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Wiskott-Aldrich Syndrome Protein Deficiency in Innate Immune Cells Leads to Mucosal Immune Dysregulation and Colitis in Mice

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Abstract

Background & Aims—Immunodeficiency and autoimmune sequelae, including colitis, develop in patients and mice deficient in Wiskott-Aldrich Syndrome protein (WASP), a hematopoietic-specific intracellular signaling molecule that regulates the actin cytoskeleton. Development of colitis in WASP-deficient mice requires lymphocytes; transfer of T cells is sufficient to induce colitis in immunodeficient mice. We investigated the interactions between innate and adaptive immune cells in mucosal regulation during development of T-cell-mediated colitis in mice with WASP-deficient cells of the innate immune system.

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Methods—Naïve and/or regulatory CD4^{+} T cells were transferred from 129 SvEv mice into RAG-2 deficient (RAG-2 KO) mice or mice lacking WASP and RAG-2 (WRDKO). Animals were observed for the development of colitis; effector and regulatory functions of innate immune and T cells were analyzed with in vivo and in vitro assays.

Results—Transfer of unfractionated CD4^{+} T cells induced severe colitis in WRDKO, but not RAG-2 KO, mice. Naïve wild-type T cells had higher levels of effector activity and regulatory T cells had reduced suppressive function when transferred into WRDKO mice compared to RAG-2 KO mice. Regulatory T-cell proliferation, generation, and maintenance of FoxP3 expression were reduced in WRDKO recipients, and associated with reduced numbers of CD103^{+} tolerogenic dendritic cells and levels of interleukin (IL)-10. Administration of IL-10 prevented induction of colitis following transfer of T cells into WRDKO mice.

Conclusions—Defective interactions between WASP-deficient innate immune cells and normal T cells disrupt mucosal regulation, potentially by altering the functions of tolerogenic dendritic cells, production of IL-10, and homeostasis of regulatory T cells.

Keywords

antigen presenting cells; inflammatory bowel disease; mouse model; immunodeficiency

Introduction

Over the past two decades, numerous murine models of inflammatory bowel disease (IBD) demonstrate a critical role for effector T cell populations in inducing intestinal inflammation[1, 2]. More recently, primary defects in the innate immune system have been shown to be sufficient to trigger colitis, either in the presence or absence of an adaptive immune system[3-10]. Moreover, primary immune deficiencies, which can affect both the innate and adaptive immune compartments, are frequently associated with intestinal inflammation[11]. However, because of the broad distribution of immune cells affected by immunodeficiencies, the precise roles of the adaptive and innate immune compartments during initiation of an inflammatory response remain unclear.

Patients with Wiskott-Aldrich Syndrome (WAS) are devoid of or have functional defects in the Wiskott-Aldrich Syndrome protein (WASP). These individuals are immunodeficient with up to 5-10% of patients developing IBD[12-14]. Similarly, WASP-deficient (WKO) animals on the 129 SvEv background also develop spontaneous colitis[15, 16]. Interestingly, polymorphisms in Arpc2, a member of the WASP-interacting Arp2/3 complex in humans that is directly activated by WASP, has been associated with increased risk of human ulcerative colitis[31].

WASP expression is restricted to hematopoietic lineages and regulates the actin cytoskeleton controlling leukocyte migration and a variety of effector functions[17]. However, for both WASP-deficient patients and mice, the specific role of WASP in adaptive and innate immune cells in disease initiation is not defined.

Colitis development in WKO mice is lymphocyte dependent as WASP/Recombination activating gene-2 (RAG) double knockout mice are devoid of colonic inflammation[16]. Moreover, WKO CD4^{+} T cells are sufficient to induce disease when transferred into immunodeficient RAG KO mice[16]. Since naturally-occurring Tregs are defective in WKO mice[26-30], colitis may be mediated in part by dysfunctional Tregs. However, since innate immune cells from WKO mice have multiple cellular abnormalities and innate immune defects in other models have been shown to influence Treg homeostasis[5], we hypothesized that WASP deficiency in innate immune cells plays a critical role in colitis development. In

this regard, WASP deficiency in myeloid cells lead to defects in processes dependent on rearrangement of the actin cytoskeleton (e.g., chemotaxis, immune synapse formation, antigen presentation)[18-25]. To test the hypothesis that WKO innate immune cell abnormalities play a critical role in disease development, we generated chimeric mice containing wild-type (WT) CD4^{+} T cells and WASP-deficient innate immune cells. These chimeric mice rapidly developed severe colitis, suggesting that WASP is required in innate immune cells to prevent intestinal inflammation. Herein, we explore the role of WASP in the crosstalk between aberrant innate immune cells and normal adaptive immune cells in the pathogenesis of colitis.

Results

WASP-deficient innate immune cells induce colitogenicity in WT CD4^{+} T cells

We previously reported that, in contrast to WKO mice, WASP^{-/-}RAG-2^{-/-} double knockout (WRDKO) mice on a 129 SvEv background do not develop spontaneous colitis[16]. We hypothesized that the colitis in WKO mice, although lymphocyte-dependent, might be initiated by defects in the innate immune compartment. To investigate whether WASP-deficient innate immune cells could induce WT CD4^{+} T cells to be colitogenic, we transferred unfractionated WT or WKO CD4^{+} T cells into WRDKO mice (Figure 1A). These chimeric mice (referred throughout the text as WT-DKO chimeric mice for WT T cells in WRDKO mice) have WT CD4^{+} T cells but WASP-deficient innate immune cells. Recipient animals of WT CD4^{+} T cells rapidly lost weight (Figure 1B) and developed severe colitis (Figure 1C and 1D) with early mortality comparable to recipients of WKO CD4^{+} T cells. This is contrast to our prior results demonstrating that transfer of WT unfractionated CD4^{+} cells into RAG KO mice, whose innate immune cells express WASP, does not induce colitis[16]. Therefore, severe colitis developed rapidly in the presence of WASP-deficient innate immune cells independent of WASP expression in CD4^{+} T cells.

Next, we determined whether T cells that were previously exposed to a WASP-deficient innate immune cell environment were permanently imprinted with colitogenic properties. CD4^{+} T cells from WT-DKO chimeric mice were used in a secondary transfer into RAG KO or WRDKO mice (Supplementary Figure 1A). While WRDKO recipients developed severe disease upon secondary transfer of CD4^{+} T cells from WT-DKO chimeric mice, there was no clinically significant disease in RAG KO recipients (Supplementary Figure 1B and S1C), indicating WASP-deficient innate immune cells do not permanently imprint WT T cells to become colitogenic.

Given autoimmune disease can develop in CD4^{+} adoptive cell transfer experiments when limited numbers of donor WT CD4^{+} cells are used (a setting in which the regulatory T cell repertoire is limited[32]), we examined whether varying the number of donor CD4^{+} cells affected disease outcome in WRDKO recipients. Irrespective of the number of donor CD4^{+} cells transferred (0.5 million to 3 million), the severity of colitis remained unchanged (Figure 1E) with disease present as early as one week after cell transfer (data not shown).

