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Regulation of CD11b by HIF-1α and the STAT3 signaling pathway contributes to the immunosuppressive function of B cells in inflammatory bowel disease

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ABSTRACT

B cells have been reported to have a suppressive function in autoimmune diseases, which appears to require an increase of CD11b expression on B cells. However, little is known how CD11b is induced in B cells to play the function. In this study, we found that the high expression of CD11b in B cells occurred not only in the mucosal immune organs, but also in systemically immune organs such as the spleen during dextran sulfate sodium (DSS)-induced colitis. Since the inflammatory lesions in mouse models of inflammatory bowel disease (IBD) were revealed to be significantly hypoxic or even anoxic, the B cells from colitic mice Peyer's patches (PP) were investigated to express higher levels of hypoxia-inducible factor-1α (HIF-1α) than naïve B cells from wildtype (WT) mice. HIF-1α siRNA transfection or HIF-1α protein inhibition led to decreased CD11b expression at both the mRNA and protein levels *in vitro*. B cells with HIF-1α specific knockdown were then adoptively transferred to Rag-$1^{-/-}$ mice. The result displayed that CD11b expression was decreased in B cells and an exacerbated colitis occurred. The bio-informatics promoter analysis and ChIP assay showed that HIF-1α was the critical transcription factor for CD11b and cooperatively formed a complex with the p-STAT3 homodimers to bind onto hypoxia-responsive element (HRE) regions, which was guaranteed by MEK/ERK pathway activation and IL-10 secretion. In conclusion, our study demonstrated the key function of the hypoxia-associated transcription factor HIF-1α together with p-STAT3 in driving CD11b transcription in B cells and controlling B cell's protective activity in experimental inflammatory bowel disease (IBD).

1. Introduction

B cells have been found to suppress inflammation in T cell-mediated autoimmune diseases by secreting immune-regulatory cytokines, including interleukin 10 (IL-10), transforming growth factor β, and IL-35 (Mauri and Blair, 2014; Rosser and Mauri, 2015; Wei et al., 2019). In mice, multiple subsets of B cells have been identified as the regulatory B cells (Bregs) to affect $CD4^+$ T cell response through the production of IL-10, such as $CD5^+CD1d^{hi}$ B10 B cells, $CD21^{hi}CD23^{hi}CD24^{hi}$ transitional 2 marginal-zone precursor cells, Tim-1^+ B cells, and $CD138^+$ plasma cells (Rosser and Mauri, 2015). Our published data demonstrated that a subset of B cells expressing CD11b displayed a regulatory function by inhibiting $CD4^+$T effect cell response in an experimental autoimmune hepatitis (EAH) model (Liu et al., 2015), and Peyer's patches (PP)-derived $CD11b^+$ B cells recruited regulatory T (Treg) cells by secreting CXCL9 in DSS-induced colitis (Wang et al., 2019), indicating that CD11b may play a regulatory function for regulatory B cells. CD11b encoded by integrin subunit alpha M (*Itgam*) is an integrin αM subunit of Mac-1 that dimerizes with CD18 and is widely expressed on the surface of various immune cells (Farokhzad et al., 1996). Multiple studies have demonstrated that CD11b has a pivotal role in suppressive regulation. CD11b expressed on antigen-

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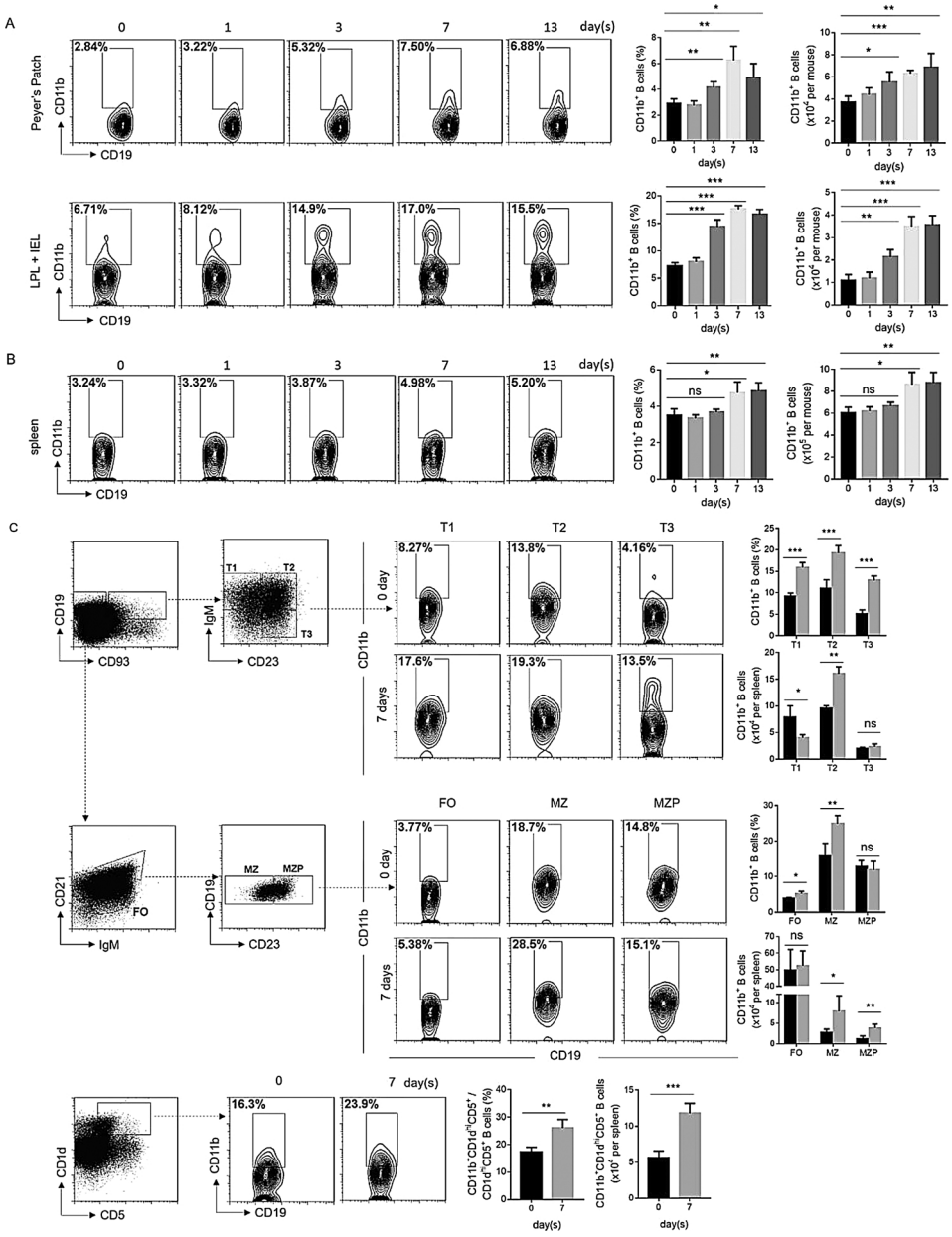

