SIGNALING THROUGH THE TYPE 2 CANNABINOID RECEPTOR REGULATES THE SEVERITY OF ACUTE AND CHRONIC GRAFT VERSUS HOST DISEASE

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Key Points:

- CB2R expression on donor T cells regulates the severity of GVHD and the accumulation of proinflammatory $CD8^+$ T cells in target organs.
- Therapeutic targeting of the CB2R is dependent upon the pharmacological agonist profile and the composition of pathogenic immune cells.

ABSTRACT

Graft versus host disease (GVHD) pathophysiology is a complex interplay between cells that comprise the adaptive and innate arms of the immune system. Effective prophylactic strategies are therefore contingent upon approaches that address contributions from both immune cell compartments. In the current study, we examined the role of the type 2 cannabinoid receptor (CB2R) which is expressed on nearly all immune cells and demonstrated that absence of the CB2R on donor $CD4^+$ or $CD8^+$ T cells, or administration of a selective CB2R pharmacological antagonist, exacerbated acute GVHD lethality. This was accompanied primarily by the expansion of proinflammatory $CD8^+$ T cells indicating that constitutive CB2R expression on T cells preferentially regulated $CD8^+$ T cell alloreactivity. Using a novel CB2R-EGFP reporter mouse, we observed significant loss of CB2R expression on T cells, but not macrophages, during acute GVHD, indicative of differential alterations in receptor expression under inflammatory conditions. Therapeutic targeting of the CB2R with the agonists, tetrahydrocannabinol (THC) and JWH-133, revealed that only THC mitigated lethal T cell-mediated acute GVHD. Conversely, only JWH-133 was effective in a sclerodermatous chronic GVHD model where macrophages contribute to disease biology. In vitro, both THC and JWH-133 induced arrestin recruitment and ERK phosphorylation via CB2R, but THC had no effect on CB2R-mediated inhibition of adenylyl cyclase. These studies demonstrate that the CB2R plays a critical role in the regulation of GVHD and suggest that effective therapeutic targeting is dependent upon agonist signaling characteristics and receptor selectivity in conjunction with the composition of pathogenic immune effector cells.

INTRODUCTION

Graft versus host disease (GVHD) remains a major cause of morbidity and mortality in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT). GVHD is classified into two phases termed acute and chronic, which are distinguishable based on temporal characteristics as well as unique clinical and pathological manifestations.[1-4] While T cells are the proximate driver of GVHD, disease progression is the result of the complex interplay between elements of the innate and adaptive arms of the immune system. In fact, it is increasingly being recognized that cells of the innate immune system (i.e. macrophages, neutrophils, etc.) play pivotal roles in the biology of this disease.[5-10] Macrophages, in particular, have been shown to be important in the pathophysiology of chronic GVHD in both murine models and humans.[6,7,10] However, in spite of this evolving mechanistic understanding, the majority of therapeutic strategies for both acute and chronic GVHD have focused on the adaptive immune system with less regard for approaches that also directly target innate immune cells.

Endocannabinoids are endogenously produced, arachidonic acid-containing bioactive lipids which act widely throughout the body as agonists for several G protein coupled receptors (GPCR).[11,12] The two primary endocannabinoids are *N*-arachidonoylethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG).[11] Both AEA and 2-AG act as agonists at the CB1 receptor (CB1R) which is expressed at high density in the CNS and a lower density in metabolic tissues, such as liver and adipose.[11] 2-AG is also a full agonist of the CB2 receptor (CB2R) which is expressed predominantly by immune cells.[13] Increasing evidence demonstrates that the CB2R mediates the effects of the plant-derived cannabinoids, Δ^9-tetrahydrocannabinol (THC), on the immune system[14] and genetic variants in the CB2R gene (*cnr2*) have been associated with inflammatory disorders in humans.[15-18] Furthermore, CB2R can regulate cells in both the innate and adaptive immune system, and has specifically been shown to regulate cytokine release,[19-21] B and T cell differentiation[22] and modulate macrophage-mediated inflammation.[23-25]

Therapeutic targeting of the CB2R has therefore emerged as an area of intense interest for a variety of inflammatory disorders;[26] however, the optimal pharmacological approach remains undefined. The goal of the current study was to delineate the role of CB2R signaling in GVHD pathobiology and determine whether pharmacological targeting of the receptor could ameliorate disease using complementary murine models in which there are pathophysiological contributions from the adaptive and innate arms of the immune system.

MATERIAL AND METHODS

Mice. C57BL/6 (B6) (H-2^b), B6.PL (Thy1.1^+), Balb/c (H-2^d), FVB/N (H-2^q), B10.D2 (H-2^d), *Rag-*$1^{-/-}$ (H-2^b) and CB2$R^{-/-}$ (B6) mice were bred in the Biomedical Resource Center at the Medical College of Wisconsin or purchased from The Jackson Laboratory (Bar Harbor, ME). CB2R^{EGFP} reporter mice were constructed by inserting an enhanced green fluorescent gene preceded by an internal ribosomal entry site (IRES) into the 3' untranslated region of the *cnr2* mouse gene.[27]

Other detailed methods. All other methods are described in the supplemental files (available on the Blood web site).

RESULTS

Donor CB2R expression regulates the severity of acute GVHD. To define the role of CB2R signaling in GVHD biology, we first conducted transplantation studies using CB2$R^{-/-}$ mice as either donor or recipients. We observed that there were no differences in the percentage of total, naïve, central memory, or effector memory splenic $CD4^+$ and $CD8^+$ T cells (Figures 1A-1D), or in the frequency of $CD4^+$ $Foxp3^+$ regulatory T cells (Figures 1B and 1D) between wild type (WT) B6 and CB2$R^{-/-}$ mice, indicating that the

transgenic environment did not result in obvious alterations in the composition of the T cell compartment. Balb/c recipients reconstituted with CB2R$^{-/-}$ grafts had accelerated GVHD lethality compared to animals transplanted with WT B6 grafts (Figure 1E). Similar results were observed in a second MHC-incompatible model (B6→FVB), indicating that results were not strain-specific (Figure 1F). Examination of GVHD target organs revealed that animals transplanted with BM and spleen cells from CB2R$^{-/-}$ donors had a significant increase in the absolute number of CD8^{+} T cells in the colon and liver when compared to mice reconstituted with WT grafts (Figure 1G). Conversely, there was no augmentation in CD4^{+} T cell numbers in these tissue sites, and an overall decrease in the lungs. In addition, mice transplanted with CB2R$^{-/-}$ grafts had increased numbers of CD8^{+} IFN-γ^{+} T cells in the colon (Figure 1H), indicating that donor CB2R expression appeared to be important for the regulation of CD8^{+} T cell alloreactivity. To account for the fact that the transgenic environment may have still resulted in unappreciated alterations in T cell development, we employed a complementary pharmacological approach to verify that inhibition of CB2R signaling exacerbated GVHD. Animals administered SR144528, which is a selective CB2R antagonist,[28] also had significantly greater mortality (Figure 1I), providing confirmation that inhibition of CB2R signaling worsened GVHD. There was no difference in survival between CB2R$^{-/-}$ and WT recipient animals receiving WT grafts (Figure 1J), indicating that only CB2R expression on donor cells regulated GVHD severity.

CB2R expression on CD4^{+} or CD8^{+} T cells primarily regulates CD8^{+} T cell alloreactivity. To further interrogate the role of CB2R expression on CD4^{+} versus CD8^{+} T cells, we performed transplants in which animals were reconstituted with mixtures of purified CD4^{+} and/or CD8^{+} T cells from either WT or CB2R$^{-/-}$ donors so that the effect of absent CB2R expression in one T cell population could be assessed in the presence of the alternate WT T cell population. We observed that absence of the CB2R on either CD4^{+} or CD8^{+} T cells resulted in significantly worse survival compared to animals reconstituted with WT CD4^{+} and WT CD8^{+} T cells (Figure 2A). Histological analysis revealed that absence of the CB2R on

$CD8^+$ T cells exacerbated pathological damage in the colon and liver, whereas transplantation with $CD4^+$ $CB2R^{-/-}$ T cells worsened pathology in the liver compared to WT controls (Figures 2B and 2C). Examination of GVHD target tissues demonstrated that absence of the CB2R on $CD4^+$ T cells resulted in an increased number of donor $CD8^+$ T cells in the colon and lung, whereas transplantation with $CD8^+$ $CB2R^{-/-}$ T cells led to an increase in the number of $CD8^+$ T cells in the colon, liver, and lung (Figure 2D). Analysis of proinflammatory cytokine production revealed a significantly greater number of $CD8^+$ T cells that produced either IFN-γ, GM-CSF or TNF-α in the lung, liver and colon in mice transplanted with $CD8^+$ $CB2R^{-/-}$ T cells (Figures 2E-2G). Notably, absence of the CB2R on $CD4^+$ and/or $CD8^+$ T cells had no effect on the number of $CD4^+$ T cells producing IFN-γ, GM-CSF or TNF-α in any tissue when compared to WT controls (Supplemental Figure 1). Thus, CB2R expression primarily on $CD8^+$ T cells was critical for the regulation of $CD8^+$ T cell expansion and inflammatory cytokine production in GVHD target organs.

GVHD results in significant loss of CB2R expression on donor T cells. Given the importance of the CB2R in the regulation of GVHD within target organs, we sought to specifically identify receptor-expressing cells in these tissues. A long-standing problem in the field of cannabinoid biology, however, has been the inability to accurately identify $CB2R^+$ cells because of antibody non-specificity and low receptor expression levels.[29] Therefore, we created and utilized a $CB2R^{EGFP}$ reporter mouse in which expression of eGFP is regulated by CB2R promotor activity through an IRES.[27] Immunofluorescent staining of the spleen from these mice demonstrated the presence of eGFP in $CD3^+$ T and $CD19^+$ B cells in merged images, indicative of receptor expression in these populations (Figures 3A). In addition, flow cytometric analysis of splenocytes from $CB2R^{EGFP}$ and WT mice revealed that B cells had the highest expression of the receptor with nearly all cells (i.e. ~97%) expressing eGFP (Figures 3B and 3C) which is consistent with prior reports utilizing other methods.[30] High expression of eGFP was also observed on splenic $CD8^+$ T cells (>90%), whereas expression was significantly lower on $CD4^+$ T cells (~20%).

