Regulation of Alternative Macrophage Activation by Galectin-3


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Regulation of Alternative Macrophage Activation by Galectin-3

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Alternative macrophage activation is implicated in diverse disease pathologies such as asthma, organ fibrosis, and granulomatous diseases, but the mechanisms underlying macrophage programming are not fully understood. Galectin-3 is a carbohydrate-binding lectin present on macrophages. We show that disruption of the galectin-3 gene in 129sv mice specifically restrains IL-4/IL-13-induced alternative macrophage activation in bone marrow-derived macrophages in vitro and in resident lung and recruited peritoneal macrophages in vivo without affecting IFN-γ/LPS-induced classical activation or IL-10-induced deactivation. IL-4-mediated alternative macrophage activation is inhibited by siRNA-targeted deletion of galectin-3 or its membrane receptor CD98 and by inhibition of PI3K. Increased galectin-3 expression and secretion is a feature of alternative macrophage activation. IL-4 stimulates galectin-3 expression and release in parallel with other phenotypic markers of alternative macrophage activation. By contrast, classical macrophage activation with LPS inhibits galectin-3 expression and release. Galectin-3 binds to CD98, and exogenous galectin-3 or cross-linking CD98 with the mAb 4F2 stimulates PI3K activation and alternative activation. IL-4-induced alternative activation is blocked by bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane, a specific inhibitor of extracellular galectin-3 carbohydrate binding. These results demonstrate that a galectin-3 feedback loop drives alternative macrophage activation. Pharmacological modulation of galectin-3 function represents a novel therapeutic strategy in pathologies associated with alternatively activated macrophages. The Journal of Immunology, 2008, 180: 2650–2658.
stabilizing atherosclerotic plaques. To exploit M1 and M2 macrophages for future anti-inflammatory and anti-cancer therapies, it is important to understand the mechanism and extracellular ligands that determine M1 and M2 macrophage programming.

Galectin-3 is a β-galactoside-binding lectin of ~30 kDa that has been implicated in inflammation and fibrosis (23–27). Galectin-3 is highly expressed and secreted by macrophages, and recent data suggest that galectin-3 plays a significant role in many facets of macrophage biology. Galectin-3 is up-regulated when monocytes differentiate into macrophages (28) and down-regulated when macrophages differentiate into dendritic cells (29). Galectin-3 also promotes monocyte–monocyte interactions that ultimately lead to polykaryon (multinucleated giant cell) formation, a phenotype-associated with alternative macrophage activation (30), and chronic inflammatory and fibrotic diseases (31). Our previous work has demonstrated that mice deficient in galectin-3 exhibit reduced hepatic fibrosis following chronic administration of CCl4 (32).

CD98 is a disulfide-linked 125-kDa heterodimeric type II transmembrane glycoprotein composed of a glycosylated 85-kDa H chain (designated CD98) and a nonglycosylated 40-kDa L chain implicated in inflammation (33). CD98 is highly expressed on macrophages and has been shown to be a receptor for galectin-3 (34). CD98 constitutively associates with β1 integrins, and cross-linking with the 4F2 Ab stimulates integrin-mediated increases in focal adhesion kinase and PI3K activation (35, 36). In this study, we have examined the role of galectin-3 and CD98 on macrophage activation phenotype.

Materials and Methods
Tissue culture reagents were purchased from Life Technologies. Cytokines and recombinant mouse and human galectin-3 were purchased from R&D Systems and PeproTech. The cytometric bead array mouse inflammation kit was from BD Biosciences. The hybridoma cell line 4F2 was purchased from the American Type Culture Collection, and secreted Ab was purified using protein A affinity chromatography. The galectin-3 inhibitor bis-(3-deoxy-3-(3-methoxybenzamido)-d-glucopyranosyl) sulfate was provided by U. Nilsson and H. Leffler (37). Cynaropicrin was a kind gift from Dr. J. Cho (Kangwon National University).

Animals
Generation of galectin-3−/− 129sv mice by gene-targeting technology has been described previously (38). As control, age- and sex-matched wild-type (WT) mice were used. All procedures were performed in accordance with U.K. Home Office guidelines (Animals (Scientific Procedures) Act 1986). All mice used were 8–10 wk old at the start of the experiments and were maintained in the animal facilities at the University of Edinburgh.

