SEM. $^{*}p < 0.05$.



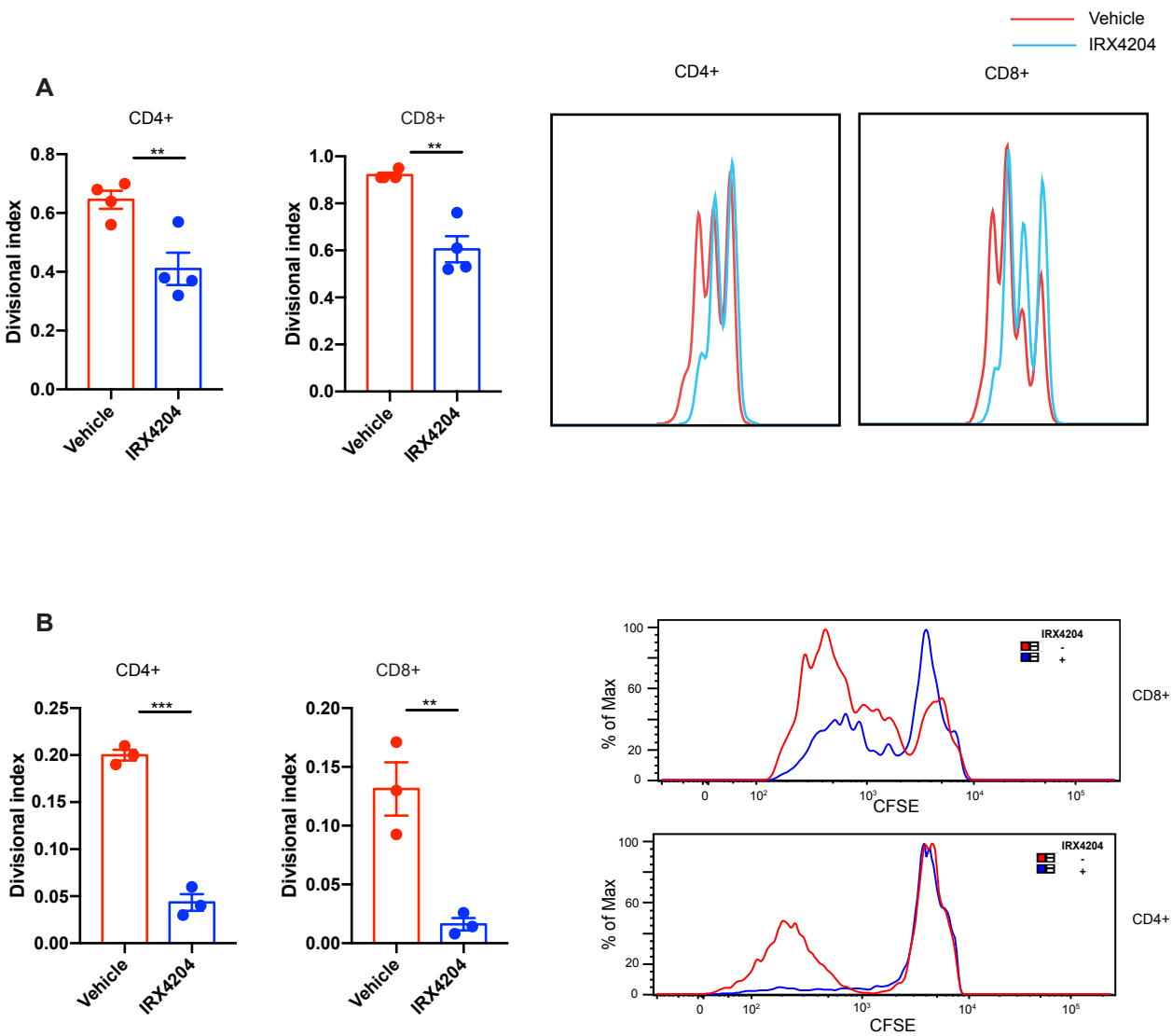




Supplemental Figure 1



Supplemental Figure 2



