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NLRP6 function in inflammatory monocytes reduces susceptibility to chemically-induced intestinal injury

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Abstract

NLRP6 is a member of the Nod like receptor family, whose members are involved in the recognition of microbes and/or tissue injury. NLRP6 was previously demonstrated to regulate the production of IL-18 and is important for protecting mice against chemically-induced intestinal injury and colitis-associated colon cancer. However, the cellular mechanisms by which NLRP6 reduces susceptibility to colonic inflammation remain unclear. Here, we determined that NLRP6 expression is specifically upregulated in Ly6C^{hi} inflammatory monocytes that infiltrate into the colon during dextran sulfate sodium (DSS)-induced inflammation. Adoptive transfer of WT Ly6C^{hi} inflammatory monocytes into *Nlrp6*^{-/-} mice was sufficient to protect them from mortality, significantly reducing intestinal permeability and damage. NLRP6-deficient inflammatory monocytes were defective in TNF α production, which was important for reducing DSS-induced mortality and was dependent on autocrine IL-18 signaling by inflammatory monocytes. Our data reveal a previously unappreciated role for NLRP6 in inflammatory monocytes, which are recruited after DSS-induced intestinal injury to promote barrier function and limit bacteria-driven inflammation. This study highlights the importance of early cytokine responses, particularly NLRP6-dependent and IL-18-dependent TNF α production, in preventing chronic dysregulated inflammation.

Keywords

colitis; inflammatory monocytes; TNF α ; NLRP6

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Disclosure

The authors have declared that no conflict of interest exists.

Introduction

Inflammatory bowel disease (IBD) is a major health problem that results in significant morbidity and mortality due to recurrent episodes of bloody diarrhea, weight loss, and complications from chronic intestinal inflammation, such as cancer. Current approved therapies for IBD rely primarily on symptom management and inhibiting inflammation, but do not address the underlying mechanism behind IBD pathogenesis. The current prevailing model for the pathogenesis of IBD is the development of an imbalance in the microbial community structure within the intestine, or dysbiosis, and/or aberrant immune responses to commensal bacteria in a genetically susceptible host.¹ A better understanding of events that lead to dysregulated inflammation is needed to identify targets for treatment and cure.

Recently, the Nod like receptor (NLR), NLRP6, has been shown to be important for promoting intestinal homeostasis and for protection against the development of colitis and colitis associated tumorigenesis in mice.¹⁻⁴ The mechanism by which NLRP6 protects against colitis remains to be fully elucidated. Gene ontology analyses have established a link between NLRP6 and intestinal epithelial cell (IEC) repair.⁴ NLRP6 also regulates IL-18 production, which is important for epithelial repair and protection against inflammation-associated colon tumorigenesis.^{1, 2, 4-6} NLRP6 is highly expressed in IECs, and bone marrow (BM) chimera experiments demonstrated that IL-18 production by epithelial cells is important for reduced susceptibility to DSS-induced intestinal injury and inflammation, suggesting that NLRP6 functions primarily in epithelial cells to promote IL-18 production and epithelial repair. NLRP6 also regulates autophagy within intestinal goblet cells to affect mucus secretion, which contributes to intestinal homeostasis.⁷ However, NLRP6 is also expressed in hematopoietic cell populations, and we have previously demonstrated that NLRP6 functions in BM-derived cells to limit colitis-associated tumorigenesis.¹ Thus, the specific cell type that is important for NLRP6-mediated protection against the development of colitis and tumorigenesis has not been definitively identified.

In this study, we examined the expression of NLRP6 in various hematopoietic cell populations within the lamina propria (LP) during DSS-induced intestinal injury and determined that inflammatory monocytes, defined as CD11b⁺Ly6G^{int}Ly6C^{hi} cells, induce NLRP6 expression in response to DSS. More importantly, the adoptive transfer of wildtype (WT) Ly6C^{hi} monocytes into *Nlrp6*^{-/-} mice improves survival after DSS treatment. NLRP6-deficient Ly6C^{hi} monocytes had impaired production of TNF α and reactive oxygen species (ROS), and the injection of recombinant TNF α (rTNF α) into *Nlrp6*^{-/-} mice early during DSS treatment was sufficient for protection against mortality. We further demonstrate that *Il18*^{-/-} and *Il18r1*^{-/-} Ly6C^{hi} inflammatory monocytes have similar defects in TNF α production, and their adoptive transfer into *Nlrp6*^{-/-} mice failed to rescue DSS-induced mortality, suggesting that autocrine IL-18 signaling by inflammatory monocytes is important for TNF α production and protection against acute intestinal injury. Altogether, these studies reveal a protective role for early TNF α production by inflammatory monocytes, which is at least, in part, IL-18- and NLRP6-dependent and is critical for limiting dysregulated commensal-driven intestinal inflammation.

Results

NLRP6 function in Ly6C^{hi} inflammatory monocytes reduces susceptibility to DSS-induced intestinal injury

We previously demonstrated that *Nlrp6*^{-/-} mice are more susceptible to DSS-induced colitis as well as colitis-associated tumorigenesis after treatment with the carcinogen azoxymethane (AOM) and DSS.^{1, 2, 4} Furthermore, NLRP6 activity in BM-derived cells was important for limiting inflammation-associated tumors.¹ To identify the cell type responsible for the protective effects of NLRP6, expression of NLRP6 was measured in different cell populations in the BM and colon LP before and after DSS treatment. We analyzed NLRP6 mRNA expression in IECs, intraepithelial lymphocytes (IELs), BM, and LP cells from WT mice on day 0 and day 10 (at the end of 5 days of 2% DSS) in the AOM/DSS model of colitis-associated tumorigenesis.¹ We determined that NLRP6 expression was upregulated in the LP, but not IEC, IEL or BM cells in response to DSS (Figure 1a). Within the LP, NLRP6 was specifically increased in myeloid cells, and in particular, Ly6C^{hi} monocytes and neutrophils, after DSS treatment with the highest induction in Ly6C^{hi} inflammatory monocytes (Figure 1b). In contrast, NLRP6 expression did not change in T cells and was undetectable in B cells (Figure 1b). We confirmed the upregulation of NLRP6 expression in LP cells and myeloid cells within the LP in WT mice treated with DSS only, indicating that the observed change in NLRP6 expression is not dependent on AOM (Supplementary Figure 1).

Upregulation of NLRP6 in response to DSS in Ly6C^{hi} inflammatory monocytes prompted us to investigate if NLRP6 function in this population of cells was important for maintaining intestinal homeostasis. WT Ly6C^{hi} monocytes (CD3⁻ CD11b⁺ Ly6C^{hi} Ly6G⁻) were FACS-sorted to approximately 99% purity (Figure 1c), and were adoptively transferred into *Nlrp6*^{-/-} mice on day 3.5 of a 7-day course of high dose DSS (3.5%). In contrast to mock treated *Nlrp6*^{-/-} mice, which had a 15% survival rate after DSS treatment, *Nlrp6*^{-/-} mice that received Ly6C^{hi} monocytes were protected from lethality with a survival rate of approximately 70% (Figure 1c). Consistent with improved survival, *Nlrp6*^{-/-} mice harboring WT Ly6C^{hi} monocytes also showed significantly less weight loss (Figure 1d). These results strongly suggest that NLRP6 is upregulated in inflammatory monocytes that are important for reducing DSS-induced mortality. To demonstrate that donor Ly6C^{hi} monocytes can be recruited to the intestine during DSS treatment, we adoptively transferred Ly6C^{hi} monocytes isolated from transgenic mice that express green fluorescent protein (GFP) into *Nlrp6*^{-/-} mice during DSS treatment, and detected GFP⁺ Ly6C^{hi} monocytes in the LP of recipient mice (Supplementary Figure 2a). As confirmation that the recruitment of inflammatory monocytes into the intestinal LP is important for NLRP6-mediated protection against DSS-induced injury and inflammation, we adoptively transferred *Nlrp6*^{-/-} mice with Ly6C^{hi} monocytes isolated from mice deficient in CCR2, which is expressed by inflammatory monocytes and is required for their recruitment to sites of inflammation,⁸ and observed no improvement in weights or survival with DSS treatment (Supplementary Figure 2b-d).