To determine whether CB2R expression was similar on immune cell populations resident in non-lymphoid tissues, we performed a similar analysis on cells isolated from the liver, lung, and colon, as well as the peripheral blood. These data revealed that B cells expressed high levels of the CB2R regardless of tissue site. However, whereas CB2R expression was low on $CD4^+$ T cells isolated from the spleen, we observed that it was substantially higher on $CD4^+$ T cells in the lung, liver, and the peripheral blood (Supplemental Figure 2). Conversely, there was also significant variability in CB2R expression on $CD8^+$ T cells where expression was high on splenic $CD8^+$ T cells but significantly lower on cells isolated from the liver and colon. Thus, these data demonstrate that EGFP faithfully marks CB2R expression on immune cells, and that there is substantial variability in receptor expression on adaptive immune cells in diverse tissue sites.

Interestingly, we observed that there was significant loss of eGFP expression in both $CD4^+$ and $CD8^+$ T cells isolated from GVHD tissue sites when compared to expression of eGFP in the same cell populations in the spleen of naïve mice (Figures 3D). This was most marked in $CD8^+$ T cells which had higher expression of eGFP in nontransplanted reporter mice (Figure 3B). Notably, similar loss of expression was observed in BM control animals that did not have clinically evident GVHD. Since it was formally possible that loss of eGFP expression in BM control mice could have been due to a lower level of alloantigen induced proliferation as a consequence of small numbers of mature donor T cells in the marrow graft, we conducted experiments using syngeneic recipients, where donor T cell expansion occurs only though homeostatic proliferation, in order to ascertain the strength of activation required to induce loss of the fluorescent signal. Interestingly, we observed that there was a significantly higher percentage of $CD4^+$ T cells in the spleen and $CD8^+$ $eGFP^+$ T cells in all tissues of mice transplanted with syngeneic grafts (Figure 3E), indicating the homeostatic expansion was much less effective at inducing loss of eGFP expression. As additional confirmation, we activated T cells from $CB2R^{EGFP}$ animals in vitro under strong polyclonal and alloantigen-specific conditions which demonstrated a marked reduction in eGFP

expression under both conditions, although loss of expression was more pronounced when T cells with stimulated with alloantigen (Figure 3F). Since there is an intervening IRES site between the CB2R and eGFP genes in the targeting construct of the reporter mouse,[27] it was conceivable that loss of eGFP did not signify a commensurate loss of CB2R gene expression. To address this possibility, we examined CB2R mRNA levels in $CD4^+$ and $CD8^+$ T cells under polyclonal activating conditions. These experiments demonstrated a significant reduction in CB2R mRNA levels in both $CD4^+$ and $CD8^+$ T cells (Figure 3G). We did not carry out these studies in DC co-culture experiments because of the potential for confounding effects from contaminating dendritic cell mRNA. Collectively these studies support the premise that strong T cell activation, as occurs in GVHD, results in a substantial loss of CB2R expression on donor T cells.

Agonistic signaling through the CB2R mitigates the severity of acute GVHD. Given that loss or blockade of the CB2R worsened acute GVHD, we sought to determine whether targeted CB2R activation could mitigate GVHD severity. We first examined levels of 2-AG and AEA, which are the natural ligands for the CB2R,[31] to determine if GVHD deleteriously affected endocannabinoid concentrations in target organs. We observed that endocannabinoid concentrations in tissues obtained from BM control or GVHD animals were no different or slightly higher than levels seen in normal mice except for significantly lower 2-AG levels in the spleen (Figure 4A). Notably, quantitation of 2-AG and AEA levels revealed no difference in the concentration of either ligand in the colon, liver, or lung in GVHD when compared to BM control animals. Thus, GVHD did not result in the diminished production of these natural ligands. The effects of chronic treatment with two CB2R agonists, JWH-133 and THC, on GVHD severity were then examined to determine if GVHD could be ameliorated. JWH-133 is a synthetic, CB2R selective cannabinoid that has been shown to be a highly potent and efficacious in mice,[28] whereas THC is a phytocannabinoid that is less selective as it binds to both the CB1R and CB2R and has lower affinity than JWH-133.[31] Cohorts of mice were treated with low (1.5 mg/kg) and high doses (20 mg/kg) of JWH-133 over the first 14 days post transplantation which was the same treatment schedule we employed using

the CB2R antagonist, SR144528 (Figure 1I). These studies demonstrated that neither JWH-133 dosing regimen resulted in improved survival (Figure 4B). Additionally, a more protracted (i.e. 35 days) course of therapy also failed to protect animals from lethal GVHD (Figure 4C), as did abbreviated regimens designed to determine whether transient agonist exposure in the setting of reduced receptor expression could mitigate disease severity (Supplemental Figure 3). In contrast, prolonged administration (i.e. 40 days) of THC resulted in significantly improved survival relative to vehicle-treated control animals (Figure 4D). Since THC can bind to the CB1R and CB2R, it was possible that the protective effects of this agent could be mediated, in part, by binding to the CB1R. However, there was no difference in survival between THC- and vehicle-treated mice transplanted with $CB2R^{-/-}$ marrow grafts (Figure 4E), indicating that the beneficial effect of THC was mediated through the CB2R.

THC reduces the accumulation of proinflammatory T cells in GVHD target tissues. While THC was effective at reducing GVHD severity via the CB2R signaling pathway, the highly efficacious agonist, JWH-133 was without effect. To explore this paradox, we compared the signaling of these agonists using an *in vitro* assay. The CB2R is coupled to heterotrimeric G proteins; thus the primary signaling pathways downstream of CB2R include inhibition of adenylyl cyclase, extracellular regulated kinase (ERK) phosphorylation, and ß-arrestin recruitment.[32,33] We observed that THC and JWH-133 were equally efficacious at increasing pERK (Figure 5A, Table 1, and Supplemental Figure 4). With respect to ß-arrestin recruitment, both JWH-133 and THC exhibited agonist effects; however, the Emax achieved by THC was significantly lower than that of JWH-133, indicating that THC is a partial agonist in this signaling pathway (Figure 5B and Table 1). Notably, JWH-133 was significantly more potent than THC in both these assays. Only JWH-133, however, exhibited agonistic activity to inhibit forskolin-induced cAMP accumulation. THC did not significantly inhibit cAMP accumulation at concentrations up to 30 μM, and even exhibited a slight increase in cAMP accumulation (Figure 5C and Table 1). These findings suggest that the ability of THC to reduce GVHD lethality is due to a signaling bias that results in a lack of effect on cAMP homeostasis while other CB2R signaling pathways are engaged.

Since cAMP is a mechanism by which Tregs mediate suppressive effects[34] and is able to inhibit effector T cell responses,[35] we examined how THC ameliorated GVHD by quantifying the number of proinflammatory and regulatory T cells in the primary GVHD target tissues affected by CB2R signaling (i.e. colon and liver) (Figure 2). Mice treated with THC had a significant reduction in the absolute number of donor $CD4^+$ and $CD8^+$ T cells in the colon and liver (Figure 5D). In addition, there was a statistically significant reduction in the number of $CD4^+$ and $CD8^+$ T cells that produced IFN-γ in the liver and colon (Figure 5E), and $CD4^+$ and $CD8^+$ T cells that made GM-CSF in the GI tract (Figure 5F). Notably, there was no effect on Treg numbers (Figure 5G), indicating that THC reduced proinflammatory T cell accumulation in target organs but did not augment Treg reconstitution.

Signaling through the CB2R regulates sclerodermatous chronic skin GVHD. Whereas acute GVHD is driven primarily by alloreactive donor T cells, chronic GVHD is characterized by additional critical contributions from the innate immune system. Macrophages, in particular, have a pivotal role in the development of fibrosis which is a cardinal feature of this disease.[4,8,36] We observed that the CB2R was also expressed on macrophages from naïve $CB2R^{EGFP}$ animals, albeit with some variability in different tissue sites (Figures 6A and 6B). To determine the role of CB2R signaling in chronic GVHD, we employed two murine sclerodermatous chronic GVHD models.[4,37,38] We first used a pharmacological approach which demonstrated that mice administered the CB2R antagonist, SR144528, had a significant increase in overall pathological score (Figure 6C) as evidenced by hyperkeratosis in the epidermis along with increased inflammatory cell infiltrates in the dermis (Figure 6D). Notably, immunohistochemical staining revealed that dermal infiltration was attributable to the accumulation of both $CD3^+$ T cells and $F4/80^+$ macrophages in antagonist-treated mice (Figure 6E), indicating that there were contributions from both cell populations in pathologically involved tissue. Given the presence of macrophages in the skin, we isolated these cells from more easily accessible sites (i.e. lung and peritoneal cavity) to determine whether administration of a CB2R antagonist altered macrophage phenotype and function. We observed

that there was no difference in expression of class II or costimulatory molecules (Supplemental Figure 5A), or production of IL-1β or GM-CSF (Supplemental Figure 5B) in macrophages obtained from antagonist versus vehicle control treated mice. We then obtained skin samples from these mice and examined genes that are expressed by macrophages and have been shown to be important in the pathophysiology of fibrosis in non-transplant models.[39-41] These studies demonstrated that GVHD control mice had a significant increase in mRNA levels of TGF-β, galectin 3 (GAL-3), and alpha smooth muscle (α-SMA) compared to BM animals (Figure 6F). Notably, the administration of a CB2R antagonist resulted in a further increase in the expression of galectin 3 when compared to vehicle-treated mice, indicating that inhibition of CB2R signaling promoted skin expression of a gene implicated in the development of fibrosis.