Fig. 1. CD11b expression is up-regulated systemically in B cells during the process of colitis. (A, B) B cells were isolated from the PP, LPL, IEL, and spleen of WT (day 0) and mice with DSS-induced colitis on days 1, 3, 7, and 13. (C) A FCM analysis of CD11b expression on various B-cell subsets in the spleen from WT and colitic (day 7) mice. Data are the mean ± standard error of the mean (SEM) from one experiment with six mice, which was repeated at least three times with similar results. *P < 0.05, **P < 0.01, ***P < 0.001.

presenting cells (APCs) negatively regulated T cell activation (Wang et al., 2015) and T-helper 17 (Th17) differentiation (Ehirchiou et al., 2007). Furthermore, CD11b expressed on APCs inhibited inflammatory responses through Toll-like receptor (TLR) suppression (Ding et al., 2013; Rosetti and Mayadas, 2016; Wang et al., 2010) and provoke innate anti-tumor immunity (Schmid et al., 2018) while expressed on macrophages. CD11b-null mice exhibited severer colitis compared to the wildtype (WT) mice (Abdelbaqi et al., 2006). Furthermore, CD11b activation reduced TLR-dependent inflammation, reduced type I interferon response, and protected mice from severe sepsis in systemic lupus erythematosus (Faridi et al., 2017). Regarding B cells, CD11b was reported to regulate immunoglobulin heavy chain class switch recombination via the induced expression of AID in activated B2 B cells (Park et al., 2017). CD11b expression on B cells was up-regulated in liver and spleen during the pathological process of EAH (Liu et al., 2015) and in the PPs in IBD (Wang et al., 2019). These studies imply that CD11b, as an integrin subunit, not only is a surface marker of Breg cells but may also function as a regulatory molecule in B cell-mediated suppressive response. However, it is still unknown whether CD11b is systemically expressed in inflammatory bowel disease (IBD) and how CD11b is induced to express on B cells in gut-related autoimmune diseases.

In autoimmune diseases, lymphocytes penetrate into the inflammatory lesions of tissues, where it is prominently hypoxic or even anoxic, and utilize glycolysis for an energy supply after activation or under hypoxia (Sitkovsky and Lukashev, 2005; Palazon et al., 2014), which is modulated by the oxygensensing HIF transcription factors. HIFs are heterodimeric proteins of the basic helix-loop-helix family and consist of the oxygen-dependent α subunit and the constitutively expressed β subunit (Balamurugan, 2016). Under normal oxygen tensions, HIFα subunits are hydroxylated by prolyl hydroxylases and are subsequently subjected to oxygen-dependent proteasomal degradation

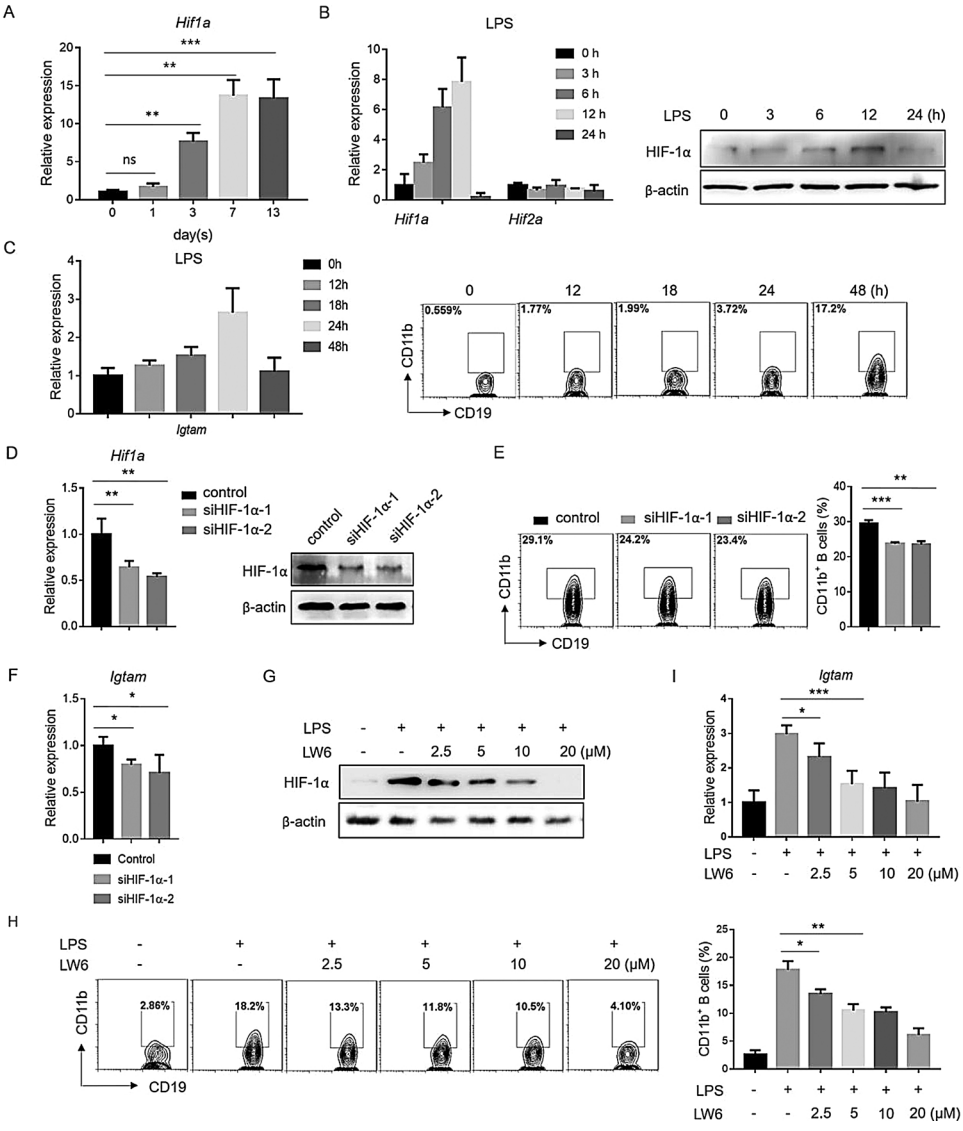

Fig. 2. *In vitro,* CD11b expression is decreased by HIF-1α inhibition. (A) A qPCR analysis of HIF-1α expression during the process of colitis. WT splenic B cells were stimulated with lipopolysaccharide (LPS) at indicated time points, and HIF-1α, HIF-2α (B) and CD11b (C) expression were assessed by qPCR or western blot. (D) HIF-1α expression in splenic B cells transfected with HIF-1α siRNA or negative control (NC) siRNA were analyzed by qPCR (12 h) or western blot (24 h). (E, F) qPCR and FCM analysis were used to check CD11b expression on B cells. WT splenic B cells were co-treated with LPS and the HIF-1α inhibitor LW6 at indicated concentrations. HIF-1α (G) and CD11b (H, I) expression were measured by western blot, flow cytometry, and qPCR. The data shown are means ± SEM and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

mediated by the von Hippel–Lindau tumor-suppressor protein (VHL) (Ivan et al., 2001; Jaakkola et al., 2001). However, the HIFα subunit can stably translocate to the nucleus and dimerize with the HIF-1β subunit to drive the transcription of targets under hypoxic conditions (Cramer et al., 2003; Peyssonnaux et al., 2005). A large body of research has shown that HIF-1α triggers the transcription of many glycolytic genes and plays a vital role in innate and adaptive immune response (Brown and Taylor, 2018; Ratcliffe, 2007). HIF-1α increases macrophage aggregation, invasion, and motility and derives the expression of pro-inflammatory cytokines. In neutrophils, HIF-1α increases cell survival by inhibiting apoptosis and triggers NF-κB dependent neutrophilic inflammation (Walmsley et al., 2005). HIF-1α in dendritic cells is necessary for the activation of Tregs to control intestinal inflammation (Fluck et al., 2016). HIF-1α increases the expression of RORγt, which in turn promotes IL-17 production and Th17 cell development and supports Treg cell function (Higashiyama et al., 2012). HIF-1α has also been reported to induce IL-12p40 to suppress mucosal Th1 and Th17 responses, which protects against colitis (Marks et al., 2017). Although HIF-1α has been widely studied in T cells and myeloid cells, its function in B cells is largely unexplored and was only known to contribute to IL-10 production by B cells (Meng et al., 2018).