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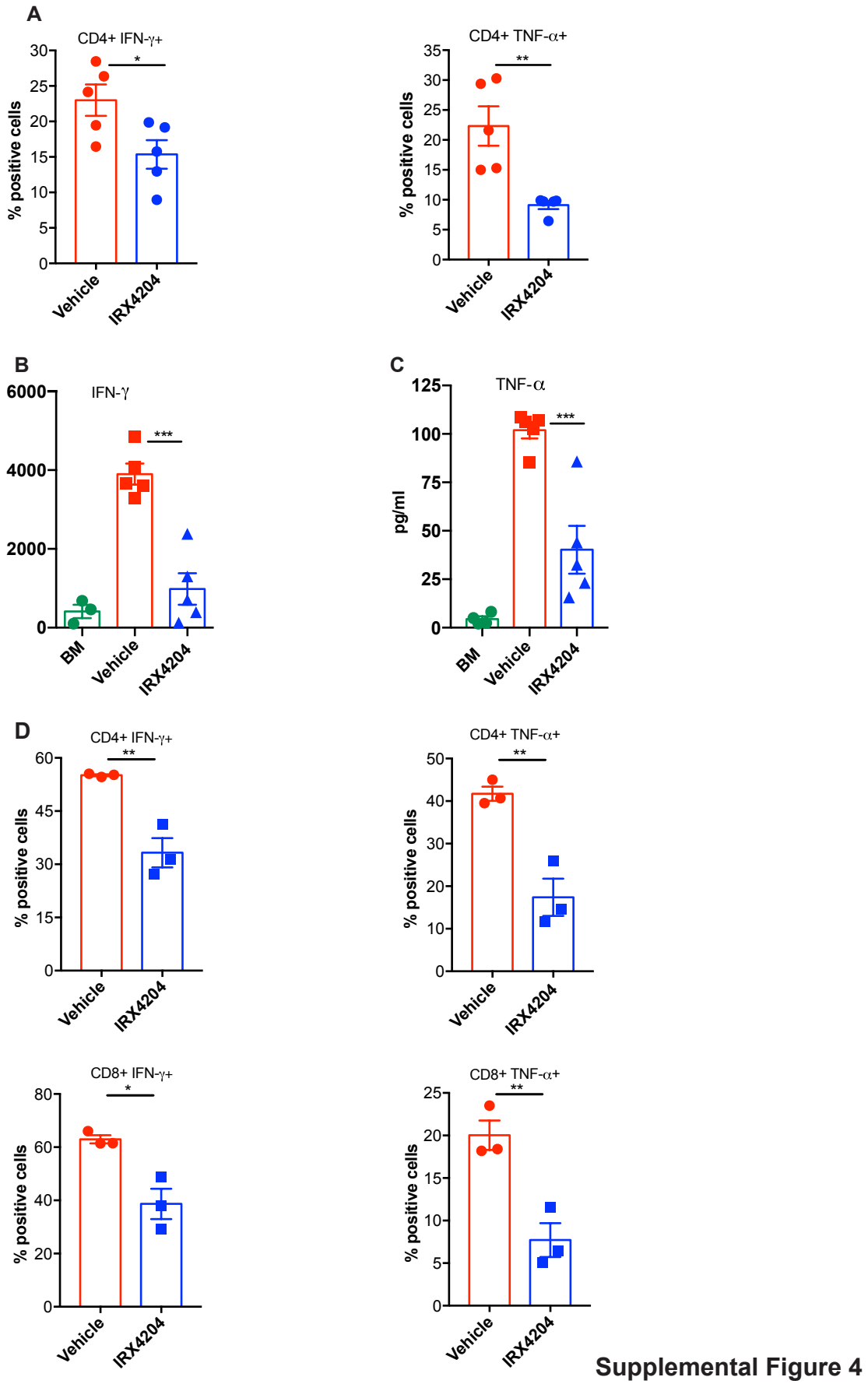