We then employed a complementary genetic approach in which Balb/c mice were transplanted with *Rag-1*$^{-/-}$ BM and a low dose of T cells from either WT B6 or CB2R$^{-/-}$ animals.[38] We observed increased pathological damage in the skin of recipient mice transplanted with CB2R$^{-/-}$ marrow grafts as evidenced by dermal fibrosis, inflammatory infiltrates and loss of hair follicles (Figures 6G). Trichrome staining also demonstrated augmented collagen deposition in these animals (Figure 6H). Overall, there was a significant increase in pathological damage (Figure 6I) when compared to GVHD controls. In addition, animals reconstituted with CB2R$^{-/-}$ marrow grafts had augmented mRNA levels of TGF-β, galectin-3 and α-SMA relative to mice transplanted with WT grafts (Figure 6J). Collectively, these results demonstrated that pharmacological and genetic inhibition of CB2R signaling exacerbated sclerodermatous chronic GVHD.

Agonistic signaling through the CB2R mitigates the severity of chronic GVHD. Based on these results, we examined whether administration of a CB2R agonist could mitigate chronic GVHD in a model which is characterized by pathophysiological contributions from both T cells and macrophages (Figure

6E). Interestingly, we observed that there was no loss of eGFP expression on macrophages isolated from the spleen, lung, or liver of GVHD animals compared to nontransplant or BM control CB2R^{EGFP} mice (Figures 7A and 7B). Consistent with that finding in reporter mice, mRNA levels of CB2R in flow-sorted macrophages obtained from the spleen were no different between BM controls and GVHD WT mice (Figure 7C). Thus, CB2R expression appeared more stable on macrophages, than on T cells, under inflammatory conditions. Given this disparity, we examined the relative efficacy of THC versus JWH-133 for mitigating disease severity. In contrast to what we observed during acute GVHD, JWH-133 administration resulted in a significant decrease in pathological damage, whereas THC had no protective effect in chronic GVHD (Figures 7D and 7E). In addition, there was a commensurate reduction in gene expression of TGF-β and α-SMA in the skin of JWH-133-treated mice, whereas THC administration had no effect on gene expression relative to GVHD control animals (Figure 7F). Thus, these studies demonstrated that JWH-133 was more efficacious than THC in a chronic GVHD model, and that these agonists had differential effects on acute and chronic GVHD.

DISCUSSION

The complex interplay between cells of the adaptive and innate arms of the immune system is a critical aspect of GVHD pathophysiology. While T cells have long been accepted as primary drivers of GVHD biology, innate immune cells are increasingly being recognized as having important roles in acute and, particularly, chronic GVHD.[4,5,8,9] Therefore, therapeutic strategies that target immune cells resident in both compartments have the potential to have a salutary impact on this disease. In the current study, we examined the functional role of the CB2R which is expressed on both T cells and macrophages in complementary murine models where both cell types contribute to disease pathogenesis. These results demonstrated that the CB2R plays an important role in the regulation of GVHD and that the administration of CB2R agonists can be employed as a therapeutic strategy to mitigate disease severity.

The fact that the CB2R was differentially expressed on $CD8^+$ versus $CD4^+$ T cells (i.e. ~90% versus ~20%), suggested that the receptor might be disproportionately important in the regulation of $CD8^+$ T cell alloreactivity. This was apparent from acute GVHD studies in which transplantation with $CB2R^{-/-}$ marrow grafts resulted only in an increased accumulation of $CD8^+$ T cells with a proinflammatory phenotype, with no effect on $CD4^+$ T cells. Surprisingly, when we examined the specific contribution of CB2R expression on $CD4^+$ versus $CD8^+$ T cells, we observed that absence of the receptor on either population was sufficient to exacerbate GVHD lethality in the presence of the corresponding WT population. However, this was not attributable to any discernible alteration in the proinflammatory profile of $CD4^+$ T cells as neither transplantation with $CD4^+$ $CB2R^{-/-}$ nor $CD8^+$ $CB2R^{-/-}$ T cells promoted $CD4^+$ T expansion or inflammatory cytokine production. Rather, absence of CB2R expression on either T cell population only promoted the accumulation and production of inflammatory cytokines by $CD8^+$ T cells within GVHD target tissues. Interestingly, histological examination did reveal that absence of the CB2R on $CD4^+$ T cells resulted in worse pathological damage in the liver. The increased expression of the receptor on $CD4^+$ T cells in the liver, relative to other tissue sites, is a potential explanation for this finding and may be indicative of an important role for the CB2R in regulating immune responses within this tissue. Overall, however, the predominant observation from these studies is that CB2R expression, particularly on $CD8^+$ T cells, was critical for the regulation of $CD8^+$ T cell alloreactivity during acute GVHD.

Our data demonstrated substantial loss of EGFP expression and a significant reduction in CB2R mRNA levels during acute GVHD, both of which were consistent with a loss of CB2R expression on the cell surface of T cells. However, our findings that treatment with an antagonist or the complete genetic loss of donor CB2R exacerbated GVHD suggest that sufficient CB2 receptors remain on T cells that could potentially be activated to reduce disease by an appropriate agonist. To test this hypothesis, we examined

the ability of two CB2R agonists, JWH-133 and THC, to ameliorate GVHD severity. JWH-133, a synthetic cannabinoid, was employed since it is reported to be a selective CB2R full agonist with minimal off target effects, and relatively balanced activation of signal transduction pathways except for little activity in the recruitment of β-arrestin, suggesting it might not further down-regulate CB2R function.[28] THC, a phytocannabinoid, with much lower affinity for CB2R than JWH-133 but efficacy in at least some signaling assays[28] had previously been shown to reduce weight loss and splenomegaly in a nonirradiated GVHD model.[42] Surprisingly, we observed that JWH-133 failed to protect animals from lethal acute GVHD despite using multiple dosing and administration schedules. Conversely, GVHD mice treated with THC exhibited a significant increase in survival when compared to vehicle treated GVHD control animals. Since THC can bind to both CB1 and CB2 receptors, we considered that binding to the CB1R could have played a role in mitigating inflammation. However, the administration of THC to mice reconstituted with $CB2R^{-/-}$ grafts failed to prolong survival, indicating that THC exerted immune suppressive effects through the CB2R.

Biased agonism is a characteristic of CB2R agonists whereby the primary intracellular signaling pathways; β-arrestin signaling, inhibition of adenylyl cyclase, and activation of ERK are selectively engaged which can lead to differential biological effects.[32] To understand why JWH-133 and THC administration had divergent results with respect to protection from acute GVHD, we performed *in vitro* assays using an engineered cell line expressing human CB2R to compare the effects of JWH-133 and THC on these major signaling pathways. Our data demonstrated that both JWH-133 and THC effectively activated CB2R signaling cascades that resulted in ß-arrestin recruitment and activation of ERK. We observed that JWH-133 was considerably more potent than THC, and that THC exhibited the characteristic of a partial agonist in the ß-arrestin recruitment assay. Notably, whereas JWH-133 inhibited adenylyl cyclase, THC had no measurable effect on this pathway which is in accord with earlier studies.[43] Our data that THC was a full agonist in some CB2R-mediated signaling assays, but had very low efficacy in others, suggests that it is a protean ligand. Protean ligands are receptor agonists that can

activate signaling cascades that are inactive when the receptor is unbound; however, the efficacy of these ligands is too low to affect signaling cascades that are constitutively active.[44] Mechanistically, from a GVHD perspective, adenylyl cyclase is responsible for the synthesis of cAMP which is critical for maintaining immune regulation[35] and is a primary pathway by which regulatory T cells mediate their suppressive effects.[34] Furthermore, cAMP is also capable of inhibiting effector T cell-mediated inflammatory responses.[35] One potential mechanism for the superiority of THC over JWH-133 is that, while both agonists activate CB2R-mediated signaling in T cells that result in anti-inflammatory effects, JWH-133 also reduces cAMP-mediated signaling, which exerts proinflammatory effects in opposition to the positive effects of CB2R activation. Since THC does not inhibit adenylyl cyclase, it can activate the beneficial signaling cascade without the opposing effect to reduce cAMP concentrations. An alternative explanation is that the two ligands result is different rates of removal and replacement of CB2R on the cell surface. These and other potential mechanistic insights await further study.

Numerous studies in nontransplant murine models of liver and dermal fibrosis have demonstrated that endocannabinoids that signal through the CB2R can exert anti-fibrotic effects.[45-47] Consistent with these data, we observed that the genetic absence of the CB2R on donor T cells as well as CB2R antagonism exacerbated disease severity in two sclerodermatous chronic GVHD models. Notably, however, the administration of THC, which prolonged survival in acute GVHD, had no salutary effect in chronic GVHD. Conversely, JWH-133 resulted in significantly reduced inflammation as determined by pathological assessment and quantification of genes associated with the development of fibrosis, despite its lack of efficacy in acute GVHD. There are several possible explanations for these divergent results. One is that whereas acute GVHD is primarily a T cell-dependent process, the pathophysiology of chronic GVHD is characterized by prominent contributions from cells of the innate immune system, particularly macrophages. To that end, our studies revealed that macrophages, unlike T cells, retained CB2R expression when directly isolated from GVHD mice. It is therefore possible that the signaling cascade elicited by JWH-133 is more effective than THC in macrophages. An alternative perhaps more likely

explanation, however, is that THC binds to both the CB1R and the CB2R. Whereas ligation of the CB2R is anti-fibrogenic, binding of agonists to the CB1R promotes fibrogenesis,[48,49] in part by activation of IRF5.[50,51] Thus, non-selectivity of THC for the CB2R could explain its lack of observed efficacy in comparison to JWH-133 in chronic GVHD.