Ly6C^{hi} monocytes can be further characterized by their CX3CR1 expression. During intestinal inflammation, Ly6C^{hi} CX3CR1^{int} inflammatory monocytes are recruited to the

LP.⁹ Consistently after 5 days of DSS, all Ly6C^{hi} monocytes in the LP of WT and *Nlrp6*^{-/-} mice express CX3CR1 (Supplementary Figure 3a). To determine CX3CR1 expression in Ly6C^{hi} donor monocytes in adoptive transfer experiments and track their recruitment in the intestine during DSS treatment, we used CX3CR1-GFP mice, in which CX3CR1-expressing cells are labeled with GFP, as donors and *Ccr2*^{-/-} mice as recipients. Ly6C^{hi} donor monocytes were mostly CX3CR1^{int} (~80%), (Supplementary Figure 3b), but after transfer into *Ccr2*^{-/-} mice treated with DSS, all donor CCR2⁺ cells in the LP were CX3CR1^{int} (Supplementary Figure 3c). These results strongly suggest that Ly6C^{hi} monocytes that migrate into the colon in this model and have protective effects in *Nlrp6*^{-/-} mice represent a relatively homogenous population of CX3CR1^{int} inflammatory monocytes.

WT Ly6C^{hi} monocytes limit tumor growth in *Nlrp6*^{-/-} mice

We had previously reported that *Nlrp6*^{-/-} mice developed more colon tumors compared to WT mice in the AOM/DSS model of inflammation-associated tumorigenesis.¹ To determine whether NLRP6 function in inflammatory monocytes is important for reducing inflammation-associated tumorigenesis, we adoptively transferred WT Ly6C^{hi} monocytes into *Nlrp6*^{-/-} mice 3.5 days after the start of each cycle of 2% DSS (Figure 2a). As previously published, *Nlrp6*^{-/-} mice developed more tumors compared to WT mice (Figure 2b).¹ Adoptive transfer of WT Ly6C^{hi} monocytes resulted in fewer tumors; however, this did not reach statistical significance (Figure 2b). The transfer of WT Ly6C^{hi} monocytes into *Nlrp6*^{-/-} mice did result in a significant reduction in tumor sizes with fewer tumors greater than 2 mm in size compared to that in *Nlrp6*^{-/-} mice (Figure 2c), and reduced weight loss early during AOM/DSS treatment (days 14-26, Figure 2d).

NLRP6 function in Ly6C^{hi} monocytes promotes resistance against DSS-induced damage

To determine the effects of WT Ly6C^{hi} monocytes within the colon of *Nlrp6*^{-/-} mice during the acute inflammatory response to DSS, we looked specifically on day 10 of the AOM/DSS model of tumorigenesis when mice have completed 5 days of 2% DSS prior to the onset of chronic, dysregulated inflammation in *Nlrp6*^{-/-} mice.¹ Increased intestinal permeability is a classical hallmark of DSS induced IEC damage.^{1, 2, 4} To determine if the adoptive transfer of WT Ly6C^{hi} monocytes affected intestinal permeability, mice were gavaged with FITC-dextran and its passage into the peripheral circulation was measured. As demonstrated previously,¹ naïve WT and *Nlrp6*^{-/-} mice had very low levels of serum fluorescence indicative of an intact epithelial barrier, whereas DSS-treated mice, especially *Nlrp6*^{-/-} mice, had higher levels of serum fluorescence suggesting increased intestinal permeability (Figure 3a).¹ Importantly, the intestinal permeability of *Nlrp6*^{-/-} mice adoptively transferred with WT Ly6C^{hi} monocytes was similar to that of WT mice after exposure to DSS, and was significantly reduced compared to that of mock-treated *Nlrp6*^{-/-} mice (Figure 3a). Consistently, there was a reduction in bacterial translocation into the mesenteric lymph nodes (MLNs) as measured by qPCR in *Nlrp6*^{-/-} mice adoptively transferred with WT Ly6C^{hi} monocytes (Figure 3b). Levels of fecal lipocalin-2, a surrogate marker of intestinal epithelial damage,¹⁰ were also reduced in *Nlrp6*^{-/-} mice adoptively transferred with WT Ly6C^{hi} monocytes (Figure 3c). Histological scoring of colitis demonstrated reduced intestinal epithelial damage and inflammation in the presence WT Ly6C^{hi} monocytes

(Figure 3d). Thus, NLRP6 activity in Ly6C^{hi} inflammatory monocytes is important in limiting DSS-induced damage.

***Nlrp6*^{-/-} Ly6C^{hi} inflammatory monocytes have impaired TNF α and ROS production**

Inflammatory Ly6C^{hi} monocytes perform critical functions during inflammation, including phagocytosis, production of ROS, and secretion of cytokines.¹¹ These functions limit bacterial translocation, promote tissue repair, and ultimately, help to resolve inflammation. To determine whether *Nlrp6*^{-/-} Ly6C^{hi} inflammatory monocytes have a defect in phagocytosis, Ly6C^{hi} monocytes were isolated from naïve and AOM/DSS-treated WT and *Nlrp6*^{-/-} mice (day 10).¹ In agreement with previous studies, we observed no defect in phagocytosis as BM-sorted Ly6C^{hi} monocytes from WT and *Nlrp6*^{-/-} mice exhibited equivalent levels of internalized GFP⁺ *E.coli* (Figure 4A).¹² However, ROS production was significantly reduced in BM-derived and LP-isolated *Nlrp6*^{-/-} Ly6C^{hi} monocytes as compared to WT Ly6C^{hi} monocytes after DSS treatment (Figure 4b, c). We next measured levels of various cytokines produced by LP inflammatory Ly6C^{hi} monocytes isolated from AOM/DSS-treated *Nlrp6*^{-/-} mice and cultured overnight. *Nlrp6*^{-/-} LP-derived inflammatory monocytes exhibited decreased early production of TNF α at the completion of DSS, whereas other cytokines such as IL-6 and IL-1 β were produced at levels similar to that by WT Ly6C^{hi} monocytes (Figure 4d). Consistently, TNF α mRNA expression was also significantly reduced in *Nlrp6*^{-/-} LP Ly6C^{hi} monocytes (Figure 4e). By day 16, however, approximately one week after completion of DSS, TNF α levels were no longer significantly different between WT and *Nlrp6*^{-/-} inflammatory monocytes, suggesting that NLRP6-independent pathways may be compensating (Figure 4d). The defect in TNF α production was specific to *Nlrp6*^{-/-} inflammatory monocytes as *Nlrp6*^{-/-} resident LP macrophages, defined as CD11b⁺Ly6C^{low/-} F4/80^{high}Ly6G⁻ cells,⁹ exhibited no differences in cytokine production compared to WT (Supplementary Figure 4). In mice treated with 5 days of DSS alone, we observed similar defects in TNF α production, but not IL-1 β , by *Nlrp6*^{-/-} Ly6C^{hi} monocytes associated with increased fecal lipocalin levels in *Nlrp6*^{-/-} mice (Supplementary Figure 5).

To determine if NLRP6 upregulation is associated with TNF α production, we sorted Ly6C^{hi} monocytes from the BM of WT mice, and stimulated them with rosiglitazone, which was previously demonstrated to increase NLRP6 expression in colon epithelial cells.¹³ We indeed observed upregulation of NLRP6 expression when WT monocytes were treated with rosiglitazone (Supplementary Figure 6a) along with increased TNF α production in WT macrophages, which did not occur to the same extent in *Nlrp6*^{-/-} Ly6C^{hi} monocytes (Supplementary Figure 6b), suggesting that the TNF α response is indeed partially NLRP6 dependent. We next determined whether a defect in the recruitment of *Nlrp6*^{-/-} inflammatory monocytes into the LP was associated with decreased TNF α production. However, examination of monocyte populations in the LP of WT and *Nlrp6*^{-/-} mice on day 0 and day 10 after completion of 5 days of DSS showed, in fact, increased numbers of Ly6C^{hi} monocytes recruited to the LP of *Nlrp6*^{-/-} mice (Supplementary Figure 7). In order to determine if the impairment in TNF α production by *Nlrp6*^{-/-} Ly6C^{hi} monocytes was not a result of global deficiency in cytokine responses, we isolated inflammatory Ly6C^{hi} monocytes from the LP of WT and *Nlrp6*^{-/-} mice on day 10 of AOM/DSS treatment and

performed gene expression analysis by microarray. Functional pathway analysis revealed increased mRNA expression of several cytokines and chemokines in *Nlrp6*^{-/-} Ly6C^{hi} cells, while TNF α was one of the few cytokines that was the most reduced (Supplemental Figure 8).