In the present study, we found that the expression of CD11b on B cells was systemically increased in the spleen and gut-associated lymphoid tissues (GALT) during the DSS-induced colitis process. Compared to WT cells, B cells from colitic mice had a greater expression of the transcriptional regulator HIF-1α. Knockdown or inhibition of HIF-1α led to the down-expression of CD11b in B cells *in vitro*. Furthermore, Rag-1$^{-/-}$ mice adoptively transferred with HIF-1α-knockdown B cells exhibited a severer colitic phenotype and has the decreased expression of CD11b in B cells than those transferred with WT B cells. The CD11b transcription was then identified to be regulated by a HIF-1α and p-STAT3 complex *via* binding onto the hypoxia-responsive element (HRE) regions of *Itgam* promoter, which was guaranteed by the MEK/ERK pathway activation and IL-10 secretion. Taken together, these findings reveal HIF-1α as a vital transcription factor for B cells expressing CD11b to exert a protective function in colitis.

2. Results

2.1. CD11b expression on B cells is increased in the DSS-induced colitis process

To determine CD11b expression on B cells in mucosal related diseases, we first induced a mouse colitis model by feeding WT C57BL/6 mice with 2.5% DSS and detected CD11b expression in B cells in gut-associated lymphoid tissue at different time points by flow cytometric (FCM) analysis. The percentage and absolute number of CD11b$^+$ B cells were steadily increased during the colitis process in PP B cells (Fig. 1A).

Both the intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) are effect sites of the colitis. IEL are mainly $CD8^+$ T cells, but there are a certain proportion of B cells in the IEL (Supplementary Fig S1A). We checked their immuno-regulatory functions together and found that CD11b expression was also increased in the B cells of IEL and LPL (Fig. 1A). Although DSS-induced colitis is a well-known chronic immune-mediated mucosal inflammation, the mice suffering colitis also systemically induced CD11b expression in splenic B cells, which occurred more slowly than that in GALT B cells (Fig.1B). The induction of CD11b expression on B cells was also induced in the subpopulation of splenic B cells (Fig.1C), including transitional types 1, 2, and 3, follicular B cells, marginal zone (MZ), marginal zone precursor (MZP) and $CD1d^{hi}CD5^+$ B cells during the colitis disease process, implying that CD11b might be crucial for the regulatory function of B cells. Taken together, these data suggest that B cells are induced to generally increase CD11b expression in the course of colitis.

2.2. Induction of CD11b by LPS was HIF-1α dependent in vitro

In colitis models, B cells were activated to produce IL10 to suppress autoimmunity (Rosser and Mauri, 2015; Matsumoto et al., 2014). During the process of colitis, B cells isolated from PPs expressed increased *ki67* (Supplementary Fig. S2A), implying that the B cells were active and proliferative. It has been reported that these B lymphocytes converted to a highly activated state in response to antigen challenge and were concomitant with a high rate of glycolysis to support cell proliferation (Dufort et al., 2014; Pearce and Pearce, 2013). HIF-1α has been widely reported to be involved in glycolytic regulation by mediating hypoxia-responsive protective pathways and targeting genes as a key transcription factor. We next examined HIF-1α expression in PP B cells and found that during the colitis process, B cells were induced to express increased HIF-1α (Fig. 2A), implying that HIF-1α might be associated with B cell activation.

To investigate whether HIF-1α was related to CD11b expression and the suppressive function of B cells, we next stimulated the splenic B cells of C57BL6 WT mice with lipopolysaccharide (LPS) under normoxia *in vitro* and determined that HIF-1α was induced by LPS stimulation, while HIF-2α, another member of the HIF-α family, was not expressed (Fig. 2B). Both the mRNA and protein levels of HIF-1α increased before 12 h but were undetectable at 24 h, which was consistent with the expression pattern of CD11b (Fig. 2C). We then silenced HIF-1α expression by siRNA transfection into WT splenic B cells (Fig. 2D). In agreement with the *in vivo* results showing that HIF-1α was associated with CD11b expression of B cells, HIF-1α silence led to the inhibition of CD11b expression in B cells *in vitro* (Fig. 2E, F). To identify whether the HIF-1α protein took part in the regulation of CD11b expression, WT splenic B cells were further treated with the HIF-1α inhibitor LW6 at different concentrations for 12 h, which inhibited HIF-1α accumulation *via* proteasomal degradation mediated by VHL (Fig. 2G) but had no effect on *Hif-1a* mRNA expression (Supplementary Fig. S2B). HIF-1α degradation by LW6 was shown to inhibit the induction of CD11b by LPS in B cells (Fig. 2H, I) but had no effect on B cell apoptosis until it reached a 20 μM concentration (Supplementary Fig. 2C). Taken together, these data support the notion that HIF-1α significantly influences the production of CD11b by B lymphocytes.

A previous study demonstrated that LPS stimulated IL-$10^{-/-}$ B cells expressed less CD11b than IL-$10^{+/+}$B cells (Liu et al., 2015). To investigate whether HIF-1α regulates CD11b expression *via* IL-10 signaling, we examined IL-10 production by B cells under the treatment of HIF-1α inhibitor LW6. The results from flow cytometry, qPCR and ELISA revealed that IL-10 production and secretion was inhibited upon LW6 treatment (Supplementary Fig. 3A–C). In the IL-$10^{-/-}$ model, CD11b expression could be rescued by additional IL-10 supplement in a dose-dependent manner (Liu et al., 2015). To explore whether additional IL-10 could break down the inhibition of LW6 on CD11b expression, we supplied B cells with recombinant mouse IL-10 together with the HIF-1α inhibitor LW6 (Supplementary Fig. 3D) and examined CD11b expression. However, additional IL-10 supplement failed to rescue CD11b expression (Supplementary Fig. 3E, F), implying that IL-10 indeed regulated CD11b expression but this regulation was HIF-1α-dependent. Collectively, these results indicate that HIF-1α is indispensable for CD11b expression.