In summary, these studies identify the CB2R as a GPCR expressed on cells of the adaptive and innate arms of the immune system that can regulate the severity of acute and chronic GVHD. Effective therapeutic targeting of the CB2R was dependent upon agonist signaling characteristics and receptor selectivity in conjunction with the specific composition of pathogenic immune effector cells in involved tissues. The growing interest in the development of selective and specialized ligands that target the CB2R raises the possibility that pharmacological agonists will be available to mitigate GVHD-associated inflammation in human allogeneic HSCT patients. In addition, the availability of dronabinol as an agent to treat chemotherapy-induced nausea and function as an appetite stimulant, may provide the opportunity for this drug to be re-purposed to determine whether THC can prevent acute GVHD in carefully selected patient populations undergoing HSCT.

AUTHOR CONTRIBUTIONS

C-Y.Y. conducted and designed experiments and wrote the manuscript. V.Z. and T.S. performed experiments. G.S. conducted mass spectrometry studies. R.K. performed blinded pathological analysis. A.L., R.M.T., and J.R. conducted immunofluorescence analyses. C.H. designed experiments, supervised studies, and edited the paper. W.R.D. designed experiments, supervised the study, and wrote the manuscript. The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1: **Absence of donor CB2R expression exacerbates the severity of acute GVHD.** (A). Percentage of $CD4^+$ and $CD8^+$ T cells in the spleen of wild type (n=5) and $CB2R^{-/-}$ (n=6) mice. (B, C). Percentage of $CD4^+$ $Foxp3^+$, naïve ($CD62L^{hi}$, $CD44^{lo}$), central memory ($CD62L^{hi}$ $CD44^{hi}$), and effector memory ($CD62L^{lo}$ $CD44^{hi}$) $CD4^+$ T cells (panel B), and naïve, central memory, and effector memory $CD8^+$ T cells (panel C) in the spleen of WT B6 and $CB2R^{-/-}$ mice. Data are cumulative results from 5-6 mice/group and are presented as the mean ± SD. (D). Representative dot plots of $CD4^+$ and $CD8^+$ T cell populations are shown together with the gating approach that was used for the data in panels B and C. (E). Lethally irradiated (900 cGy) Balb/c recipients were transplanted with BM alone from B6 mice (5 x 10^6) (n=9) or BM and spleen cells (adjusted to yield an αβ T cell dose of 0.6 x 10^6 cells) from B6 (n=15) or $CB2R^{-/-}$ (n=15) animals. Overall survival is depicted. Results are from three experiments. (F). Lethally irradiated (1100 cGy) FVB mice were transplanted with B6 BM alone (n=9), B6 BM and spleen cells (n=17), or $CB2R^{-/-}$ BM and spleen cells (n=19) (adjusted to yield an αβ T cell dose of 0.85 x 10^6 cells). Overall survival is depicted. Results are from three experiments. (G, H). Lethally irradiated Balb/c mice were transplanted with BM and spleen cells (adjusted to yield an $\alpha\beta^+$ T cell dose of 0.6-0.8 x 10^6) from B6 or $CB2R^{-/-}$ animals. Animals transplanted with B6 BM alone served as controls. The absolute number of $CD4^+$ and $CD8^+$ T cells in the colon, liver, and lung 14 days post transplantation are depicted in panel G (n=12-20 per group) and absolute number of $CD4^+$ or $CD8^+$ IFN-γ^+ T cells in the same organs are shown in panel H (n=9-15 per group). Data are presented as the mean ± SD. Results are from 3-4 experiments. (I). Irradiated Balb/c recipients were transplanted with B6 BM and spleen cells (adjusted to yield an αβ T cell dose of 0.8 x 10^6 cells). Animals were treated with a CB2R antagonist, SR144528 (3 mg/kg) (n=10) or an equivalent amount of vehicle (n=10) for 14 days beginning on the day of transplant. Balb/c mice transplanted with B6 BM alone and then treated with either vehicle (n=6) or a SR144528 (n=3) served as controls. Results are from two experiments. (J). Lethally irradiated (1100

cGy) B6 (n=15) or CB2R$^{-/-}$ (n=15) animals were transplanted with Balb/c BM and spleen cells (adjusted to yield an αβ T cell dose of 5-5.5 x 10^6 cells). Balb/c mice transplanted with B6 BM alone (n=9) served as controls. Results are from three experiments. Statistics: *p<0.05, ** p<0.01, ****p<0.0001.

Figure 2: **Absence of CB2R expression on either CD4$^+$ or CD8$^+$ T cells exacerbates GVHD mortality.** (A-G). Irradiated Balb/c mice were transplanted with B6 *Rag-1*$^{-/-}$ BM (5 x 10^6)(n=7), *Rag-1*$^{-/-}$ BM plus purified CD4$^+$ (1 x 10^6) and CD8$^+$ (0.55 x 10^6) B6 T cells (n=10), *Rag-1*$^{-/-}$ BM plus B6 CD4$^+$ and CD8$^+$ CB2R$^{-/-}$ T cells (n=10), and *Rag-1*$^{-/-}$ BM plus CD4$^+$ CB2R$^{-/-}$ and B6 CD8$^+$ T cells (n=10). Overall survival is shown. Data are from two experiments. (B, C). Representative hematoxylin and eosin-stained sections of the lung, liver, and colon of animals 14 days post transplantation are depicted in panel B. Original magnification is 100X for photomicrographs. Pathological scores of colon, liver, and lung from mice transplanted are shown in panel C. Data are from three experiments with 9-12 mice per group. (D-G). The absolute number of CD4$^+$ and CD8$^+$ T cells in the liver, lung and colon is shown in panel D. The absolute number of CD8$^+$ T cells that produced IFN-γ, GM-CSF and TNF-α in the colon, liver and lung 14 days post transplantation are shown in panels E-G. Data are from four experiments with 11-17 mice per group. Statistics: *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

Figure 3: **GVHD induces a loss of CB2R EGFP expression in T cells isolated from target organs.** (A). Spleen sections from CB2R^{EGFP} reporter mice were stained with fluorescence-labeled antibodies directed against eGFP, CD19 or CD3. Topro served as a nuclear stain. Merged results are shown in final panels in each row. (B, C). eGFP expression on CD4$^+$ T cells, CD8$^+$ T cells, and B cells derived from the spleens of normal CB2R^{EGFP} mice (n=16-17) are shown in panel B. Representative dot plots showing eGFP expression on these immune cell populations is depicted in panel C. (D). Lethally irradiated Balb/c mice were transplanted with CB2R^{EGFP} BM alone (n=4) or together with CB2R^{EGFP} spleen cells (adjusted to yield an αβ$^+$ T cell dose of 0.6 x 10^6) (n=5). Animals were euthanized 14 days post

transplantation. The percent eGFP expression on $CD4^+$ and $CD8^+$ T cells obtained from spleen, liver, lung, and colon of animals transplanted with BM alone (BM) or together with adjunctive spleen cells (GVHD) is depicted. The percent eGFP expression on comparable cells obtained from the spleen of normal nontransplanted $CB2R^{EGFP}$ mice (labeled "naïve") (n=5) is shown for comparison. Statistical comparison is from all tissue sites relative to splenic $CD4^+$ and $CD8^+$ T cells from naïve mice. (E). Lethally irradiated Balb/c or B6.PL mice were transplanted with $CB2R^{EGFP}$ BM and spleen cells (adjusted to yield an $\alpha\beta^+$ T cell dose of 0.6 x 10^6). The percent eGFP expression on $CD4^+$ and $CD8^+$ T cells obtained from spleen, liver, and lung of animals transplanted with syngeneic versus allogeneic marrow grafts 14 days post transplantation is depicted. Data are from three experiments with a total of 15 mice per group. The percent eGFP expression on T cells obtained from the spleen of normal $CB2R^{EGFP}$ mice is shown for comparison. (F). Magnetically purified $CD4^+$ or $CD8^+$ T cells (1 x 10^5) from CB2R-eGFP mice were cultured with anti-CD3 (2.5ug/mL) and anti-CD28 (5ug/mL) antibody for three days. Magnetically purified pan T cells (1 x 10^5) from CB2R-eGFP mice were cultured with allogeneic Balb/c CD11c-enriched dendritic cells (5 x 10^4) for four days. Data depict the percentage of $CD4^+$ T cells and $CD8^+$ T cells expressing eGFP following stimulation. Results are from two experiments with 7-14 mice per group. (G). CB2R mRNA expression in B6 naïve and anti-CD3/anti-CD28 antibody activated $CD4^+$ or $CD8^+$ T cells. Data are presented as fold expression above 18S ribosomal RNA control and are derived from 10-13 mice per group. Results in panels B, D-G are presented as the mean ± SD. Statistics: *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

Figure 4: **Signaling through the CB2R by THC mitigates the severity of acute GVHD.** (A). Endocannabinoid levels of AEA and 2-AG in the liver, lung, colon, and spleen of lethally irradiated Balb/c mice transplanted with B6 BM alone or together with B6 spleen cells (adjusted to yield an αβ T cell dose of 0.6 x 10^6 cells) and then assessed 14 days post transplantation. Data are from three experiments with 14-15 mice per group. AEA and 2-AG levels in the same tissues from naïve nontransplanted Balb/c mice is also depicted (n=10). Data are presented as mean ± SD. (B). Lethally

irradiated Balb/c mice were transplanted with B6 BM and spleen cells (adjusted to yield an αβ T cell dose of 0.85-0.90 x 10^6 cells), and then treated with a vehicle control (n=20), or the CB2R agonist, JWH-133, at a low (1.5 mg/kg)(n=10) or high dose (20 mg/kg)(n=10) for 14 consecutive days beginning on day 0. Balb/c mice transplanted with B6 BM alone served as controls (n=12). Overall survival is depicted. Data are cumulative results from four experiments. (C). Irradiated Balb/c mice were transplanted with B6 BM and spleen cells, and then treated with a vehicle control (n=10), or the CB2R agonist, JWH-133 (1.5 mg/kg)(n=10) for 35 consecutive days beginning on day 0. Balb/c mice transplanted with B6 BM alone served as controls (n=6). Results are from two experiments. (D). Lethally irradiated Balb/c recipients were transplanted with B6 BM and spleen cells (adjusted to yield an αβ T cell dose of 0.85-0.9 x 10^6 cells). Animals were then treated with THC (20 mg/kg) (n=20) or vehicle (n=20) for 40 days beginning on day 0. Balb/c mice transplanted with B6 BM alone and treated with either vehicle or THC served as controls (n=12 per group). Data are from four experiments. (E). Irradiated Balb/c mice were transplanted with $CB2R^{-/-}$ BM alone (n=6) or together with $CB2R^{-/-}$ spleen cells (adjusted to yield an αβ T cell dose of 0.85-0.9 x 10^6 cells). Animals that received adjunctive spleen cells were treated with either THC (n=10) or a vehicle control (n=10) daily beginning on day 0 until demise. Survival is depicted. Data are from two experiments. Statistics: *$p<0.05$, ****$p<0.0001$.

