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A Small Molecule Inhibitor of ITK and RLK Impairs Th1 Differentiation and Prevents Colitis Disease Progression

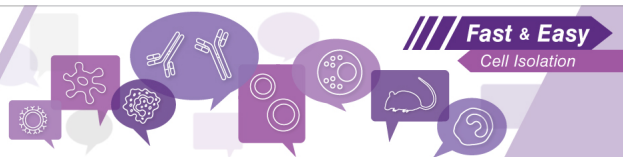
Item Type	Journal Article
Authors	Cho, Hyoung-Soo;Shin, HyunMu;Haberstock-Debic, Helena;Xing, Yan;Owens, Timothy D.;Funk, Jens Oliver.;Hill, Ronald J.;Bradshaw, J. Michael;Berg, Leslie J.
Citation	J Immunol. 2015 Nov 15;195(10):4822-31. doi: 10.4049/jimmunol.1501828. Link to article on publisher's site.Epub 2015 Oct 14.
DOI	10.4049/jimmunol.1501828
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Link to Item	https://hdl.handle.net/20.500.14038/39867

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This information is current as of February 26, 2016.

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J Immunol 2015; 195:4822-4831; Prepublished online 14 October 2015;

doi: 10.4049/jimmunol.1501828

<http://www.jimmunol.org/content/195/10/4822>

Supplementary Material <http://www.jimmunol.org/content/suppl/2015/10/14/jimmunol.1501828.DCSupplemental.html>

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A Small Molecule Inhibitor of ITK and RLK Impairs Th1 Differentiation and Prevents Colitis Disease Progression

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In T cells, the Tec kinases IL-2-inducible T cell kinase (ITK) and resting lymphocyte kinase (RLK) are activated by TCR stimulation and are required for optimal downstream signaling. Studies of CD4⁺ T cells from *Itk*^{-/-} and *Itk*^{-/-}*Rlk*^{-/-} mice have indicated differential roles of ITK and RLK in Th1, Th2, and Th17 differentiation and cytokine production. However, these findings are confounded by the complex T cell developmental defects in these mice. In this study, we examine the consequences of ITK and RLK inhibition using a highly selective and potent small molecule covalent inhibitor PRN694. In vitro Th polarization experiments indicate that PRN694 is a potent inhibitor of Th1 and Th17 differentiation and cytokine production. Using a T cell adoptive transfer model of colitis, we find that in vivo administration of PRN694 markedly reduces disease progression, T cell infiltration into the intestinal lamina propria, and IFN- γ production by colitogenic CD4⁺ T cells. Consistent with these findings, Th1 and Th17 cells differentiated in the presence of PRN694 show reduced P-selectin binding and impaired migration to CXCL11 and CCL20, respectively. Taken together, these data indicate that ITK plus RLK inhibition may have therapeutic potential in Th1-mediated inflammatory diseases. *The Journal of Immunology*, 2015, 195: 4822–4831.

The Tec family tyrosine kinases play a key role in Ag receptor-mediated signaling pathways in lymphocytes. Among these kinase family members, T cells express IL-2-inducible kinase (ITK), resting lymphocyte kinase (RLK), and tyrosine kinase expressed in hepatocellular carcinoma (1). Although each of these kinases is expressed in mature naive T cells, ITK is the most predominant. Based on mRNA analysis, RLK is expressed at 3- to 10-fold lower levels than ITK, and Tec is 30- to 100-fold reduced compared with ITK (2, 3). Following TCR stimulation, ITK is activated and directly phosphorylates phospholipase C (PLC) γ 1. Activated PLC γ 1 hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce inositol triphosphate and diacylglycerol, secondary messengers that lead to Ca²⁺ influx and MAPK and protein kinase C activation (4). As a consequence, *Itk*^{-/-} T cells have significant defects in T cell activation and differentiation (5–8). For RLK, some evidence supports a role in TCR signaling, as *Itk*^{-/-}*Rlk*^{-/-} double-deficient T cells are more impaired than those lacking only ITK (5,

9). Nonetheless, based on present data, the precise functions of RLK and tyrosine kinase expressed in hepatocellular carcinoma in T cell activation are unclear.

To elucidate the role of Tec kinases in TCR signaling, several studies have addressed the impact of a deficiency in ITK, or ITK plus RLK, in CD4⁺ Th cell differentiation and function. Initial studies showed that *Itk*^{-/-} mice exhibited impaired Th2 differentiation and Th2-biased responses to parasitic infection, with little effect on protective Th1 responses to intracellular protozoans (2, 10). These data were further supported by controlled in vitro studies that demonstrated that naive *Itk*^{-/-} CD4⁺ T cells were defective in Th2 but not Th1 differentiation, in part due to the fact that differentiated Th2 cells fail to express any RLK protein, as do Th1 cells (2). Additionally, ITK and RLK functions in Th cells are at least partially redundant, as RLK overexpression in *Itk*^{-/-} mice was able to restore Th2 responses in animal models of allergic asthma and schistosome egg-induced lung granuloma formation (11). Nonetheless, it has been difficult to distinguish which phenotypes observed in these mice are due to the functions of ITK and/or RLK in mature naive CD4⁺ T cells, and which are the consequence of altered T cell development generating an abnormal cytokine environment in the *Itk*^{-/-} or *Itk*^{-/-}*Rlk*^{-/-} mice.

More recently, studies by Schwartzberg and colleagues (12, 13) have indicated an additional role for ITK in Th17 differentiation. Specifically, *Itk*^{-/-} T cells showed reduced IL-17A production and increased Foxp3 expression following in vitro polarization. Additionally, *Itk*^{-/-} T cells provided enhanced regulatory T cell (Treg)-mediated protection in an adoptive transfer model of colitis owing to their increased potential to upregulate Foxp3 (13), although another study found that *Itk*^{-/-} Tregs were unable to protect against T cell-mediated colitis (14). Despite some disparities between studies, in general, these findings have provided impetus for the development of small-molecule ITK kinase inhibitors, with the intent of using them as treatments for atopic diseases, as well as for their potential as immunosuppressants to block graft rejection or autoimmunity.

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Received for publication August 14, 2015. Accepted for publication September 21, 2015.

This work was supported by National Institutes of Health Grants AI084987 and AI083505 (both to L.J.B.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: IEL, intraepithelial lymphocyte; ITK, IL-2-inducible T cell kinase; iTreg, induced regulatory T cell; LP, lamina propria; mLN, mesenteric lymph node; PLC, phospholipase C; RLK, resting lymphocyte kinase; Tg, transgenic; Treg, regulatory T cell; WT, wild-type.

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The complex phenotype of *Itk*^{-/-} mice, including defects in T cell development, activation, differentiation, and effector function, has made it difficult to precisely assess the function of ITK in each lineage of T cells and at different stages of an immune response. It has also been challenging to distinguish functions of ITK in T cell activation and differentiation from effects due to altered T cell development in *Itk*^{-/-} mice. A more direct strategy to address ITK and/or RLK function in T cells is to use a selective small-molecule inhibitor of these Tec kinases. PRN694 is a small molecule that forms an irreversible covalent bond with C442 in ITK or C350 in RLK, and it has recently been shown to selectively inhibit ITK and RLK in T cells (15). To date, the inhibitory effects of PRN694 on CD4⁺ Th cell differentiation and effector function have not been tested.

In this study, we examined the effects of PRN694 on CD4⁺ T cell differentiation and function in vitro and in vivo. Surprisingly, we found that PRN694 showed potent inhibitory effects on Th1 differentiation and IFN- γ production as well as on Th17 differentiation and IL-17A production, with decreased potency on Th2 differentiation. To test the relevance of this inhibitory activity in vivo, we used the T cell adoptive transfer model of colitis, an inflammatory condition mediated by IFN- γ -producing Th1 cells (16, 17). Consistent with our in vitro data, PRN694 administration ameliorated colitis disease progression and markedly inhibited colonic inflammation. Taken together, these data indicate that simultaneous inhibition of ITK and RLK by a small-molecule inhibitor may be an effective treatment for Th1-biased inflammatory diseases.

Materials and Methods

Mice

C57BL/6 wild-type (WT), C57BL/6 *Rag2*^{-/-}, C57BL/6 OT-II *Rag1*^{-/-} TCR transgenic (Tg), and B10.A 5C.C7 *Rag2*^{-/-} TCR Tg mice were purchased from Taconic Biosciences and housed in specific pathogen-free conditions at the University of Massachusetts Medical School in accordance with Institutional Animal Care and Use Committee guidelines. CD-1 mice were purchased from Charles River Laboratories; all housing and procedures with these mice were in accordance with the guidelines approved by Principia Biopharma's Institutional Animal Care and Use Committee.

Th cell polarization and CFSE dilution assay

Naive CD4⁺ T cells (3.0×10^5) from B10.A 5C.C7 *Rag2*^{-/-} TCR Tg or C57BL/6 OT-II *Rag1*^{-/-} TCR Tg mice were isolated using CD4 (L3T4) microbeads (Miltenyi Biotec). Isolated cells were plated in 12- or 24-well plates and activated by plate-bound anti-CD3 (1.0 μ g/ml) and anti-CD28 (4.0 μ g/ml) (BD Biosciences) for 72 h in the presence of the following conditions: Th0 (anti-CD3/CD28 with no cytokines), Th1 (IL-12, 10 ng/ml plus anti-IL-4, 10 μ g/ml), Th2 (IL-4, 10 ng/ml plus anti-IFN- γ , 10 μ g/ml), Th17 (IL-6, 20 ng/ml, TGF- β , 5.0 ng/ml, IL-1 β , 20 ng/ml plus anti-IFN- γ , anti-IL-4, and anti-IL-2, each at 10 μ g/ml) (all anti-cytokine Abs were purchased from R&D Systems and BD Biosciences). For CFSE dilution assay, CD4⁺ T cells isolated by magnetic separation were resuspended in 1.0 ml 1 \times PBS with 0.1% BSA at 5.0×10^6 cells/ml. Then, cells were stained with CFSE at a final concentration of 2.0 μ M at 37°C for 10 min. After incubation, stained cells were quenched by adding 5 vol ice-cold RPMI 1640 complete medium (10% FBS) for 5 min on ice. Cells were washed three times with complete medium and then plated for Th polarization for 72 h.

Cytokine and transcription factor analysis

T cells were stimulated with PMA (50 ng/ml) and ionomycin (1.0 μ g/ml) for 5 h with protein transport inhibitors, GolgiStop and GolgiPlug (BD Biosciences), each at 1.0 μ g/ml. All cells were first stained with Abs to CD4 and CD44 (BD Biosciences) and a Live/Dead fixable aqua dead cell stain kit (Life Technologies). Stained cells were then fixed and permeabilized by using a BD Cytofix/Cytoperm kit (BD Biosciences). For cytokine staining, cells were stained with Abs to IFN- γ , IL-4, IL-17A, or IL-2 (BD Biosciences). For transcription factor staining, cells were fixed and permeabilized using a Foxp3/transcription factor staining buffer set

(eBioscience) and stained with Abs to T-bet, GATA-3, retinoic acid-related orphan receptor γ t, or Foxp3 (BD Biosciences). Cells were analyzed on an LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo (Tree Star).