2.3. B cell-specific knockdown of HIF-1α led to decreased CD11b expression and severer inflammation in DSS-induced colitis

We next sought to address the effect of HIF-1α on the regulation of CD11b expression and on the suppressive function of B cells by using an in vivo model. Rag-$1^{-/-}$ mice were co-transferred with $CD3^+$ T cells and HIF-1α siRNA or control siRNA transfected B cells, and then colitis was induced by DSS treatment for 7 days. The HIF-1α expression was confirmed to be knocked down in splenic B cells, PP B cells and intestinal B cells of Rag-$1^{-/-}$ mice transferred with HIF-1α siRNA-transfected B cells (Fig. 3A, Supplementary Fig. S4A). Interestingly, we found a decreased expression of CD11b in the splenic B cells and PP B cells of Rag-$1^{-/-}$ mice with adoptive transfer of HIF-1α knockdown B cells (Fig. 3B, C) as well as in LPL and IEL (Fig. 3C), which implied that HIF-1α mediated B cell response to antigen. It has been identified that HIF-1α-induced response played a protective role in murine models of IBD (Brown and Taylor, 2018; Fluck et al., 2016; Marks et al., 2017). In agreement with previous reports, these Rag-$1^{-/-}$ mice with adoptive transfer of HIF-1α knockdown B cells suffered severer colitis, with increased weight loss (Fig. 3D), decreased colon length (Fig. 3E), and increased intestinal permeability (Fig. 3F) in comparison to the mice with transfer of control siRNA-transfected B cells, confirming that HIF-1α is necessary for the suppressive response of B cells to inflammation. Together, these data suggested that the induced expression of HIF-1α during B cell activation is responsible for the upregulation of CD11b expression in DSS-induced colitis.

2.4. HIF-1α promoted CD11b transcription by binding onto its promoter regions

Given that the increased expression of CD11b during mucosal inflammation or LPS stimulation was related to HIF-1α activation, we investigated the mechanism by which HIF-1α regulated CD11b expression on B cells. Since HIF-1α inhibition down-regulated CD11b expression at both the mRNA and protein levels, and HIF-1α was acknowledged to be a transcription factor for multiple genes by binding to their HREs in the promoter region, we posited that CD11b induction was directly related to HIF transcriptional activity. Through a bio-informatic analysis with JASPAR, we identified that there were several potential HIF-1α binding sites on the murine *Itgam* promoter (Fig. 4A). A chromatin immunoprecipitation (ChIP) assay was performed to determine whether HIF-1α bound to the HRE regions on the *Itgam* promoter. The result suggested that the potential HIF-1α binding sites II and IV of the HRE regions were responsible for HIF-1α binding (Fig. 4B). In addition, HIF-1β was also detected to bind to the HRE II and IV regions on the *Itgam* promoter in B cells after LPS stimulation (Fig. 4C), whereas no binding of the control IgG was detected, which suggested that these two regions were in a transcriptionally active status. A luciferase assay was carried out with co-transfection of putative HRE constructs and the HIF-1α negative control plasmid (NC) or HIF-1α transcription factor (TF) into 293 T cells. Consistent with ChIP result, the luciferase activity of the HRE II and IV constructs was increased with transfection of HIF-1α TF (Fig. 4D), indicating that HIF-1α bound effectively to the HRE II and IV in the CD11b promoter region and regulates its expression. These results imply that HIF-1α directly targets HRE regions on the *Itgam* promoter to promote CD11b expression on B cells.

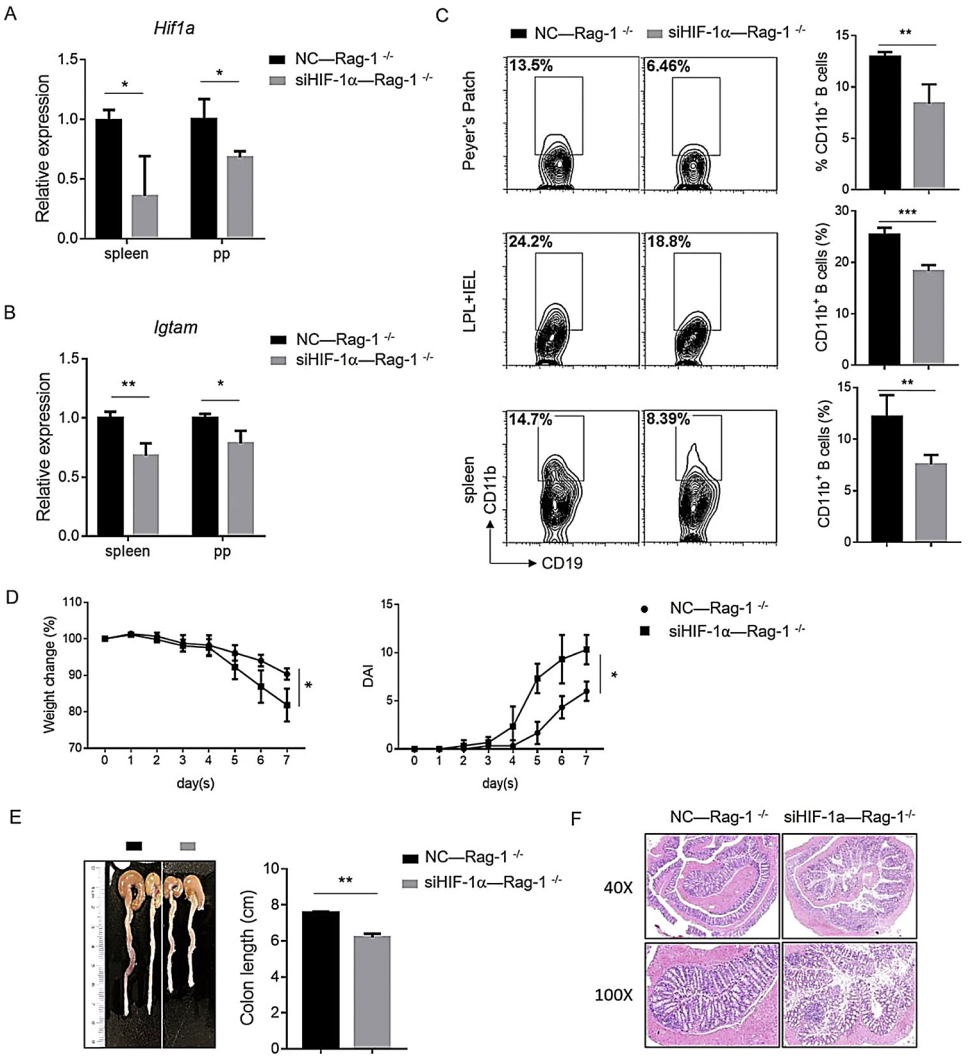

Fig. 3. Decreased CD11b expression by HIF-1α knockdown in B cells induces severer colitis. Rag-1$^{-/-}$ mice were co-transferred with CD3^{+} T cells and HIF-1α siRNA or NC siRNA transfected B cells, and then colitis was induced by DSS treatment for 7 days. The mRNA expression of HIF-1α (A) and CD11b (B) in splenic and PP's B cells were examined by qPCR. (C) FCM analysis of CD11b expression on B cells isolated from the PP, LPL, IEL, and spleen in colitic mice on the 7th day. The severity of intestinal injury was evaluated by body weight, disease activity index (DAI) scores (D), colon length (E), and histological analysis (x40, x100) (F). Data are the mean ± SEM from one experiment with four mice and was repeated twice with similar results. *P < 0.05, **P < 0.01, ***P < 0.001.