WT naïve T cells but not Tregs induce colitis in WRDKO recipients

As described above, the transfer of unfractionated CD4^{+} T cells induces rapid and severe colonic inflammation in WRDKO mice. In order to assess whether specific T cell subsets were responsible for the observed phenotype, we examined whether fractionating CD4^{+} cells into naïve (Tnaïve: CD4^{+}CD45RB^{hi}CD25^{-}) and regulatory (Treg: CD4^{+}CD45RB^{lo}CD25^{+}) T cell subsets would alter disease severity following cell transfer (Figure 2A). When WRDKO mice received only WT Tnaïves, recipient animals lost weight rapidly within 2 weeks associated with severe colitis and early mortality (Figure 2B and 2C).

WRDKO recipients of WT Tregs demonstrated no disease (Figure 2B and 2C). Similar transfer of WT Tnaïves into RAG KO mice, which have WASP-expressing innate immune cells, did not induce clinically significant disease in the timeframe of our experiments (Figure 2D-2F), consistent with previously published data[33], and emphasizing the profound colitogenicity seen in WRDKO hosts. These data demonstrate that WT Tnaïves, but not Tregs, become colitogenic after interacting with WASP-deficient innate immune cells.

T cell expansion, but not apoptosis, is altered in WRDKO hosts

To elucidate the mechanism by which WT-DKO chimeric mice develop severe and early onset colitis, we investigated CD4$^+$ T cell expansion and survival in the setting of WASP-deficient innate immune cells. We observed a higher absolute number of CD4$^+$ cells in the lamina propria (LP) of WRDKO compared to RAG KO recipients of WT Tnaïves (Supplementary Figure 2A). To assess directly the role of WASP-deficient innate immune cells on T cell proliferation, RAG KO and WRDKO recipients of WT Tnaïves were injected with BrdU two weeks post T cell transfer and lymphoid organs were harvested 16 hours following BrdU administration. There was a significant increase in the BrdU$^+$ CD4$^+$ population from LP in WRDKO mice compared to RAG KO mice (Supplementary Figure 2B and S2C). Lastly, we investigated whether a decrease in T cell apoptosis might account for the higher CD4$^+$ T cell counts in the LP of WRDKO recipients of WT CD4$^+$ T cells. The percent of apoptotic cells (Annexin V$^+$7AAD$^-$) in the LP were similar in both WRDKO and RAG KO recipients (data not shown). In summary, the increased LP T cell numbers observed in WRDKO recipients correlated with an increase in early T cell proliferation after Tnaïve transfer without evidence of decreased apoptosis.

WT Tregs fail to suppress normally *in vivo* in the presence of WASP-deficient innate immune cells

We next expanded our T cell analysis to include regulatory T cell activity and sought to address whether WT Tregs were functionally suppressive in the setting of WASP-deficient innate immune cells. We transferred WT Tregs along with WT Tnaïves at a 1:1 ratio into WRDKO recipients (the Treg:Tnaïve ratio used in the standard CD45RB transfer model is 1:4) (Figure 3A). Nevertheless, despite the higher Treg:Tnaïve ratio, there was no protection from colitis (Figure 3B-3D). Partial protection was observed only when the Treg:Tnaïve ratio was 8 times the usual required ratio (i.e., 2:1, Figure 3B-3D). Of note, similar Treg:Tnaïve 1:1 transfer into RAG KO mice demonstrated full protection of colitis with a mean histologic colitis score of 0.45 out of 8 (n = 11). Overall, these data indicate that WT Tregs can suppress only when present at increased numbers in the setting of WASP-deficient innate immune cells.

WASP-deficient innate immune cells lead to defects in Treg homeostasis

We hypothesized that the aberrant Treg suppressive activity noted *in vivo* might result from impaired Treg expansion, survival, and/or adaptive Treg generation in WRDKO mice. We therefore assessed the proportion of Tregs in WRDKO and RAG KO mouse recipients of WT Tnaïves alone and noted that the proportion of generated Tregs was significantly lower in the MLN and the LP of WRDKO mice compared to RAG KO recipients (Figure 4A and 4B). To assess *in vitro* whether WASP-deficient innate immune cells, specifically DCs, demonstrate defects in facilitating Treg induction, Tnaïves were cultured in the presence of MLN DCs from RAG KO or WRDKO mice after T cell transfer under conditions that promote *de novo* generation of Tregs. MLN DCs from WRDKO mice were defective in their ability to generate Tregs compared to DCs from RAG KO mice in the absence or presence of low doses of TGF-β with rescue only in the presence of high doses (Figure 4C and 4D). Thus, Treg generation was impaired in the presence of WASP-deficient innate immune cells both *in vitro* as well as *in vivo*.

In addition to defects in Treg generation, aberrant Treg expansion, survival, or maintenance could also contribute to defective Treg function observed in WRDKO recipient mice of Treg:Tnaïve co-transfer. To assess globally the outcome of these processes, we examined the percent of Tregs in recipients of unfractionated WT CD4$^+$ T cells and found decreased Treg proportions in WRDKO compared to RAG KO recipients (Figure 5A and 5B). To assess for a specific defect in Treg maintenance, we transferred Tregs expressing GFP under the Foxp3 promoter into RAG KO or WRDKO mice and assessed for the percentage of CD4$^+$Foxp3$^+$ cells in MLN and LP two weeks after transfer. In this context, Foxp3-expressing cells were reduced in the MLN and LP of WRDKO compared to RAG KO mice (Figure 5C and 5D). Since this defect in Treg maintenance in the LP may be due to a decrease in Treg proliferation or an increase in Treg apoptosis, we assessed these parameters employing BrdU and Annexin V/7AAD staining, respectively, within the Foxp3GFP$^+$ population. While there was no increase in apoptosis (data not shown), proliferation was significantly impaired in WRDKO compared to RAG KO recipients of Tregs correlating with the reduction in Treg maintenance (Figure 5E and5F). Taken together, these data indicate that Treg generation and proliferation are defective in the presence of WASP-deficient innate immune cells.

Reduction in CD11b$^+$CD103$^+$ DC subset in WRDKO Mice

WASP-deficient DCs have known defects in chemotaxis, podosome formation, immune synapse formation, and antigen presentation[18-20, 24, 25]. Since DCs can regulate T cell activation, we hypothesized that WASP-deficient DCs may be the critically altered innate immune cell population affecting T cell homeostasis. We analyzed mucosal and peripheral DC populations in RAG KO and WRDKO mice after T cell transfer. To delineate macrophages and DC subsets, we defined plasmacytoid DCs (pDCs) as MHCII^{mid}PDCA-1$^+$, conventional DCs (cDCs) as CD11c$^+$CD11b$^{-/lo}$, and monocytes/macrophages (Mφ) as CD11c$^{-/lo}$CD11b^{hi} (Figure 6A, gating strategy depicted in Supplementary Figure 3A). Following these criteria, there were no significant differences in the percentages of Mφ, cDCs, or pDCs in SPL, MLN, and LP of WRDKO mice when compared with RAG KO mice (Figure 6B-D). Given that CD103$^+$ DCs have tolerogenic properties[34], we analyzed the proportion of cDCs that are CD103$^+$ and found decreased proportions in WRDKO in both the MLN as well as LP (Supplementary Figure 3B). Moreover, recent data demonstrate that Treg and gut homing induction capacity reside within the CD8α$^-$ (which are CD11b$^+$) subset of CD103$^+$ DCs[35]. Further analysis showed a decrease in the proportion of CD11b$^+$CD103$^+$ cDCs in the LP and MLN of WRDKO mice (Figure 6E). Thus, colitis in WT-DKO chimeric mice may be associated with a decrease in tolerogenic DCs.