Early production of TNF α is important for resistance against DSS-induced intestinal injury

Elevated levels of TNF α have frequently been associated with disease severity in chronic IBD patients;¹⁴ however, TNF α also promotes epithelial restitution after DSS-induced injury.¹⁵ To determine whether the early production of TNF α by inflammatory monocytes is important for protection against intestinal damage and DSS-induced mortality, *Nlrp6*^{-/-} mice were injected with rTNF α on days 3-7 during a 7 day course of high dose 3.5% DSS. As expected, the administration of rTNF α into WT mice had no effect on their survival (Figure 5a-b). In contrast, *Nlrp6*^{-/-} mice that received rTNF α showed significant improvement in survival with decreased weight loss compared to *Nlrp6*^{-/-} mice that did not receive supplemental rTNF α . There was reduced colonic inflammation and damage as suggested by decreased lipocalin-2 levels as well as less bacterial translocation into the MLNs in *Nlrp6*^{-/-} mice that received rTNF α (Figure 5c-d). Finally, histologic examination of colons revealed a significant improvement in inflammatory scores for *Nlrp6*^{-/-} mice that received rTNF α after 5 days of 2% DSS (day 10 AOM/DSS model), reflecting reduced levels of epithelial damage, hyperplasia, and inflammation (Figure 5e). Altogether, these results suggest that NLRP6 dependent production of TNF α by WT inflammatory monocytes is important for limiting intestinal damage and bacterial translocation, thereby reducing DSS-induced inflammation and mortality.

Production of TNF α by Ly6C^{hi} inflammatory monocytes is dependent on IL-18 signaling

We have previously demonstrated that *Nlrp6*^{-/-} mice have impaired production of mature IL-18, which is important for epithelial repair to limit commensal-driven inflammation.¹ Although it was previously shown that IL-18 is predominantly produced by the intestinal epithelium,³ LP cells are also capable of secreting IL-18.¹⁶ Importantly, we also observed reduced levels of IL-18 production by Ly6C^{hi} inflammatory monocytes isolated from DSS-treated *Nlrp6*^{-/-} mice compared to that from WT (Supplementary Figure 9). To determine if IL-18 production by Ly6C^{hi} monocytes is important for resistance to DSS induced mortality, *Nlrp6*^{-/-} mice were adoptively transferred with *Il18*^{-/-} Ly6C^{hi} monocytes, and survival of these mice was compared to that of mock-transferred *Nlrp6*^{-/-} and WT mice after 7 days of 3.5% DSS (Figure 6a). *Nlrp6*^{-/-} mice that received *Il18*^{-/-} monocytes had similar levels of mortality and weight loss compared to *Nlrp6*^{-/-} mice (Figure 6a,b). Interestingly, Ly6C^{hi} monocytes isolated from DSS treated *Il18*^{-/-} mice also had impaired production of TNF α , but not IL-1 β , indicating that TNF α production by Ly6C^{hi} monocytes is, in part, IL-18 dependent (Figure 6c).

To determine whether inflammatory monocytes respond to IL-18 to promote TNF α production and resistance to DSS, *Nlrp6*^{-/-} mice were also adoptively transferred with *Il-18R*^{-/-} monocytes and survival measured after treatment with high-dose DSS. *Nlrp6*^{-/-} mice that received *Il-18R*^{-/-} monocytes were equally susceptible to DSS induced mortality and weight loss as mock-treated *Nlrp6*^{-/-} mice (Figure 6d, e). Furthermore, cultures of

Ly6C^{hi} cells isolated from DSS-treated *Il18r1*^{-/-} mice also exhibited a defect in TNF α production, while secretion of IL-1 β was actually increased rather than decreased (Figure 6f). These data strongly suggest that autocrine IL-18 signaling by inflammatory monocytes is important for regulating TNF α production early during the acute inflammatory response to DSS to promote resistance to intestinal injury and lethality.

Discussion

NLRP6 is a member of the NLR family that is important for maintaining intestinal homeostasis.³ Our studies provide important insights into the mechanism by which NLRP6 protects against the development of colitis. We show that NLRP6 functions particularly in Ly6C^{hi} inflammatory monocytes that are recruited to the intestinal LP during inflammation to protect the host against DSS-induced epithelial injury and subsequent life-threatening immunopathology. NLRP6 deficiency in inflammatory monocytes was also associated with defective TNF α production early in response to DSS, which, in turn, is important for reducing mortality. Our data further suggest that TNF α production is mediated by autocrine IL-18 signaling by Ly6C^{hi} inflammatory monocytes. Therefore, we propose a model in which NLRP6 activation in inflammatory monocytes recruited in response to DSS-induced inflammation results in IL-18 secretion that upregulates their production of TNF α , which promotes epithelial repair and timely resolution of damage.¹⁵

Although IL-18 levels can be elevated in patients with active inflammatory disease,^{16, 17} studies in mice have also demonstrated the protective effects of IL-18 as *Il18*^{-/-} and *Il18r1*^{-/-} mice are more susceptible to colitis and colitis-associated colon tumorigenesis.¹⁸ This discrepancy may reflect differential roles of IL-18 that is dependent on timing and context. The production of IL-18 early during DSS-induced inflammation is important for epithelial restitution and resolution of inflammation, but in the setting of chronic, dysregulated inflammation, excessive IL-18 production can exacerbate disease.¹⁹ Consistently, impairment in IL-18 production during DSS-induced colitis by NLRP6-deficient mice is observed only early on during and after DSS treatment before the development of significant intestinal damage and inflammation.¹ Our data demonstrates that *Nlrp6*^{-/-} Ly6C^{hi} monocytes in the LP also have impaired early IL-18 production. However, it is not currently known whether the reduced IL-18 secretion is due to decreased inflammasome-mediated caspase-1 activation or reflects a defect in a non-canonical mechanism that remains to be identified. In addition, the mechanism for IL-18-mediated protection is unclear. Previous studies suggested that IL-18 is predominantly expressed by the epithelium and that epithelial-derived IL-18 is important for protection against DSS-induced damage.^{3, 20} IL-18 production is also associated with upregulation of IL-22, which is implicated in epithelial repair.²¹ Our study suggests an additional mechanism for IL-18 function, which is to upregulate TNF α production in inflammatory monocytes.

TNF α levels can be significantly elevated in IBD patients, and TNF α antagonism is an approved therapy for IBD. The excessive production of TNF α observed in patients with IBD and in mouse models of colitis likely reflects active disease that is already chronic and dysregulated. However, similar to IL-18, early production of TNF α is important for tissue repair and maintaining intestinal homeostasis.^{15, 22-24} Germ free mice or mice that lack the

adaptor protein MyD88, which functions downstream of TLR-mediated bacterial sensing, have significant DSS-induced epithelial damage associated with the lack of induction of reparative cytokines, including TNF α .^{15, 25} In addition, TNF α blockade in WT mice results in increased susceptibility to colitis.^{22, 24} *Tnfr1*^{-/-} or *Tnfr2*^{-/-} mice as well as *Tnfa*^{-/-} mice also develop more severe DSS-induced colitis with elevated levels of pro-inflammatory cytokines, increased epithelial damage, and increased mortality as compared to WT mice.^{24, 26-28} Although there has been conflicting data that demonstrate a pathogenic role for TNFR2 in DSS-induced colitis,²⁹ inflammatory monocytes are more likely to express TNFR1, while TNFR2 upregulation in colonic epithelial cells is observed in patients with inflammatory bowel disease.^{30, 31} Altogether, these studies are consistent with our data since *Nlrp6*^{-/-} mice that receive rTNF α early during DSS treatment exhibit reduced intestinal inflammation and bacterial translocation.

We previously demonstrated that NLRP6 functions in a BM-derived cell to limit inflammation-associated tumorigenesis.¹ In the current study, we show that NLRP6 was induced specifically in myeloid cells within the LP during DSS-induced colitis. Furthermore, NLRP6 activity in Ly6C^{hi} monocytes is important for reducing susceptibility to DSS-induced colitis. Despite the protection against the detrimental effects of inflammation, we did not see a statistically significant reduction in the number of tumors. Rather, the size of tumors was significantly decreased, suggesting that NLRP6 function in monocytes limits inflammation that primarily drives tumor growth. Consistently, the downregulation of inflammatory responses by removal of NF- κ B activity in myeloid cells also resulted in reduced tumor size rather than tumor number.³³ However, it is also possible that the administration of Ly6C^{hi} monocytes only once with each DSS cycle is insufficient to affect tumor number, or that another BM-derived cell in addition to monocytes is necessary for protection.