Figure 5: **THC reduces proinflammatory T cell accumulation in GVHD target organs.** (A-C). PathHunterTM Chinese hamster ovary (CHO)-K1 CNR2 cells stably expressing the human CB2 receptor were used for signaling assays. Ratio of phosphorylated ERK to total ERK (panel A), percentage of β-arrestin recruitment (panel B), and percent inhibition of forskolin-induced cAMP production (panel C) as a function of varying concentrations of JWH-133 and THC are depicted. The EC50/IC50 and maximum effect (Emax) values calculated from these curves are reported in Table 1. Symbols in panels A-C represent the mean from 3-6 determinations per assay. Lines drawn are the least squares best fit of the data to the non-linear, three parameter log concentration-response relationship where bottom (for P-ERK/ERK and β-arrestin) or top (for cAMP) values were constrained to 100. (D-G). Lethally irradiated

Balb/c recipients were transplanted with B6 BM and spleen cells (adjusted to yield an αβ T cell dose of 0.85-0.9 x 10^6 cells). Animals were treated with THC (20 mg/kg) (n=9-14) or vehicle control (n=10-15) for 21 days beginning on day 0. Balb/c mice transplanted with B6 BM alone served as controls (n=7-10). The total number of $CD4^+$ and $CD8^+$ T cells in the colon and liver 21 days post transplantation is shown in panel D. The absolute number of $CD4^+$ or $CD8^+$ T cells that produced IFN-γ (panel E) or GM-CSF (panel F) is depicted in these tissue sites. Total number of $CD4^+$ $Foxp3^+$ T cells in the colon or liver (panel G). Data are presented as the mean ± SD and are cumulative results from 2-3 experiments. Statistics: *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

Figure 6: **Signaling through the CB2R regulates the severity of chronic GVHD.** (A, B). The percentage of EGFP expression on macrophages derived from the liver, lung or spleen of normal $CB2R^{EGFP}$ mice (n=20-23) is shown. Representative dot plots showing eGFP expression on macrophages from these tissue sites is shown in panel B. (C, D). Lethally irradiated Balb/c mice were transplanted with B10.D2 BM alone (n=9) or together with 15 x 10^6 B10.D2 spleen cells. Animals that received spleen cells were treated with the CB2R antagonist, SR144528 (3 mg/kg) (n=24), or a vehicle control (n=22) for 14 days beginning on the day of transplant. Cumulative pathological chronic GVHD skin scores of mice 30-40 days post transplantation are depicted in panel C. Representative hematoxylin and eosin-stained sections of the skin on day 35 are shown in panel D. Original magnification is 100X for photomicrographs. Data are derived from 5 experiments. (E). Immunohistochemical staining depicting CD3 and F4/80 positive cells (brown coloration) in the skin of mice treated with SR144528. (F). mRNA expression of TGF-β, GAL-3, and α-SMA in the skin of animals 30-40 days post transplantation. Results are from 3 experiments with 6-15 mice per group. (G-J). Lethally irradiated (850 cGy) Balb/c mice were transplanted with B6 *Rag-1*$^{-/-}$ BM alone, B6 *Rag-1*$^{-/-}$ BM and B6 spleen cells (adjusted to yield an αβ T cell dose of 0.4 x 10^6) , or $CB2R^{-/-}$ BM and spleen cells (adjusted to yield the same αβ T cell dose). Representative hematoxylin and eosin and trichrome stained sections of the skin on day 45 are shown in

panels G and H. Original magnification is 100X for photomicrographs. (I). Pathology scores of the skin 40-45 days post transplantation in chronic GVHD mice. Results are from three experiments with 7-13 mice per group. (J). mRNA expression of TGF-β, GAL-3, and α-SMA in the skin of animals (8-15 mice per group) 40-45 days post transplantation. Results are from three experiments. Data in panels in A,C,F, I and J are presented. Statistics: *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

Figure 7: **JWH-133, but not THC, mitigates the severity of chronic GVHD.** (A-C). Lethally irradiated Balb/c mice were transplanted with $CB2R^{EGFP}$ BM alone or together with spleen cells (adjusted to yield an αβ T cell dose of 0.6 x 10^6). Macrophages were isolated from the liver, lung, and spleen of mice 14 days post transplantation. eGFP expression on macrophages from BM control and GVHD mice is shown in panel A. Nontransplanted naïve $CB2R^{EGFP}$ mice are shown for comparison. Results are from 4-5 mice per group. Representative histogram of CB2R-EGFP expression on splenic macrophages from mice in panel B. Normal B6 and nontransplanted naïve $CB2R^{EGFP}$ mice are shown for comparison. CB2R mRNA expression from flow sorted $CD11b^+$ $F4/80^+$ macrophages obtained from the spleen on day 14 is depicted in panel C. Results for GVHD mice were pooled from 2-3 mice per data point. (D-F). Lethally irradiated Balb/c mice were transplanted with B10.D2 BM alone or with 20 x 10^6 spleen cells. Animals that received spleen cells were treated with JWH-133 (1.5 mg/kg), THC (20 mg/kg), or a vehicle control for 35-40 days beginning on day 0. Representative hematoxylin and eosin-stained sections of the skin on day 35 is shown in panel D and cumulative pathological skin scores from replicate animals 35-40 days post transplantation is depicted in panel E. Original magnification is 100X for photomicrographs. mRNA expression of TGF-β, GAL-3, and α-SMA in the skin of animals (7-15 mice per group) 35-40 days post transplantation (panel F). Results are from six experiments. Data in panels A, C, E and F are presented as the mean ± SD. Statistics: *p<0.05, ** p<0.01, ***p<0.001.

Table 1. Potency and efficacy of JWH-133 and THC in CB2R signaling response assays

	ERK Phosphorylation		ß-Arrestin$_2$ Recruitment		Inhibition of cAMP	
	EC50****	Emax	EC50****	Emax**	IC50	Max inhibition
JWH-133	19 ± 2 nM	340 ± 63%	49 ± 1 nM	177 ± 6%	16 ± 1 nM	35 ± 4 %
THC	23 ± 3 μM	475 ± 158%	13 ± 1 μM	144 ± 3%	Inactive	Inactive

CHO cells were stably transfected with the human CB2 receptor and then employed in Path-Hunter assays to assess the effect of varying concentrations of JWH-133 and THC on specified signaling pathways. Data are from 3-6 replicates per assay and are presented as the mean ± SEM. Statistics: ** $p<0.01$; **** $p<0.0001$