Measurements of cytokine production by human PBMCs and in-mouse plasma

Human PBMCs isolated from whole blood were incubated for 1 h with and without PRN694 and stimulated with plate-bound anti-CD3 (2.5 μ g/ml) and soluble anti-CD28 (1.0 μ g/ml) (BD Biosciences) for 18 h. Supernatants were collected, frozen, and analyzed using the human InflammationMAP v.1.0 biomarker panel (Myriad RBM). For the IL-2 inhibition dose response, PBMCs were incubated for 1 h with a concentration range of PRN694 and stimulated either with anti-CD3/CD28 or 10 μ M thapsigargin (Sigma-Aldrich) for 18 h. Supernatants were analyzed for IL-2 using the AlphaLISA IL-2 kit (PerkinElmer). For cytokine inhibition in vivo, triplicate CD-1 mice were administered a 20 mg/kg i.p. dose of PRN694 followed either 1 or 6 h later by administration of 10 μ g/mouse anti-CD3 (R&D Systems). Plasma was collected 2 h after anti-CD3 injection and analyzed for cytokines using the RodentMAP v.3.0 biomarker panel (Myriad RBM).

Mouse adoptive transfer colitis model and PRN694 administration

CD4⁺CD45RB^{hi} T cells were sorted from spleens of C57BL/6 WT mice, and 4.0×10^5 cells were injected i.p. into *Rag2*^{-/-} hosts. Recipient mice were weighed daily and sacrificed 7 wk posttransfer, at which time colons were removed for length measurement, histologic analysis, and lymphocyte isolation, and lymphoid organs were harvested for flow cytometry analysis. Vehicle (5% ethanol/95% Capterx 355 EP/NF, ABITEC) or PRN694 was orally administered according to the following regimen: once a day (40 mg/kg) for weeks 0–2 and 4–7, and twice a day (20 mg/kg) for weeks 2–4.

Histologic examination of colon

Colon specimens obtained from vehicle-treated or PRN694-treated hosts were fixed in 10% buffered formalin and stained with H&E. To assess the severity of colitis, histologic scores of the proximal, middle, and distal colon were examined.

In vivo PRN694 target engagement assay and assessments of toxicity

Naive C57BL/6 mice were dosed with vehicle or PRN694 (40 mg/kg). After 2 or 6 h of oral administration, the dosed mice were sacrificed and CD4⁺ T cells were isolated from the spleen by using an EasySep mouse CD4⁺ T cell isolation kit (StemCell Technologies). Then, isolated CD4⁺ T cells were stimulated with anti-CD3 (10 μ g/ml) and anti-CD28 (5.0 μ g/ml) for 5 min at 37°C. After stimulation, CD4⁺ T cells (4.0×10^6) were lysed by CellLytic M buffer (Sigma-Aldrich) containing proteinase and phosphatase inhibitor. The expression of phosphorylated PLC γ 1 and total PLC γ 1 was examined by Western blot using anti-p-PLC γ 1 (Tyr⁷⁸³) mAb and anti-PLC γ -1 mAb (D9H10) (Cell Signaling Technology) at 1:1000 dilution. The secondary Ab was Alexa Fluor 647-conjugated anti-rabbit IgG (H+L) (Invitrogen) at 1:1000 dilution, and the signal was evaluated using a Typhoon biomolecular imager (GE Healthcare Life Sciences) with signal quantification using ImageQuant TL 7.0 software (GE Healthcare Life Sciences). Following long-term in vivo administration, we noted that the mice treated with PRN694 gained weight comparable to control untreated *Rag2*^{-/-} controls and showed no behavioral indications of drug-mediated toxicity. Furthermore, to address whether PRN694 might be generally cytotoxic, we examined the inhibition of proliferation of the HCT116 colorectal carcinoma cell line in the presence or absence of PRN694 and found an IC₅₀ of 10 μ M in this assay. Because this is a concentration higher than the maximum concentration of PRN694 in plasma following a 40 mg/kg oral dose, we concluded that PRN694 had no apparent toxicity during the 7-wk dosing time frame.

Isolation of intraepithelial lymphocytes and lamina propria lymphocytes from the colon

Colons were opened longitudinally and then cut into 1- to 2-cm pieces. After incubation with EDTA and DTT in HBSS at 37°C for 20 min with vigorous vortexing, the cell suspension was passed through a 70- μ m strainer, and the flow-through containing intraepithelial lymphocytes (IELs) was collected by centrifugation. For lamina propria (LP) lymphocytes, the remaining tissues were digested with collagenase D (Roche), Disperse

II (Roche), and DNase I (Roche) at 37°C for 20 min and then vortexed vigorously. Final cell suspensions were isolated using 40/80% Percoll (GE Healthcare Life Sciences) density gradients, and the viability of extracted cells was tested using trypan blue.

P-selectin glycoprotein ligand-1 binding and cell migration assays

Naive CD4⁺ T cells were isolated with CD4 (L3T4) microbeads (Miltenyi Biotec) and then cultured in the presence or absence of PRN694 (25 or 50 nM) for 72 h in each Th polarization condition as described above. For P-selectin glycoprotein ligand 1 binding assay, cultured and polarized CD4⁺ T cells were first treated with 2.4G2 Fc Block (anti-CD16/CD32) for 10 min at 4°C and then washed with FACS buffer (1× PBS containing 5.0% FBS). Recombinant P-selectin-human IgG fusion protein (BD Biosciences) was added to the cells (1:200 dilution) at 4°C for 30 min. Cells were washed and then stained with allophycocyanin-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories) and surface marker Abs. The cell migration assay was performed by using HTS Transwell-96 well permeable supports with 3.0-μm pores (Corning). Cultured CD4⁺ T cells were washed twice and resuspended at 2.5×10^6 cells/ml with serum-free RPMI 1640 containing 0.1% BSA. Lower chambers were loaded with 200 μl diluted chemokines (CXCL11, 100 ng/ml; CCL20, 100 ng/ml; CCL25, 300 ng/ml) (PeproTech and R&D Systems). Twenty-five microliters cell suspension (5.0×10^5 cells) was loaded in the upper chamber of a polycarbonate filter. Cell migration was performed at 37°C, 5% CO₂ for 3 h, and non-migrated cells on the upper chamber were rinsed off with 1× PBS containing 0.1% BSA. After centrifugation (1500 rpm for 10 min), the upper chamber was removed and the migrated cells were resuspended in 1× PBS with 0.1% BSA. The numbers of migrated cells were counted by the flow cytometer. To calculate the chemotaxis index, the numbers of cells migrated in response to each chemokine were divided by the numbers of spontaneously migrated cells.

Statistical analysis

Data are represented as mean ± SEM. Statistically significant differences were determined by a Student *t* test.

Results

Inhibition of ITK and RLK kinases potently inhibits CD4⁺ Th cell differentiation

To determine the consequences of Tec kinase inhibition on Th differentiation, we used a recently reported small-molecule covalent inhibitor of ITK and RLK, PRN694 (Fig. 1A) (15). Based on *in vitro* kinase assays, PRN694 is a potent inhibitor of all three Tec kinases expressed in T cells, demonstrates less potency toward the other Tec kinases BTK and BMX (Fig. 1B), and shows excellent kinome-wide selectivity (15). As reported, PRN694 binds covalently to a conserved cysteine residue in the ATP binding sites of ITK and RLK (ITK C442, RLK C350) and is highly selective for these two kinases in T cells (15). Unlike its binding to ITK and RLK, PRN694 does not appear to bind covalently to BTK and hence displays a limited duration of BTK inhibition biochemically and in cells (15). Furthermore, PRN694 is 63- to 320-fold more potent in inhibiting ITK than previously described ITK inhibitors, for example, BMS-509744 and BMS-488516 (Fig. 1B) (18–22).

To assess the potential effect of PRN694 on CD4⁺ Th cell differentiation, we used naive CD4⁺ T cells from B10.A 5C.C7 *Rag2*^{-/-} TCR Tg mice and stimulated these cells under Th1-, Th2-, or Th17-polarizing conditions. Cells were cultured for 3 d in the presence of increasing doses of PRN694 and then analyzed for cytokine production after restimulation with PMA and ionomycin for 5 h. Among these conditions, PRN694 inhibited Th1 differentiation more potently than was observed for Th2 and Th17 differentiation. Whereas a low concentration of PRN694 (25 nM) inhibited IFN-γ production by Th1 cells by >50%, 50–100 nM PRN694 was required to achieve a similar level of inhibition of IL-17A production by Th17 cells (Fig. 1C, 1D). Furthermore, none of the doses tested achieved 50% inhibition of IL-4 production by Th2 cells. The effects of PRN694 on cytokine pro-

duction did not correlate with effects on cell proliferation (Supplemental Fig. 1A, 1B), indicating that the inhibition of IFN-γ production by Th1 cells was not due to reduced proliferation of these cells in the presence of PRN694.

We also examined the expression of Th cell lineage-determining transcription factors. Overall, the expression levels of all transcription factors tested (T-bet, GATA-3, and retinoic acid–related orphan receptor γt) were decreased upon the inhibitor treatment; however, the modest differences observed between the three Th cell lineages did not correlate precisely with diminished cytokine production (Fig. 1E). Collectively, these data indicate that PRN694 specifically targets ITK and RLK kinase activity and also impairs CD4⁺ Th cell differentiation and cytokine production, with a range of potency as follows: Th1 > Th17 > Th2.

PRN694 inhibits TCR-induced cytokine production from human PBMCs and in mouse plasma

To further study the effect of dual ITK/RLK inhibition on T cell function, human PBMCs were stimulated with anti-CD3/CD28 in both the absence and presence of PRN694, and the cytokines produced after 18 h were measured using a biomarker panel. We used both a moderate (20 nM) and high (5.0 μM) concentration of PRN694 and focused on cytokines that were robustly induced in this short-term assay, including IFN-γ, IL-6, and IL-2. The increase in each of these cytokines induced by anti-CD3/CD28 stimulation was completely blocked by 5.0 μM PRN694 and was strongly inhibited by 20 nM PRN694 (Fig. 2A, Supplemental Table I). To quantify the potency of inhibition of IL-2 production, a 12-concentration dose range of PRN694 was tested. These data indicated an IC₅₀ value of 8.7 ± 2.1 nM (Fig. 2B). To confirm that the inhibition of IL-2 production was due to selective ITK and RLK inhibition, we bypassed the requirement for these kinases by stimulating PBMCs with the calcium pump inhibitor thapsigargin, and we observed that the ability of PRN694 to inhibit IL-2 production was strongly impaired (Fig. 2B). To determine whether PRN694 inhibited these cytokine responses *in vivo*, mice were injected with a 20 mg/kg *i.p.* dose of PRN694, followed by injection of anti-CD3 either 1 or 6 h later to stimulate cytokine production. Plasma was collected 2 h after anti-CD3 injection, and cytokines were analyzed using a cytokine biomarker panel (Fig. 2C). The robust increase in IFN-γ, IL-6, and IL-2 induced by anti-CD3 stimulation was inhibited by PRN694 at both time points (Fig. 2C, Supplemental Table II).