2.5. HIF-1α and p-STAT3 cooperatively modulated CD11b transcription

The CD11b expression in LPS-stimulated IL-10$^{-/-}$ B cells was much lower than that in IL-10$^{+/+}$B cells (Liu et al., 2015) and IL-10 promoted CD11b expression (Supplementary Fig. S5C, D), implying that the cytokine IL-10 was indispensable for promoting CD11b expression. Therefore, we posited that IL-10 might participate in the HIF-1α-mediated regulation of CD11b expression. We first detected the signaling pathways that might be involved in the LPS stimulation *in vitro*, such as MEK/ERK, p38/MAPK, JNK, NF-kB, as well as JAK/STAT3 pathways (Roy et al., 2016; Liu et al., 2014). The cells were treated with the inhibitors of the signaling pathways, and the results of a flow cytometry analysis showed that CD11b expression on B cells only decreased upon treatment with the MEK/ERK or STAT3 inhibitors (Supplementary Fig. S5A, Fig. 5A), which implied that MEK/ERK and STAT3 might be involved in the regulation of CD11b expression. STAT3 is known to be phosphorylated on serine 727 (S727) by the mitogen-activated protein (MAP) kinase pathway to obtain maximal activation (Wen et al., 1995). Correspondingly, the phosphorylation of STAT3 on S727 was suppressed by the MEK inhibitor Trametinib while the STAT3 inhibitor Stattic had no effect on the MEK/ERK signaling pathway (Fig. 5B), which indicated that the activation of the ERK signaling pathway is indispensable for STAT3 activation. Since HIF-1α is a target of p-STAT3^{727} via binding to HIF-1α promoter (Meng et al., 2018; Dang et al., 2011), B cells treated with specific inhibitors of MEK/ERK or JAK/STAT3 had less HIF-1α expression (Fig. 5C), and less expression of HIF-1α's target IL-10 (Fig. 5D, E). STAT3 has been demonstrated to be activated by secreted IL-10 cytokine through phosphorylation on tyrosine 705 residue (Y705) (Nakamura et al., 2015; Li et al., 2018; Ouyang et al., 2011). Indeed, recombinant mouse IL-10 increased STAT3 phosphorylation of Y705 (Supplementary Fig. S5C). Interestingly, the phosphorylation of Y705 occurred later than that of S727 under LPS stimulation (Supplementary Fig. S5B). Collectively, these data imply that LPS stimulation triggers ERK activation to phosphorylate STAT3 on S727 and then increased secretion of IL-10 activates STAT3 on Y705 for dimer formation and DNA binding.

Furthermore, STAT3 has been reported to be specifically required for the activation of HIF-1α target genes (Pawlus et al., 2013). We hypothesized that HIF-1α and activated p-STAT3 might cooperatively bind onto HRE regions in the *Itgam* promoter to facilitate *Itgam* transcription. A ChIP assay was performed to determine whether p-STAT3 together with HIF-1α would bind onto the HRE regions in the *Itgam* gene. The pattern of p-STAT3 binding was similar to that of HIF-1α or HIF-1β binding shown in Fig. 4, and this binding was attenuated by treatment with the HIF-1α inhibitor LW6 (Fig. 5F), which indicates that p-STAT3 is a transcriptional co-activator of HIF-1α binding to the HRE regions of the *Itgam* gene. Taken together, our results illustrate that HIF-1α forms a complex with the p-STAT3 homodimers and cooperatively binds onto HRE regions to modulate *Itgam* transcription, which was guaranteed by ERK activation.

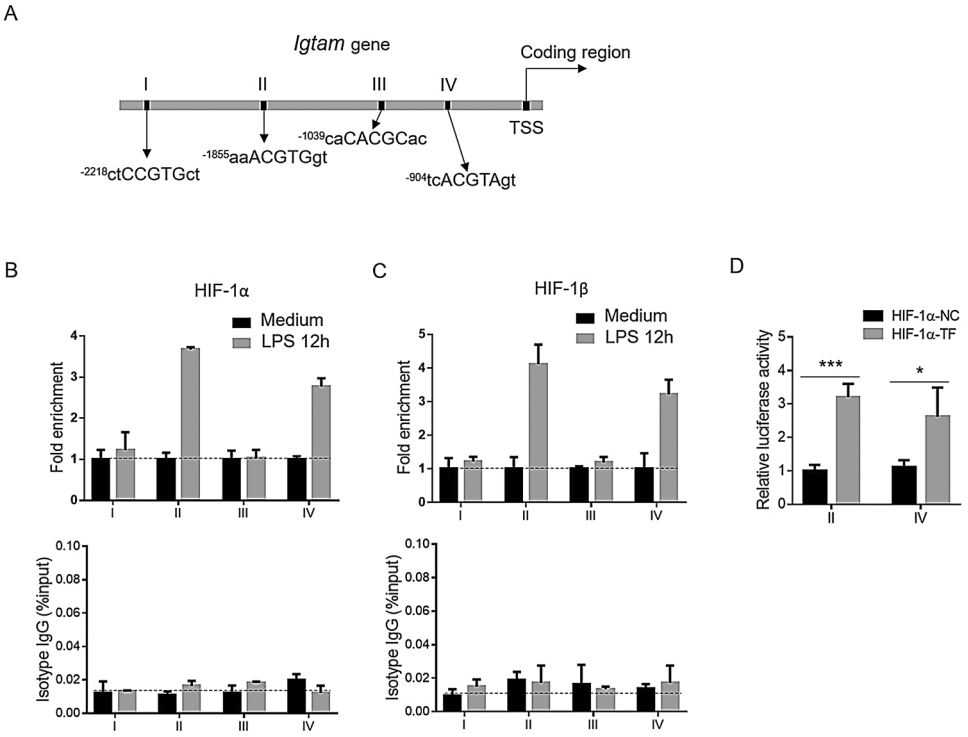

Fig. 4. *Itgam* transcription is promoted by HIF-1α binding onto its promoter regions. (A) Scheme of the predicted HRE regions (I, II, III, and IV) in the *Itgam* promoter. ChIP assays were used to measure the recruitment of HIF-1α (B) and HIF-1β (C) on HRE regions of the *Itgam* promoter in B cells after LPS stimulation for 12 h (n = 4 per group). (D) 293 T cells were co-transfected with HRE II and HIF-1α-NC or HIF-1α-TF, and with HRE IV and HIF-1α-NC or HIF-1α-TF for 24 h, and the relative luciferase activity was measured and normalized to Renilla activity. *P < 0.05, ***P < 0.001. Data represent are mean ± SEM from three independent experiments.