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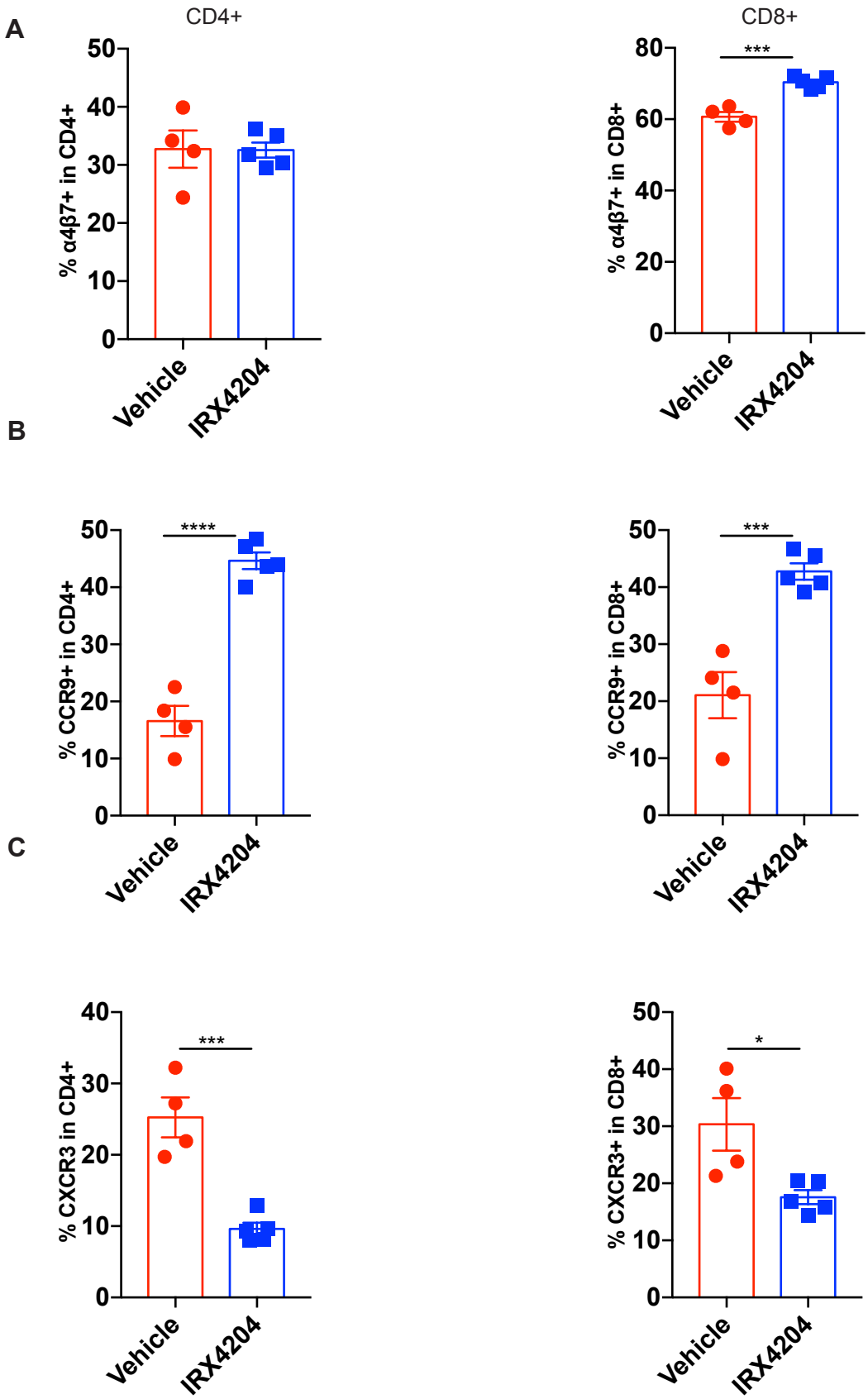