*PRN694 treatment *in vivo* ameliorates symptoms in the CD4⁺ CD45RB^{hi} T cell transfer model of colitis*

Owing to the potent inhibitory effect of PRN694 on IFN-γ production by Th1 cells in *in vitro* culture experiments, we considered whether PRN694 might function *in vivo* to suppress a Th1/IFN-γ-mediated disease. To test this hypothesis, we adoptively transferred WT colitogenic CD4⁺CD45RB^{hi} T cells from C57BL/6 mice into RAG2-deficient hosts and monitored the mice for weight loss as a surrogate for disease progression. Previous studies have demonstrated that the colitis induced in this model is due to Th1-mediated inflammation, with little involvement of Th2 or Th17 effector responses (16, 23, 24). In addition to the transferred cells, mice received vehicle alone or PRN694 by oral gavage. Based on studies of ITK target occupancy in thymocytes following *in vivo* administration of PRN694 in mice (15), we dosed mice daily and, for a 2-wk period (weeks 2–4), twice daily (Fig. 3A). As expected, recipient mice receiving vehicle alone failed to gain weight and progressively lost weight, beginning 4 wk after T cell transfer. In contrast, PRN694-treated mice exhibited no weight loss and remained similar to control RAG2-deficient mice that did not receive a colitogenic T cell transfer (Fig. 3B). Consistent

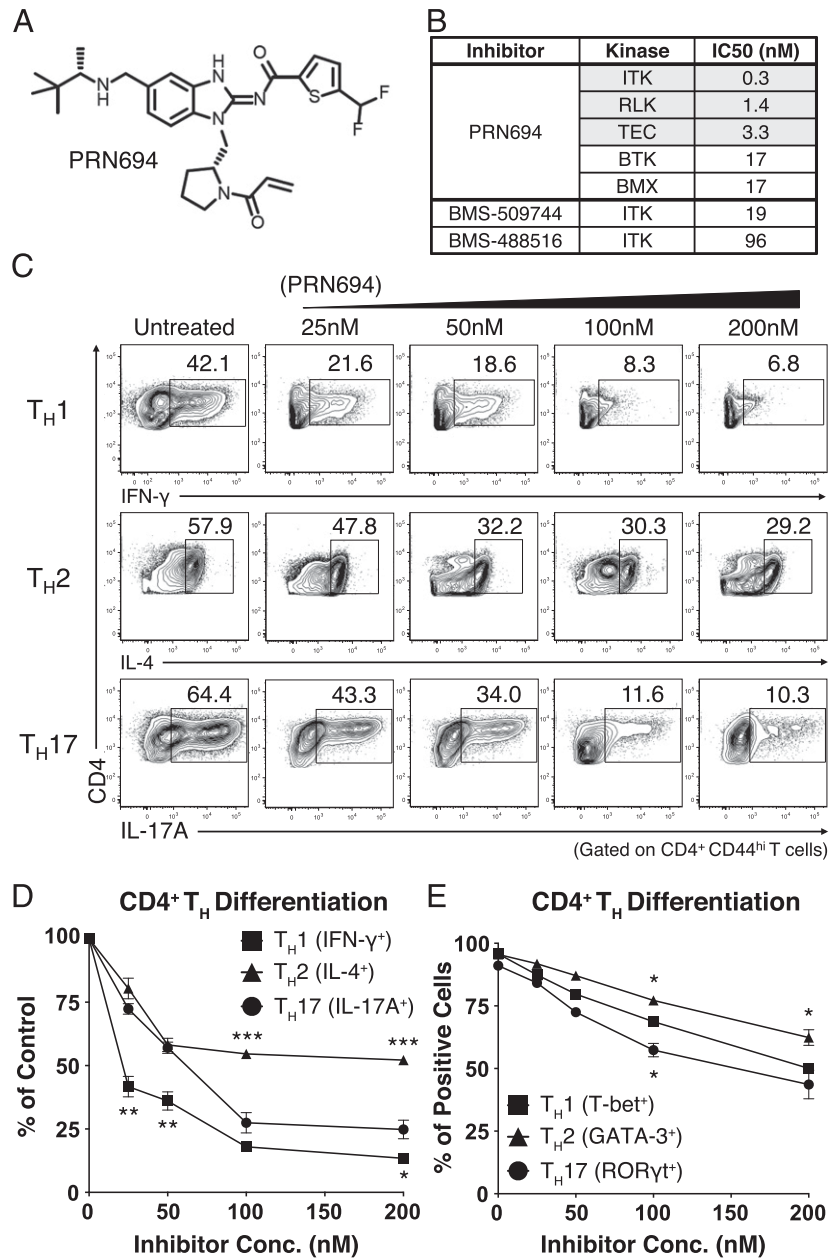


FIGURE 1. PRN694 inhibits CD4⁺ Th cell differentiation. **(A)** Chemical structure of PRN694. **(B)** The selectivity and potency of PRN694 were examined by in vitro kinase assay. The table shows the IC₅₀ values for the kinases indicated; for the complete dataset, see Zhong et al. (15). **(C and D)** Purified naive mouse splenic CD4⁺ T cells were stimulated in Th-polarizing conditions in the presence of PRN694 at the indicated concentrations for 72 h. CD4⁺ T cells were then restimulated with PMA and ionomycin for 5 h and analyzed by intracellular cytokine staining. The untreated controls were cultured in the presence of DMSO. The percentages of cytokine-producing CD4⁺ T cells (gated on CD4⁺CD44^{hi} cells) are shown (C). Compilation of data from three independent experiments indicating the inhibitory effect of PRN694 on the cytokine production are shown. For each cytokine, the data were normalized to the percentage of cytokine-producing cells in the absence of inhibitor. **(E)** Data from three experiments were compiled, and for each Th transcription factor, the data were normalized to the percentage of positive cells in the absence of inhibitor. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

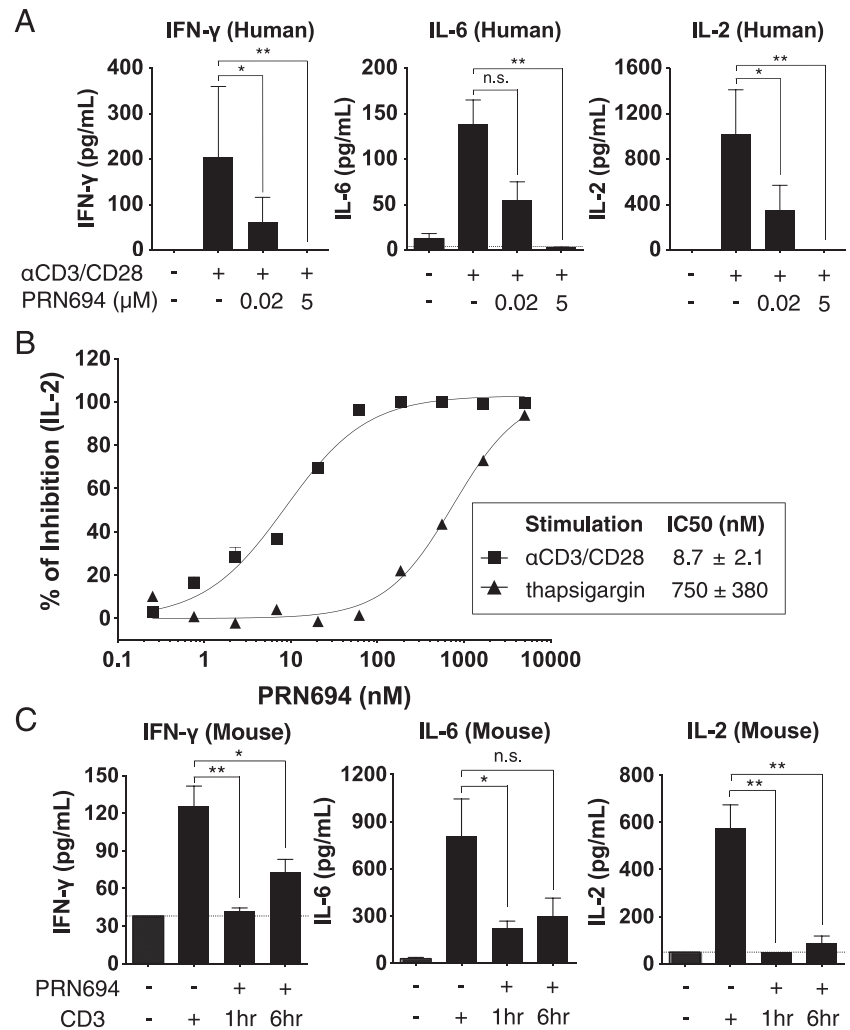
with these data, analysis of colon length at 7 wk posttransfer indicated that the reduced colon length seen in the vehicle-treated mice was prevented in the PRN694-treated mice (Fig. 3C). Additionally, histological analysis of the colonic epithelium revealed lymphocytic infiltration in the colon of the vehicle-treated mice (Fig. 3D), in contrast to the reduced inflammation seen in the colon of PRN694-treated mice. To further confirm target engagement of PRN694 with ITK in vivo, splenic CD4⁺ T cells were isolated from vehicle-dosed or PRN694-dosed mice (2 or 6 h posttreatment) and then stimulated with anti-CD3/CD28 mAb to examine PLCγ1 phosphorylation. As shown, the phosphorylation of PLCγ1 was completely blocked at both time points following PRN694 administration (Supplemental Fig. 1C). Taken together, these data demonstrate that PRN694 reduces T cell-mediated colonic inflammation by inhibiting ITK/RLK.

PRN694 impairs IFN-γ-mediated Th1 responses and prevents T cell migration to the inflamed colon

To determine the basis of PRN694-mediated inhibition of colitis, we analyzed the T cell populations in the spleen, mesenteric lymph

node (mLN), intestinal epithelium (intraepithelial lymphocytes [IELs]), and LP of recipient *Rag2*^{-/-} mice at 7 wk posttransfer. Assessment of CD4⁺ T cell proportions did not reveal any significant differences in the spleens or mLNs between the vehicle- or PRN694-treated mice, although a modest but significant difference in absolute numbers of CD4⁺ T cells was observed in mLNs (Fig. 4A, 4B). However, CD4⁺ T cell proportions in intestinal epithelium and LP of PRN694-treated mice were significantly less than those seen in the vehicle-treated mice, along with a statistically significant difference in absolute numbers in the LP (Fig. 4A, 4B). To test whether PRN694 exerted any inhibitory effect on T cell function, we examined cytokine production from transferred colitogenic T cells in several different organs after a brief in vitro stimulation. As expected, CD4⁺ T cells showed robust IFN-γ production in all analyzed sites of vehicle-treated mice, whereas no IL-17A production was observed (Fig. 4C). Interestingly, PRN694 administration markedly reduced the proportions of CD4⁺ T cells producing IFN-γ in mLNs, IELs, and LP, although this effect was less prominent in the spleen (Fig. 4C, 4D). There was also a large decrease in the numbers of IFN-γ-producing CD4⁺ T cells in all

FIGURE 2. PRN694 inhibits TCR-induced cytokine production from human PBMCs and in mouse plasma. **(A)** Human PBMCs were stimulated in vitro with anti-CD3/CD28 for 18 h in the absence or presence of PRN694 at 20 nM or 5.0 μ M, and levels of IFN- γ , IL-6, and IL-2 in the supernatants were measured as part of the human InflammationMAP v.1.0 biomarker panel. A complete list of all biomarkers of the panel is found in Supplemental Table I. Data are shown as means \pm SEM ($n = 3$ /group). **(B)** A dose response of PRN694 was used to assess the potency of inhibition of IL-2 production by PBMCs. Cells were stimulated with anti-CD3/CD28 (■) or thapsigargin (▲). IL-2 was quantified using an AlphaLISA IL-2 immunoassay. **(C)** Mice were injected i.p. with PRN694 (20 mg/kg) followed by anti-CD3 (10 μ g/mouse) 1 or 6 h later. Two hours after anti-CD3 injection, plasma was collected and cytokines were measured as part of the RodentMAP v.3.0 biomarker panel. A complete list of all biomarkers of the panel is found in Supplemental Table II. Dotted lines indicate the detection limit for each cytokine in the assay. Data are shown as means \pm SEM ($n = 3$ /group). * $p < 0.05$, ** $p < 0.01$.



organs following PRN694 treatment, although the suppression did not reach significance in the LP (Fig. 4E). Consistent with our in vitro studies, the reduced production of IFN- γ by T cells in the PRN694-treated mice could not be accounted for simply by defects in expression of the Th1 transcription factor, T-bet (Supplemental Fig. 2A, 2B).

