WASP-mediated regulation of anti-inflammatory macrophages is IL-10 dependent and is critical for intestinal homeostasis

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Mutations in Wiskott–Aldrich syndrome protein (WASP) cause autoimmune sequelae including colitis. Yet, how WASP mediates mucosal homeostasis is not fully understood. Here we show that WASP-mediated regulation of anti-inflammatory macrophages is critical for mucosal homeostasis and immune tolerance. The generation and function of anti-inflammatory macrophages are defective in both human and mice in the absence of WASP. Expression of WASP specifically in macrophages, but not in dendritic cells, is critical for regulation of colitis development. Importantly, transfer of WT anti-inflammatory macrophages prevents the development of colitis. DOCK8-deficient macrophages phenocopy the altered macrophage properties associated with WASP deficiency. Mechanistically, we show that both WASP and DOCK8 regulates macrophage function by modulating IL-10-dependent STAT3 phosphorylation. Overall, our study indicates that anti-inflammatory macrophage function and mucosal immune tolerance require both WASP and DOCK8, and that IL-10 signalling modulates a WASP-DOCK8 complex.
A large genome-wide association study among inflammatory bowel disease (IBD) patients identified over 163 loci associated with IBD risk. A Bayesian network analysis containing these risk loci as well as gene expression data identified an IBD sub-network that includes several genes (e.g., IL10, NOD2, HCK and WAS) that are highly enriched in bone-marrow-derived macrophages (BMDMs) and point to a possible role for this IBD sub-network in regulating anti-inflammatory macrophage development and/or function. The Wiskott–Aldrich syndrome (WAS) gene, encoding the actin cytoskeletal protein WAS protein (WASP), is one of the genes identified within this sub-network along with other IBD-associated genes. Patients with WAS typically manifest recurrent infections, thrombocytopenia and eczema. In addition, 10% of patients develop IBD and 100% of Was−/− mice on the 129SvEv background develop spontaneous colitis. WASP expression is restricted to haematopoietic lineages and broad defects are observed in most WASP-deficient leukocytes. WASP regulates cytoskeleton-dependent functions, including podosome formation, migration, phagocytosis and antigen uptake in a variety of innate immune cells. WASP regulates macrophage function and differentiation, we compared the phenotype of colonic macrophages from pre-colitic 5-week-old Was−/− and wild-type (WT) mice. In these Was−/− mice we observed a significant increase in the percentage of P2 pro-inflammatory macrophages (**p < 0.001, Student’s t-test) and a concomitant decrease in the percentage of P3, P4 anti-inflammatory macrophages (**p < 0.001, Student’s t-test) compared with WT mice (Fig. 1a). These alterations were more pronounced in 12-week-old Was−/− mice (Fig. 1b). Although the frequency of P2 versus P3/P4 macrophages was inverted in Was−/− mice compared with WT animals, the absolute number of all macrophages subset was greater in Was−/− mice compared with control animals, which is most likely due to increased recruitment of circulating monocytes in the setting of inflammation. Altered macrophage populations were also apparent in Was−/−Bag2−/− mice (129SvEv background), which in the absence of T-cell transfer do not develop colonic inflammation (Supplementary Fig. 1b). To further characterize functionally LP macrophages, we evaluated the expression of pro- and anti-inflammatory genes expression in sorted P3 + P4 macrophages isolated from 5-week-old Was−/− and WT mice. The expression of anti-inflammatory genes including Arg1, Fizz1, Ym1 and Il10 was lower in Was−/− P3 + P4 macrophages compared with WT macrophages (Fig. 1c). Interestingly P3 + P4 macrophages from Was−/− mice was more pro-inflammatory in nature as evidenced by higher expression of inflammatory genes including Il1b, Il12, Tnf and Il12 (Fig. 1c). The per cell expression of inflammatory genes in P2 macrophages were comparable between WT and Was−/− mice (Supplementary Fig. 1c).

To determine whether the aberrant differentiation of LP macrophages observed in Was−/− mice was due to a cell-intrinsic defect caused by the absence of WASP, we used a mixed chimera approach to study macrophages differentiation. Bone marrow from WT CD45.1+ mice and Was−/− CD45.2+ mice were transferred at a 1:1 ratio into irradiated CD45.2+ Was−/− mice and analysed 8 weeks after reconstitution. Flow cytometric analysis of gated WT (CD45.1) and Was−/− (CD45.2) cells from LP showed an increase in the percentage of pro-inflammatory macrophages and a concomitant decrease in anti-inflammatory macrophages in Was−/− compared with WT compartments (Fig. 1d). Moreover, the P3/P4 macrophages from Was−/− (CD45.2) compartment were more pro- and less anti-inflammatory in nature compared with WT (CD45.1) compartment (Fig. 1e). Taken together, these results indicate that WASP regulates the development of LP anti-inflammatory macrophages in a cell intrinsic manner.