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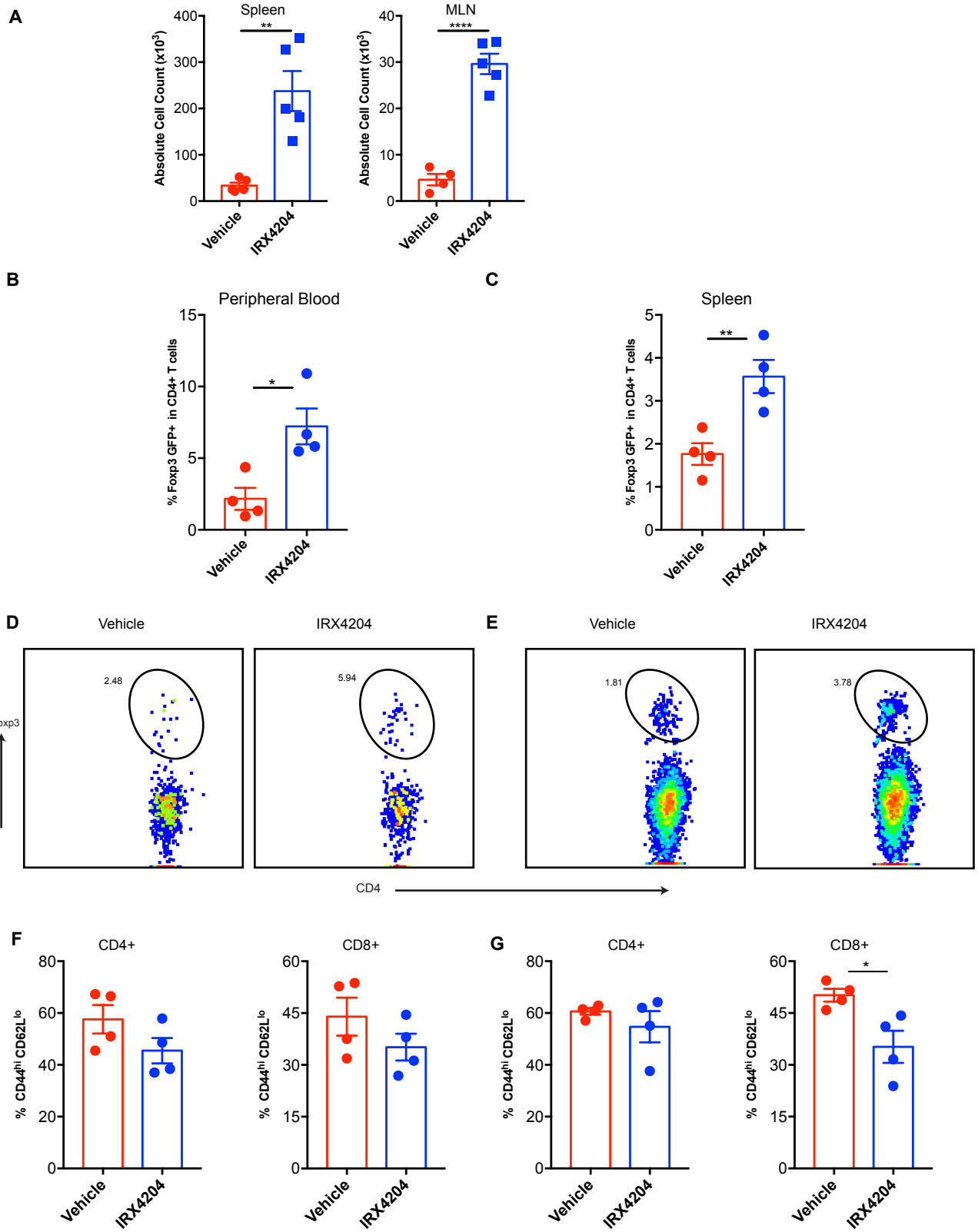