Owing to the reduced proportion of CD4⁺ T cells in the inflamed intestines of PRN694-treated mice, we also investigated expression of gut-homing receptors, integrin $\alpha_4\beta_7$ (LPAM-1) and CCR6, on colitogenic T cells (25–27). Consistent with the proportions of gut-infiltrating CD4⁺ T cells, expression of both integrin $\alpha_4\beta_7$ and CCR6 were reduced on intestinal epithelial CD4⁺ T cells when PRN694 was administered (Fig. 4F). In contrast, we did not detect any significant level of expression of these receptors on LP-isolated CD4⁺ T cells from either group of mice (Fig. 4F). Taken together, these data strongly suggest that PRN694 attenuates in vivo Th1-biased intestinal inflammation through the inhibition of IFN- γ production and the expression of intestine-homing surface receptors.

PRN694 impairs induced Treg differentiation and IL-2 production by transferred colitogenic CD4⁺ T cells

A recent study has shown that *Itk*^{-/-} CD4⁺ T cells have an increased propensity to upregulate Foxp3 and differentiate into induced Tregs (iTregs) when stimulated under Th17-polarizing conditions (13). We therefore considered whether the inhibitory effect of PRN694 on colitis might be due to enhanced differen-

tiation of Foxp3⁺ iTregs in the inhibitor-treated mice. Instead, analysis of T cells at 7 wk posttransfer indicated that PRN694 significantly reduced the proportions of CD4⁺ T cells expressing Foxp3 compared with controls, a result consistent with the findings of Huang et al. (14). This was evident in all organs examined (Fig. 5A, 5B). Examination of surface markers commonly found on most Tregs, including CTLA-4, PD-1, and GITR, also showed reduced expression on Foxp3⁺CD4⁺ T cells in PRN694-treated mice (Supplemental Fig. 2C–E).

Because upregulation of Foxp3 and iTreg differentiation are dependent on IL-2 (28–31), we examined the effects of PRN694 on IL-2 production by in vitro polarized CD4⁺ T cells. As shown, PRN694 inhibited IL-2 production by all three lineages of Th cells (Fig. 5C, 5D). Consistent with these data, T cells from the adoptive transfer colitis studies also showed reduced proportions of cells capable of producing IL-2 following treatment with PRN694 (Fig. 5E, 5F). These data indicate that reduced disease progression in mice treated with PRN694 is not a consequence of enhanced differentiation of Foxp3⁺ iTreg cells, but rather a result of inhibition of differentiation and activation of the IFN- γ -producing Th1 effector cell population.

PRN694 inhibits P-selectin binding activity and chemokine-induced migration of polarized Th1 and Th17 cells

Our results in the adoptive transfer colitis studies showed reduced numbers of CD4⁺ T cells present in the colonic epithelium of PRN694-treated mice compared with controls. As previous studies

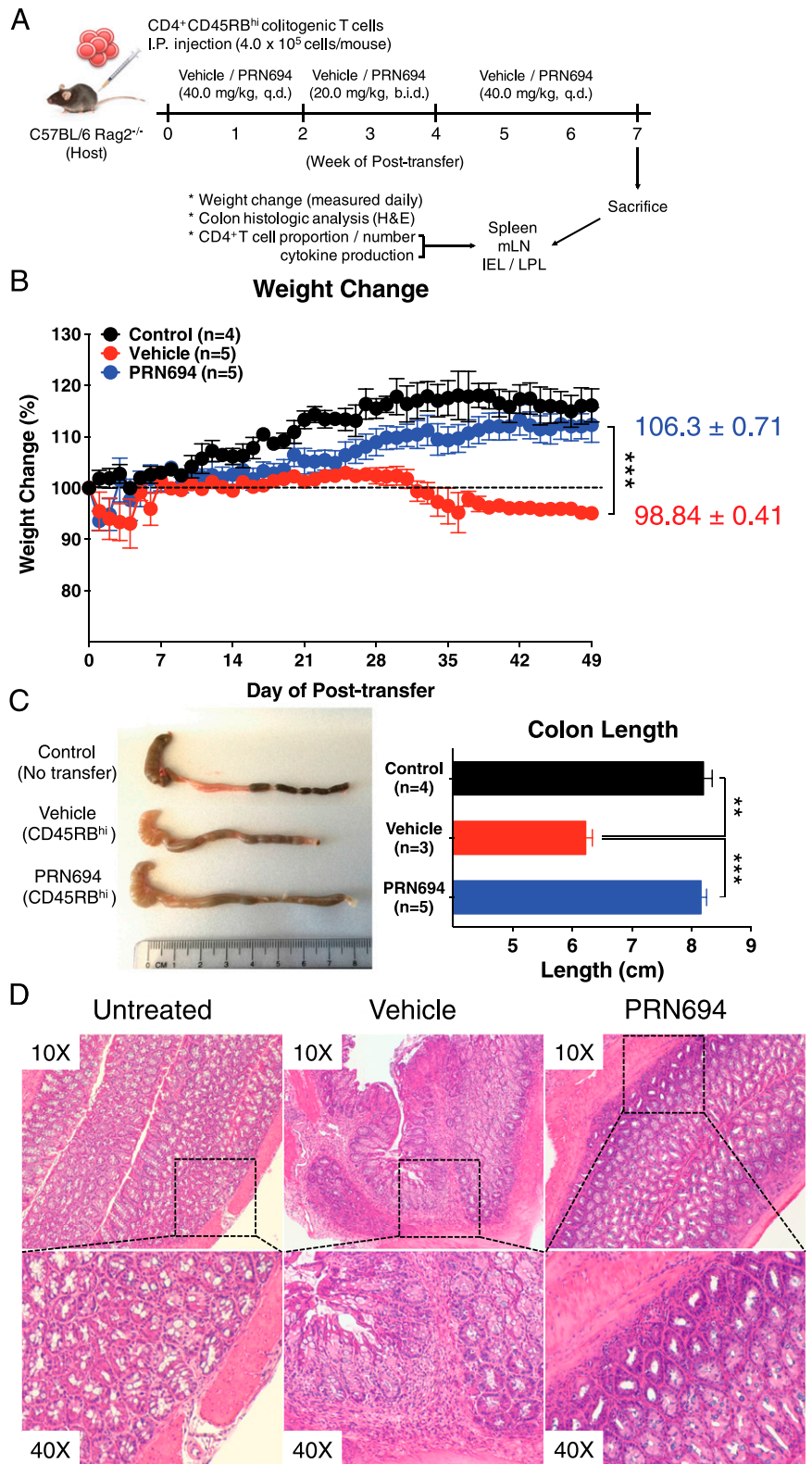


FIGURE 3. PRN694 administration ameliorates colitis disease progression. (**A–D**) Colitogenic CD4⁺CD45RB^{hi} splenic T cells (4.0×10^5 cells/mouse) from C57BL/6 WT mice were injected i.p. into C57BL/6 Rag2^{-/-} hosts. Recipients were treated with vehicle (red, $n = 5$) or PRN694 (blue, $n = 5$) with the indicated regimen (**A**), and disease progression of dosed recipients and untreated (no CD4⁺CD45RB^{hi} T cell transfer) Rag2^{-/-} controls (black, $n = 4$) was monitored by weight change (**B**). Data are shown as means \pm SEM. At 7 wk posttransfer, untreated Rag2^{-/-} control, vehicle-dosed, and PRN694-dosed mice were sacrificed for the measurement of colon length (**C**) and histologic analysis using H&E staining (**D**). Data were compiled from two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

using naive T cells from *Itk*^{-/-} and *Itk*^{-/-}*Rlk*^{-/-} mice had shown a role for these kinases in CCL19- and CXCL12-induced migration (7, 32), we considered whether CD4⁺ effector T cells might also require ITK and RLK for efficient migration into tissues. Using CD4⁺ T cells polarized in vitro to Th1, Th2, or Th17 lineages in the presence or absence of PRN694, we first examined binding to P-selectin, a ligand expressed on activated endothelium (33). Both Th1 and Th17 cells showed reduced P-selectin binding

following differentiation in the presence of both doses of PRN694 tested; in contrast, Th2 cells showed only a modest reduction, and this reduction was only visible in the higher dose of inhibitor (Fig. 6A, 6B).

As a second approach, we tested migration of CD4⁺ effector T cells to chemokines implicated in trafficking to the gastrointestinal epithelium. We focused on responses to CXCL11, a ligand for CXCR3 commonly found on effector Th1 cells (34), CCL20, a