Figure 1

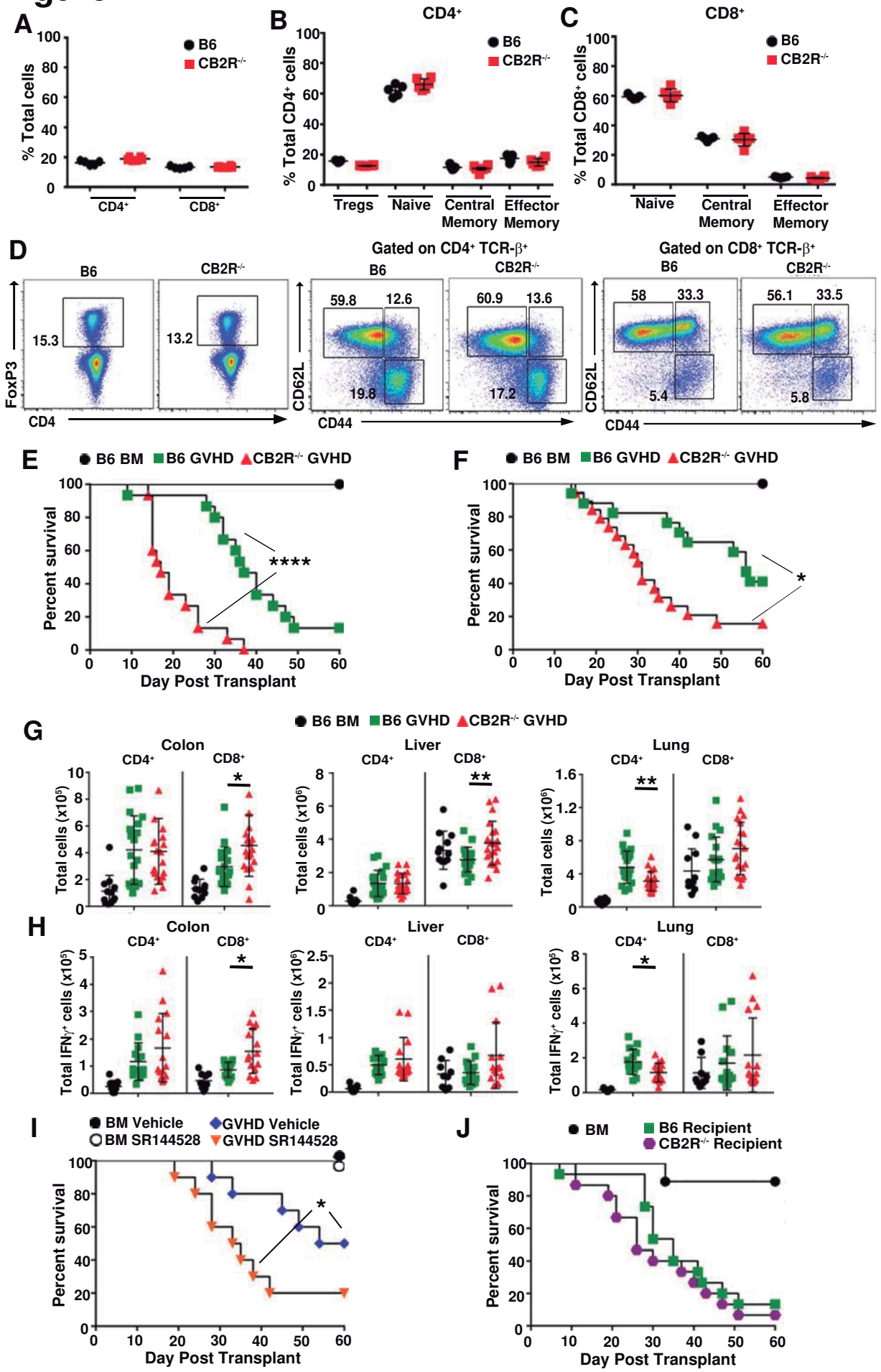

A
% Total cells
B6
CB2R-/-
CD4+
CD8+
B
CD4+
% Total CD4+ cells
Tregs
Naive
Central Memory
Effector Memory
C
CD8+
% Total CD8+ cells
Naive
Central Memory
Effector Memory
D
Gated on CD4+ TCR-β+
Gated on CD8+ TCR-β+
FoxP3
CD4
CD62L
CD44
15.3
13.2
59.8
12.6
19.8
60.9
13.6
17.2
58
33.3
5.4
56.1
33.5
5.8
E
B6 BM B6 GVHD CB2R-/- GVHD
Percent survival
Day Post Transplant
****
F
B6 BM B6 GVHD CB2R-/- GVHD
Percent survival
Day Post Transplant
*
G
B6 BM B6 GVHD CB2R-/- GVHD
Colon
Liver
Lung
CD4+
CD8+
Total cells (x10^6)
H
Colon
Liver
Lung
CD4+
CD8+
Total IFNγ+ cells (x10^6)
I
BM Vehicle
BM SR144528
GVHD Vehicle
GVHD SR144528
Percent survival
Day Post Transplant
J
BM
B6 Recipient
CB2R-/- Recipient
Percent survival
Day Post Transplant

Figure 2

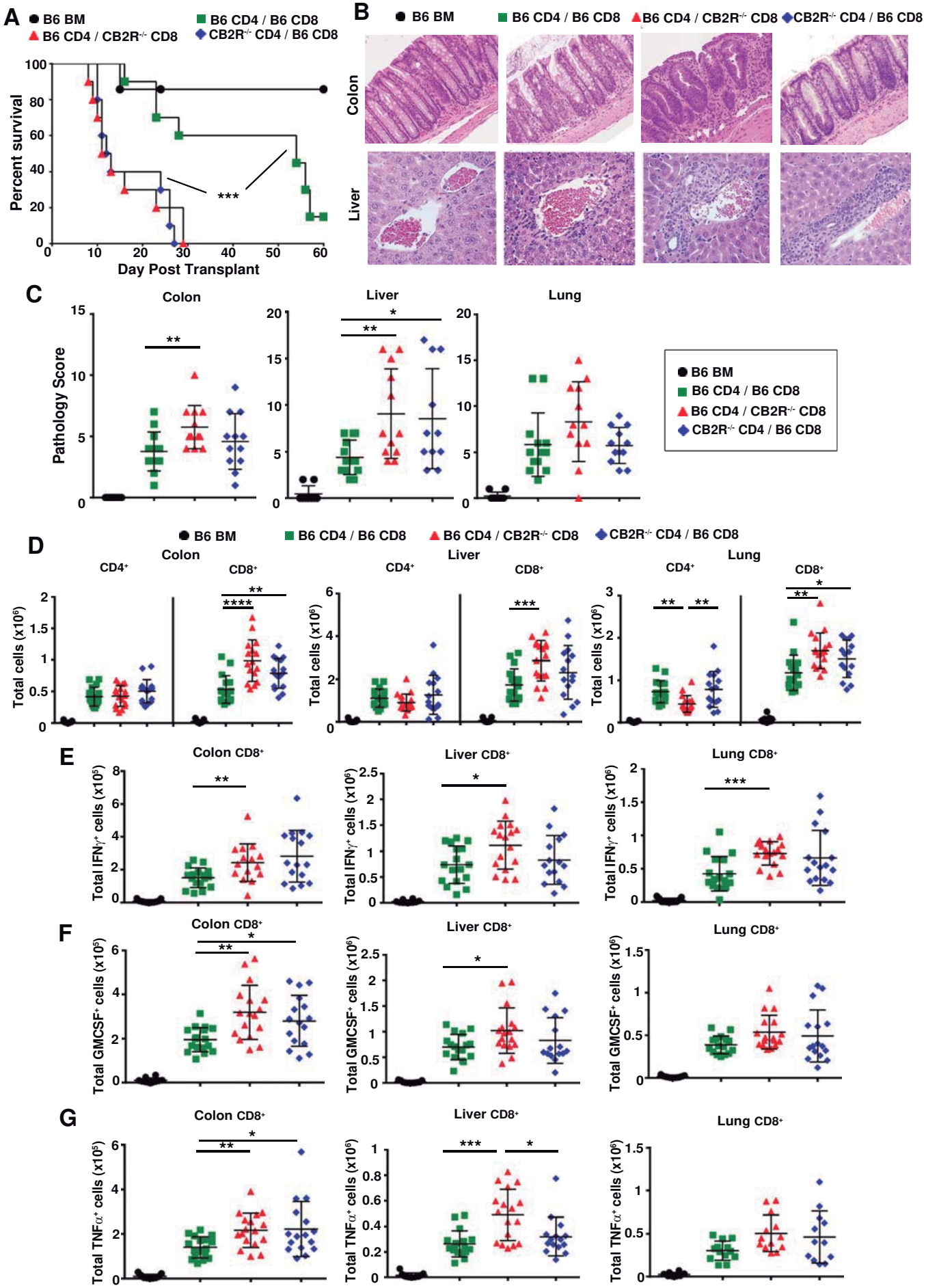

A
B6 BM
B6 CD4 / B6 CD8
B6 CD4 / CB2R-/- CD8
CB2R-/- CD4 / B6 CD8
Percent survival
Day Post Transplant
***
B
Colon
Liver
C
Colon
Liver
Lung
Pathology Score
D
CD4+
CD8+
Total cells (x10^6)
E
Colon CD8+
Liver CD8+
Lung CD8+
Total IFNγ+ cells (x10^6)
F
Total GMCSF+ cells (x10^6)
G
Total TNFα+ cells (x10^6)