**Results**

**WASP regulates macrophage function and differentiation.** We sought to investigate the role of WASP in macrophages differentiation in both mucosal and non-mucosal sites. In the LP, monocytes undergo several stages of development during differentiation and can be categorized into four different groups based on the expression of Ly6c and major histocompatibility complex (MHC) II: P1 (Ly6chigh MHCII−), P2 (Ly6clow MHCII−) and P3 + P4 (Ly6clow MHCII−, P4 CX3CR1+) (Supplementary Fig. 1a). P2 LP macrophages have pro-inflammatory characteristics, whereas P3 and P4 LP macrophages have anti-inflammatory properties. To examine whether WASP regulates LP macrophage differentiation and function, and to minimize any effect that inflammation may have on skewing of macrophage differentiation, we compared the phenotype of colonic macrophages from pre-colitic 5-week-old Was−/− and wild-type (WT) mice. In these Was−/− mice we observed a significant increase in the percentage of P2 pro-inflammatory macrophages (**p < 0.001, Student’s t-test) and a concomitant decrease in the percentage of P3, P4 anti-inflammatory macrophages (**p < 0.001, Student’s t-test) compared with WT mice (Fig. 1a). These alterations were more pronounced in 12-week-old Was−/− mice (Fig. 1b). Although the frequency of P2 versus P3/P4 macrophages was inverted in Was−/− mice compared with WT animals, the absolute number of all macrophages subset was greater in Was−/− mice compared with control animals, which is most likely due to increased recruitment of circulating monocytes in the setting of inflammation. Altered macrophage populations were also apparent in Was−/−Bag2−/− mice (129SvEv background), which in the absence of T-cell transfer do not develop colonic inflammation (Supplementary Fig. 1b). To further characterize functionally LP macrophages, we evaluated the expression of pro- and anti-inflammatory genes expression in sorted P3 + P4 macrophages isolated from 5-week-old Was−/− and WT mice. The expression of anti-inflammatory genes including Arg1, Fizz1, Ym1 and Il10 was lower in Was−/− P3 + P4 macrophages compared with WT macrophages (Fig. 1c). Interestingly P3 + P4 macrophages from Was−/− mice was more pro-inflammatory in nature as evidenced by higher expression of inflammatory genes including Il1b, Il12, Tnf and Il12 (Fig. 1c). The per cell expression of inflammatory genes in P2 macrophages were comparable between WT and Was−/− mice (Supplementary Fig. 1c).

**WASP expression in macrophages regulates colitis.** To determine the macrophage- and DC-intrinsic role of WASP in mucosal homeostasis, we examined the impact of selective...
Fig. 1 Defective anti-inflammatory macrophage differentiation and function in the colon of Was−/− mice. Flow cytometric analysis of LP macrophage in mice at a 5 (WT n = 6; Was−/− n = 8) and b 12 (WT n = 6; Was−/− n = 6) weeks of age followed by quantification of pro- (P2) and anti-inflammatory (P3 + P4) subsets. Macrophages were gated as live CD45+ CD11b+ CD103+ CD64+ cells. Data are cumulative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t-test). c Expression of pro- and anti-inflammatory genes in sorted P3 + P4 macrophages (WT n = 12; Was−/− n = 12). P3 + P4 cells from three mice were pooled together. Data are cumulative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t-test). d CD45.1+ (WT) and CD45.2+ (Was−/−) bone marrow cells were transferred at the ratio of 1:1 into lethally irradiated CD45.2− recipient. LP macrophage was analysed after 10 weeks. FACS plot shows the gating strategy. Graph shows the quantification of P2 and P3/P4 cells in the WT (n = 6) and Was−/− (n = 6) compartment of recipient mice. Data are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t-test). e Expression of pro- and anti-inflammatory genes in sorted P3 + P4 macrophages in mice (n = 8). P3 + P4 cells from two mice were pooled together. *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant (Student’s t-test). All graphs shows mean ± SEM.
105) from wild-type (WT) mice were transferred i.p. into
and frequency of TNF
homogenates at 6 weeks post transfer. Data are cumulative of two independent experiments (n=17, includes Exp 1, 2, 3, 4; Was
Rag
n=17, includes Exp 1, 2, 3, 4, Was
Rag
n=7, includes Exp 3, 4). Difference was not significant between Was
Rag
−/− cohorts. *p<0.01, ****p<0.0001 (two-way ANOVA). b Representative photomicrographs of H&E-stained colonic section and histological score after naive T-cell transfer. Scale bars: 200 μm. c Colon length at 6 weeks post transfer (Rag−/− n=8; Was
Rag
−/− n=9). d Cytokines expression in colonic homogenates at 6 weeks post transfer. Data are cumulative of two independent experiments (Rag−/− n=6; Was
Rag
−/− n=6). e Absolute number and frequency of TNF
, IFN-γ
, IL-17A
 and IFN-γ
IL-17A
 helper T cells in the LP was determined by flow cytometry (Rag−/− n=9; Was
Rag
−/− n=9). Data are cumulative of three independent experiments. f Percentage of Treg cells (CD45
 TCRβ
CD4
FoxP3
) in the LP was determined by flow cytometry (Rag−/− n=5; Was
Rag
−/− n=5). Data are cumulative of two independent experiments. Data shown in b-f are mean ± SEM and P-value was obtained by Student’s t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
deletion of WASP in macrophages and DCs. We first generated mice with macrophage- Wasfl/LysMCre (WasDel) and DC-Wasfl/ItgaxCre (WasdcDel) selective deletion of WASP on either the Rag1−/− or Rag2−/− background. Spontaneous inflammation was not observed in either WasDelRag1−/− or WasdcDel Rag2−/− mice at homeostasis. To evaluate whether WASP expression in DCs contributes to disease pathogenesis, we transferred WT naive CD45RBhiCD4+ T cells into either WasdcDelRag2−/− or Rag2−/− mice. After naive CD4+ T cells transfer, both WasdcDelRag2−/− and Rag2−/− mice developed severe colitis (Supplementary Fig. 2b-2c). Only 10 weeks after transfer were significant differences in weight loss observed between WasdcDelRag2−/− and
The role of IL-23 in driving disease development, we transferred either Il23r−/− or WT naive T cells into Was−/−Rag1−/− mice. Mice that received Il23r−/− naive T cells were completely protected from colitis compared with WT naive T-cell recipients with maintenance of body weight, colonic length and reduced colonic inflammation (Fig. 3d–f). There was a reduction in the absolute number of IL-17, IL-17–IFN-γ double-positive and IFN-γ-producing cells in the colon of Was−/−Rag1−/− mice receiving Il23r−/− T cells compared with WT T cells (Fig. 3g). However, in vivo Treg generation was comparable between mice that received either Il23r−/− T cells or WT T cells (Supplementary Fig. 4b). These data support the hypothesis that macrophage-derived IL-23 and IL-1β drive disease development in Was−/−Rag1−/− mice after T-cell transfer.