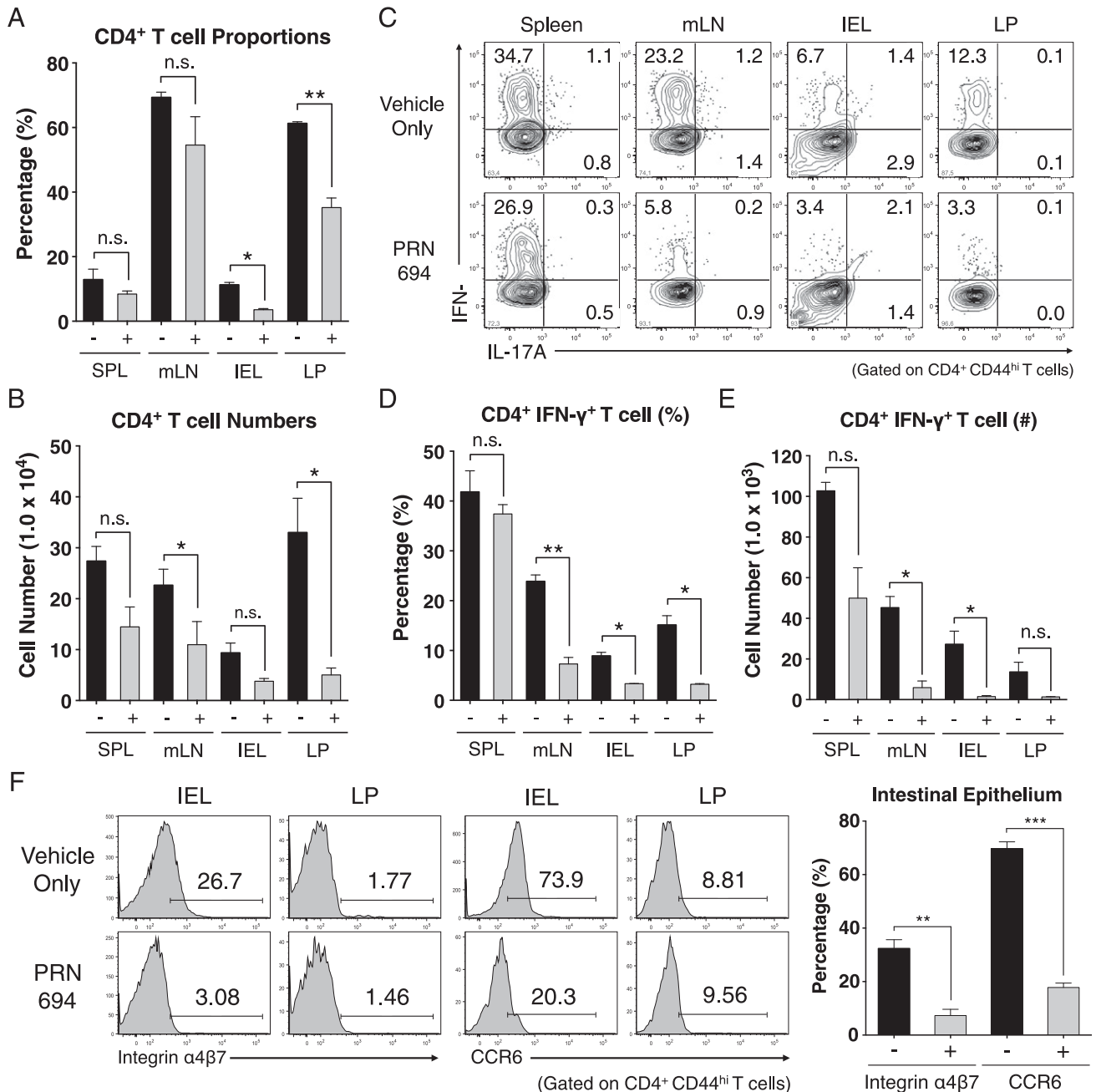


FIGURE 4. PRN694 treatment reduces colitogenic T cell proportions and IFN- γ production in the intestinal epithelium. **(A and B)** The proportions and absolute numbers of CD4⁺ T cells in spleen, mLN, intestinal epithelium, and colon LP are shown. **(C–E)** Isolated lymphocytes from various sites were stimulated with PMA and ionomycin and analyzed for IL-17A and IFN- γ production. Dot plots show gated CD4⁺CD44^{hi} T cells (C). The proportions (D) and numbers (E) of IFN- γ -producing CD4⁺ T cells are shown. Data are compiled from two independent experiments. **(F)** Histograms show the expression of gut-homing receptors, integrin $\alpha_4\beta_7$ (LPAM-1) and CCR6, on colonic IELs and LP CD4⁺CD44^{hi} T cells. Numbers indicate the percentages of cells in each region. Graph shows a compilation of data from three to five mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

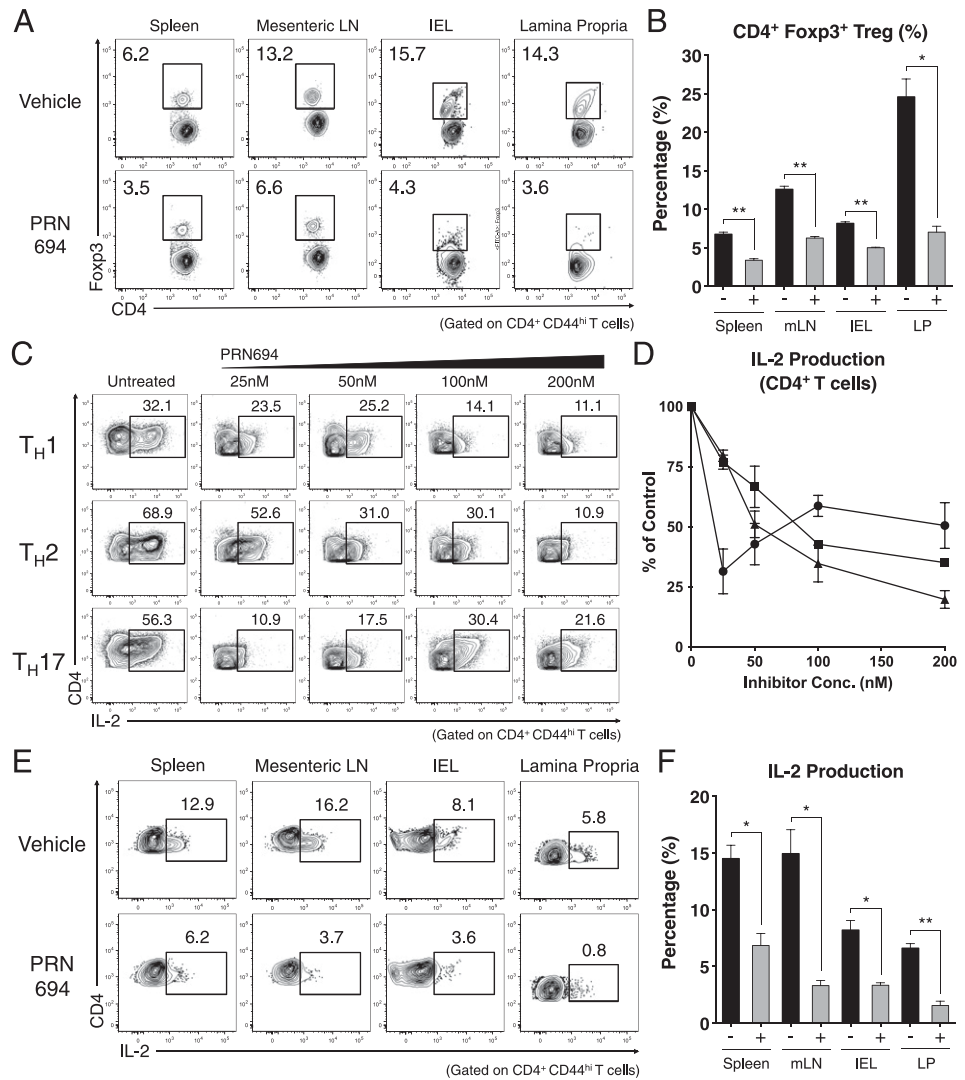
ligand for CCR6 found on Th17 cells (35, 36), and CCL25, a ligand for CCR9 (37, 38), which is expressed on gut-resident T cells. Using a Transwell migration assay, we found that PRN694 inhibited Th1 migration to CXCL11, with little migration observed by other subsets to this chemokine. CCL20 induced a modest degree of migration of Th17 cells, and this migration was reduced ~2-fold by PRN694. Th0 and Th1 cells showed fewer cells migrating in response to CCL20, and these responses were further reduced by PRN694. Overall, few cells of any lineage migrated in response to CCL25, with significant inhibition seen only in Th1 and Th0 cells. Polarized Th2 cells showed no migration to any of the chemokines tested (not shown). Overall, these data confirm that PRN694 im-

pacts differentiating Th1 and Th17 cells to reduce their ability to bind to activated endothelium and to migrate to inflammatory chemokines.

Discussion

In this study, we show that inhibition of ITK and RLK by PRN694 leads to a significant impairment in CD4⁺ Th cell differentiation and to protection from Th1-mediated colitis. Although PRN694 treatment inhibited the differentiation of all CD4⁺ Th subsets in vitro, the most potent inhibitory effect we observed was on Th1 differentiation. These findings are consistent with a recent study using an allele-sensitive mutant of ITK coupled with a selective

FIGURE 5. PRN694 treatment decreases Treg frequencies and IL-2 production from CD4⁺ T cells in vitro and in vivo. **(A and B)** CD4⁺ T cells from spleen, mLNs, IELs, and LP from vehicle-treated or PRN694-treated mice at 7 wk posttransfer were examined for Foxp3 expression. **(A)** Dot plots show CD4 versus Foxp3 staining, with numbers indicating the percentages of CD4⁺Foxp3⁺ Tregs, and **(B)** the graph shows a compilation of data from three to five mice in each group. **(C and D)** Naive CD4⁺ T cells were stimulated in Th-polarizing conditions with PRN694 at the indicated concentrations for 72 h. Cells were then restimulated with PMA and ionomycin for 5 h and then analyzed for IL-2 production. **(C)** The percentages of IL-2-producing CD4⁺ T cells (gated on CD4⁺CD44^{hi}) are shown. **(D)** Compilation of data from three independent experiments is shown, with the data for each Th subset normalized to the percentage of positive cells in the absence of inhibitor. **(E and F)** Isolated lymphocytes from vehicle-treated or PRN694-treated mice at 7 wk posttransfer were stimulated with PMA and ionomycin and analyzed for IL-2 production. Plots show gated CD4⁺CD44^{hi} T cells, and the graph shows a compilation of data from three to five mice in each group. **p* < 0.05, ***p* < 0.01.



inhibitor, which also revealed an important role for ITK kinase activity in Th1 differentiation and cytokine production (39). Our data with PRN694 are also consistent with previous studies showing that *Itk*^{-/-}*Rlk*^{-/-} mice mounted a normal protective type II cytokine response against *Schistosoma mansoni*, but were highly susceptible to the intracellular protozoan *Toxoplasma gondii* (5, 6). Furthermore, these results strongly suggest that a dual inhibitor of ITK and RLK would likely have a different effect on Th2-skewed inflammation such as asthma or atopic diseases compared with an inhibitor that was selective for ITK alone.

Also consistent with previous studies using *Itk*^{-/-} T cells (12), we observed that PRN694 was a potent inhibitor of Th17 differentiation and IL-17A production in vitro. Nonetheless, it is unlikely that inhibition of Th17 responses can account for the effects of PRN694 in ameliorating disease in our colitis experiments. Although some studies have shown a role for Th17 cells in colitis (40–42), these effects were seen either when colitogenic BALB/c T cells were transferred to syngeneic hosts (41) or when the cecal bacterial Ag-specific C3H/HeJBir CD4⁺ T cell lines were transferred to C3H.SCID hosts and then restimulated for 10 d in the presence of bacterial Ag-pulsed dendritic cells and with or without IL-23 prior to analysis (42). In contrast, a study using a protocol most similar to our own found that colitis was actually enhanced when Th17 responses were eliminated (40), and the transfer of naive CD4⁺

T cells from *Il17a*^{-/-} mice still induced a severe colitis (41). Furthermore, several other reports suggest that Th17 responses arise early on during colitis disease progression and that in vitro polarized Th17 cells are eventually converted into Th1 cells after adoptive transfer to lymphopenic hosts (43, 44). Using this CD4⁺CD45RB^{hi} adoptive transfer model of T cell-mediated colitis, we were unable to detect IL-17A production from colitogenic T cells in mice that were treated with vehicle alone, suggesting that Th17 responses were not contributing to disease progression in our studies. Thus, we were unable to assess the efficacy of PRN694 to inhibit IL-17A production in vivo in this disease model.

In the present study, we cannot rule out an effect of PRN694 on the Tec kinase family member Tec. Previous studies have indicated that *Tec* mRNA expression is negligible in primary T cells examined ex vivo, including memory phenotype cells (45) (<http://www.immgen.org>), and that only modest increases in Tec protein expression (<2-fold) are observed even after Th1 polarization in vitro (46). Furthermore, studies of T cell responses in *Tec*^{-/-} mice have failed to reveal any significant defects due to the absence of Tec. Therefore, it seems unlikely that the inhibitory effects of PRN694 observed in our studies are due to its effect on Tec activity in T cells.