Decreased IL-10 production is associated with WASP deficiency

The anti-inflammatory cytokines TGF-β and IL-10 are important for the generation and/or function of regulatory T cells[4, 5, 36]. Therefore, we investigated the expression levels of these two down-regulatory cytokines in our colitis model. We first analyzed cytokine transcript levels in the MLN and LP of RAG KO and WRDKO recipients following transfer with WT Tnaïve cells. As expected, quantitative real-time PCR analysis showed elevated transcript levels of pro-inflammatory cytokines IL-23p19, TNF-α, and IL-6 in the colon of WRDKO mice but surprisingly not in the MLN. However, transcripts of IL-10 were markedly reduced in the MLN, but not in the colon, of WRDKO recipient mice (Figure 7A and 7B). Although elevated IL-10 transcripts were detected in the colons of WRDKO mice, this finding may be attributed to the increased T cell numbers resulting from severe colitis.

We then evaluated the production of these cytokines by WASP-deficient MLN DCs. WASP-deficient DCs secreted similar amounts of active TGF-β under stimulatory conditions and were able to activate exogenous latent TGF-β to a similar degree as control DCs (data not

shown). In contrast, WASP-deficient MLN DCs transcribed and secreted less IL-10 *in vitro* under stimulatory conditions compared to WASP-expressing DCs (Figure 7C). Interestingly there was no statistically significant alteration in TNF-α, IFN-γ, IL-6, or IL-12 at the transcript or protein level (data not shown). Hence, these data suggest that CD4+ T cells reside in an altered cytokine environment in the MLN with reduced IL-10, which may influence their developmental program or function in WRDKO mice.

Colitis in WT-DKO chimeric mice results from a loss-of-function defect that can be prevented with IL-10 administration

Since WASP-deficient DCs exhibited defects in IL-10 secretion and may be associated with colitis development in WT-DKO mice, we determined whether colitis could be prevented with WASP+ innate immune cells by generating mixed bone marrow chimeras. Lethally irradiated WT recipients received WRDKO bone marrow cells with or without RAG KO bone marrow cells (which express WASP) followed five weeks later by the transfer of WT Tnaïve cells (Supplementary Figure 4). Chimeric animals receiving both WRDKO and RAG KO bone marrow cells did not develop disease (Figure 7D) despite equal reconstitution of WASP-deficient and WASP-sufficient bone marrow (data not shown). In contrast, control mice that received only WRDKO bone marrow cells developed colitis (Figure 7D). Protection against colitis induction by WASP-expressing innate immune cells suggests that colitis in WT-DKO chimeric mice results from a loss–of-function defect.

The observed reduction in IL-10 levels produced by WRDKO MLN DCs *in vitro* and by MLNs in WT-DKO chimeric mice *in vivo* prompted us to determine whether colitis in WT-DKO mice could be rescued by exogenous administration of IL-10. WT Tnaïve cells were transferred into WRDKO mice receiving exogenous IL-10-Ig or control Ig. In contrast to animals that had received control Ig, IL-10-Ig-treated animals were protected from colitis induction with no weight loss or histologic evidence of inflammation (Figure 7E and 7F). Collectively, these data show that the colitis susceptibility in WT-DKO mice is attributed to a loss-of-function defect in innate immune cells and can be rescued by administration of exogenous IL-10. Experiments addressing whether IL-10-Ig is acting directly upon innate immune cells or T cells are ongoing.

Discussion

WASP deficiency in humans and mice is associated with both immunodeficiency and autoimmune sequelae[11]. Inflammatory diseases in the setting of a primary genetic immunodeficiency, while perhaps seeming paradoxical, are quite common[37]. Since WASP expression is essential for the function of most leukocyte subtypes, the specific contribution of the adaptive and innate immune system to the pathogenesis of the associated autoimmunity has been unclear. Although Treg dysfunction has been noted in WASP-deficient T cells[26-29] and may contribute to autoimmunity, in this current work, we demonstrated that WASP deficiency, when limited to the innate immune compartment, is sufficient to promote severe colitis development in the presence of WT CD4+ T cells.

Myeloid cells have been increasingly recognized as critical regulators of gut homeostasis[6-10]. Moreover, specific subsets (e.g., DCs) can have differential roles in immune modulation. Thus, appropriate crosstalk between the innate and adaptive immune system is often an essential component for maintaining mucosal homeostasis. Here we demonstrate that severe colonic inflammation occurs with rapid onset in WRDKO but not RAG KO recipients of WT CD4+ T cells. Our data demonstrate that WASP-deficient innate immune cells alter both regulatory and effector T cell arms of the adaptive immune system.

WASP-deficient innate immune cells enhanced WT effector T cell function since WT naïve T cells without co-transfer of Tregs caused more severe and earlier onset disease when transferred into WRDKO compared to RAG KO recipients. WASP-deficient innate immune cells also led to increased CD4^{+} T cell proliferation after T cell transfer. This enhanced early T cell proliferation correlates temporally with the spontaneous phase of lymphopenia-induced proliferation that can be associated with effector T cell generation and autoreactivity[38].

Nevertheless, it is possible that the observed enhanced T cell effector function (colitogenicty) and proliferation is secondary to altered Treg homeostasis in WRDKO hosts. The suppressive function of WT Tregs was markedly reduced *in vivo* in WRDKO hosts, despite co-transfer of sufficient number of WT Tregs. Likewise, Treg generation and proliferation were also depressed. Interestingly, Snuffner *et al.* demonstrated that homeostatic proliferation of Tregs in a lymphopenic host is hampered by deletion of DCs[39] consistent with the altered Treg homeostasis and reduction in CD103^{+} DCs, a population of DCs critical for *de novo* Treg generation[34], in WRDKO hosts. In particular, the CD8α^{-} subset of CD103^{+} DCs, which express CD11b and found to be reduced in WRDKO mice, also appears to be the specific DC subset with Treg-inducing potential[35]. While we and others demonstrated that expression of WASP is required for differentiation/maturation of specific leukocyte subsets and leukocyte migration[20, 21, 23,24, 30, 40], it remains unknown whether WASP is uniquely required for the differentiation/migration of CD103^{+} DCs in mucosal compartments.