3. Discussion

In this study, we first found that CD11b expression on B cells systemically increased in gut-associated lymphoid tissue and the spleen during the process of experimental colitis. B cells in colitis had a greater expression of the important transcription factor HIF-1α than naïve B cells from WT mice. We then demonstrated that HIF-1α knockdown by siRNA impaired the B cells' protective activity in colitis *in vivo* and reduced the CD11b expression in B cells *in vitro*, and the HRE II and IV of four putative HRE regions in the *Itgam* promoter were responsible for HIF-1α binding to promote *Itgam* transcription. Finally, the MEK/ERK and STAT3 signaling pathways were shown to be involved in LPS- and colitis-induced CD11b upregulation. Our study indicates that the transcriptional complex of HIF-1α and p-STAT3 was activated by the MEK/ERK pathway and secreted IL-10, and consequently activated HRE regions of the *Itgam* promoter (Fig. 6).

Multiple subsets of Breg cells in mice have been identified that could suppress the T cell-mediated response. However, a lack of consensus surface markers for Breg cells made it difficult to judge whether Breg cells are a special B cell subset or whether all B cells have an immunoregulatory function under inflammation induction, and it impossible to apply this important immunosuppressive B cell to clinical practice. Therefore, investigating the mechanisms of Breg induction and finding its specific transcription factors have become the most important challenge in the study of Breg. It has been reported that the B cells that act as the regulatory cells in colitis was $CD1d^+$ B cells in MLN (Mizoguchi et al., 2002) or the splenic $CD5^+CD1d^+$ B cells (Yanaba et al., 2011). In the studies of our group, we have identified $CD11b^+$ B cells as the regulatory B cells that played important roles in the suppression of inflammatory immune responses in autoimmune diseases, such as IBD and EAH. CD11b, as an integrin subunit, combines with the β2 chain CD18 to form Mac-1, which is an integrin heterodimer with important effects on myeloid cell migration and adherence to stimulated tissue (Pawlus et al., 2013; Abram and Lowell, 2009). Studies also highlighted the protective role of CD11b in B cells following activation during inflammatory responses, including the maintenance of autoreactive B cell tolerance (Watanabe et al., 2000), inhibition of T cell activity (Liu et al., 2015), recruitment of Treg cells (Wang et al., 2019), as well as regulation of TLR4-dependent inflammatory responses (Faridi et al., 2017). Although best known for its expression on B1 cells (Griffin and Rothstein, 2011), our previous study of a chronic liver disease model showed that CD11b was universally expressed on all of the B cell subsets (Liu et al., 2015), and we first suggested CD11b as a surface marker and regulatory molecule for Breg cells. In this study, we found that during the process of colitis CD11b expression was widely increased in B cells of gut-associated lymphoid tissue, the splenic B cells, as well as several reported regulatory B cells, such as marginal zone (MZ), marginal zone precursor (MZP) and $CD1d^{hi}$ $CD5^+$ B cells (Fig. 1), implying that CD11b might be crucial for the regulatory function of B cells. Although IEL mainly consists of $CD8^+$ T cells, there is also a certain proportion of B cells. It has been reported that after the activation, B cells in peyer's patches differentiate into germinal center B cells and plasmablasts, and then traffic into the intestinal lamina propria. Both the IEL and LPL are effect site of the colitis, so we checked their immuno-regulatory functions together. Furthermore, the down-expression of CD11b led to severer colitis (Fig. 3), and CD11b-knockout B cells lost their regulatory function in the EAH model (Liu et al., 2015). Our data supports the notion that $CD19^+CD11b^+$B cells are likely to be a subset of B cells with immuno-regulatory functions in autoimmune diseases.

In the models of colitis, B cells have been reported to be activated to produce IL10 to suppress inflammation (Rosser and Mauri, 2015; Mizoguchi et al., 2002). Activated B cells responding to antigen stimuli convert to a highly activated state and led to increased rates of glycolysis to support cell proliferation (Kunisawa, 2017). Our published data in an EAE model suggested that CD11b expression in B cells might be related to B cell activation, and $CD11b^+$ B cells are proliferative (Liu et al., 2015). During the process of colitis, the B cells isolated from PPs expressed increased *ki67* (Supplementary Fig. S2A), implying that the B cells in colitic mice were active and proliferative. The major finding in this paper is that HIF-1α is a promoter of *Itgam* gene transcription. The mucosal inflammatory lesions in mouse IBD models were revealed to be profoundly hypoxic or even anoxic (Colgan and Taylor, 2010; Taylor and Colgan, 2017), which could promote the activity and survival of mucosal immune cells through HIF-1 activation. HIF-1α has been reported to play a protective role in IBD (Fluck et al., 2016; Marks et al.,