**WASP regulates M1 and M2 macrophages.** We sought to explore whether BMDMs differentiation and function is also dependent on WASP. BMDMs can be differentiated in vitro into either pro-inflammatory M1 or anti-inflammatory M2 macrophages using different combinations of polarizing agents. LPS (lipopolysaccharide) and IFN-γ treatment of BMDM generates M1-type macrophage, whereas the combination of IL-4, IL-13, TGF-β and IL-10 promotes the generation of M2-type macrophages.38,29. Others and we reported the generation of highly immunosuppressive M2r type macrophages using a combination regimen of IL-4, TGF-β and IL-10. These M2r macrophages express programmed death ligand 1 (PD-L1) and PD-L2, secrete IL-10 and TGF-β, suppress T-cell proliferation and are capable of preventing diabetes in NOD mice.29 Compared with WT M2r, Was−/− M2r macrophages expressed lesser amount of M2-specific genes including Arg1, Ym1, Fizz1 and Il10 (Fig. 4a). The observed difference in M2 polarization in Was−/− mice is not due to any difference in the bone marrow progenitor population or in cultured BMDM prior to M2 differentiation (i.e., M0 cells), as they were comparable between WT and Was−/− mice both quantitatively and qualitatively. In the absence of TLR (toll-like receptor) stimulation, WT M2r macrophages express negligible amount of inflammatory mediators; upon LPS stimulation, WT M2r macrophages produce inflammatory cytokines but at significantly reduced levels when compared with M1 macrophages.30 After restimulation with LPS, Was−/− M2r macrophages expressed higher amount of inflammatory cytokines including Il1β, Il6, Tnf and Il23 compared with WT M2r macrophages (Fig. 4b). Moreover, functionally Was−/− M2r macrophages induced higher T-cell proliferation and induced less iTreg cell generation compared with WT M2r macrophages (Fig. 4c, d). Increased proliferation observed in presence of Was−/− M2r macrophages could be due to reduced expression of Arg1 as...
described by Van de Velde et al.31. In M1 polarizing conditions, Was−/− macrophages express higher amounts of M1-specific genes including Il6, Nos2 and Il1β in comparison with WT macrophages (Fig. 4e). Was−/− M1 macrophages, upon coculture with naive CD4+ T cells, induced higher T-cell proliferation compared with WT M1 macrophages (Fig. 4f). Collectively, these data indicate that WASP is critical for the differentiation and function of tolerogenic BMDM.
M2r macrophages protect Was−/−Rag2−/− mice from colitis.