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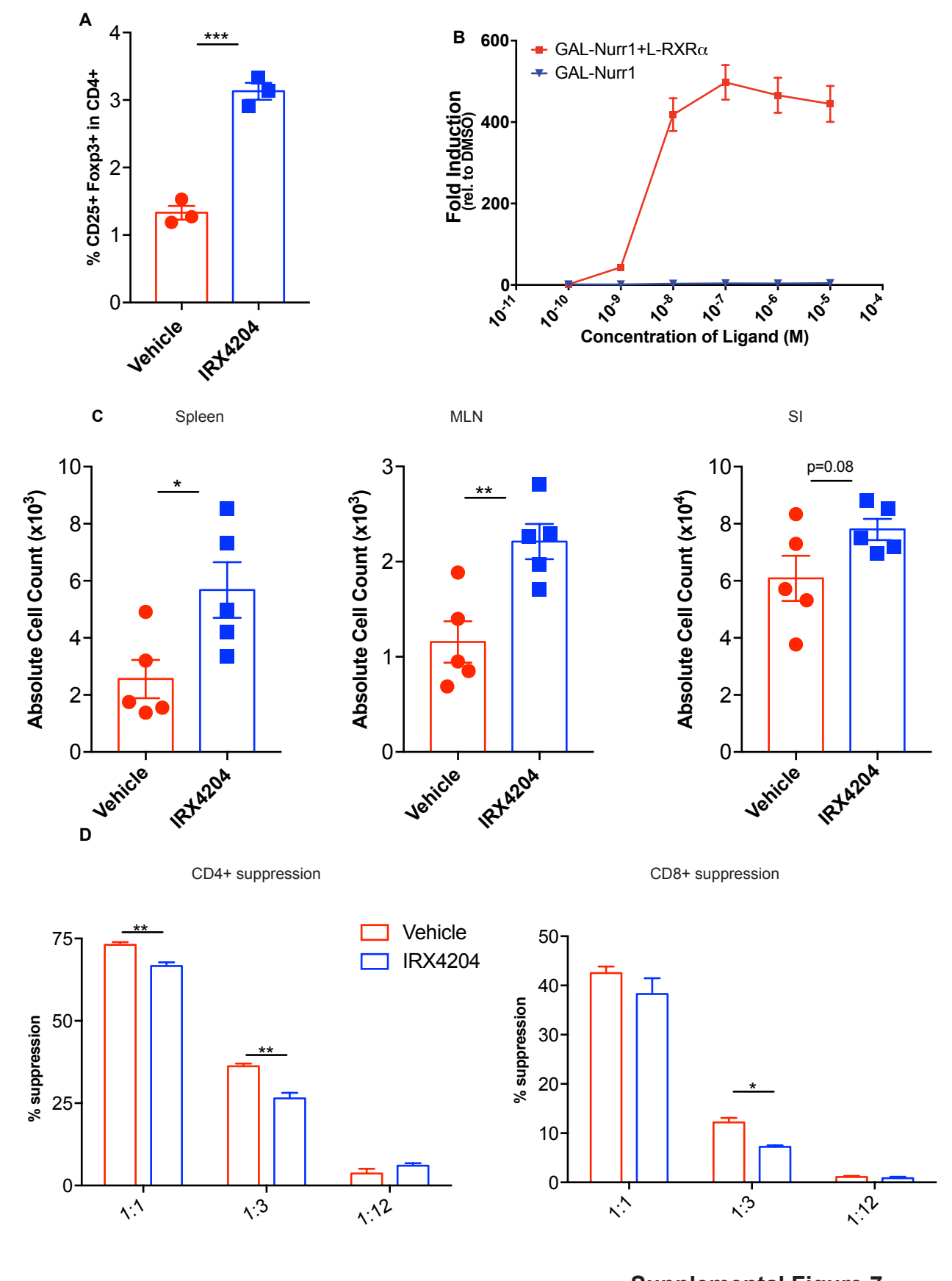

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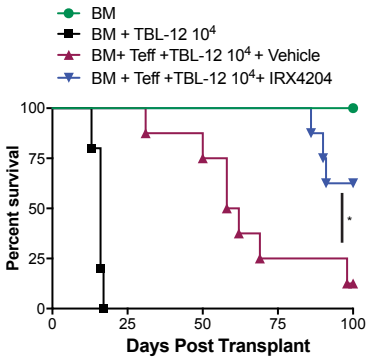