Tissue culture and transfections
Bone marrow-derived macrophages (BMDMs) were prepared from WT and galectin-3−− mice by maturing bone marrow cells in DMEM containing 10% FBS and 20% L929 conditioned media for 7–9 days (39). In vivo-derived macrophages were obtained from lung and peritoneal lavage and separated by adhesion onto tissue culture plastic. Human peripheral blood monocytes were prepared as previously described (40), and they were cultured for 5 days in Iscove’s medium containing 5% autologous serum. THP-1 cells were obtained from the American Tissue Culture Collection and were maintained in RPMI 1640 medium supplemented with 10% FBS. Cells were differentiated with 10 ng/ml PMA for 24 h, washed, and transfected with 100 pmol siRNA duplexes using Oligofectamine (Invitrogen). Cells were incubated for 48 h in complete media before addition of 10% or 4F2. Initial experiments were conducted using four duplexes directed against human CD98 (SLC3A2) as supplied in CD98 SMARTpool (Dharmacon). All additional experiments used siRNA duplexes (Dharmacon) directed to the target sequence AGAATG GCATCCTGGTACAGA. Initial experiments to target galectin-3 used siRNA duplexes directed against the target sequences: 1) GGAAGAAGACAGTC GGTTC, 2) GCAATACAAAGCTGGATAA, 3) GTACAATCATCGGG TAAAG, and 4) CAGTACAATCATCGGGTTA.

All additional experiments were conducted using duplex 1. Control duplex was siCONTROL nontargeting siRNA no. 2 (Dharmacon). siRNA duplexes directed against human STAT6 were purchased from Santa Cruz Biotechnology (catalog no. sc-29497). Cytokine/nitrite analysis
Cytokine release from macrophage supernatants was determined by cyto- metric bead array. Human TNF-α was measured by ELISA (BD Biosciences). NO production was determined by measurement of nitrite release using the Griess reaction (Sigma-Aldrich).

Arginase assay
Arginase activity was assessed by the production of urea generated by the arginase-dependent hydrolysis of L-arginine as described (41).

Real-time RT-PCR
Total RNA from BMDMs or THP-1 cells (4 × 106 cells/sample) was prepared using RNeasy kits (Qiagen) and reverse transcribed into cDNA using random hexamers (Applied Biosystems). For analysis of alternative activation markers, cDNA was analyzed using a SYBR green-based quantitative fluorescence method (Applied Biosystems). The following primer pairs were used: mouse β-actin: forward 5′-AGAGGGAAATCTGCGTG ACG-3′, reverse 5′-CAATAGTGATGACCTGGCCGT-3′; mouse FIZZ1: forward 5′-CGGTGGTGAAGA-3′, reverse 5′-ATTACAATCATCGGGTACT-3′; reverse 5′-TATCCCTGGTGTCCTGGTTAC-3′, reverse 5′-GATCTGGTGTCTCCTGGT-3′; mouse arginase-1: forward 5′-TTGTGGTGCTGTCAC-TG-3′, reverse 5′-TTGCGCATGCAATCCTCC-3′; mouse mannose receptor: forward 5′-CATGACCCCTCTCCTGCTCT-3′, reverse 5′-TTGGCTCTCTGAACTGATG-3′; mouse NOS2: forward 5′-ACCTTGAAAGTAGGCGTT-3′, reverse 5′-TTGGAAACACGTTCCTGAGGAG-3′; human β-actin: forward 5′-CATCACCATTGCGCAATGAGC-3′, reverse 5′-CGATTCACAC GGAGTACTTG-3′; human mannose receptor: forward 5′-GCCCAATTG AGGAATTGTGAGG-3′, reverse 5′-CAGAAGCCATTGTTGAAACCG-3′.

Immunofluorescence
Alveolar macrophages were fixed in 3% paraformaldehyde and stained with rabbit anti-Ym1 (Professor J. Allen, Edinburgh) followed by species-specific Alexa 488-conjugated secondary Abs and fluorescence microscopy (Leica).

Immunoprecipitation and Western blotting
Cell lysates were resolved by SDS-PAGE (under nonreducing conditions for mannose receptor blots) and transferred onto nitrocellulose. Blots were probed with the following primary Abs: mouse monoclonal anti-human mannose receptor Ab clone 15–2 (MCA2155, Serotec), rat anti-mouse mannose receptor Ab clone 5D3 (MCA2235GA, Serotec), monoclonal anti-galectin-3 Ab clone A3A12 (Alexis Biochemicals), goat polyclonal anti-CD98 (sc-7095, Santa Cruz Biotechnology), rabbit polyclonal anti-β-actin Ab (Sigma-Aldrich), rabbit anti-PKβ and anti-pPKβ (S473) (Invitrogen: IgSource), rabbit anti-STAT6 and anti-pSTAT6 (T641) (Cell Signaling Technology), rabbit anti-mouse IL-4Rα (sc-686, Santa Cruz Biotechnology), and rabbit polyclonal anti Ym1 (from Professor J. Allen).