Interestingly, we observed reduced differentiation of Foxp3⁺ iTreg cells in PRN694-treated mice compared with controls. In

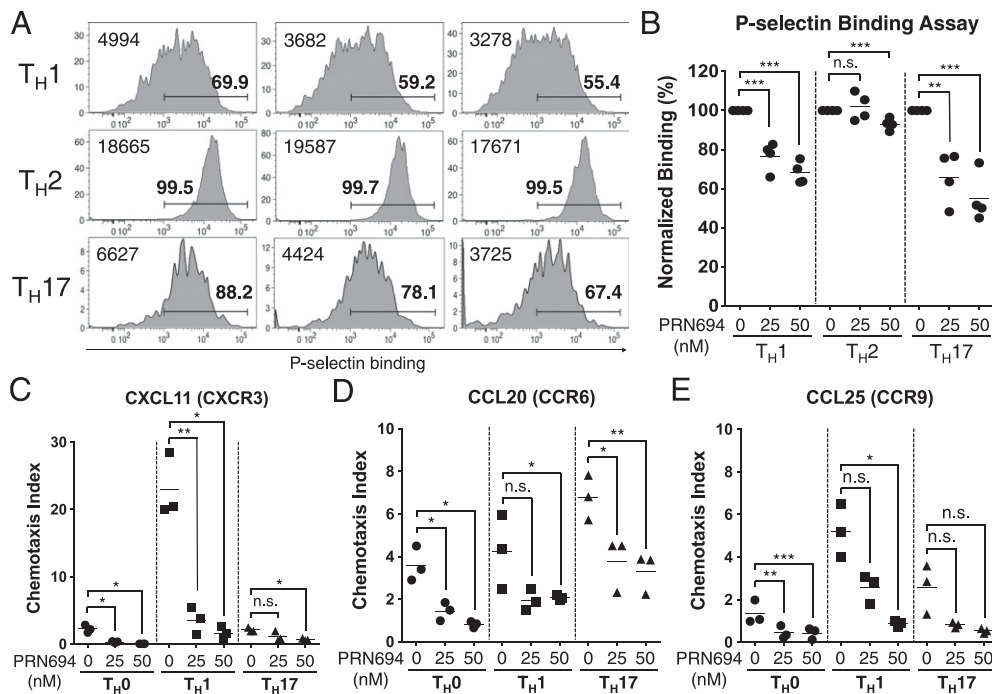


FIGURE 6. PRN694 leads to impaired P-selectin binding and inhibits chemokine-induced CD4⁺ T cell migration. (A–E) Isolated naive CD4⁺ T cells were cultured in the presence or absence of PRN694 (25 or 50 nM) for 72 h in each Th polarization condition. (A) Histograms show polarized CD4⁺ T cells stained with recombinant P-selectin–human IgG fusion protein as an assay for P-selectin glycoprotein ligand 1 binding. Bold numbers above region indicate the percentages of cells staining positively for P-selectin–Ig based on comparison with an isotype control stain; numbers at the left of each histogram indicate the mean fluorescence intensity of P-selectin–Ig staining in each panel. (B) Graph shows a compilation of data from two independent experiments. The data were normalized to the percentage of P-selectin–binding cells among each Th-polarizing condition in the absence of inhibitor. (C–E) Cultured CD4⁺ T cells (5.0×10^5 cells) were plated in the upper chamber of HTS Transwell-96 well permeable supports (3.0- μ m pore); bottom chambers were loaded with diluted CXCL11 (100 ng/ml) (C), CCL20 (100 ng/ml) (D), or CCL25 (300 ng/ml) (E). After 3 h at 37°C, the numbers of migrated cells were counted by the flow cytometer. The chemotaxis index was calculated by dividing the number of cells that migrated in response to each chemokine by the number of spontaneously migrated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

contrast, a previous study showed increased differentiation of *Itk*^{-/-} naive CD4⁺ cells into Foxp3⁺ iTreg cells in a similar adoptive transfer model of colitis (13). The difference in these two sets of findings could be due to a number of factors. First, the numbers of cells transferred and the time points analyzed were each different in the two studies. More importantly, there may be an unappreciated difference in the starting populations of CD4⁺ CD45RB^{hi}CD25⁻ T cells isolated from WT mice, as in our studies, compared with those isolated from *Itk*^{-/-} mice. Additionally, PRN694 is a potent inhibitor of both ITK and RLK, and this dual inhibition may lead to a different outcome than the single genetic deficiency in *Itk*. It is also important to consider that a small-molecule kinase inhibitor might have a different effect than a genetic deficiency that causes a complete absence of ITK protein, as the former situation would not abolish any kinase-independent functions of ITK. Finally, it is important to consider the possibility that mice housed in two different environments have significant differences in their microbiota, and that these differences may impact iTreg differentiation in vivo.

Our studies indicate that PRN694 treatment reduces the numbers of colitogenic CD4⁺ T cells in the intestinal epithelium at 7 wk posttransfer. It is possible that one component of this decrease is reduced proliferation of the T cells in the presence of PRN694, although our in vitro studies examining proliferation suggest that this explanation is unlikely. Instead, we propose that impaired T cell migration to the gastrointestinal tract is contributing to this deficit. We find that PRN694 treatment during Th cell polarization leads to reduced P-selectin binding on Th1 and Th17 cells, an alteration that could impact T cell rolling on the activated endo-

thelium prior to extravasation into the gastrointestinal tract. This possibility is consistent with our previous studies showing impaired tissue homing and transendothelial migration of *Itk*^{-/-} T cells in an autoimmune disease model (47). Furthermore, we observed reduced expression of integrin $\alpha 4\beta 7$ and CCR6, as well as reduced in vitro chemotaxis to inflammatory chemokines, by PRN694-treated CD4⁺ T cells. Taken together, these data provide strong support for the conclusion that ITK/RLK inhibition leads to impaired T cell migration into the gastrointestinal tissue.

Overall, we show that PRN694 inhibits CD4⁺ effector T cell differentiation and cytokine production, particularly for Th1 and Th17 lineage cells. Together with the inhibitory effect of PRN694 on T cell migration and on colitis disease progression, the data presented in the present study provide strong support for the development of a combined ITK/RLK inhibitor as a potential therapeutic strategy for Th1-mediated, and possibly Th17-mediated, inflammatory diseases.

Acknowledgments

We thank all members of the Berg Laboratory, especially Regina Whitehead and Sharlene Hubbard, for technical assistance. We also thank the University of Massachusetts Medical School's Department of Animal Medicine for the maintenance of mouse colonies and Flow Cytometry Core Facility for assistance in cell sorting.

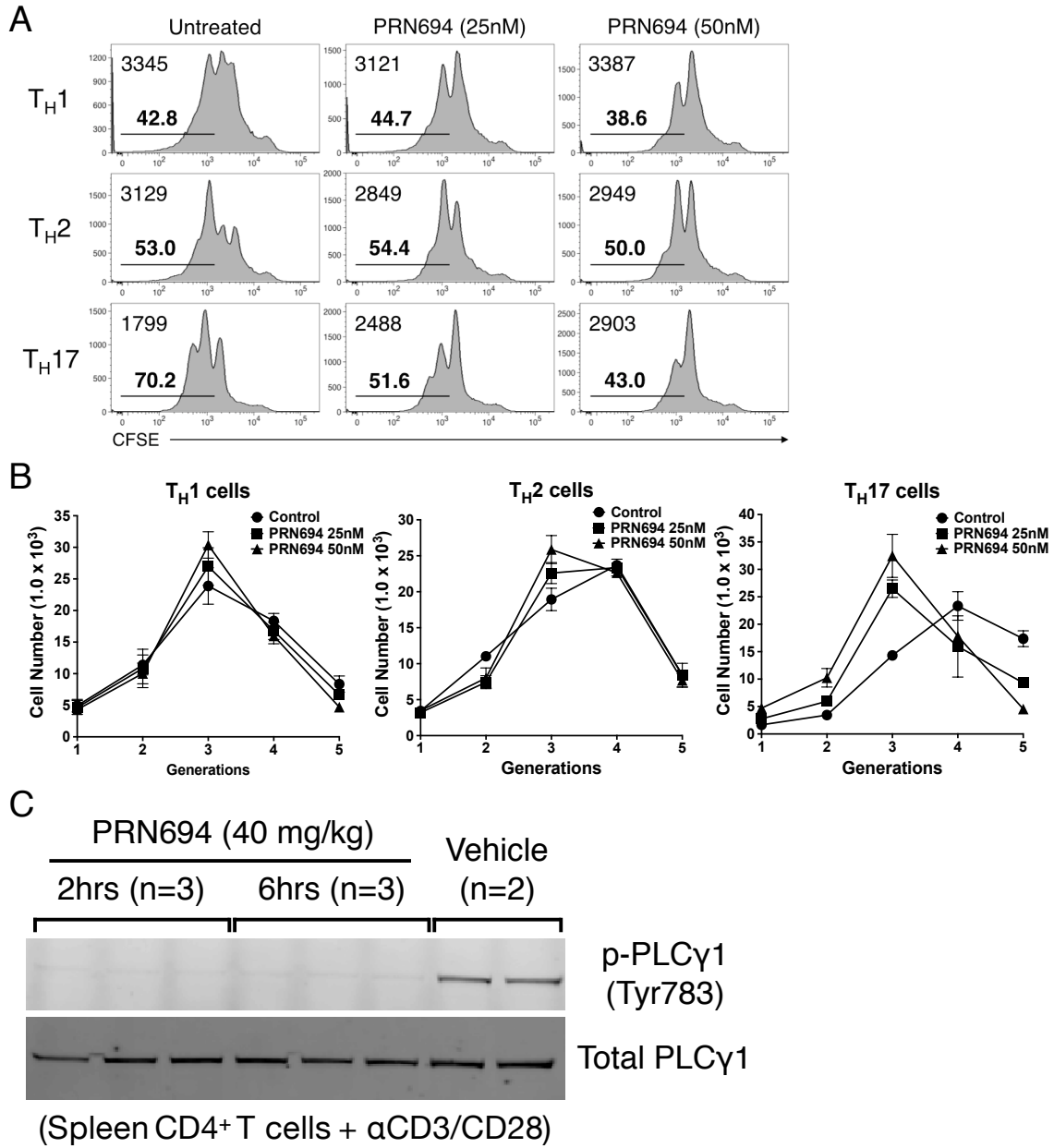
Disclosures

H.H.-D., T.D.O., J.O.F., R.J.H., and J.M.B. are members of a biotechnology company developing molecules for inflammatory and autoimmune diseases. The other authors have no financial conflicts of interest.

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Supplemental Figure 1



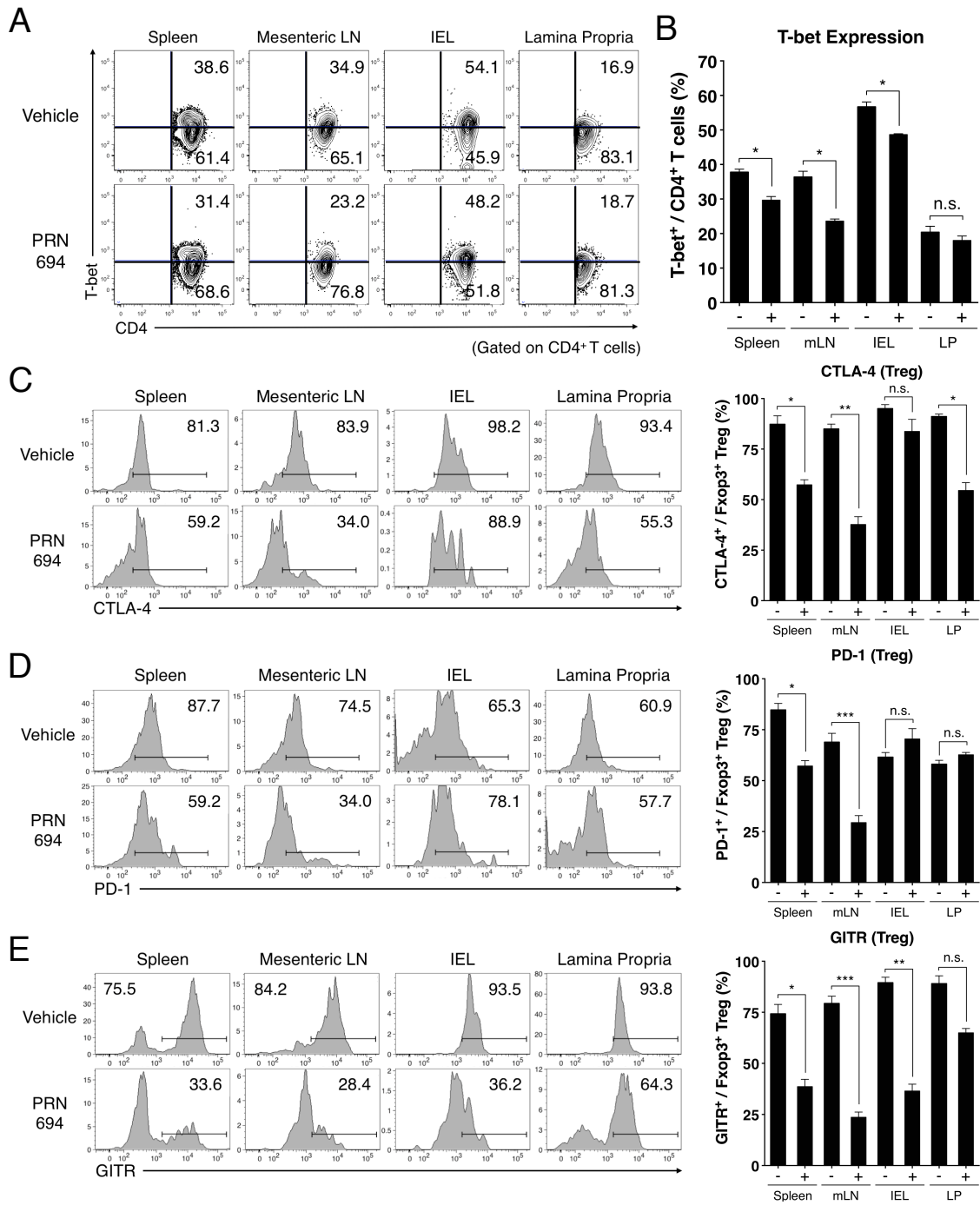
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2 Supplemental Figure 1. PRN694 effect on CD4⁺ T cell proliferation *in vitro* and
 3 phosphorylation of PLCγ1 *in vivo*.