Taken together, our in vivo and in vitro data suggest that macrophages lose their tolerogenic function in the absence of WASP, thereby inducing robust effector T-cell expansion and colitis. We wanted to explore whether adoptive transfer of tolerogenic M2r macrophages could prevent T-cell transfer induced colitis in Was−/−Rag2−/− mice. We previously reported that Was−/−Rag2−/− mice when transferred with unfractionated total CD4+ T cells develop severe colonic inflammation within 3 weeks. To evaluate the function of M2r macrophages, we transferred either WT or Was−/− M2r BMDM 1 day before CD4+ T-cell transfer and confirmed their presence in the LP after 7 days (Supplementary Fig. 5a). After CD4+ T-cell transfer, Was−/−Rag2−/− mice treated with WT M2r BMDM lost less body weight compared with mock-treated Was−/−Rag2−/− mice (Fig. 5a). In contrast, Was−/− M2r BMDM failed to protect Was−/−Rag2−/− mice from weight loss. Histopathology showed significant reduction in colonic inflammation in Was−/−Rag2−/− mice (***p < 0.01, ****p < 0.001, Student’s t-test) treated with WT M2r BMDM but not with Was−/− M2r BMDM (Fig. 5b). Evaluation of colonic Th cells showed a reduction in percentage of IL-17A, IFN-γ and TNF-α producing cells in WT M2r BMDM treated compared with mock-treated Was−/−Rag2−/− mice (Fig. 5c). We did not observe an increase in the percentage and number of Tregs in Was−/−Rag2−/− mice transferred with WT M2r macrophages (Fig. 5c).

Colonic tissue expression of inflammatory genes was also reduced in Was−/−Rag2−/− mice treated with WT M2r macrophages (Supplementary Fig. 5). These results indicate that colitis development in Was−/−Rag2−/− mice can be improved by restoring tolerogenic macrophage population.
Il-10 signalling is impaired in Was−/− macrophages. Differentiation of macrophages into anti-inflammatory M2-type cells is predominantly driven by transcription factors signal transducer and activator of transcription 6 (STAT6) and STAT3 downstream of IL-4, IL-13 and IL-10 signalling.29,30,32 As described above, M2r polarization of Was−/− macrophages was impaired in the presence of IL-4, IL-10 and TGF-β. The expression of IL-10R, IL-4R and TGF-βR was comparable between WT and Was−/− macrophages at homeostasis (Supplementary Fig. 6a and d). Given the role of these cytokine in macrophages differentiation, we hypothesized that WASP may have a role in the regulation of IL-4 or IL-10 signalling, or both. Upon evaluation of IL-4 signalling in BMDM we did not find any difference in STAT6 phosphorylation between WT and Was−/− BMDM, indicating functional IL-4 signalling (Fig. 6a and Supplementary Fig. 6b). To assess the role of WASP in IL-10-mediated STAT3 activation, we measured the expression of certain genes associated with M2 polarization in IL-4 and IL-10 differentiation. As shown in (g), the expression of Ym1, Arg1, and Fizz1 was significantly increased in Wild-type M2r compared to Dock8−/− M2r, indicating a functional role of WASP in M2 differentiation. Additionally, we assessed the role of WASP in the proliferation of M2r macrophages upon treatment with IL-4 and IL-10. As shown in (i), the proliferation of M2r macrophages was significantly increased in the absence of M6 and Dock8−/− M2r, indicating a functional role of WASP in M2r proliferation.
phosphorylation, we treated BMDM with IL-10 and analysed STAT3 phosphorylation at different time points. IL-10-mediated STAT3 phosphorylation was delayed and reduced in Was/−/− BMDM (Fig. 6b). However, the defect in STAT3 phosphorylation was restricted to lower concentrations of IL-10, as we could rescue STAT3 phosphorylation at increased IL-10 concentrations (Fig. 6c). In addition, we found that the differential expression of M2-specific markers in Was/−/− M2r macrophages is predominantly due to defect(s) in IL-10 signalling (Supplementary Fig. 6c). Expression of IL-10 target genes, including IL-4R and SOCS3, was also significantly reduced in Was/−/−/−/− macrophages (**p < 0.01, ***p < 0.001, Student's t-test) (Supplementary Fig. 6d and e). Taken together, our data indicate that WASP is involved in IL-10-mediated STAT3 phosphorylation and suggest that defective STAT3 activation after IL-10R binding may be responsible for aberrant M2 macrophages polarization.