There are two major populations of monocytes in the murine intestinal LP, classical Ly6C^{hi} inflammatory monocytes and Ly6C⁻ monocytes that differentiate into macrophages.¹¹ Ly6C^{hi} monocytes can be further differentiated by their expression level of CX3CR1.^{34, 35} LP Ly6C^{hi} monocytes can differentiate into CX3CR1^{hi} resident macrophages or effector CX3CR1^{int} inflammatory monocytes whose primary function is to rapidly produce pro-inflammatory cytokines after their recruitment to sites of inflammation and injury. Our data strongly indicates that during DSS-induced inflammation, the majority of Ly6C^{hi} monocytes that are recruited to the LP express CX3CR1 and adoptively transferred Ly6C^{hi} inflammatory monocytes recruited to the intestinal LP are predominantly Ly6C^{hi}CX3CR1^{int} (Supplementary Figure 3), consistent with previous studies.³⁶ Although inflammatory monocytes have been implicated in exacerbating colitis since they are capable of producing pro-inflammatory factors, and are found in increased numbers in persistently inflamed tissue,^{11, 32} they also play important roles in restoring homeostasis and limiting damage. This is largely through their ability to be early responders to tissue injury and produce cytokines, including TNF α and IL18 that have reparative functions thereby allowing epithelial restitution and resolution of inflammation.⁹ Consistently, depletion of intestinal monocytes in WT mice resulted in increased susceptibility to colitis.³⁷ Our data further highlight a positive impact by Ly6C^{hi} monocytes via NLRP6 as an early source of TNF α to prevent uncontrolled inflammation in response to epithelial damage. NLRP6-deficiency in

Ly6C^{hi} monocytes is also associated with defective ROS production, which may further limit translocation of bacteria and exacerbation of intestinal inflammation.³⁸ Indeed, the adoptive transfer of Ly6C^{hi} monocytes resulted in reduced bacterial translocation and improved barrier function. It is possible that the decreased ROS production is related to defective production of TNF α , which is needed to promote the assembly of the NADPH oxidase complex that produces ROS.³⁹ Nonetheless, these results are somewhat surprising in light of the study by Anand *et al.*, which suggests that NLRP6 negatively regulates cytokine responses, including TNF α , in BM-derived macrophages (BMDMs) after exposure to bacterial ligands.¹² One possible explanation for this disparate finding is that BMDMs cultured *in vitro* represent a population distinct from inflammatory monocytes. In fact, we demonstrate that not all monocyte populations regulate TNF α production via NLRP6 as we observed no difference in TNF α production between WT and *Nlrp6*^{-/-} LP resident macrophages. Thus, it is likely that differential TNF α responses reflect not only differences in the particular cell population and location, but also the context in which inflammation occurs.

How NLRP6 promotes TNF α production in inflammatory monocytes remains to be fully elucidated. The lack of a known NLRP6 receptor agonist has made the identification of cognate signaling pathways challenging. However, our data suggest that the mechanism may be mediated indirectly through the regulation of IL-18 production in monocytes. The adoptive transfer of *Il18*^{-/-} or *Il18r1*^{-/-} monocytes into *Nlrp6*^{-/-} mice failed to rescue DSS-induced mortality, suggesting that autocrine IL-18 signaling by inflammatory monocytes is important for protection against DSS-induced colitis. Furthermore, both *Il18*^{-/-} and *Il18r1*^{-/-} monocytes isolated from DSS-treated mice have impaired production of TNF α similar to that observed with *Nlrp6*^{-/-} monocytes, consistent with a role for NLRP6 in regulating TNF α production in monocytes via IL-18. Although IL-18 is typically associated with the upregulation of Th1 responses, and in particular, IFN γ ,⁴⁰ IL-18 has also been shown to activate monocytes to enhance TNF α production,^{41, 42} consistent with our data.

In summary, our studies highlight a previously unrecognized role for NLRP6 in modulating IL-18-dependent TNF α production in inflammatory Ly6C^{hi} monocytes that can act as early responders to an inflammatory insult, thereby limiting bacterial translocation, promoting timely epithelial repair, and preventing the onset of dysregulated inflammation. Our data additionally suggest that early production of IL-18 to promote resistance against DSS-induced colitis is mediated not only by the intestinal epithelium, but also by inflammatory monocytes through NLRP6. Together, these results suggest that modulation of NLRP6 activity may be a potential strategy for the prevention and treatment of inflammatory bowel disease.

Materials and Methods

Animal procedures

Adult 7-11 weeks old C57BL/6 WT, *Nlrp6*^{-/-}, *Il18*^{-/-} or *Il18r1*^{-/-}, and GFP⁺ transgenic C57BL/6 (C57BL/6-Tg(UBC-GFP)30Scha/J) mice were bred in-house under specific pathogen free (SPF) conditions at the University of Michigan. WT, *Il18*^{-/-} and *Il18r1*^{-/-}, GFP⁺, CCR2^{-/-} and CX3CR1^{gfp} mice (all B6 background) were originally

purchased from Jackson. *Nlrp6*^{-/-} in B6 background were previously described.¹ Animal studies were conducted under protocols approved by the University of Michigan Committee on the Use and Care of Animals.

DSS-induced colitis and AOM/DSS-induced tumorigenesis

To induce colitis and lethality, mice were treated with 3.5% DSS (MP Biomedicals; m.w. 36,000–50,000 Da) in regular drinking water for 7 days. To develop colitis-associated tumors, mice were first injected with 10 mg/kg AOM (Sigma) i.p., followed by 5 days of 2% DSS starting on day 5. Mice were allowed to recover for 16 days with regular drinking water, and then treated with two additional cycles of 2% DSS (Figure 2A). Mice were sacrificed after the last cycle of DSS for tumor counting and size measurement by calipers.

Isolation of lamina propria cells

LP cells were isolated as previously described.¹ Briefly, colons were cut into small pieces in HBSS buffer (Gibco) supplemented with 2.5% heat-inactivated FBS (Gibco) (HBSS+), and washed with magnetic stirring at 37°C. Colon pieces were then incubated in HBSS+/1 mM DTT at 37°C followed by additional washes and incubation in HBSS+/1 mM EDTA. The supernatant containing IECs and IELs was saved for further analysis. The remaining colon pieces were further digested with 400 I.U./mL Type III collagenase (Worthington) and 10 µg/mL DNase I (Worthington) for two hours at 37°C. When all tissue was digested, the cell suspension was filtered through a 70 micron filter, before running on a 75%/40% Percoll gradient to collect enriched LP cells from the interface.

Flow Cytometry

Cell suspensions were treated with anti CD16/CD32 (2.4G2) and then surface stained with combinations of the following fluorochrome-conjugated antibodies: From Biolegend: CD3e-FITC (clone: 145 2C11), Ly6C-FITC or APC/Cy7 (clone: HK1.4), Ly6G-PE/Cy7 (clone: 1A8), CD3e-APC/Cy7 (clone: 145 2C11), CD45R/B220-Pacific Blue (clone: RA3 6B2), CCR2-AlexaFluor647 (clone: SA203G11), CX3CR1-APC (clone: SA011F11), IgG2A,κ-APC isotype control; from BD Biosciences: NK1.1-PerCP/Cy5.5 (clone: PK136); from Ebiosciences: F4/80-APC (clone: BM8), CD4 (L3T4)-FITC (clone: RM4 5), CD11b-FITC (clone: M1/70), CD11b-PE (clone: M1/70), CD4-APC (clone: GK1.5). Cells were acquired on FACSCanto or AriaIII flow cytometers using FACSDiVa software, and data were analyzed using FlowJo software (Tree Star).

Adoptive transfer experiments

WT, *Ccr2*^{-/-} or CX3CR1^{gfp} mice were used as donors. Single-cell suspensions were prepared from flushed BM in fully supplemented RPMI followed by lysis of red blood cells and isolation of Ly6C^{hi} monocytes (CD3⁻ CD11b⁺ Ly6C^{hi} Ly6G⁻) by FACs sorting. Two million cells were injected i.v. into *Nlrp6*^{-/-}, WT or *Ccr2*^{-/-} recipient mice.

Treatment of mice with recombinant TNF α

WT or *Nlrp6*^{-/-} mice were administered 1 μ g of sterilely filtered recombinant mouse TNF α (PreproTech) i.p. on days 3-7 in the 3.5% DSS colitis model (days 3-7) or days 8-10 in the AOM/ DSS colitis and tumorigenesis model.

Assessment of colon inflammation

Colons were flushed free of feces, opened longitudinally, and jelly-rolled for formalin fixation and paraffin embedding. 5-micron sections were used for H&E staining. Histological assessment was performed in a blinded fashion by a pathologist (KE) using a previously described scoring system¹ with modifications. Entire colon sections were assessed for three main parameters: inflammatory cell infiltration, hyperplasia and epithelial damage that were individually scored (0 to 4), (Supplementary Table I). For each mouse, a weighted percent average based on the number of fields with a certain score was calculated for each parameter and then summed to obtain the final score. Assessment of colon weight after DSS treatment was performed by measuring the colon weight (excluding the cecum) after removal of feces normalized by its length (cm).