4 (A-B) CFSE-labeled naïve CD4⁺ T cells were stimulated with anti-CD3/CD28 under
 5 different T_H polarization conditions for 72hr. Histograms are shown to analyze the

6 magnitude of CD4⁺ T cell proliferation. Mean fluorescence intensity of CFSE (Top, left)
7 and percentages of more-divided (diluted) CD4⁺ T cells (Bottom, left) are shown (A).
8 Calculated CD4⁺ T cell numbers of each cell division (Generation #1-5) by FlowJo
9 software are shown. Data from three experiments were compiled (B). (C) Splenic CD4⁺ T
10 cells isolated from vehicle-dosed (n=2) or PRN694-dosed (40 mg/kg) (2hr post-treatment,
11 n=3; 6hr post-treatment, n=3) mice were stimulated with anti-CD3/CD28 for 5min and
12 then lysed to examine the phosphorylation of PLCγ1 (Tyr783) by western blot. Both p-
13 PLCγ1 (Top) and total PLCγ1 (Bottom) are shown.

Supplemental Figure 2



14

15 **Supplemental Figure 2. Transcription factor and surface marker expression on**
 16 **transferred colitogenic CD4⁺ T cells from vehicle-treated and PRN694-treated mice.**

17 (A-B) Isolated CD4⁺ T cells from each different organ of vehicle-treated or PRN694-
18 treated mice at 7 weeks of post-transfer were analyzed for T-bet expression, and the
19 percentages of T-bet⁺ CD4⁺ T cells are shown. Graph shows a compilation of data from 3-
20 5 mice in each group. (C-E) Histograms show the expression of CTLA-4 (C), PD-1 (D),
21 and GITR (E) on CD4⁺ Foxp3⁺ Treg cells isolated from spleen, mesenteric LN, intestinal
22 epithelium (IEL), and lamina propria tissue of vehicle-treated (-) or PRN694-treated (+)
23 mice. Graphs shown each panel represent the compiled data from 3-5 mice in each group.

Supplemental Table 1

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ	Samples											
				PRN694											
				Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
				-			-			20nM			5µM		
				-			+			+			+		
Alpha-1-Antitrypsin (AAT)	mg/mL	3.8E-08	8.2E-08	0.000004	0.000002	0.0000019	0.0000046	0.0000038	0.0000028	0.0000049	0.0000034	0.0000029	0.0000044	0.0000024	0.0000021
Alpha-2-Macroglobulin (A2Macro)	mg/mL	0.000098	0.000087	0.00045	0.00051	0.00052	0.00053	0.00052	0.00045	0.00053	0.00054	0.00053	0.00044	0.00053	0.00050
Beta-2-Microglobulin (B2M)	ug/mL	0.000045	0.000051	0.0066	0.012	0.0082	0.014	0.029	0.021	0.014	0.016	0.020	0.0047	0.012	0.0067
Brain-Derived Neurotrophic Factor (BDNF)	ng/mL	0.0046	0.0072	<0.0072	0.0098	<0.0072	<0.0072	<0.0072	<0.0072	<0.0072	<0.0072	<0.0072	<0.0072	<0.0072	<0.0072
C-Reactive Protein (CRP)	ug/mL	0.000034	7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06	<7.8E-06
Complement C3 (C3)	mg/mL	1.6E-08	1E-07	0.000025	0.000016	0.000001	0.000024	0.0000019	0.0000086	0.000025	0.0000016	0.0000097	0.000026	0.000014	0.0000074
Eotaxin-1	pg/mL	1.9	29	<29	<29	<29	<29	<29	<29	<29	<29	<29	<29	<29	<29
Factor VII	ng/mL	1.2	0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77	<0.77
Ferritin (FRTN)	ng/mL	0.013	0.021	0.24	0.42	0.20	0.44	0.78	0.45	0.49	0.72	0.51	0.29	0.48	0.35
Fibrinogen	mg/mL	0.0000022	2.4E-07	0.000091	0.000047	0.000051	0.000082	0.000047	0.000058	0.000086	0.000047	0.000058	0.000087	0.000034	0.000052
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	pg/mL	3.2	18	<18	<18	<18	56	297	49	46	257	38	<18	<18	<18
Haptoglobin	mg/mL	0.0000012	3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07	<3.2E-07
Intercellular Adhesion Molecule 1 (ICAM-1)	ng/mL	0.35	0.26	<0.26	<0.26	<0.26	<0.26	0.28	<0.26	<0.26	0.28	<0.26	<0.26	<0.26	<0.26
Interferon gamma (IFN-gamma)	pg/mL	0.49	0.30	<0.30	<0.30	<0.30	42	516	52	11	170	5.3	<0.30	<0.30	<0.30
Interleukin-1 alpha (IL-1 alpha)	ng/mL	0.00030	0.00040	<0.0040	<0.0040	<0.0040	0.0023	0.0054	0.0032	0.00085	0.0042	0.00047	<0.0040	<0.0040	<0.0040
Interleukin-1 beta (IL-1 beta)	pg/mL	0.30	0.57	2.8	.57	1.7	44	126	47	9.8	90	11	.57	.57	.57
Interleukin-1 receptor antagonist (IL-1ra)	pg/mL	9.9	19	<19	<19	<19	184	218	211	406	417	652	<19	46	<19
Interleukin-2 (IL-2)	pg/mL	1.0	1.7	1.7	1.7	1.7	594	1800	660	174	787	83	1.7	1.7	1.7
Interleukin-3 (IL-3)	ng/mL	0.0011	0.0032	<0.0032	<0.0032	<0.0032	0.0036	0.0059	0.0038	<0.0032	<0.0032	<0.0032	<0.0032	<0.0032	<0.0032
Interleukin-4 (IL-4)	pg/mL	4.4	5.9	5.9	5.9	5.9	75	131	86	33	97	16	5.9	5.9	5.9
Interleukin-5 (IL-5)	pg/mL	1.0	2.6	2.6	2.6	2.6	5.6	19	7.2	3.1	13	2.6	2.6	2.6	2.6
Interleukin-6 (IL-6)	pg/mL	0.84	2.2	2.2	2.2	13	104	192	117	18	58	88	2.2	2.2	3.7
Interleukin-7 (IL-7)	pg/mL	2.1	1.8	<1.8	<1.8	<1.8	<1.8	<1.8	<1.8	<1.8	<1.8	2.5	<1.8	<1.8	<1.8
Interleukin-8 (IL-8)	pg/mL	0.63	0.79	2630	489	1810	18500	15900	16700	14100	16100	1100	1540	1650	1650
Interleukin-10 (IL-10)	pg/mL	1.1	1.4	<1.4	<1.4	<1.4	59	97	97	18	29	9.4	<1.4	<1.4	<1.4
Interleukin-12 Subunit p40 (IL-12p40)	ng/mL	0.016	0.055	<0.055	<0.055	<0.055	<0.055	0.21	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055
Interleukin-12 Subunit p70 (IL-12p70)	ng/mL	5.9	9.7	<9.7	<9.7	<9.7	132	132	10	<9.7	24	<9.7	<9.7	<9.7	<9.7
Interleukin-15 (IL-15)	ng/mL	0.12	0.079	<0.079	<0.079	<0.079	<0.079	<0.079	<0.079	<0.079	0.080	<0.079	<0.079	<0.079	<0.079
Interleukin-17 (IL-17)	pg/mL	1.2	0.99	.99	.99	.99	12	8.6	10	5.0	3.7	1.6	.99	.99	.99
Interleukin-18 (IL-18)	pg/mL	3.7	8.3	<8.3	<8.3	<8.3	<8.3	16	8.3	<8.3	12	<8.3	<8.3	<8.3	<8.3
Interleukin-23 (IL-23)	ng/mL	0.12	0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080
Macrophage Inflammatory Protein-1 alpha (MIP-1 alpha)	pg/mL	8.2	8.4	198	22	206	4790	5810	4040	6320	11800	5220	53	83	138
Macrophage Inflammatory Protein-1 beta (MIP-1 beta)	pg/mL	4.0	6.2	1350	257	1460	22600	27900	23500	20300	39300	17400	297	391	651
Matrix Metalloproteinase-2 (MMP-2)	ng/mL	1.3	0.87	1.4	<0.87	<0.87	6.1	7.9	7.7	5.0	9.9	7.1	<0.87	<0.87	<0.87
Matrix Metalloproteinase-3 (MMP-3)	ng/mL	0.0066	0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012
Matrix Metalloproteinase-9 (MMP-9)	ng/mL	5.0	7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9
Monocyte Chemoattractant Protein 1 (MCP-1)	pg/mL	4.6	9.0	508	206	298	1710	2760	2680	1550	3690	2150	51	144	50
Stem Cell Factor (SCF)	pg/mL	18	23	<23	<23	<23	<23	<23	<23	<23	<23	<23	<23	<23	<23
T-Cell-Specific Protein RANTES (RANTES)	ng/mL	0.00018	0.0010	0.26	0.13	0.18	0.48	0.30	0.41	0.51	0.28	0.40	0.32	0.066	0.24
Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)	ng/mL	0.010	0.016	3.0	2.1	2.0	2.1	1.9	1.8	2.2	1.6	2.1	1.1	1.3	0.75
Tumor Necrosis Factor alpha (TNF-alpha)	pg/mL	2.9	4.5	42	6.9	53	3830	7680	3270	2920	6880	2340	9.0	21	38
Tumor Necrosis Factor beta (TNF-beta)	pg/mL	2.3	1.9	<1.9	<1.9	<1.9	11	34	13	5.3	24	3.5	<1.9	<1.9	<1.9
Tumor necrosis factor receptor 2 (TNFR2)	ng/mL	0.0011	0.0057	0.077	0.071	0.053	0.21	0.24	0.22	0.23	0.26	0.26	0.053	0.057	0.052
Vascular Cell Adhesion Molecule-1 (VCAM-1)	ng/mL	0.0090	0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057	<0.057
Vascular Endothelial Growth Factor (VEGF)	pg/mL	12	6.8	12	13	8.3	10	14	10	<6.8	10	<6.8	8.3	14	<6.8
Vitamin D-Binding Protein (VDBP)	ug/mL	0.00002	0.000028	0.00037	0.000078	0.00014	0.00039	0.00013	0.00016	0.00039	0.000078	0.00018	0.00036	0.000066	0.00014
von Willebrand Factor (vWF)	ug/mL	0.0016	0.0034	0.018	0.017	0.014	0.016	0.015	0.015	0.015	0.015	0.014	0.015	0.011	0.014

24

25 **Supplemental Table 1. Inhibition of cytokines upon anti-CD3/CD28 stimulation of**
 26 **human PBMC by PRN694 using Human InflammationMap® v.1.0 biomarker**
 27 **panel.**

28 Shown are the levels of cytokine measured at each condition. The least detectable dose
 29 (LDD) was determined as the mean + 3 standard deviations of 20 blank readings. The
 30 LLOQ (Lower Limit of Quantitation) is the lowest concentration of an analyte in a
 31 sample that can be reliably detected.