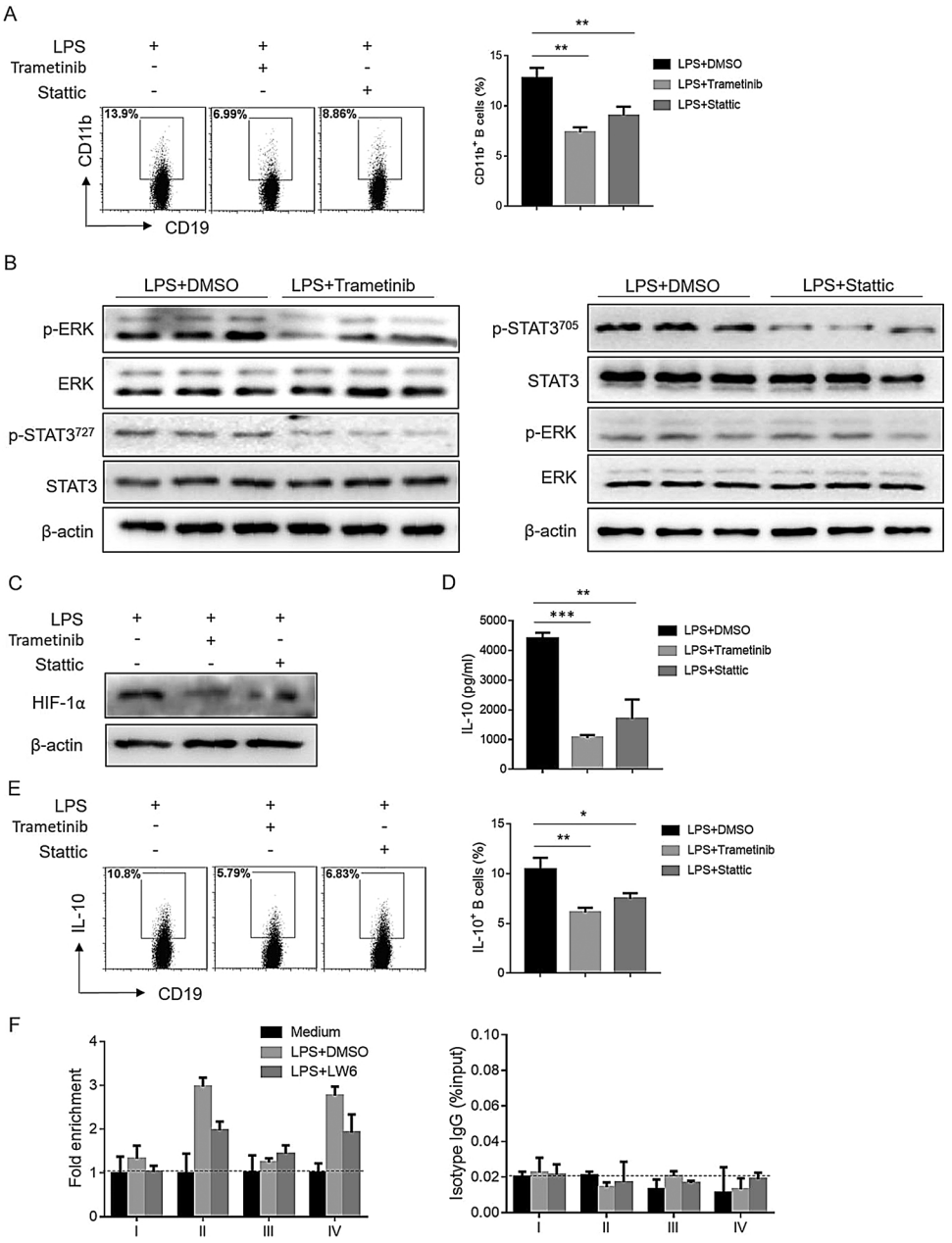

Fig. 5. *Itgam* transcription is cooperatively regulated by HIF-1α and p-STAT3. (A) WT splenic B cells were treated with the MEK/ERK inhibitor trametinib or the STAT3 inhibitor Stattic for 48 h, and CD11b expression was measured by a FCM analysis. (B) phosphorylation levels of ERK and STAT3 upon LPS stimulation at indicated time points were checked by western blot. (C) HIF-1α expression under trametinib or Stattic treatment for 12 h were checked by western blot. (D, E) WT splenic B cells were treated with Trametinib or Stattic for 4 h. IL-10 secretion and production in splenic B cell was checked by ELISA and FCM analysis. (F) ChIP assay was used to measure the recruitment of p-STAT3 on HRE regions of the *Itgam* promoter in B cells after LPS stimulation or LPS plus LW6 treatment for 12 h (n = 4 per group). The data shown are means ± SEM and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

2017; Wang et al., 2017). It seems that HIF-1α is involved in the suppressive function of CD11b in B cells. We found that B cells from colitic mice had a greater expression of the transcriptional regulator HIF-1α than those naïve B cells (Fig. 2A). These findings are consistent with a study demonstrating that IgA plasma cells in the iLP seem to prefer using glycolysis as an energy supply, compared to naïve B cells in the PPs (Kunisawa, 2017). Knockdown with siRNA or protein inhibition of HIF-1α led to the down-expression of CD11b in B cells (Fig. 2), which implies that HIF-1α might be involved in the transcriptional regulation of CD11b. The transcriptional regulation of CD11b is very important in its expression. The transcription factors Sp1, Sp3, and PU.1 have been shown to bind to CD11b promoter to induce its expression during monocytic lineage differentiation (Shelley and Arnaout, 1991; Pahl et al., 1993; Chen et al., 1993). MS-2, another putative transcription factor, was also reported to prime the expression of CD11b (Farokhzad et al., 1996). Furthermore, ZBP-89, as a zinc finger transcription factor, was demonstrated to repress CD11b gene transcription and subsequently inhibit monocytes differentiating into macrophages *in vitro* (Park et al., 2003). HIF-1α is a key transcription factor for regulating the expression of target genes by binding to HRE elements in the promoter region (Palazon et al., 2014), and our results showed that HIF-1α bound onto the HRE II or HRE IV regions in the *Itgam* promoter to promote its transcription. Nevertheless, HIF-1α inhibition or knockdown did not absolutely suppress CD11b transcription, which implies that LPS or inflammation might first trigger an inflammatory response, cause HIF-1α activity and accumulation, and subsequently promote CD11b transcription, but there might be other factors involved in the regulation of CD11b expression.

Upon LPS stimulation, ERK and STAT3 signaling pathways were activated in a time-dependent manner, and the phosphorylation of Y705 occurred later than that of S727 (Supplementary Fig. S5B). STAT3 is known to be phosphorylated on serine 727 (S727) by the mitogen-activated protein (MAP) kinase pathway to obtain maximal activation (Wen et al., 1995). Correspondingly, the phosphorylation of STAT3 on S727 was found to be suppressed by the MEK/ERK inhibitor while the STAT3 inhibitor had no effect on the MEK/ERK signaling pathway (Fig. 5B), implying that LPS stimulation might trigger the ERK activation to phosphorylate STAT3 on S727. Previous study has reported that

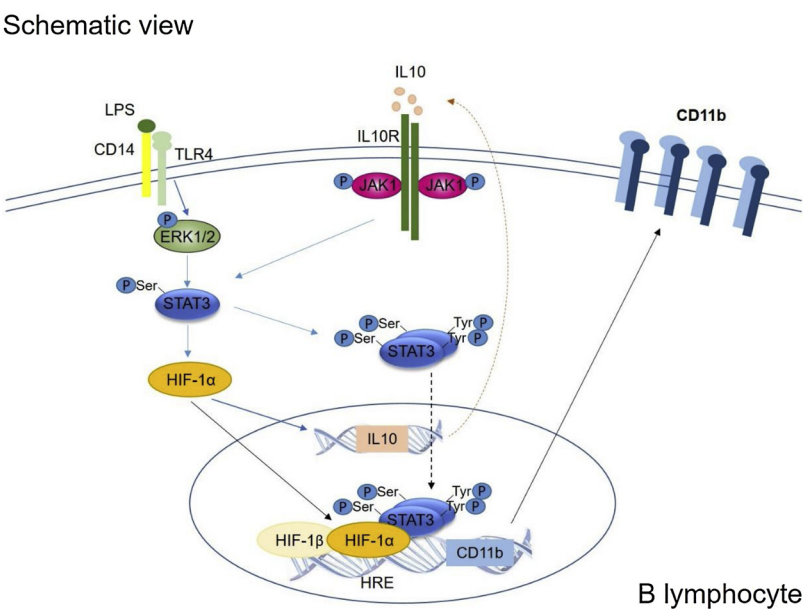

Fig. 6. A schematic view of the regulatory role of HIF-1α and p-STAT3 on CD11b transcription in B cells under LPS stimulation. LPS stimulation or mucosal inflammation might trigger ERK phosphorylation, activating STAT3 on S727. HIF-1α was then activated by p-STAT3^{727} and enhanced IL-10 production and secretion which subsequently phosphorylated STAT3 on Y705 to promote p-STAT3 to homodimerize. Finally, the p-STAT3 homodimer translocated to the nucleus and formed a complex with the HIF-1α and HIF-1β heterodimer to bind onto the HRE regions to facilitate the transcriptional regulation of CD11b.