The reduction in Treg generation, proliferation, and maintenance in the setting of WASP-deficient innate immune cells correlates with decreased IL-10 production by WASP-deficient DCs. Innate immune cell-derived IL-10 may be important for Treg generation[4] and function of Foxp3-expressing Tregs[5]. In addition, pathogenicity of WT CD4^{+} T cells is enhanced in mice devoid of IL-10 in innate immune cell compartments[4] implicating a shared pathogenic mechanism between IL-10/RAG DKO and WRDKO recipients of WT T cells. Thus, we speculate that defective Treg homeostasis in WRDKO hosts and the associated intestinal inflammation are due, at least in part, to impairment in IL-10 production. In our studies, reduced IL-10 transcription in the MLN of WT-DKO hosts may be related to the decrease in CD103^{+} DCs in the MLN since these cells are known to secrete IL-10[41]. In support of this, our data show that MLN DCs isolated from WRDKO mice produce less IL-10. The mechanistic underpinnings for defective IL-10 secretion in WASP-deficient DCs remains elusive, but regardless of the specific mechanism, a role for deficient IL-10 production in colitis development in WRDKO hosts is consistent with our bone marrow chimeric studies revealing a loss-of-function defect of WASP-deficient innate immune cells and the observed prevention of disease with exogenous IL-10-Ig treatment.

In summary, our data indicate that WASP-deficient aberrant innate immune cells can render WT T cells severely colitogenic. We speculate that WASP deficiency is associated with reductions in the generation, migration, and/or function of tolerogenic CD103^{+} DCs that lead to decreased IL-10 production in the MLN. This reduction in IL-10 results in both enhanced effector T cell activity as well as defects in Treg generation, expansion, and suppressive function. Taken together, intestinal inflammation in mice and humans resulting from WASP deficiency may arise, at least in part, from primary innate immune abnormalities resulting in aberrant innate-adaptive immune cell crosstalk.

Materials and Methods

Mice

WT, RAG KO, WKO[15], and WRDKO mice[16], all on pure 129 SvEv background, were maintained in SPF animal facilities at Massachusetts General Hospital (Boston, MA). Foxp3GFP transgenic mice were obtained from Jackson Laboratories, Inc. All experiments were conducted upon approval and according to regulations of the Subcommittee on Research Animal Care of the Massachusetts General Hospital.

Cell Transfer, Analysis of CD4^{+}Expansion, DC isolation, Stimulation of DCs, Quantitative RT-PCR, Treg generation and suppression assays, Histologic colitis score, Bone Marrow Transplantation, Flow Cytometry, Antibodies, and Exogenous IL-10 Administration: Please see Supplemental Information.

Statistical Analysis

Given they are non-parametric values, histologic scores were displayed in scatter plots showing median values ± interquartile range and compared using Mann Whitney U test or Kriskal-Wallis analysis (if 2 groups) with post-hoc Dunn's multiple comparison test. For parametric values, an unpaired two-tailed Student's t-test was used (unless otherwise indicated) with error bars representing STD if graph is from one representative experiment or SEM if graph reflects data pooled from multiple experiments. We assigned the number of asterisks as follows: $0.01 < *p < 0.05$; $0.001 < ** p$ 0.01; $*** p$ 0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard abbreviations

WAS	Wiskott-Aldrich Syndrome
WASP	Wiskott-Aldrich Syndrome Protein
KO	knockout
WT	wild-type
WKO	WASP KO
RAG KO	Recombination activating gene-2 KO
WRDKO	WASP/RAG-2 double KO
LP	lamina propria
MLN	mesenteric lymph node
SPL	spleen
WT-DKO chimeric	WT CD4^{+}→WRDKO
Tnaïve	naïve CD4^{+} T cells