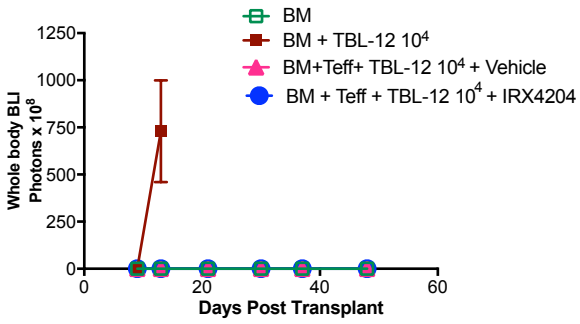

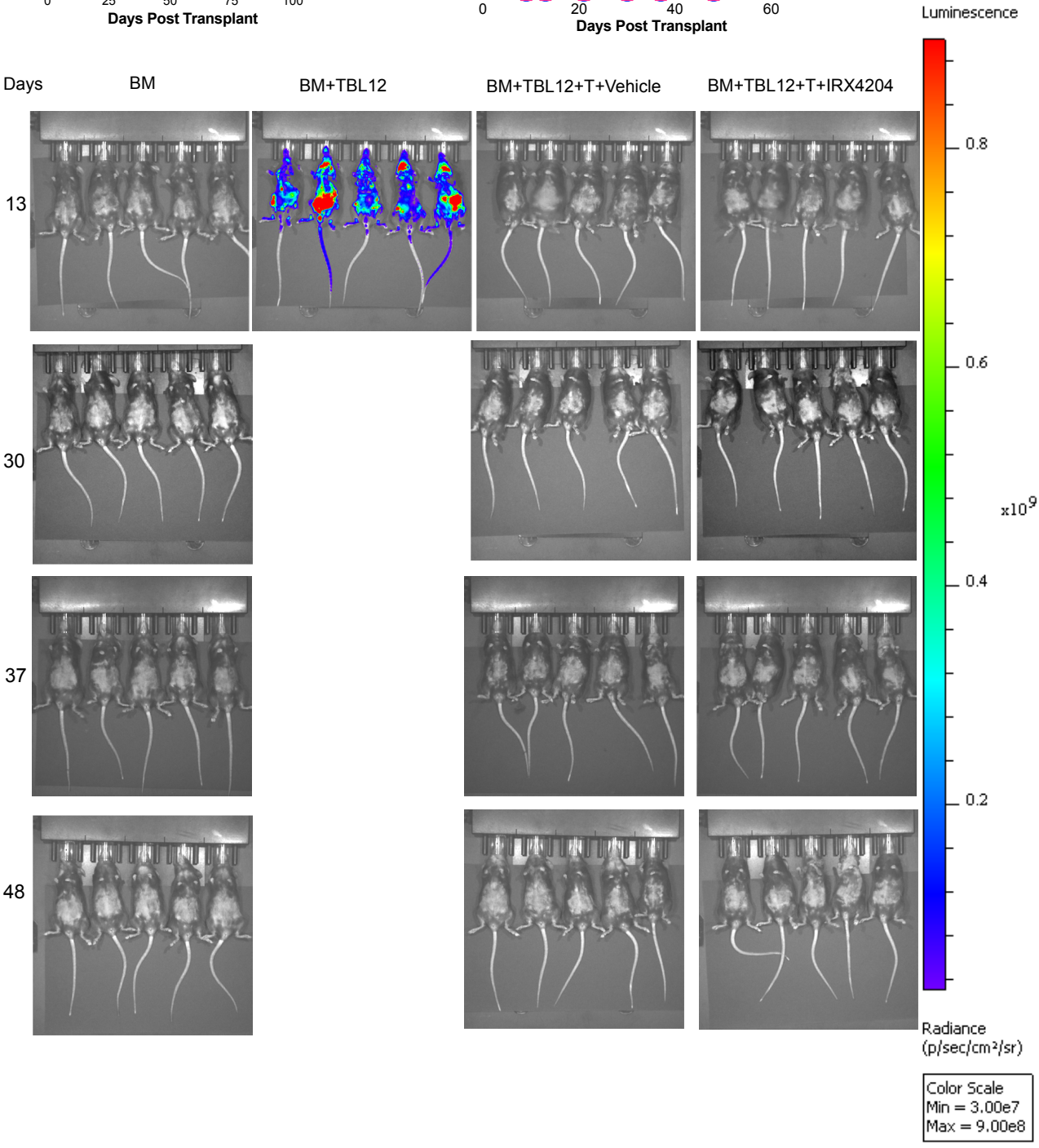

Supplemental Figure 8