Supplemental Table 2

Analytes	Units	Myriad RIM LDD	Myriad RIM LLOQ	Samples	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	Mouse 8	Mouse 9	Mouse 10	Mouse 11	Mouse 12
				PRN694	-			-			+			+		
				anti-CD3	-			+			1hr			6hr		
Apolipoprotein A-1 (Apo A-1)	ug/mL	2.3	20		357	543	291	353	244	289	315	312	263	250	193	307
C-Reactive Protein Mouse (CRP Mouse)	ug/mL	0.75	2.1		5.6	4.9	3.8	7.0	5.5	4.4	4.0	4.7	4.3	6.4	7.2	8.0
CD40 (CD40)	pg/mL	6.7	13		44	47	38	48	56	81	61	69	62	48	72	116
CD40 Ligand (CD40-L)	pg/mL	370	428		1040	787	443	1480	1310	1840	1130	1480	1310	3740	3150	2960
Eotaxin	pg/mL	3.5	6.2		953	1100	1200	2770	3050	3910	2160	3160	2270	6940	8140	6050
Epidermal Growth Factor Mouse (EGF Mouse)	pg/mL	18	15		20	17	20	35	40	44	31	29	35	51	60	49
Factor VII	ng/mL	25	19		25	38	27	54	52	68	38	51	47	82	96	70
Fibrinogen	ug/mL	231	510		73100	89600	70100	66000	76900	62300	73900	92600	74100	75900	80600	117000
Fibroblast Growth Factor 9 (FGF-9)	ng/mL	2.4	2.9		6.6	4.2	5.2	16	16	18	9.3	14	14	12	14	16
Fibroblast Growth Factor basic (FGF-basic)	ng/mL	12	15		<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15	<15
Granulocyte Chemotactic Protein-2 Mouse (GCP-2 Mouse)	ng/mL	0.16	0.49		1.4	1.4	1.3	3.6	3.4	3.2	2.2	2.4	1.8	4.1	4.8	4.1
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	pg/mL	4.1	6.8		<6.8	<6.8	<6.8	77	281	114	18	33	37	19	32	19
Growth Hormone (GH)	ng/mL	1.9	1.6		5.7	3.0	3.0	3.0	3.5	3.5	3.5	3.3	3.0	6.0	4.2	2.6
Growth-Regulated Alpha Protein (KC/GRO)	ng/mL	0.012	0.026		0.48	0.74	1.3	14	11	14	5.0	7.4	7.0	6.4	8.0	6.2
Haptoglobin	ug/mL	118	121		131	160	126	125	125	125	127	125	127	144	146	150
Immunoglobulin A (IgA)	ug/mL	18	19		258	1460	117	419	342	169	368	144	431	218	258	346
Insulin	uIU/mL	4.1	6.3		<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3
Interferon gamma (IFN-gamma)	pg/mL	15	38		<38	<38	<38	128	152	98	<38	40	47	54	96	80
Interferon gamma Induced Protein 10 (IP-10)	pg/mL	6.6	7.1		292	1740	165	2940	3910	2780	737	2010	1490	3450	3470	4190
Interleukin-1 alpha (IL-1 alpha)	pg/mL	108	132		156	<132	<132	327	312	265	219	281	281	327	296	357
Interleukin-1 beta (IL-1 beta)	ng/mL	6.5	3.5		<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
Interleukin-2 (IL-2)	pg/mL	25	49		<49	<49	<49	428	761	541	<49	<49	<49	69	146	<49
Interleukin-3 (IL-3)	pg/mL	2.9	4.3		<4.3	<4.3	<4.3	9.4	14	8.4	<4.3	4.5	5.4	<4.3	6.4	5.4
Interleukin-4 (IL-4)	pg/mL	52	68		<68	<68	<68	106	633	224	<68	73	87	<68	100	<68
Interleukin-5 (IL-5)	ng/mL	0.47	0.75		.75	.75	.75	0.92	0.76	0.82	1.3	<0.75	<0.75	3.6	3.7	0.87
Interleukin-6 (IL-6)	pg/mL	2.6	3.8		45	21	17	700	1260	461	206	307	152	119	510	269
Interleukin-7 (IL-7)	ng/mL	0.086	0.12		0.14	<0.12	0.14	0.54	0.48	0.69	0.24	0.36	0.39	0.55	0.48	0.53
Interleukin-10 (IL-10)	pg/mL	91	220		220	220	220	762	1090	979	271	440	504	440	590	376
Interleukin-11 (IL-11)	pg/mL	40	70		70	70	70	122	122	110	76	110	110	103	122	155
Interleukin-12 Subunit p70 (IL-12p70)	ng/mL	0.12	0.14		.14	.14	.14	0.40	0.45	0.25	0.14	0.17	0.25	0.22	0.33	0.31
Interleukin-17A (IL-17A)	ng/mL	0.0071	0.0073		<0.0073	<0.0073	<0.0073	0.16	0.16	0.13	0.029	0.037	0.042	0.042	0.056	0.045
Interleukin-18 (IL-18)	ng/mL	6.2	8.0		14	14	12	14	12	14	16	14	14	16	18	20
Leptin	ng/mL	0.026	0.053		1.1	0.87	0.44	0.25	0.58	0.90	1.3	1.0	1.3	0.92	0.64	1.7
Leukemia Inhibitory Factor (LIF)	pg/mL	218	463		487	<463	<463	1530	1250	1530	583	1010	869	1810	2360	1710
Lymphotactin	pg/mL	19	21		93	75	67	1120	1340	1420	346	517	508	713	989	919
Macrophage Colony-Stimulating Factor-1 (M-CSF-1)	ng/mL	0.033	0.051		5.7	7.4	5.5	6.1	5.4	5.1	5.9	5.1	5.8	6.7	7.3	7.0
Macrophage-Derived Chemokine (MDC)	pg/mL	34	55		1280	1220	910	2180	3000	2900	1500	1960	1500	3870	4270	3640
Macrophage Inflammatory Protein-1alpha (MIP-1 alpha)	ng/mL	4.1	3.3		3.3	3.3	3.3	4.2	3.3	4.2	3.7	3.3	3.3	5.6	6.1	6.1
Macrophage Inflammatory Protein-1 beta (MIP-1 beta)	pg/mL	4.7	53		1590	960	714	20100	22800	25200	5960	9790	10600	10200	17100	12700
Macrophage Inflammatory Protein-1 gamma (MIP-1 gamma)	ng/mL	0.45	0.67		10	11	12	14	14	11	13	13	12	19	17	18
Macrophage Inflammatory Protein-2 (MIP-2)	pg/mL	5.3	11		52	32	36	5210	3360	3420	819	2270	1040	711	1380	1520
Macrophage Inflammatory Protein-3 beta (MIP-3 beta)	ng/mL	0.36	1.2		2.1	2.5	1.5	3.1	4.0	4.5	2.5	3.4	2.4	6.2	7.0	5.4
Matrix Metalloproteinase-9 (MMP-9)	ng/mL	9.9	14		66	66	48	168	162	125	109	100	104	100	111	106
Monocyte Chemotactic Protein 1 (MCP-1)	pg/mL	4.9	7.9		1810	1460	1550	4390	3630	4680	3170	3490	3780	4110	4250	4650
Monocyte Chemotactic Protein 3 (MCP-3)	pg/mL	4.2	4.4		1800	1470	2060	3820	4150	4170	3110	3100	3610	4210	4250	4430
Monocyte Chemotactic Protein-5 (MCP-5)	pg/mL	1.7	2.3		172	68	265	1070	1180	676	381	374	739	985	1320	1080
Myeloperoxidase (MPO)	ng/mL	0.48	0.66		102	102	61	115	140	146	100	112	104	191	182	174
Myoglobin	ng/mL	5.7	6.0		81	15	9.6	380	64	32	109	45	13	103	58	156
Oncostatin-M (OSM)	ng/mL	0.097	0.21		0.42	0.22	0.27	1.0	0.96	1.1	0.62	1.0	0.88	0.98	1.2	1.2
Plasminogen Activator Inhibitor 1 (PAI-1)	ng/mL	0.015	0.034		1.5	9.2	0.73	9.0	13	7.6	3.4	10	9.3	11	12	11
Resistin	ng/mL	0.013	0.039		<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039
Serum Amyloid P-Component (SAP)	ug/mL	6.4	9.0		45	45	42	56	49	42	39	47	48	42	53	62
Stem Cell Factor (SCF)	pg/mL	153	290		1230	934	1110	3010	2470	2740	2010	2270	2410	2780	3130	2940
T-Cell-Specific Protein RANTES (RANTES)	pg/mL	0.026	0.028		0.031	<0.028	<0.028	0.27	0.20	0.29	0.059	0.049	0.092	0.10	0.18	0.12
Thrombopoietin	ng/mL	30	60		<60	<60	76	68	<60	60	68	68	60	84	99	95
Tissue Inhibitor of Metalloproteinases 1 Mouse (TIMP-1 Mouse)	ng/mL	0.021	0.084		1.5	1.6	1.3	1.8	2.0	1.2	1.2	1.3	1.1	4.6	4.5	2.7
Tumor Necrosis Factor alpha (TNF-alpha)	ng/mL	0.029	0.11		<0.11	<0.11	<0.11	0.28	0.23	0.25	0.17	0.16	0.22	0.20	0.23	0.23
Vascular Cell Adhesion Molecule-1 (VCAM-1)	ng/mL	3.3	5.4		732	894	682	970	953	814	1040	1090	877	933	1060	1360
Vascular Endothelial Growth Factor A (VEGF-A)	pg/mL	222	158		717	474	756	2080	1960	2280	1230	1750	1690	2000	2360	2320
von Willebrand factor (vWF)	ng/mL	27	45		345	351	457	385	589	446	671	578	731	639	677	655

32

33 Supplemental Table 2. Inhibition of cytokines upon anti-CD3 stimulation *in*

34 *in vivo* by PRN694 using RodentMap® v.3.0 biomarker panel.

35 Shown are the levels of cytokine measured at each condition. The least detectable dose

36 (LDD) was determined as the mean + 3 standard deviations of 20 blank readings. The

37 LLOQ (Lower Limit of Quantitation) is the lowest concentration of an analyte in a
38 sample that can be reliably detected.