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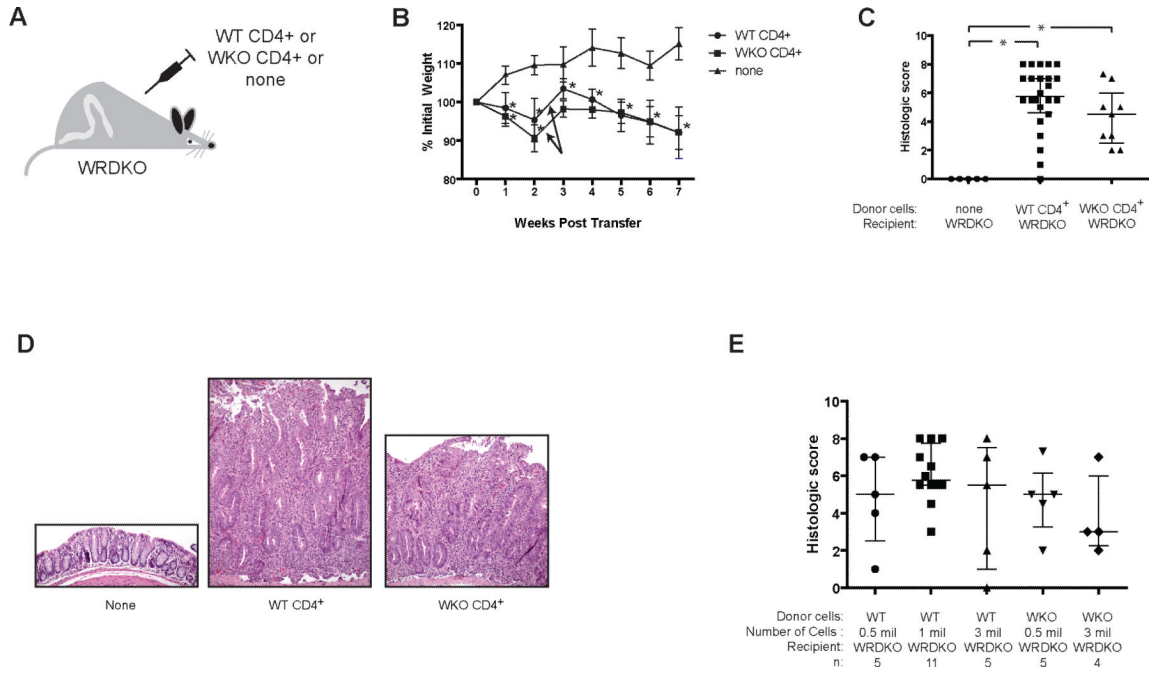
Figure 1. Transfer of WT CD4⁺T cells into WRDKO mice induced severe colitis
(A) Schematic of experimental design: WRDKO mice were injected with 0.5 - 3 million WT CD4⁺ cells (n = 22) or WKO CD4⁺ cells (n = 9), or neither (n = 7) pooled from two independent experiments. (B) Mean weights ± SEM, * p < 0.05. Arrows refer to the time point when the sickest mice in both WT CD4⁺ and WKO CD4⁺ groups died; hence, the increase in mean weights at week 3. (C) Median histologic colitis scores ± interquartile range. Kruskal-Wallis analysis with post-hoc Dunn's test showed * p < 0.05. (D) Representative H&E images of the colons (10x objective). (E) Median histologic scores ± interquartile range of WRDKO recipients of different WT or WKO donor cell numbers. No significant differences were observed using Kruskal-Wallis analysis
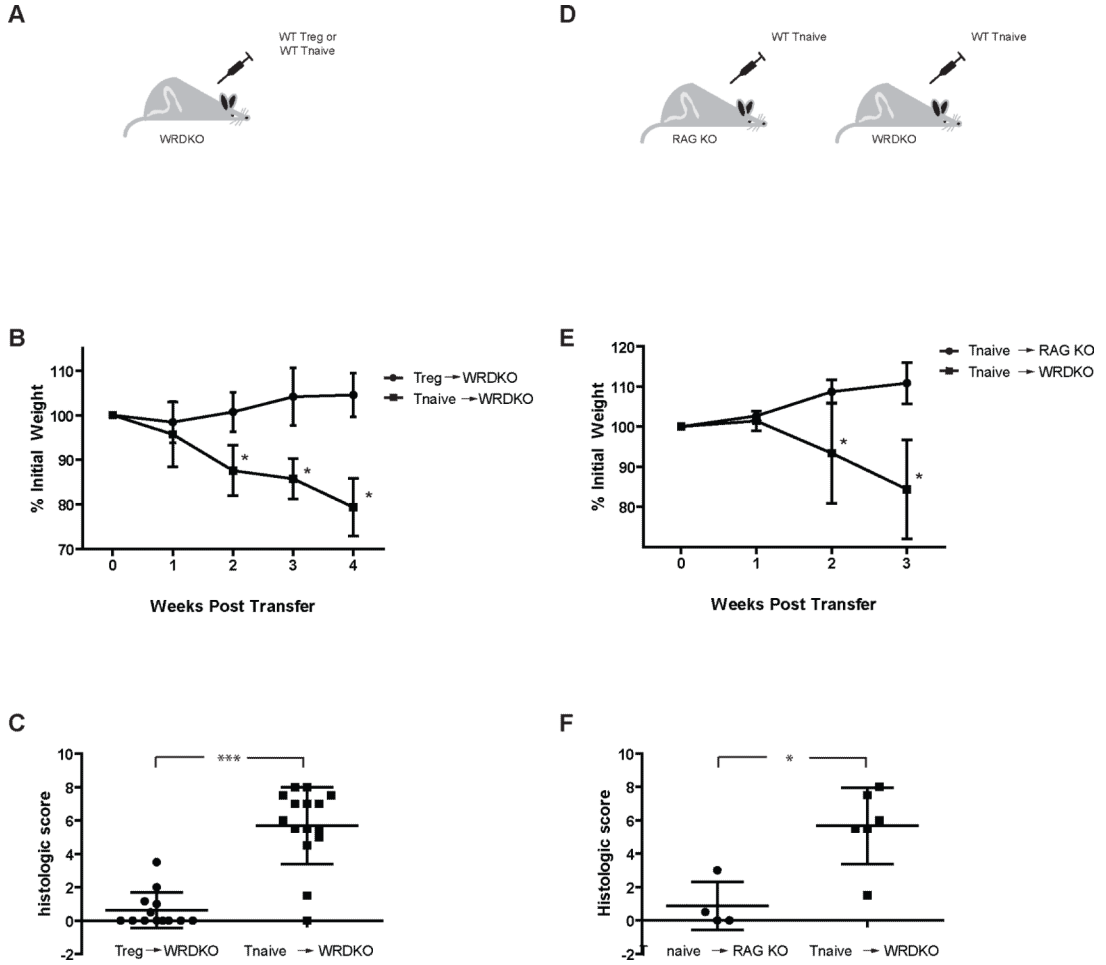
Figure 2. Transfer of WT naïve T cells, but not WT Tregs, into WRDKO mice leads to severe disease