Intestinal permeability

Mice were fasted for 8 hours with the exception of drinking water prior to the administration of 0.6 mg/kg FITC-dextran (4 kDa; Sigma-Aldrich) by oral gavage. Serum was collected 4 hours later, diluted 1:3 in PBS, and fluorescence measured using a fluorescent spectrophotometer with emission at 488 nm and absorption at 525 nm.

Reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from LP, BM cells, or from sorted populations of LP cells using the Nucleospin RNA kit (Machery-Nagel). cDNA synthesis was performed using iScript (Bio-Rad), and cDNA was used for qPCR using the SYBR Green Master Mix (Applied Biosystems) on the ABI 7900HT (Applied Biosystems). Ct values were normalized to the β -actin gene. Primer sequences are available upon request.

ELISA

To measure fecal lipocalin-2 levels, fecal samples were homogenized in PBS at 100 mg/ml and supernatant was further diluted in a range of 1:500 to 1:5000 for lipocalin-2/NGAL ELISA (R&D Systems). TNF α , IL-6 and IL-1 β ELISAs (all R&D Systems) were performed with 24-hour culture supernatants of Ly6C^{hi} monocytes.

Measurement of ROS

Total cellular ROS were measured by CellROX Deep Red reagent kit (Invitrogen) according to the manufacturer's protocol. Briefly, upon surface staining for flow cytometry, cells were resuspended in 200 μ l of pre-warmed HBSS + 2.5 mM Probenecid + CellROX, incubated for 30 minutes at 37 $^{\circ}$ C (in the dark), washed and acquired on FACSCanto or AriaIII flow cytometers (BD Bioscience). CellROX Deep Red has peak excitation and emission at 640 nm and 665 nm.

Phagocytosis assay

eGFP-expressing *Escherichia coli* (K12) was a kind gift from Gabriel Nunez (University of Michigan). Ly6C^{hi} monocytes were specifically sorted from WT or *Nlrp6*^{-/-} mice at day 10 of AOM/DSS and incubated for 1.5 hours with *E. coli* GFP at multiplicity of infection (MOI) 0, 5, 25 or 60. Zombie Violet viability dye (Biolegend) was used to identify live Ly6C^{hi} monocytes, which phagocytized GFP⁺ *E.coli* using FACSCanto or AriaIII flow cytometers (BD Bioscience).

Bacterial translocation

MLNs were homogenized in PBS by passing through a 70 micron strainer and then plated on Trypticase Soy Agar with 5% Sheep Blood (TSA II) plates (BD) to determine CFUs after 24-hour incubation under aerobic conditions or 48-hours under anaerobic conditions. Total DNA was extracted using the Nucleospin kit (Machery-Nagel), and bacterial load quantified by qPCR the universal 16S rRNA gene primers EUB-For-5'-AGAGTTTGATCCTGGCTC-3' and EUB-Rev-5'-TGCTGCCTCCCGTAGGAGT-3'.

Statistical analysis

Statistically significant differences were determined using Two-way ANOVA with a Bonferroni post-hoc test (time × genotype, $p < 0.05$), by One Way ANOVA with a Student-Newman-Keuls post-hoc test (p value < 0.05), or by 2 tailed Student's unpaired t test when only 2 groups are compared. Survival curve comparison was performed by Mantel-Cox log-rank test. Kruskal–Wallis one-way analysis of variance non-parametric test was used for non-continuous variables. Data are shown as mean ± SEM. GraphPad Prism6 software was utilized for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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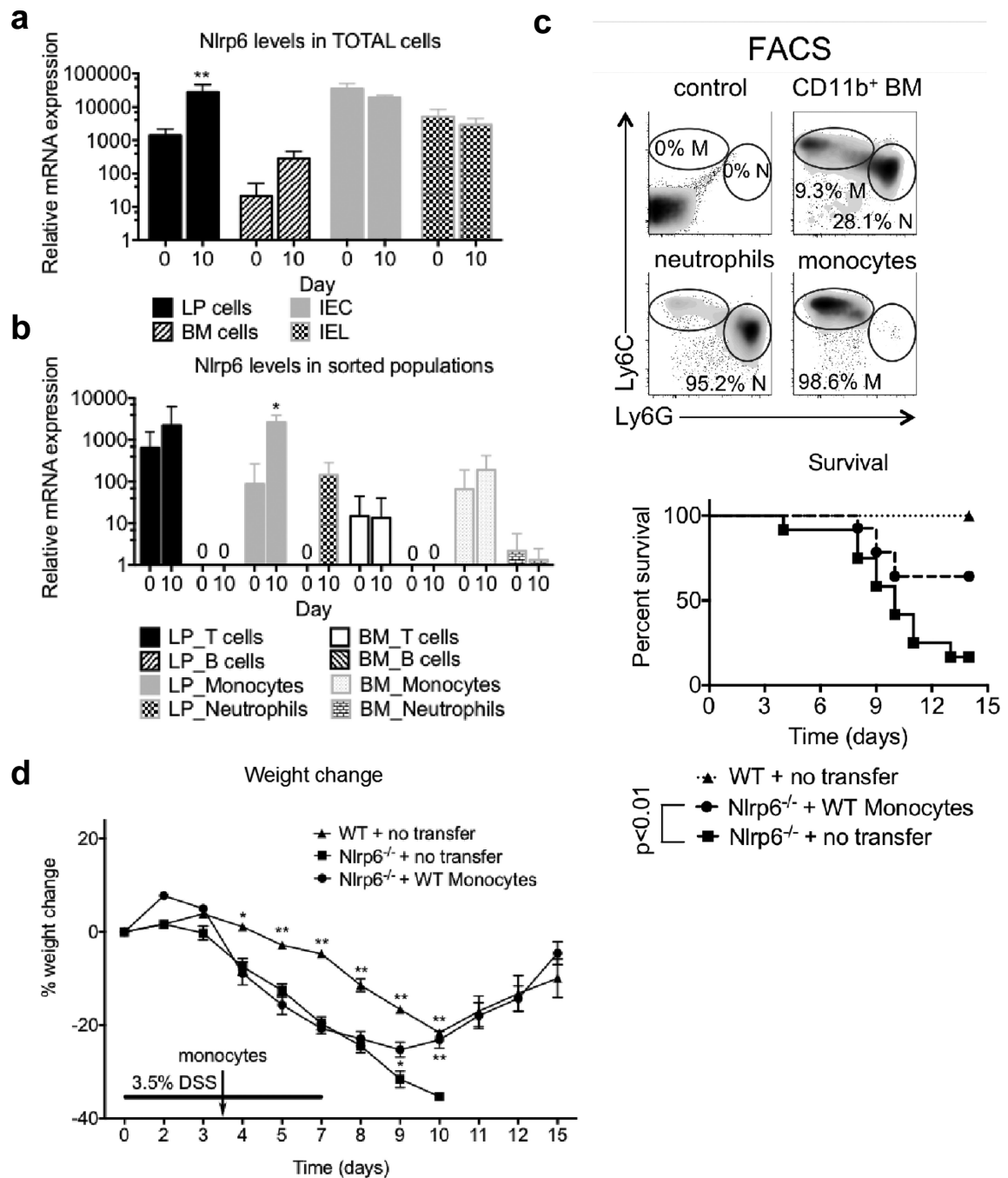


Figure 1. NLRP6 is induced in lamina propria Ly6C^{hi} monocytes during DSS induced inflammation, and is important for reducing susceptibility to colitis

(a) NLRP6 levels were measured in BM, LP, IECs and IELs of WT mice at day 0 and day 10 of AOM/DSS by qPCR. (b) NLRP6 expression was measured in CD3⁺B220⁻ CD11b⁻ T cells, CD3⁻ B220⁺CD11b⁻ B cells, CD3⁻ CD11b⁺Ly6C^{hi}Ly6G⁻ monocytes and CD3⁻ CD11b⁺Ly6C^{int}Ly6G⁺ neutrophils within the BM and LP. Data are representative of three independent experiments; n=11 for day 0, n=10 for day 10. *, ** - p<0.05, p<0.001, respectively, as compared to day 0 time point of the same genotype. (c) Representative plots of Ly6C versus Ly6G staining of CD11b⁺ BM cells (top). Kaplan–Meyer survival curves of

mice treated with 7 days of 3.5% DSS (bottom). **(d)** Percent weight change with 3.5% DSS administration. Data are representative of two independent experiments; n=15, n=24, n=14 for WT, *Nlrp6*^{-/-} and *Nlrp6*^{-/-} + WT Ly6C^{hi} monocytes groups respectively. *, ** - p<0.05, p<0.001, respectively, as compared to *Nlrp6*^{-/-}.