WASP-DOCK8 forms a complex downstream of IL-10 signalling. Recent studies have demonstrated that DOCK8 is involved in the regulation of STAT3 phosphorylation downstream of TLR9 signalling in B cells33, IL-23R signalling in innate lymphoid cells34 and IL-6R signalling in T cells35. Moreover, DOCK8 was recently shown to directly interact with STAT335. DOCK8 also constitutively interacts with WASP through WIP (WASP-interacting protein) in T cells36. We hypothesized that WASP regulates IL-10-mediated STAT3 phosphorylation through an interaction with DOCK8. We found that IL-10-mediated STAT3 phosphorylation is also reduced in Dock8/−/− compared with WT BMDM (Fig. 6d). To examine whether WASP and DOCK8 form a complex during IL-10 signalling, we immunoprecipitated WASP from untreated, IL-10, IL-4-treated BMDM. We found that WASP interacts with DOCK8 constitutively and the interaction is enriched in the presence of IL-10 and also after M2r conditioning (Fig. 6e, f). To further assess the role of DOCK8 in M2r differentiation, we differentiated Dock8/−/− BMDM into M2r macrophages. Expression of M2-specific genes including Arg1, Ym1 andFizz1 was lesser in Dock8/−/− compared with WT BMDM (Fig. 6g). Similar to Was/−/− M2r macrophages, re-stimulation of Dock8/−/− M2r macrophages with LPS, was associated with higher expression of inflammatory genes including Il6, Tnf, Il1β and Il23p19 (Fig. 6h). In a T-cell co-culture assay, Dock8/−/− M2r macrophages induced higher T-cell proliferation compared with WT (Fig. 6i). Together, these results demonstrate that similar to WASP, DOCK8 also regulates anti-inflammatory macrophages differentiation, and that IL-10 signalling induces a WASP/DOCK8 complex.

Defective macrophage function in WAS patients. We next sought to investigate whether patients with WAS also exhibit defects in macrophage polarization and function similar to Was/−/− mice. CD14+ monocytes from peripheral blood mononuclear cells (PBMCs) of seven patient and matched healthy control were differentiated into macrophages and polarized to M1 or M2r macrophages. Similar to murine Was/−/− M1 macrophages, M1 macrophages from WAS patients expressed higher quantities of M1-specific transcripts including Cxcl10 and Ccr7 compared with M1 macrophages from healthy controls (Fig. 7a). Furthermore, in an in vitro macrophage T-cell co-culture assay, WASP-deficient human M1 macrophages induced enhanced T-cell proliferation when compared with control M1 macrophages (Fig. 7b).

In contrast, expression of human M2-specific genes including CCL13, SLC38A6, MRC1, CXC4R4 and IL10 was markedly reduced in six out of seven patients compared with healthy controls (Fig. 7c). In addition, when M2 differentiated macrophages were re-stimulated with LPS, WAS patient macrophages showed higher expression of proinflammatory genes including Il6, Tnf, Il23 and Il1β analogous to findings in murine Was/−/− M2r BMDM (Fig. 7d). Co-culture of control or WAS patient M2r macrophages with naïve T cells showed higher T-cell proliferation in the presence of WAS patient-derived M2r macrophages compared with control, indicating that human WASP-deficient M2r macrophages have higher pro-inflammatory potential (Fig. 7e). Moreover, M2r macrophages derived from WAS-deficient patients induced less iTreg cell generation compared with control M2 macrophages when co-cultured with naïve CD4+CD25− T cells (Fig. 7f). Furthermore, WAS patient-derived M2r macrophages upon co-culture with naïve T cells induces more TNF production and less IL-10 production by T cells (Fig. 7g), which infers that the T cells in the presence of WASP-deficient M2r macrophages develops into more effector-type rather than regulatory-type T-helper cells. Collectively, our data suggest that the generation and function of tolerogenic macrophages requires intact WASP signalling both in mice and human.

Discussion
Tissue resident macrophages have an important role in the maintenance of immune tolerance. In the intestinal LP, resident anti-inflammatory macrophages regulate mucosal homeostasis at least in part through the generation of regulatory T cells and suppression of T-cell proliferation. Alterations in macrophage function in the intestine, due to genetic or environmental factors, can lead to abnormal activation of innate and adaptive immune responses, and result in intestinal inflammation. Recently, several groups including our own have reported that dysfunctional anti-inflammatory macrophages in the absence of IL-10 signalling result in intestinal inflammation30,37,38. An IBD focused network analysis predicted that IL-10 and other molecules including variations...
WASP and NOD2, enriched in anti-inflammatory macrophages, act in concert to regulate intestinal immune homeostasis. Although WASP has been described to have a key role in lymphocyte function, we have previously demonstrated that WASP can also regulate innate immune cell function and intestinal homeostasis. Here we investigated the role of myeloid cell-specific WASP expression in intestinal homeostasis and postulated that WASP regulates macrophages tolerogenic function.

Although WASP modulates diverse macrophage functions, the role of WASP in the regulation of anti-inflammatory function of macrophages in the context of intestinal homeostasis has not been explored. We demonstrate that WASP facilitates the differentiation of circulating monocytes into anti-inflammatory tissue resident macrophages. We observed the accumulation of inflammatory P2 macrophages and reduction in anti-inflammatory P3/P4 macrophages within the LP in the absence of WASP. The expressions of inflammatory cytokines were also elevated in P3/P4 macrophages in Was−/− mice. A key point in these studies is that the absence of WASP not only causes a reduction in the frequency of regulatory macrophages but it also dampened the regulatory potential of P3/P4 macrophages. Using mono-
Both Was<sup>mDelRag1</sup>−/− and Was<sup>mDelRag2</sup>−/− mice we found that WASP expression in macrophages but not in DCs was essential to restrict colitis development. We further identified that deletion of WASP in macrophages upregulates expression of multiple pro-inflammatory cytokines in association with expansion of pathogenic Th1/Th17 cells and disease exacerbation.