(A) Schematic of experimental design for B & C: WRDKO mice were injected with 10^5 Tregs or 10^5 Tnaïves. (B) Mean percentages of initial weight ± STD of WRDKO recipients of either Tnaïves or WT Tregs, * $p < 0.05$, representative of five independent experiments (n = 4 in each group). (C) Median histologic scores ± interquartile range four weeks post cell transfer, *** $p < 0.001$. Data are pooled from the five experiments (n = 13-15). (D) Schematic of experimental design for E & F: WRDKO (n = 7) and RAG KO mice (n = 4) were injected with 10^5 Tnaïves. (E) Mean percentages of initial weight ± STD, * $p < 0.05$. (F) Median histologic scores ± interquartile range three weeks after T cell reconstitution, * $p = 0.025$. Representative of fifteen independent experiments.
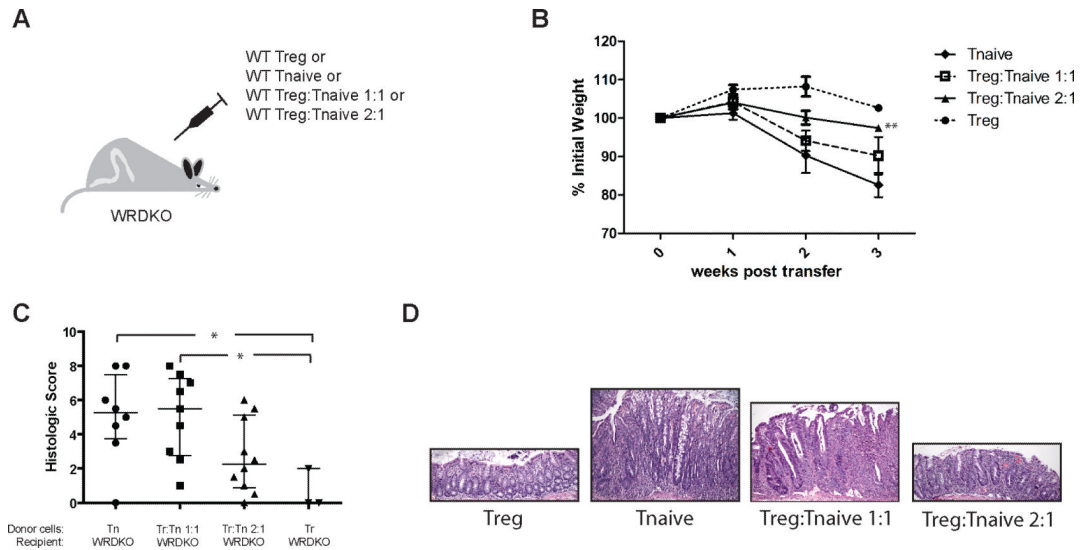
Figure 3. WT Tregs fail to suppress normally *in vivo* in the presence of WASP-deficient innate immune cells
(A) Schematic of experimental design: WRDKO were injected with Tnaïve cells (n = 9), Tregs (n = 3), Tregs:Tnaïves at 1:1 ratio (n = 10), or Tregs:Tnaïves at 2:1 ratio (n = 9), pooled from 3 independent experiments. (B) Mean percentages of initial weight ± SEM, ** p = 0.004 comparing between 2:1 and Tnaïve group and (C) median histologic scores ± interquartile range. Kruskal-Wallis analysis with post-hoc Dunn's test showed * p < 0.05, Tn = Tnaïve and Tr = Treg. (D) Representative H&E images of colons, 10x objective.
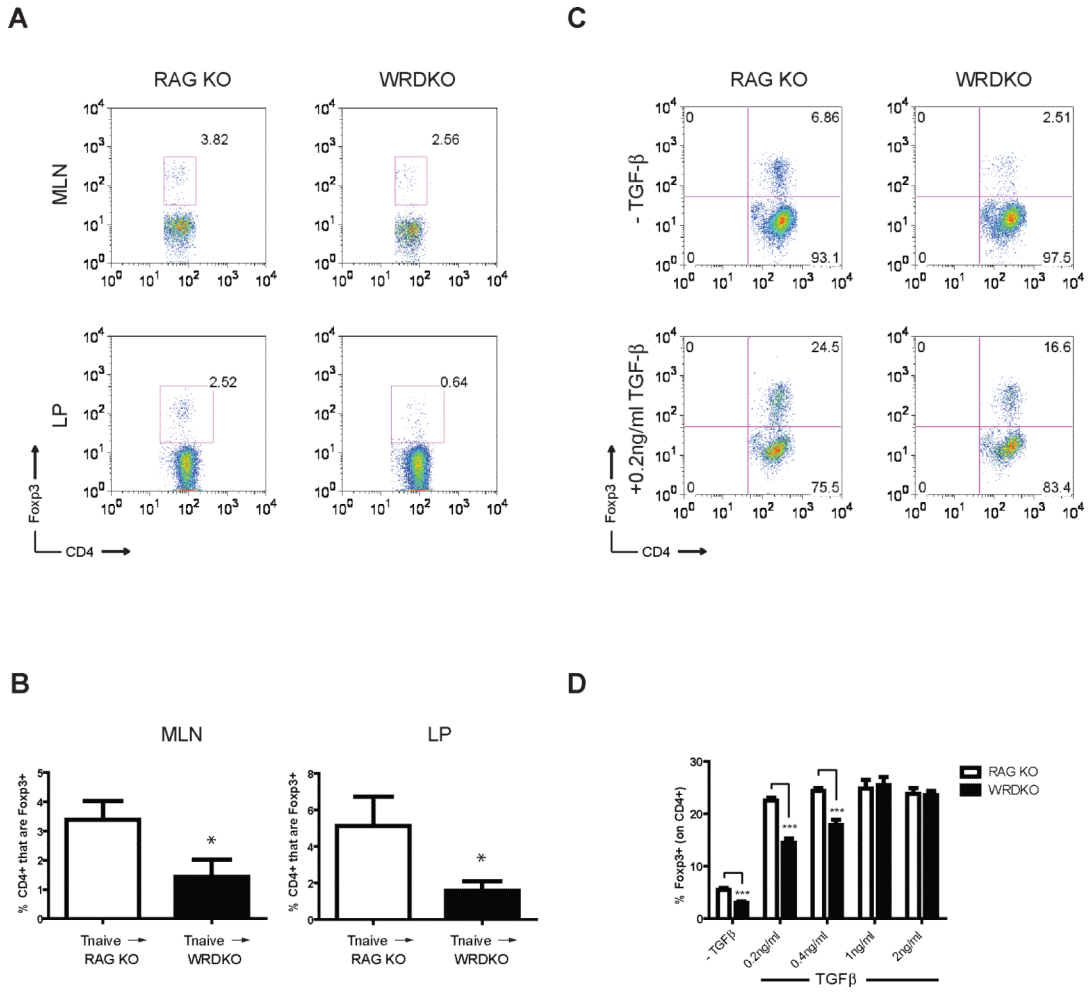
Figure 4. Treg generation is impaired *in vivo* and *in vitro* in the presence of WASP-deficient innate immune cells

(A) Three weeks after transfer of WT Tnaïve cells, WRDKO and RAG KO recipient animals were analyzed for CD4 and Foxp3 expression in mesenteric lymph node (MLN) and lamina propria (LP). Shown here are representative FACS plots of Foxp3 expression within CD4+ lymphocyte gate. (B) Mean percentages ± SEM of CD4+ cells that are Foxp3+ pooled from three independent experiments (n = 5-18), * p = 0.04 for MLN and * p = 0.01 for LP. (C) *In vitro* Treg induction assay using MLN DCs isolated from WRDKO or RAG KO mice 3 weeks after WT Tnaïve cell transfer, co-cultured with WT Tnaïve cells and anti-CD3 antibody with or without exogenous TGF-β, performed in duplicates with n = 3 for each group.Shown are representative FACS plots of Foxp3 expression within CD4+ lymphocyte gate. (D) Two-way ANOVA with repeated measures was performed to determine the effect of DC genotype on % Foxp3+ cells generated. Since the interaction of genotype with TGFβ concentration was significant (p<0.001), post-hoc t-tests were performed for each concentration. Shown are mean percentages ± STD of CD4+ cells that are Foxp3+, *** p < 0.001.
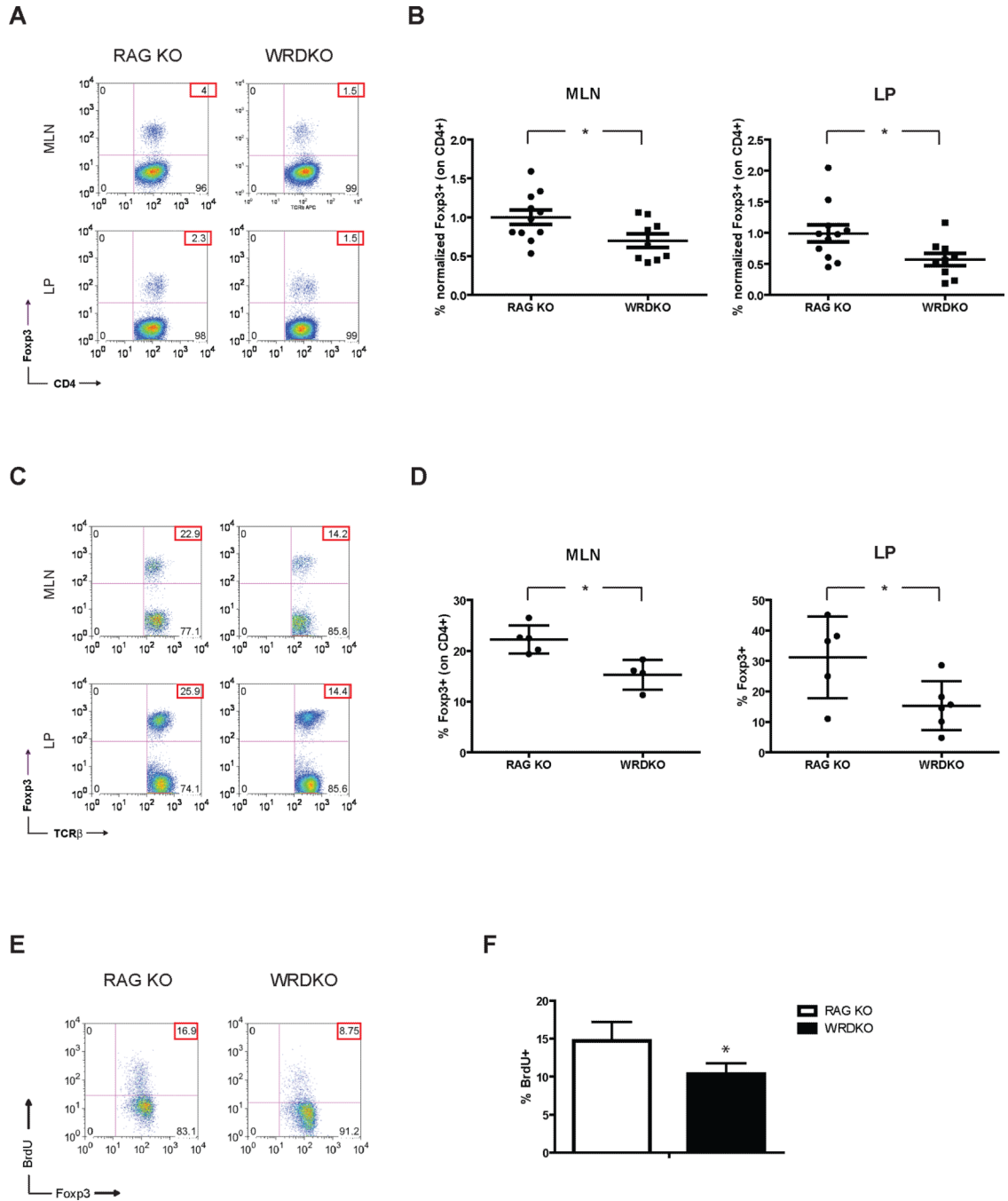
Figure 5. *In vivo* Treg maintenance and proliferation are defective in the setting of WRDKO innate immune cells