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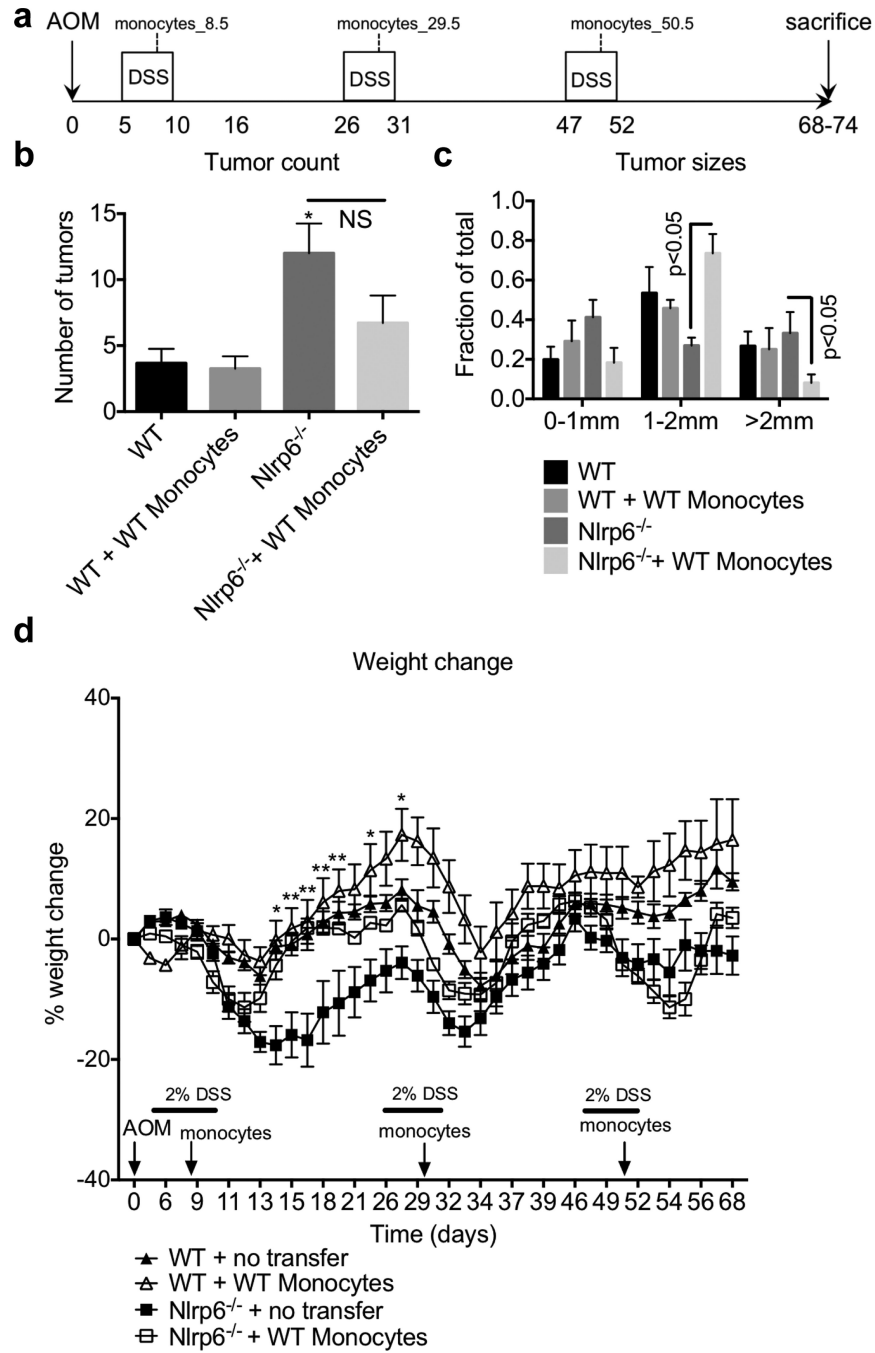


Figure 2. Adoptive transfer of WT Ly6C^{hi} monocytes into Nlrp6^{-/-} mice significantly reduces tumor growth in AOM/DSS model of colitis-associated tumorigenesis
(a) Age and sex-matched *Nlrp6*^{-/-} and WT mice were treated with AOM followed by three 5-day cycles of 2% DSS. Sorted Ly6C^{hi} monocytes were injected i.v. 3.5 days after the start of each DSS cycle. Three weeks after the last cycle of DSS mice were sacrificed and tumors grossly counted with a stereomicroscope. Number **(b)** and size **(c)** of tumors are shown. **(d)** Percent weight change during AOM/DSS treatment. Data are representative of three independent experiments, n=15, n=10, n=19, n=22 for WT, WT + WT Ly6C^{hi} monocytes,

Nlrp6^{-/-} and *Nlrp6*^{-/-} + WT Ly6C^{hi} monocytes groups respectively. *, ** - p<0.05, p<0.001, respectively, as compared to WT (b) or *Nlrp6*^{-/-} mice (d).

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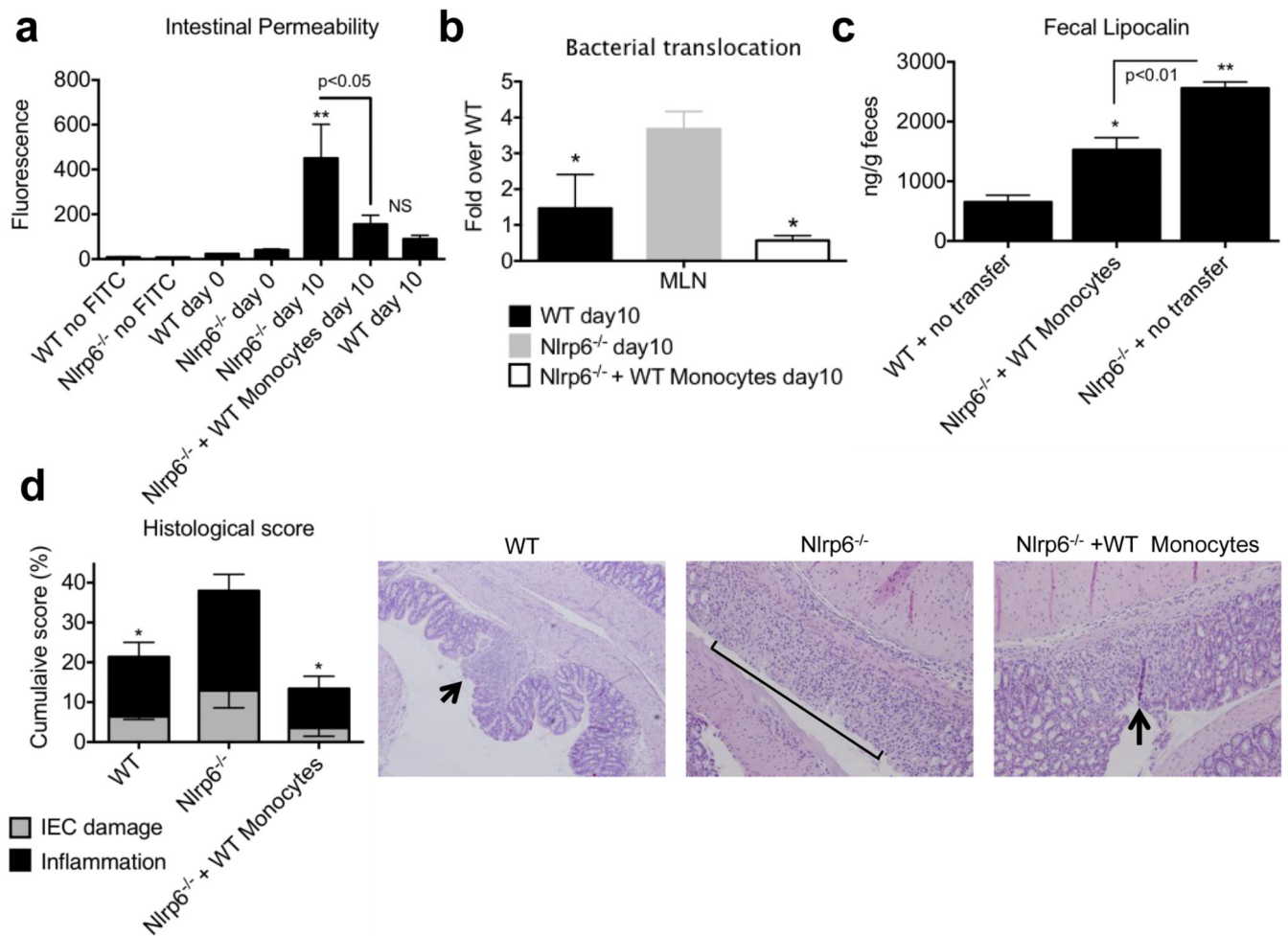