Numerous studies have identified a role of IL-1β and IL-23 in promoting T cell mediated diseases in both human and mice<sup>23,26,42</sup>. Previously, we reported elevated colonic expression of IL-1β and IL-23 in mice lacking WASP in all innate immune cells<sup>32</sup>. Here we demonstrate that macrophage specific deletion of WASP is sufficient to drive enhanced expression IL-1β and IL-23, which promotes generation of pathogenic IL-17<sup>+</sup>IFN-γ<sup>+</sup> CD4<sup>+</sup> T cells and colitis. In addition, our study, demonstrates that colitis development in the absence of WASP requires both IL-1 and IL-23. Similar to our finding, Krause et al<sup>44</sup> reported that enhanced macrophage-derived IL-23, in the absence of myeloid-specific IL-10, causes increased mortality in infectious colitis model. Our work complements a recently reported human study that describes a WAS patient with autoimmune inflammatory diseases, pan colitis and perianal abscesses who showed improvement after treatment with an IL-1 receptor antagonist<sup>44</sup>

Several prior studies have shown that WASP plays an important role in Treg function<sup>15–47</sup>. Here we observe that aberrant macrophage differentiation and function associated with selective WASP deficiency in macrophages affects Treg cell generation. Previously we demonstrated a decrease in iTreg cell generation in Was<sup>mDelRag1</sup>−/− mice transferred with CD4<sup>+</sup> T cells<sup>32</sup>. Our result using Was<sup>mDelRag1</sup>−/− mice re-confirmed these findings suggesting that the defect in iTreg generation may be due, at least in part, to defects in macrophage function. This reduction in Treg cell generation could be due to elevated colonic expression of IL-23 as described by Ahern et al<sup>26</sup>. Together we can conclude that the elevated expression of inflammatory mediators by WASP-deficient macrophages causes expansion of pathogenic T cell and reduction of Treg cells, which ultimately lead to breakdown of immune tolerance.

Similar to in vivo observations in the colon, the generation and function of M1 and M2 macrophages from BM was also aberrant. The generation and function of M1 and M2r macrophages from BM was also aberrant. Importantly, analogous to our in vivo observations in the colon, the generation and function of anti-inflammatory macrophages partially rescued colonic inflammation in Was<sup>mDelRag2</sup>−/− mice after T cell transfer. Prior studies have reported amelioration of inflammation in several models of colitis with anti-inflammatory macrophages<sup>15,20</sup>. These observations support our findings that macrophages have (WASP-dependent) tolerogenic properties. Importantly, analogous to our findings in mice, we observed defects in the generation and function of anti-inflammatory macrophages in WAS patients. Monocyte-derived M2r macrophages from WAS patients showed aberrant tolerogenic properties. These data suggest that polymorphisms that impact WASP expression may play a role in tolerogenic macrophage function and intestinal homeostasis. Indeed, about 10% of patients with WAS develop IBD<sup>48</sup>. Together, our results imply a critical role for WASP in macrophages in regulating mucosal tolerance in both mice and human.

Many studies have shown the involvement of IL-10- and STAT3-dependent signalling pathways in regulatory macrophage function. We recently reported that IL-10-mediated STAT3 phosphorylation is critical for regulatory macrophage function and intestinal homeostasis<sup>30</sup>. Moreover, selective deletion of STAT3 in macrophages causes spontaneous colitis in mice<sup>35</sup>. Here we identify a mechanistic role for WASP in specifically regulating IL-10-dependent STAT3 phosphorylation. DOCK8, a guanine nucleotide exchange factor, known to interact with WASP<sup>49–51</sup>, has been recently reported to regulate STAT3 phosphorylation in multiple cell types (including B cells, innate lymphoid cells and T cells) downstream of TLR9-, IL-23- and IL-6-dependent signals<sup>33–35</sup>, and our data specifically links WASP to STAT3 phosphorylation downstream of IL-10 signalling in macrophages. The interaction between DOCK8 and WASP in T cells appears to be mediated through WIP. Our studies indicate that WASP and DOCK8 also interact in macrophages and that this interaction is enhanced by IL-10-stimulation. Defective macrophage differentiation and IL-10-dependent STAT3 phosphorylation in DOCK8-deficient macrophages further strengthens our conclusion that WASP, together with DOCK8, regulates anti-inflammatory macrophage function and protects from colitis development. Although IL-10-dependent signalling induces the formation of a WASP-DOCK8 signalling complex, further experimentation is needed to delineate the role of a WASP-DOCK8 complex in the regulation of IL-10 signalling. Report of IBD patients with polymorphisms in DOCK8 gene<sup>50</sup> supports our notion of involvement of a WASP-DOCK8 signalling axis in the regulation of tolerogenic macrophage function and intestinal homeostasis.