Hif1a transcription was regulated by p-STAT3^{727} via binding to *Hif1a* promoter (Meng et al., 2018), and we found that HIF-1α expression was suppressed by ERK and STAT3 inhibitors (Fig. 5C), implying that HIF-1α expression was regulated by STAT3 signaling pathway. IL10 has been reported to be indispensable for CD11b expression. Upon LPS stimulation *in vitro*, CD11b expression was much lower in IL-10$^{-/-}$ B cells than that in IL-10$^{+/+}$B cells (Liu et al., 2015). We also analyzed CD11b expression in IL10^{+} or IL-10- B cells during the colitis process *in vivo*, and found that most of the IL10^{+}B cells expressed CD11b while only a small fraction of IL-10- B cells expressed CD11b (Supplementary Fig. S5D), suggesting that CD11b expression was regulated by IL-10. Furthermore, IL-10 production and secretion were suppressed under the treatment of HIF-1α inhibitor LW6 (Supplementary Fig. S3). Therefore, IL-10 was suggested to be involved in HIF-1α-regulated CD11b expression, which was verified by the treatment of ERK, STAT3 (Fig. 5D, E), implying that IL-10-regulated CD11b expression was HIF-1α- dependent. STAT3 has been identified to be activated by secreted IL-10 cytokine through phosphorylation on Y705 (Ouyang et al., 2011) and then homodimerizes and translocates to the nucleus to promote target gene transcription (Wen et al., 1995). Consistent with these findings, we found that recombinant mouse IL-10 promoted the phosphorylation of STAT3 on Y705 (Supplementary Fig. S5C). Finally, the ChIP assay suggested that p-STAT3 translocated to the nucleus and formed a complex with HIF-1α to bind onto the HRE regions to facilitate the transcriptional regulation of CD11b (Fig. 5F).

In summary, we revealed that HIF-1α may participate in lymphocyte-mediated inflammation or immune response via regulating the transcription of CD11b in B cells. Notably, HIF-1α appears to form a transcriptional complex with p-STAT3 and then binds onto HRE II or HRE IV regions in the *Itgam* promoter. This study provides more evidence for CD11b as a marker for regulatory B cells and provides a fresh therapeutic approach and insight regarding the treatment of colitis.

4. Materials and Methods

4.1. Mice

WT C57BL/6 mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). C57BL/6 background B6.129S7-Rag1tm1Mom/J (Rag-1$^{-/-}$) mice were purchased from Peking University ShenZhen Hospital (Shenzhen, China). All mice were raised to the age of 6–8 weeks in the animal facility of Fudan University (Shanghai, China) under specific pathogen-free barrier conditions. All animal experiment were conducted under the approved protocol *Guidelines for the Care and Use of Laboratory Animals.*

4.2. Mouse model

According to the previous protocol, experimental colitis was induced by feeding the mice with 2% DSS (w/v; MP Biomedicals, Santa Ana, CA, USA) in their drinking water for the indicated time.

4.3. Antibodies and FACS analysis

Mononuclear cells were first incubated with an anti-CD16/32 antibody (BD Biosciences) and then reacted with the following anti-mouse antibodies: allophycocyanin (APC) -Cy7-CD11b (M1/70), APC-CD93 (AA4.1), pacific blue (PB)-CD19 (1D3), PB-CD45 (30-F11), fluorescein (FITC)-CD19 (1D3), perCP-eFluor™ 710-IgM (11/41), APC-CD93 (AA4.1) (all from eBioscience, San Diego, CA, USA) and phycoerythrin (PE)-CD23 (B3B4) (from BD Biosciences). Concentration-matched isotype antibodies were used as negative controls. For the apoptosis assay, cells were stained with annexin V/propidium iodide and analyzed by flow cytometry. The flow-cytometric analysis was performed with the CyAn ADP Analyzer (Beckman Coulter, Carlsbad, CA, USA), and data were analyzed using the FLOWJO software (Tree Star, Ashland, OR, USA). Absolute numbers of multiple cell populations were calculated by flow cytometry according to their respective percentage.

4.4. Transfection of small interfering RNAs

Splenic B cells were purified from WT mice by the EasySep™ Mouse B Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). The purity of the isolated B cells was examined to be over 90% by using FCM analysis. The siRNAs were then transfected into the isolated splenic B cells by electroporation. Before electroporation, B cells were washed four times with Opti-MEM (Gibco, Portland, OR, USA) and re-suspended in Opti-MEM medium. Indicated doses of mRNA and B cells were then added into 100 ul of Opti-MEM medium and electroporated in a 2-mm cuvette using an ECM830 Electro Square Wave Porator (Harvard Apparatus BTX, USA)

4.5. Drug treatment

The following inhibitors were used in indicated concentration: LW6 (Absin, China), trametinib (MEK/ERKi), Stattic (JAK/STAT3i), SB202190 (p38/MAPKi), SP600125 (JNKi), and SC75741 (NF-κBi; Selleck Chemicals, Texas, USA),

4.6. Adoptive transfer assays

After being isolated and washed by Opti-MEM, 5×10^6 splenic B cells were transfected by siRNA. The transfected B cells and sorted $CD3^+$ T cells were then injected into each $Rag^{-/-}$ mouse. After 24 h, colitis was induced in the mice by feeding them 2.5% DSS water.

4.7. ChIP

ChIP was performed as previously described. The treated cells were cross-linked using 2 ml of 1% formaldehyde, lysed, and sonicated in a Covaris instrument (shearing time 30 min, 20% duty cycle, intensity 10, 200 cycles per burst, 30 s per cycle). ChIP was performed using rabbit anti-p-STAT3 antibody (1:200, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-HIF-1α (1:200, Novus, USA), rabbit anti-HIF-1β (1:200, Novus, USA), and rabbit IgG (1:200, Abcam, UK) following a standard protocol. HIF-1α binding sites were predicted by the JASPAR website and primers were designed by Primer-BLAST (Supplementary Table 1) and synthesized by Sangon Biotech.

4.8. Luciferase reporter assay

The 1 kb fragments, including the HRE II or IV region in mice CD11b promoters, was cloned between the Kpnl and Xhol cutting sites of the pGL3-control vector (Promega, Madison, WI, USA) into a pGL3-control vector. The control plasmid (HIF-1α-NC) and HIF-1α transcription factor plasmid (HIF-1α-TF) were constructed for HIF-1α over-expression. The 293 T cells were co-transfected with putative HRE regions and HIF-1α-NC or HIF-1α-TF for 24 h. The cells were then lysed and analyzed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

4.9. Quantitative real-time PCR

For the detection of mRNA, a quantitative real-time PCR (qRT-PCR) analysis was performed according to a standard protocol. Total RNA was prepared with the RNeasy kit (Qiagen, Crawley, UK), followed by DNase treatment and then reverse transcribed into cDNA. The SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) Kit (Takara, Japan) was used to measure mRNA expression. The expression was normalized to β-actin (Actb). The primers were synthesized by Sangon Biotech. All primers are listed in Table 1.

4.10. Western blotting

Proteins were extracted by using a sodium dodecyl sulfate lysis buffer (P0013 G, Beyotime, Shanghai, China) as previously reported (Qian et al., 2010). The primary antibodies used in western blotting were as follows: rabbit anti-STAT3, rabbit anti-Phospho-STAT3, rabbit anti-Phospho-ERK, rabbit anti-ERK (1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-HIF-1α (1:500, Novus, USA), and mouse anti-β-actin (1:5000, ProteinTech Group, Chicago, IL, USA).

4.11. Statistical analysis

Data were analyzed using the Graph-Pad Prism software (GraphPad Software Inc.,La Jolla, CA) and were presented as the means ± standard error of the mean (SEM). The Student's unpaired *t*-test or unpaired *t*-test with Welch's correction were used to analyze intergroup differences for two groups, ANOVA was used to analyze more than two groups, and Pearson's correlation coefficient was used to analyze the correlation between groups.

Disclosures

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.04.005.

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