Figure 3. Adoptive transfer of WT Ly6C^{hi} monocytes into *Nlrp6*^{-/-} mice limits bacterial translocation and intestinal damage

Age- and sex-matched *Nlrp6*^{-/-} and WT mice were treated with AOM followed by 5 days of 2% DSS. WT Ly6C^{hi} monocytes were adoptively transferred into *Nlrp6*^{-/-} mice 3.5 days after start of DSS. **(a)** Mice were gavaged FITC-dextran at end of 5 days of DSS followed by serum collection and measurement of fluorescence 4 hours later. **(b)** Normalized levels of total bacteria/MLN as measured by qPCR after 5 days of 2% DSS. **(c)** Fecal lipocalin-2 levels as measured by ELISA after 5 days of DSS. **(d)** Histological inflammatory scores based on extent of inflammatory cell infiltration and IEC damage; Micrographs of H&E sections are at 200x magnification. Arrow points to focal erosion with inflammatory infiltrate. Bracket indicates ulcerated epithelium with inflammatory infiltrate in LP and submucosa. Data are representative of three independent experiments, n=15, n=16, n=20 for WT, *Nlrp6*^{-/-} and *Nlrp6*^{-/-} + WT Ly6C^{hi} monocytes groups respectively. *, ** - p<0.05, p<0.001, respectively, as compared to day 0 of both genotypes (a), or as compared to *Nlrp6*^{-/-} (b,c) or as compared to WT (d).

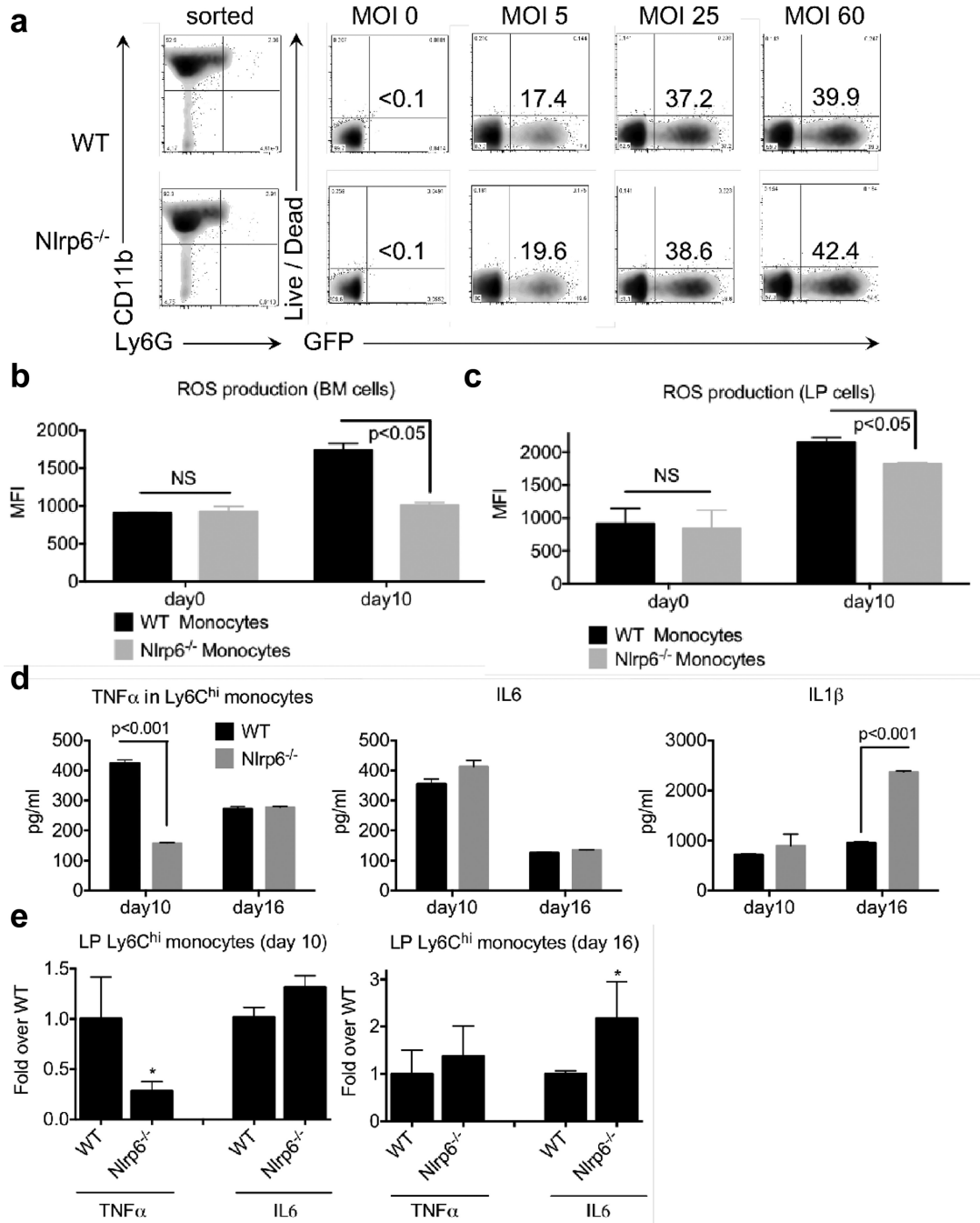


Figure 4. NLRP6 deficient Ly6C^{hi} monocytes have reduced ROS and TNF α production during the acute inflammatory response to DSS

(a) Ly6C^{hi} monocytes were sorted from WT or *Nlrp6*^{-/-} mice and incubated with GFP-labeled *E.coli* at MOI 0, 5, 25 and 60. Phagocytosis was measured by percent GFP⁺ Ly6C^{hi} monocytes. Data are representative of two independent experiments, n=6. ROS production (MFI) as measured by CellROX deep red in BM- (b) or LP-derived (c) Ly6C^{hi} monocytes at the indicated time points. Ly6C^{hi} monocytes from WT or *Nlrp6*^{-/-} mice were sorted from the LP after 5 days of 2% DSS (day 10, AOM/DSS model) or 6 days after completion of

DSS (day 16, AOM/DSS model) and cytokine levels were measured by ELISA (**d**) and by qPCR (**e**). Data are representative of at least three independent experiments, mean \pm SEM; n=14/genotype, (day 10); n=12/genotype (day 0); n=8/genotype (day 16). * - $p < 0.05$, as compared to WT.

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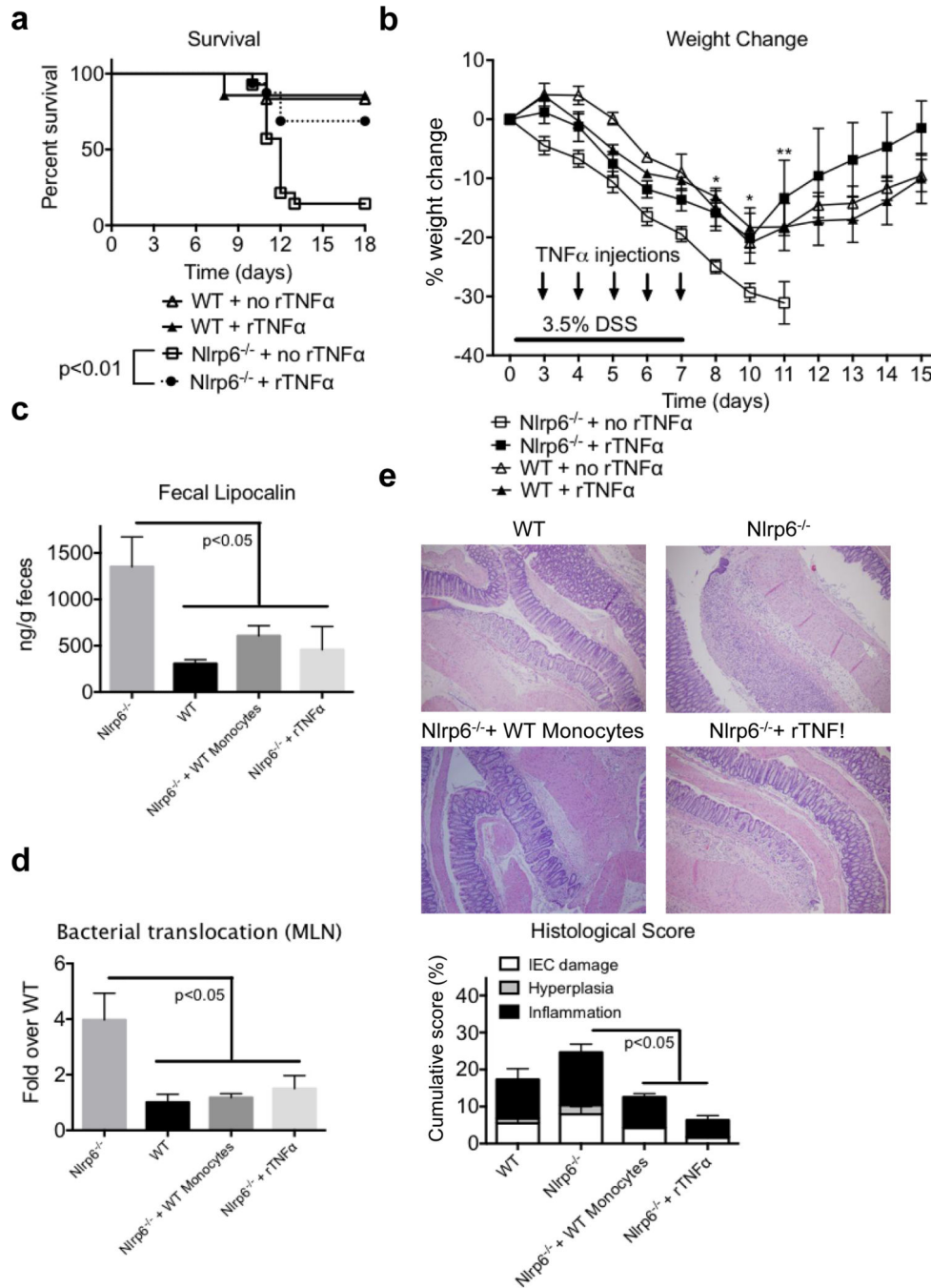