Figure 3

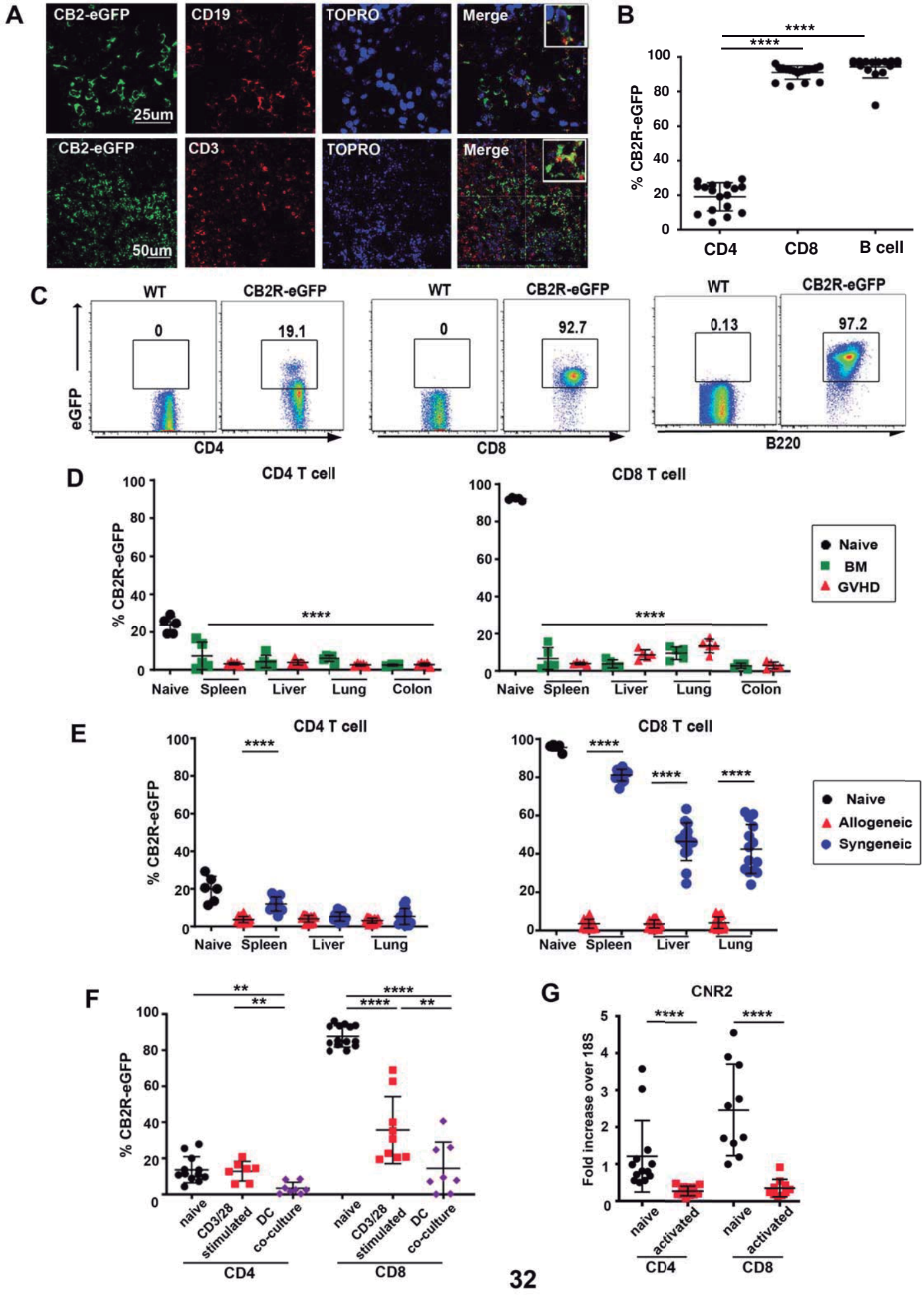

A
CB2-eGFP
CD19
TOPRO
Merge
25um
CB2-eGFP
CD3
TOPRO
Merge
50um
B
% CB2R-eGFP
CD4
CD8
B cell
****
C
WT
CB2R-eGFP
0
19.1
0
92.7
0.13
97.2
eGFP
CD4
CD8
B220
D
CD4 T cell
CD8 T cell
% CB2R-eGFP
Naive
Spleen
Liver
Lung
Colon
Naive
BM
GVHD
E
CD4 T cell
CD8 T cell
% CB2R-eGFP
Naive
Spleen
Liver
Lung
Naive
Allogeneic
Syngeneic
F
% CB2R-eGFP
naive
CD3/28 stimulated
DC co-culture
CD4
CD8
G
CNR2
Fold increase over 18S
naive
activated
CD4
CD8

Figure 4

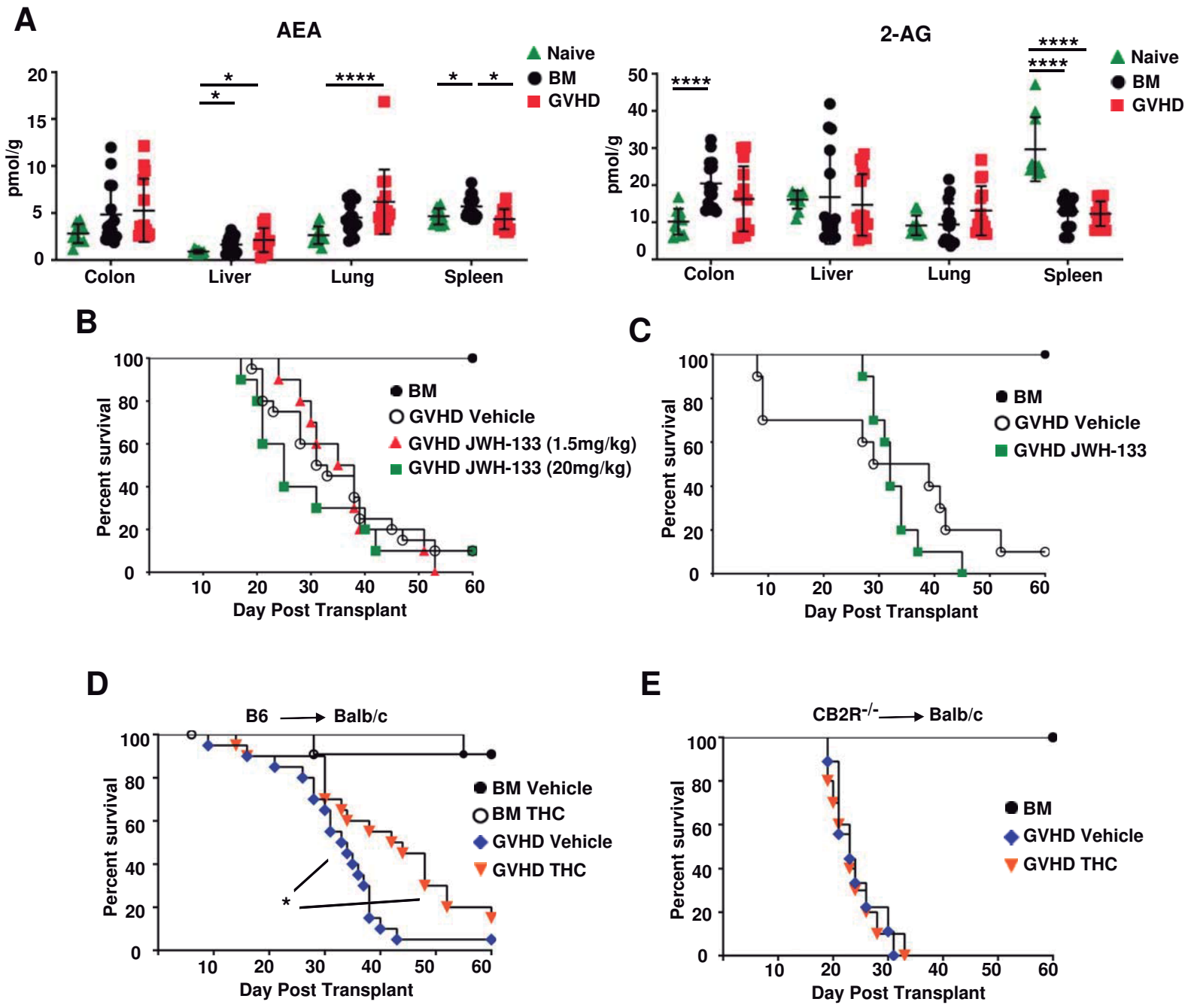

A
AEA
2-AG
Naive
BM
GVHD
pmol/g
Colon
Liver
Lung
Spleen
B
Percent survival
Day Post Transplant
BM
GVHD Vehicle
GVHD JWH-133 (1.5mg/kg)
GVHD JWH-133 (20mg/kg)
C
GVHD JWH-133
D
B6 → Balb/c
BM Vehicle
BM THC
GVHD Vehicle
GVHD THC
E
CB2R-/- → Balb/c

Figure 5

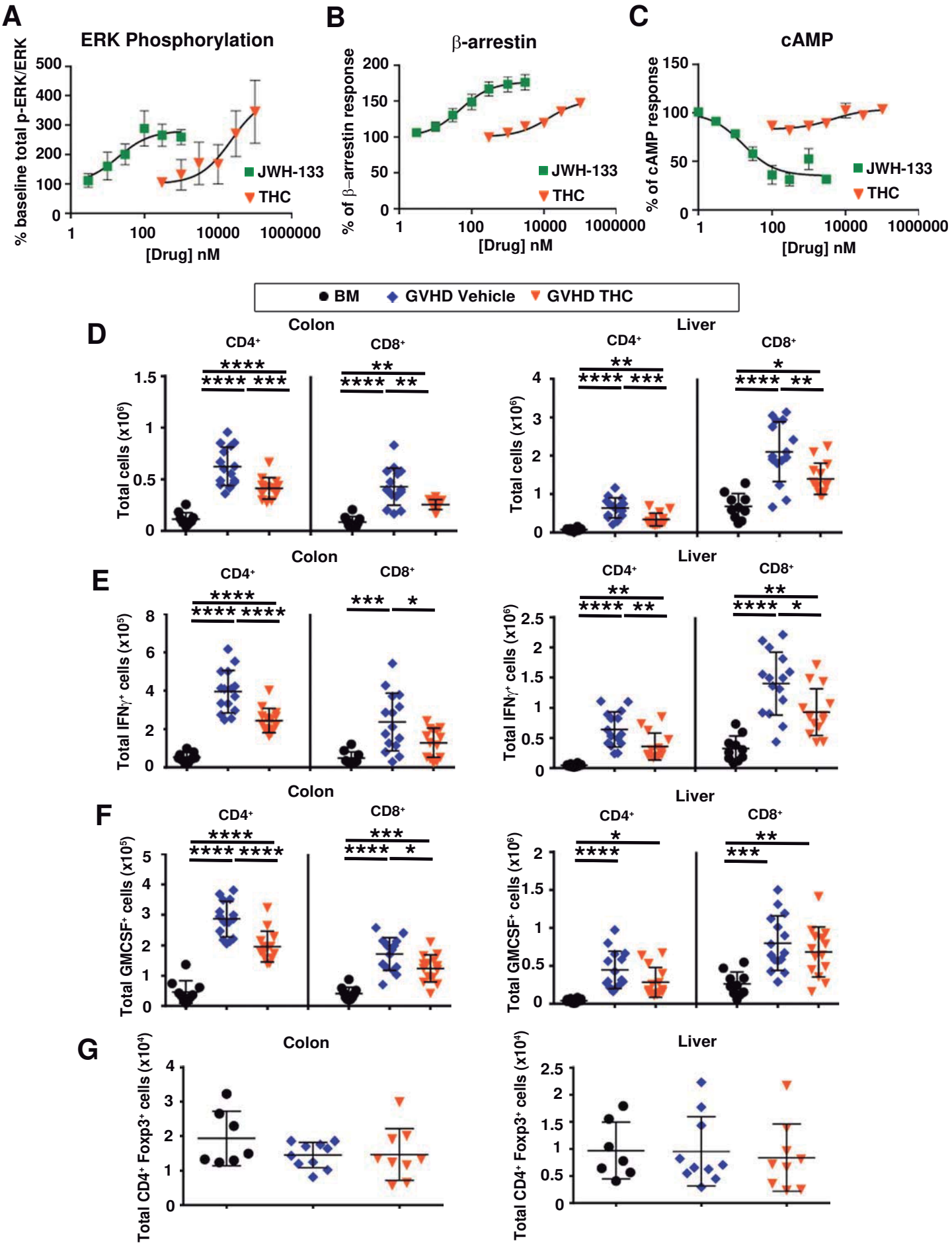

A
ERK Phosphorylation
% baseline total p-ERK/ERK
JWH-133
THC
[Drug] nM
B
β-arrestin
% of β–arrestin response
JWH-133
THC
[Drug] nM
C
cAMP
% of cAMP response
JWH-133
THC
[Drug] nM
BM
GVHD Vehicle
GVHD THC
D
Colon
Liver
CD4+
CD8+
Total cells (x10^6)
E
Total IFNγ+ cells (x10^5)
Total IFNγ+ cells (x10^6)
F
Total GMCSF+ cells (x10^5)
Total GMCSF+ cells (x10^6)
G
Total CD4+ Foxp3+ cells (x10^4)