(A) WRDKO and RAG KO recipients of WT unfractionated CD4^{+} cells were analyzed for percentages of CD4^{+} cells that express Foxp3 three weeks after cell transfer. Shown are representative FACS plots of Foxp3 expression within CD4^{+} lymphocyte gate. (B) Mean ± SEM percentages normalized to mean RAG KO percentage within each experiment, pooled from two independent experiments (n = 9-11), * p = 0.03 for MLN and * p = 0.03 for LP. (C) Two weeks after transfer of CD4^{+}Foxp3GFP^{+} T cells into WRDKO or RAG KO mice, MLN and LP were analyzed for percentages of CD4^{+} T cells that were Foxp3GFP^{+}. Shown are representative FACS plots. (D) Mean ± STD percentages in one representative

experiment out of three, n 5 recipient animals for each group, * p = 0.01 for MLN and * p = 0.02 for LP. (E) Proliferation (BrdU$^+$) of transferred Tregs (CD4$^+$Foxp3GFP$^+$ cells) that remain Foxp3$^+$ in the LP of WRDKO compared to RAG KO recipients. Shown are representative FACS plot of BrdU staining within CD4$^+$Foxp3$^+$ lymphocyte gate. (F) Mean percentages ± STD of CD4$^+$Foxp3$^+$ cells that are BrdU$^+$, * p = 0.01.
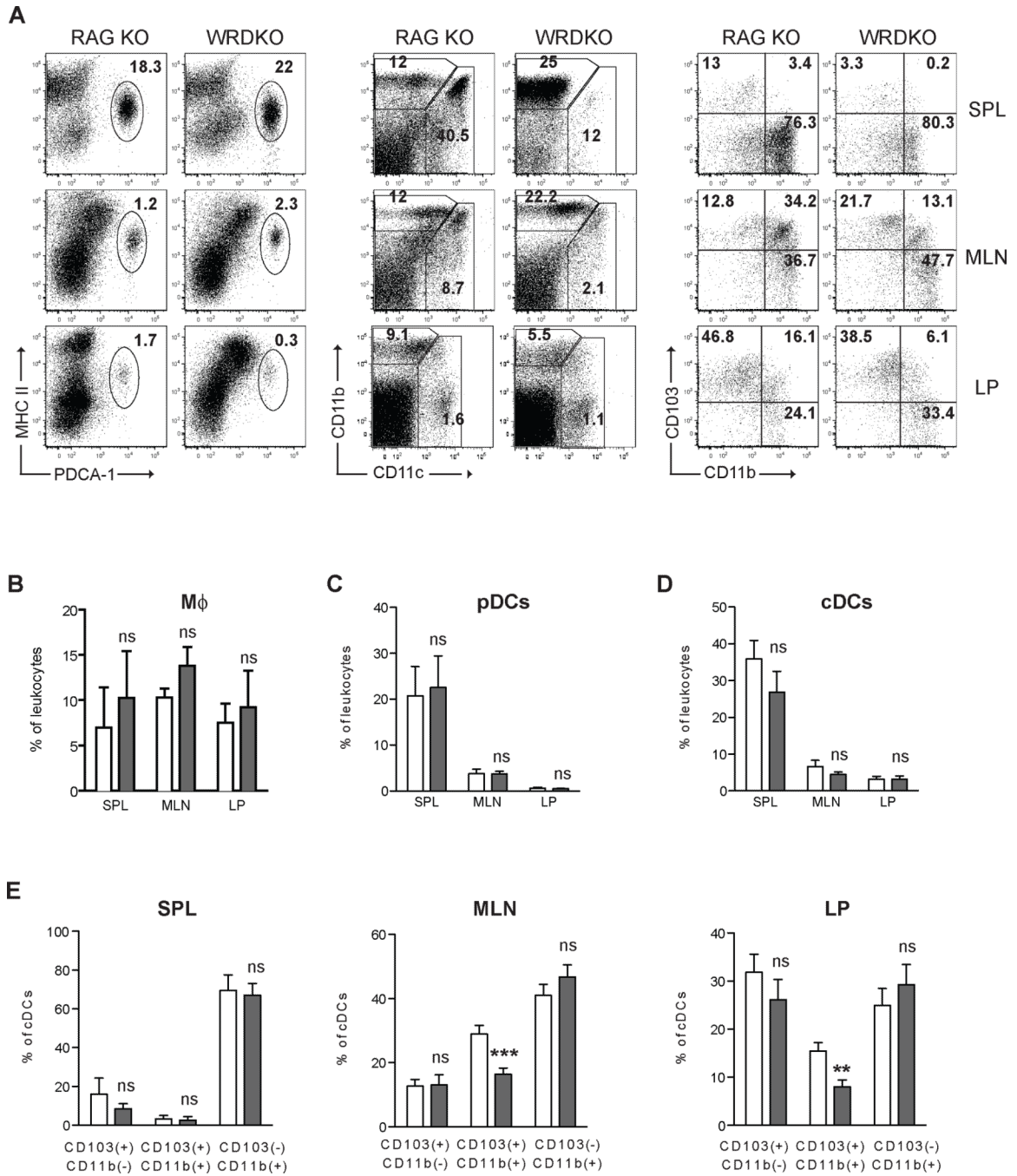
Figure 6. Decrease in CD11b^{+}CD103^{+}DCs in WRDKO mice