In summary, we have demonstrated that macrophage-selective expression of WASP is critical for the development of anti-inflammatory functions by LP and BM-derived macrophages. In WAS-deficient macrophages, expression of inflammatory cytokines is elevated leading to exacerbated Th1/Th17 helper cell response and abrogated iTreg generation. These observations support our findings that WASP-specific macrophage deficiency causes expansion of pathogenic T cells and reduction of Treg cells, which ultimately lead to breakdown of immune tolerance.

**Methods**

Mice. C57BL/6 background Was<sup>fl</sup>/<sup>fl</sup> mice have been described previously<sup>52</sup> and were crossed with LysM<sup>Cre</sup> (The Jackson Laboratory, Stock No. 004781) or Itgax<sup>Cre</sup> (The Jackson Laboratory, Stock No. 008068) mice obtained from Jackson Laboratories to generate Was<sup>fl</sup>/LysM<sup>Cre</sup> and Was<sup>fl</sup>/Itgax<sup>Cre</sup> mice. Was<sup>fl</sup>/LysM<sup>Cre</sup> and Was<sup>fl</sup>/CD11c<sup>cre</sup> mice were subsequently crossed with Rag1<sup>−/−</sup> (The Jackson Laboratory, Stock No. 002216) or Rag2<sup>−/−</sup> (Taconic, Stock No. RAGN12) mice to generate Was<sup>fl</sup>/LysM<sup>Cre</sup>Rag1<sup>−/−</sup> (Was<sup>mDelRag1</sup>−/−) and Was<sup>fl</sup>/Itgax<sup>Cre</sup>Rag2<sup>−/−</sup> (Was<sup>mDelRag2</sup>−/−) mice, respectively, for CD4<sup>+</sup> T-cell-transfer-induced colitis studies. Was<sup>−/−</sup>, Was<sup>fl</sup>/<sup>−</sup>Rag2<sup>−/−</sup> and Was<sup>−/−</sup>/Rag2<sup>−/−</sup> mice in 129SvEv background were generated as previously described<sup>48</sup>. Il1r<sup>−/−</sup> mice were obtained from Jackson Laboratories. Dr. Vijayalakshmi Raghavan and Women’s Hospital, Boston kindly provided Il23r<sup>−/−</sup> mice<sup>53</sup> and Dr. Ralph Geha at Boston Children’s provided Dock8<sup>−/−</sup> mice<sup>46</sup>. All the mice were housed in micro-isolator cages in a specific pathogen-free animal facility at Boston Children’s Hospital. Sex- and age-matched animals between 5 and 14 weeks of age were used for experiments. We did not use randomization to assign animals to experimental groups. Investigators were not informed regarding the composition of experimental groups.

**Isolation of LP cells.** Cells were isolated from LP as described previously<sup>52</sup>. Briefly, colons were removed and placed in ice-cold phosphate-buffered saline (PBS) and the intestine is cut open longitudinally. Roughly 1.5 cm pieces of colon tissues were incubated in Hank’s balanced salt solution (HBSS) containing 2% fetal bovine serum (FBS), 10 mM EDTA, 1 mM dithiothreitol and 5 mM HEPES at 37 °C with...
shaking for 30 min to remove the epithelial cell layer. After removal of the epithelial layer, tissues were washed in PBS and digested in buffer containing HBSS, 20% FBS and collagenase VIII (200 unit/ml) for 60 min. The cells from digested tissues were filtered and washed once in cold PBS before re-suspending in 40% Percoll. Cells suspension was overlaid on 80% Percoll and centrifuged for 20 min at 2000 r.p.m. at room temperature. LP cells were collected from the interface of the Percoll gradient. LP macrophages were gated as CD45+ CD11b+ CD64+ CD103− cells and positively stained (FACS). Finally recovered macrophages were distinguished based on the expression of Ly6c and MHC II (Supplementary Fig. 1a).

Generation of bone marrow chimera mice. CD45.2 Was−/− recipient mice were irradiated with 1000 rad (Gamma Cell 40, 137Ca) in 2 doses of 500 rad each 4 h apart. Bone marrow cells from both femurs and tibiae of B6.SJL (CD45.1) and Was−/− (CD45.2) donors were collected by flushing with conditions and suspended in PBS for injection. Bone marrow cells from B6.SJL (CD45.1) and Was−/− (CD45.2) mice were mixed at a 1:1 ratio and injected intravenously into each recipient mouse. The ratio of the bone marrow cells was confirmed by flow cytometry. Recipient mice were housed under specific pathogen-free conditions and were provided autoclaved water with sulphuram (trimethoprim-sulphamethoxazole) and fed autoclaved food for 3 weeks. After 3 weeks, they were provided normal food and water. Eight to 10 weeks after the injection macrophage population in the colon were examined.