Figure 5. Recombinant TNF α administration into *Nlrp6*^{-/-} mice early during DSS treatment reduces susceptibility to DSS-induced colitis

(a) Kaplan–Meyer survival curves and (b) percent weight change of age- and sex-matched WT and *Nlrp6*^{-/-} mice treated with 3.5% DSS for 7 days with or without rTNF α injections given on days 3–7. Data are representative of two independent experiments, mean \pm SEM; n=15, n=16 for *Nlrp6*^{-/-} and *Nlrp6*^{-/-} + 1 μ g/mouse rTNF α respectively; n=5 for WT and WT + 1 μ g/mouse rTNF α . (c) Fecal lipocalin-2 levels measured in WT and *Nlrp6*^{-/-} mice (day 10, AOM/DSS model) after adoptive transfer of Ly6C^{hi} monocytes or 1 μ g TNF given

on days 3-5 of DSS **(d)** Normalized levels of total bacteria/MLN as measured by qPCR after 5 days of 2% DSS (day 10 AOM/DSS model). **(e)** H&E micrographs at 100x after 5 days of 2% DSS (top) with histological scoring are shown. Bracket indicates diffuse ulceration with inflammatory infiltrate extending throughout the LP and submucosa; n=5, n=6, n=6, n=7 for WT, Nlrp6^{-/-}, Nlrp6^{-/-} + 1 µg/mouse rTNFα, and Nlrp6^{-/-} + WT Ly6C^{hi} monocytes groups respectively.

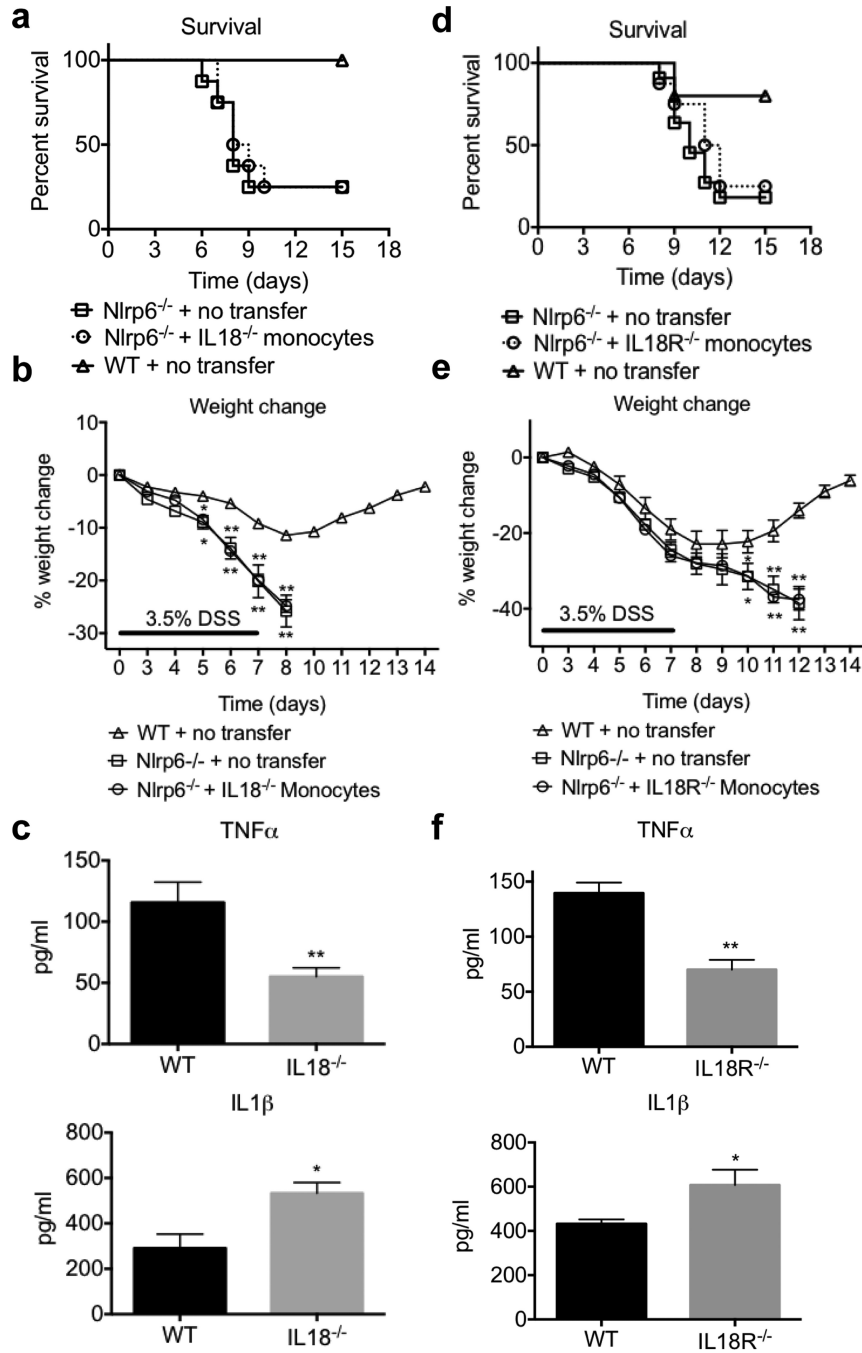


Figure 6. Autocrine IL-18 production by Ly6C^{hi} monocytes protects against DSS-induced colitis by increasing TNF α production

Ly6C^{hi} monocytes were purified from WT, *IL18*^{-/-} or *IL18r1*^{-/-} mice and i.v. injected (2×10^6 cells/mouse) into *Nlrp6*^{-/-} recipients at day 3.5 of a 7 day course of 3.5% DSS. Mice survival (a) and weight loss (b) for WT, *Nlrp6*^{-/-} and *Nlrp6*^{-/-} + *IL18*^{-/-} Ly6C^{hi} monocytes was assessed. n=8/group (c) Ly6C^{hi} monocytes were sorted from the LP after 5 days of 2% DSS (day 10 AOM/DSS model) from *IL18*^{-/-} mice, and cytokine levels in overnight cultures were measured by ELISA. n=4 per group. Mice survival (d) and weight loss (e) after

adoptive transfer of *Il18R^{-/-}* Ly6C^{hi} monocytes into *Nlrp6^{-/-}* mice. n=5 for WT, n=11 for *Nlrp6^{-/-}* and n=8 for *Nlrp6^{-/-}* + *Il18r1^{-/-}* Ly6C^{hi} monocytes (f) Cytokine levels measured from culture supernatants of WT and *Il18R^{-/-}* Ly6C^{hi} monocytes after 5 days of 2% DSS (day 10 AOM/DSS model), n=4/group. *, ** - p<0.05, p<0.001.

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