Figure 6

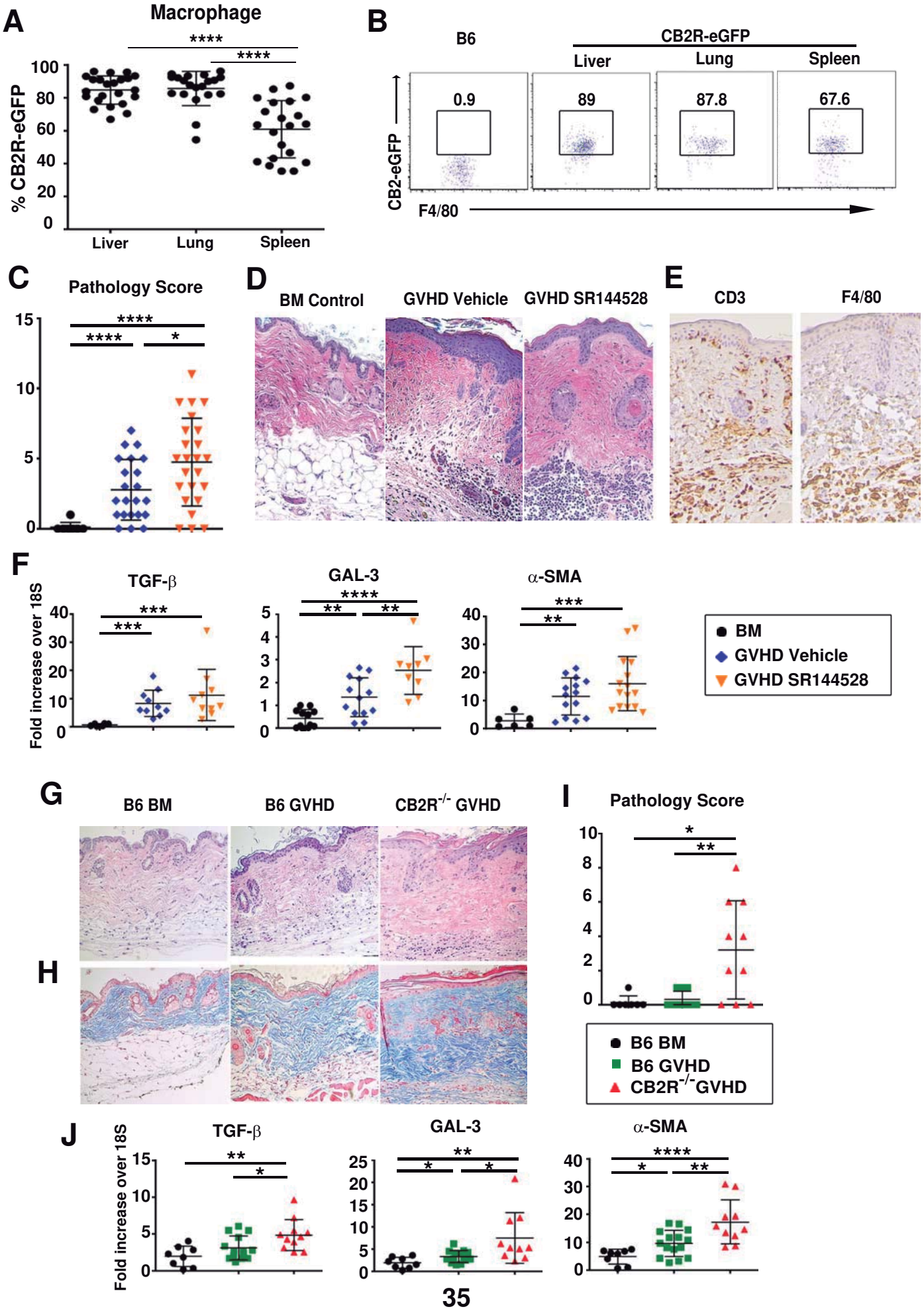
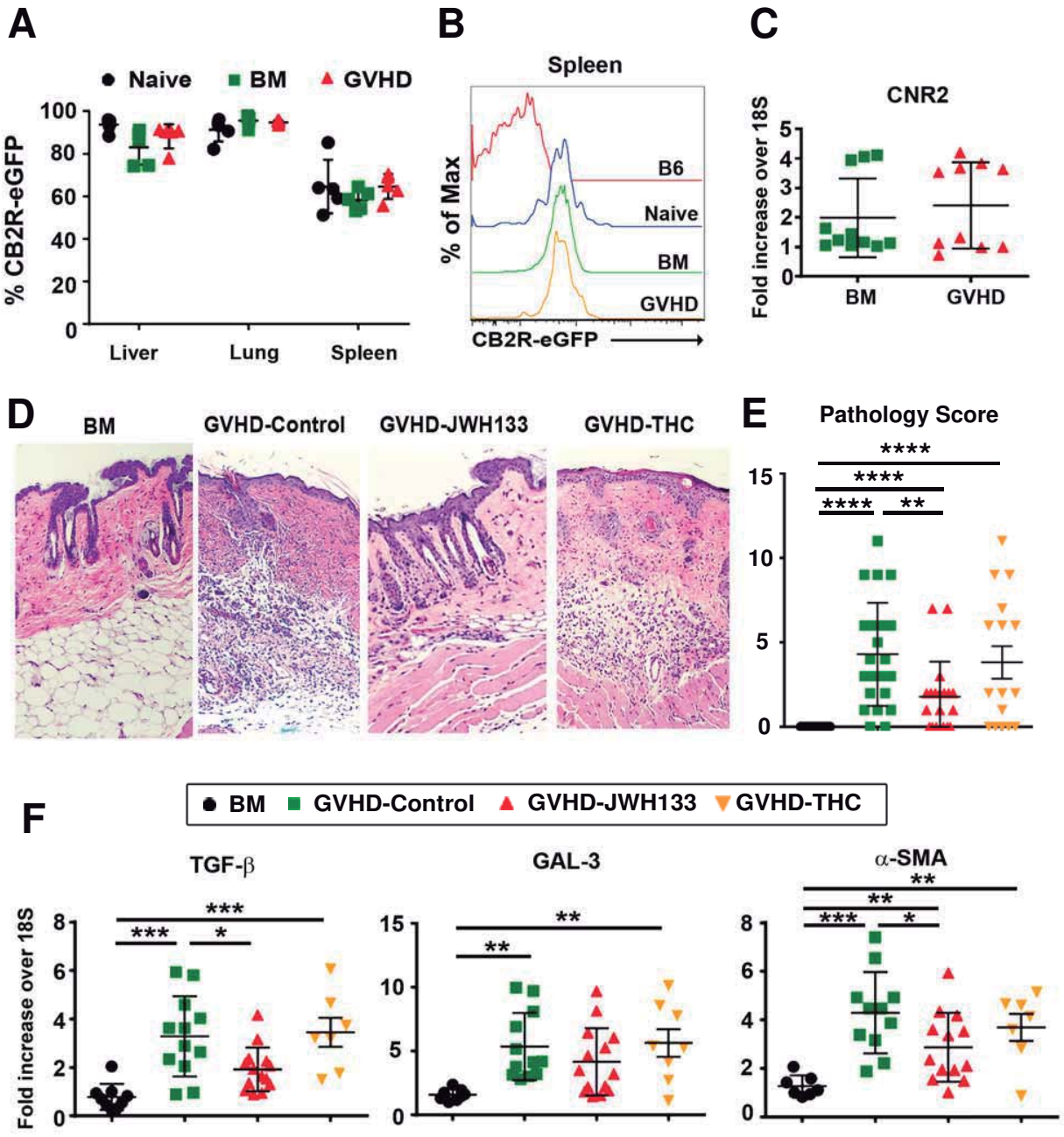

A
Macrophage
****
****
% CB2R-eGFP
100
80
60
40
20
0
Liver
Lung
Spleen
B
B6
CB2R-eGFP
Liver
Lung
Spleen
0.9
89
87.8
67.6
CB2-eGFP
F4/80
C
Pathology Score
****
****
*
15
10
5
0
D
BM Control
GVHD Vehicle
GVHD SR144528
E
CD3
F4/80
F
TGF-β
GAL-3
α-SMA
Fold increase over 18S
***
***
****
**
**
***
**
BM
GVHD Vehicle
GVHD SR144528
G
B6 BM
B6 GVHD
CB2R-/- GVHD
H
I
Pathology Score
*
**
B6 BM
B6 GVHD
CB2R-/-GVHD
J
TGF-β
GAL-3
α-SMA
Fold increase over 18S
**
*
**
*
*
****
*
**

Figure 7


A
Naive
BM
GVHD
% CB2R-eGFP
100
80
60
40
20
0
Liver
Lung
Spleen
B
Spleen
% of Max
B6
Naive
BM
GVHD
CB2R-eGFP
C
CNR2
Fold increase over 18S
5
4
3
2
1
0
BM
GVHD
D
BM
GVHD-Control
GVHD-JWH133
GVHD-THC
E
Pathology Score
****
****
****
**
15
10
5
0
F
BM
GVHD-Control
GVHD-JWH133
GVHD-THC
TGF-β
GAL-3
α-SMA
Fold increase over 18S
***
***
*
**
**
**
**
***
*
8
6
4
2
0
15
10
5
0