Induction of colitis. In this study colitis was induced by transferring naïve CD45RB+CD4 T cell in 8- to 10-week-old Rag1−/−, Rag2−/−, Was−/−Rag1−/− and Was−/−Rag2−/− mice. Splenocytes and lymph node cells from WT thymus were enriched for CD4+ T cells by magnetic-activated cell sorting (MACS) using mouse CD4+ T cells isolation kit II (Miltenyi Biotec, Catalog Number 130-104-454). Naïve CD4+ T cells (Tcrβ+CD4+CD45RB+β2m−) were fluorescence-activated cell (FACS) sorted from MACS-enriched CD4+ T cells. Purity after sorting was >98%. Naïve CD4+ T cells (3 × 105) was transferred intraperitoneally (ip) into each recipient. Body weight was monitored on a weekly basis. In the macrophage-mediated colitis rescue experiments 1 × 106 unfractionated CD4+ T cells was transferred ip. Into Was−/−Rag2−/− and Was−/− mice. One day before T-cell transfer, mice were treated with 2 × 106 macrophages form WT or Was−/− mice. Body weight was monitored on a weekly basis.

Imaging and histology scoring. An upright microscope (BX-41; Olympus) with bright-field and epifluorescence capability with SPOT imaging software and a DP70 color charge-coupled device camera were used for imaging colonic tissue. Fluorescence was assessed using a BD Canto II Flow Cytometer (BD Biosciences), and percentages of subsets and mean fluorescence intensity were analyzed with FlowJo software, versions 9 and 10.0.8. For intracellular FOXP3 staining, cells were fixed and permeabilized with the Fixation/Permeabilization Solution Kit (eBioscience) according to the manufacturer’s instructions after surface staining. Cells were stained for 30 min at room temperature with Abs and washed twice with the permeabilization buffer. For intracellular staining of cytokines (TNF, IL-17A, IL-10, and IFN-γ), cells were incubated with PMA (10 ng/ml), ionomycin (500 ng/ml) and GolgiStop (BD Biosciences) in RPMI Media 1640 (Life Technologies) supplemented with 10% FBS and antibiotics for 5 h at 37°C to stimulate cytokine production. Fluorescence-labelled Abs used were listed in Supplementary Table 1.

Western blotting and immunoprecipitation. After treating the BMDMs as mentioned above cells were lysed in RIPA buffer on ice for 1 h. For the immunoblot analysis, 30 μg of protein were resolved by 4–20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBS-T (0.1% Tween 20) for 1 h before incubation overnight at 4°C with primary antibodies. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies in 5% non-fat dry milk in TBS-T for 1 h. After successive washes, the membranes were developed using an ECL Western Chemiluminescent kit (Thermo Scientific). Immunoprecipitations with anti-WASP antibodies were performed on pre-cleared BMDM cell lysate using Protein A/G magnetic beads.
(Thermo Scientific) at 4 °C for 14–16 hr. The beads were washed three times in washing buffer (20 mM Tris-HCl pH 7.4 and 0.1% Nonidet P-40) and samples were boiled for 10 min in 25 μl of loading buffer and subjected to SDS-PAGE and immunoblot analysis. Antibodies used were listed in Supplementary Table 1.

**Statistics.** All data were analysed by Student’s t-test with 95% confidence interval or analysis of variance using GraphPad Prism version 6.0 (GraphPad Software) and presented as mean ± SEM. Normal distribution was assumed. A P-value of < 0.05 was considered statistically significant. (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001; NS, not significant).

**Study approval.** All patients provided written informed consent prior to inclusion in the study. Clinical patient samples were collected under a Boston Children’s Hospital IRB-approved research protocol (PO0000529). All animal experiments were performed in accordance with Institutional Animal Care and Use Committee-approved protocols number 14-04-2677 R (IACUC, Boston Children’s Hospital) and adhered to the National Research Council’s ‘Guide to the care and Use of Laboratory Animals’.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files, or are available upon reasonable requests to the authors.

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Author contributions
Conceived and designed the experiments: A.B. and S.B.S. Performed experiments: A.B., D.S., A.G., M.F., J.A.G., L.K., Y.H.K. and N.S.R. Acquired and analysed data: A.B. and D. S. Provided reagents and clinical patient samples: E.J., R.S.G., A.T., V.K.K., L.D.N. and S. Y.P. Wrote the manuscript: A.B. and S.B.S. Edited the manuscript: D.S. and B.H.H. Critical review of data and provided suggestions: T.C. and B.H.H. All authors approved the final version of the manuscript.

Additional information

Competing interests: The authors declare no competing interests.

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