Three weeks after WT Tnaïve cell transfer, WRDKO and RAG KO recipient animals were analyzed for innate immune cell subsets. (A) Representative plots of plasmacytoid DC (pDC) proportions (MHCII[mid]PDCA-1^{+}) within live cell gate (left panels). Within the non-pDC live cell gate, cells were analyzed for CD11c and CD11b expression to distinguish between conventional DCs (cDCs) and monocyte/macrophages (Mφ) (middle panels). Cells were stained for CD11c, CD11b, and CD103 to evaluate specific cDC subsets (right panels). Plots displayed were gated in cDC live cell gate. (B-D) Graph represents mean percentages ± SEM of leukocytes that are (B) Mφ, (C) pDCs, and (D) cDCs. (E) Mean percentages ± SEM of cDCs that are CD103^{+}CD11b^{-}, CD103^{+}CD11b^{+}, or CD103^{-} CD11b^{+}, *** p =

0.0008 for MLN, ** p = 0.0023 for LP. Entire figure is representative of three independent experiments, n = 6 for SPL and n = 10 for both MLN and LP.
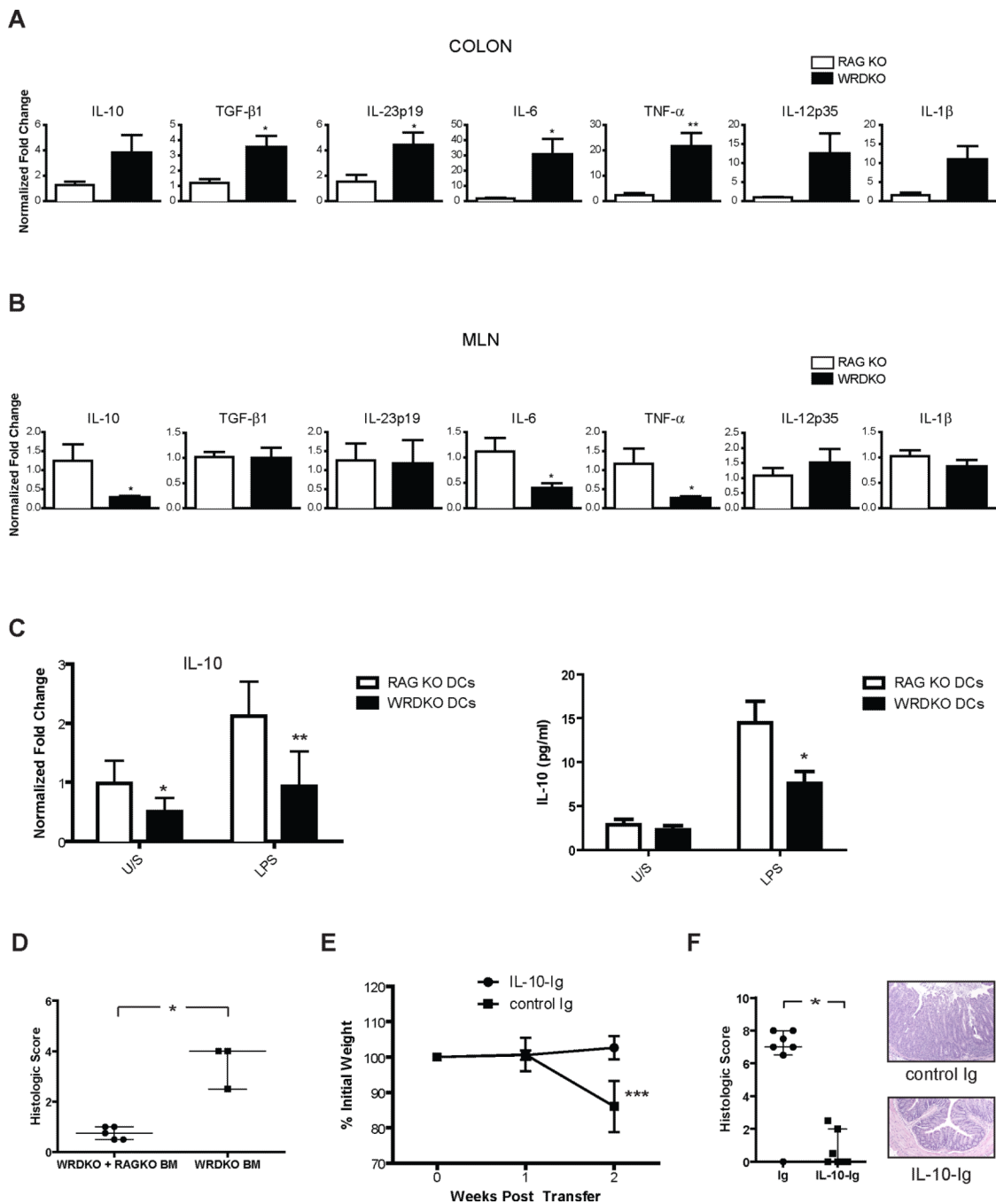
Figure 7. WASP deficiency is associated with reduced IL-10 production and administration of exogenous IL-10 or presence of WASP-expressing innate immune cells prevent colitis in WT-DKO chimeric mice

(A) Cytokine mRNA levels by qRT-PCR on whole colons of RAG KO and WRDKO recipients of WT Tnaïves, n = 7 for RAG KO; n = 9 for WRDKO with * p for TGF-β1 = 0.02, * p for IL-23p19 = 0.04, * p for IL-6 = 0.02, ** p for TNF-α = 9×10^{-3}. (B) Cytokine mRNA levels on whole MLNs with n = 4 for RAG KO; n = 6 for WRDKO with * p for IL-10 = 0.02, * p for IL-6 =0.02, * p for TNF-α = 0.02. (C) Mean IL-10 mRNA levels ± SEM detected by qRT-PCR (left) and mean IL-10 protein levels ± SEM (right) of RAG KO and WRDKO MLN DCs stimulated *in vitro* with or without LPS (U/S), n = 5-6 pooled from

3-6 independent experiments, * p = 0.03 and ** p = 0.009. (D) Median histologic scores ± interquartile range demonstrating protective effect of WASP-expressing innate immune cells in mixed bone marrow chimeric mice, * p = 0.035. Representative of two independent experiments. Refer to Supplementary Figure 4 for details. (E) WRDKO mice were transferred 1 million unfractionated CD4$^+$ cells and given 1 μg IL-10-Ig-containing serum (n = 7) or control IgG (n = 6) pretreatment 2 days before T cell transfer and twice weekly treatment for 2 weeks. Displayed are the mean weights ± STD, * p = 0.0002. (F) Left: median histologic colitis scores ± interquartile range at the end of two weeks, * p = 0.01; right: representative H&E images of colons, 10x objective.