CD98 was immunoprecipitated from THP-1 cell lysates (0.5 mg protein) with 2 μg goat polyclonal anti-CD98 (sc-7095, Santa Cruz Biotechnology) or 2 μg control goat IgG overnight at 4°C. Immune complexes were captured with protein A/G-Sepharose, and washed immunoprecipitates were resolved by SDS-PAGE and blotted for CD98 or galectin-3.

Statistical analysis
Results are presented as means ± SEM. Significance of the differences between means was assessed using one-way ANOVA or two-tailed Student’s t test. Values of p < 0.05 were considered significant. Unless stated otherwise, studies were performed on three to six independent occasions.

Results
Disruption of the galectin-3 gene in macrophages causes a specific defect in alternative activation of in vitro- and in vivo-differentiated macrophages
To investigate the role of galectin-3 in macrophage activation, we used in vitro generated BMDM from WT and galectin-3−− mice. We treated BMDMs with either IFN-γ/LPS (classical M1 activation) or IL-4/IL-13 (alternative M2 activation) and measured the
release of proinflammatory cytokines (TNF-α and IL-6), NO (nitrite), and arginase activity. We found that IFN-γ and LPS induced a similar release of TNF-α and IL-6 in both WT and galectin-3−/− BMDMs (Fig. 1, A and B). Treatment of BMDMs with IL-4 or IL-13 alone had no effect on cytokine release but significantly reduced LPS-stimulated TNF-α and IL-6 release in WT but not galectin-3−/− BMDMs (Fig. 1, C and D). Arginase-1 competes with NOS2 and mediates the alternative metabolic pathway of l-arginine to L-ornithine and urea (10). Classical activation with IFN-γ and LPS produced a similar increase in NO production from both WT and galectin-3−/− BMDMs (Fig. 1E). In contrast, IL-4/IL-13 (alternative activation) induced a >5-fold stimulation in arginase-1 activity in WT macrophages. However, in galectin-3−/− BMDMs, IL-4 or IL-13 induced significantly less arginase activity compared with WT BMDMs (Figs. 1F and 2A). IL-10 (true deactivation) inhibited TNF-α and IL-6 release by LPS in both WT and galectin-3−/− BMDMs (Fig. 1C–E).

Real time RT-PCR analysis of untreated, IL-4-stimulated, or LPS-stimulated BMDMs showed equal expression of the IL-4/IL-13 common receptor, IL-4Rα, in WT and galectin-3−/− macrophages (Fig. 2B). Western blots showed equal protein expression of IL-4Rα. To further characterize alternative activation in response to IL-4, BMDMs were treated with IL-4 in the presence or absence of LPS, and markers of alternative activation were analyzed by real-time RT-PCR. IL-4 produced a significant increase in the ratio of arginase/NOS2 expression in WT BMDMs. However, the increased ratio was significantly less in galectin-3−/− BMDMs (Fig. 2C). LPS alone caused a marked increase in NOS2 expression, decreasing the arginase/NOS2 ratio, which was not significantly different between WT and galectin-3−/− BMDMs (Fig. 2C).

Expression of mannose receptor, Ym1, and FIZZ1 was significantly increased in response to IL-4 in WT BMDMs. However, galectin-3−/− macrophages showed significantly less increase in expression of alternative activation markers (mannose receptor, FIZZ1, and Ym1) in response to IL-4 (Fig. 2C). LPS treatment alone or in combination with IL-4 reduced alternative activation marker expression in WT BMDMs.

We then compared the NO and cytokine response of in vivo-differentiated WT and galectin-3−/− macrophages to classical stimulation with IFN-γ/LPS. There was no significant difference in TNF-α, IL-6, and NO release in response to IFN-γ/LPS in vivo-differentiated peritoneal macrophages isolated from galectin-3−/− or WT mice (Fig. 3A–C). Similar results were also seen in alveolar macrophages (results not shown). As with BMDMs, in vivo-derived peritoneal macrophages from galectin-3−/− mice showed reduced IL-4- or IL-13-stimulated arginase activity (Fig. 3D). Bronchoalveolar lavage fluid from galectin-3−/− mice demonstrated a decrease in expression of the alternative activation marker Ym1 compared with WT (Fig. 3E). Furthermore, WT alveolar macrophages demonstrated dense intracellular Ym1 immunofluorescence, which was not observed in alveolar macrophages from galectin-3−/− mice.
Galectin-3 autocrine loop promotes alternative macrophage activation and is mediated by galectin-3-dependent arginase activation.

**FIGURE 3.** Galectin 3−/− peritoneal and alveolar macrophages display normal classical activation but impaired alternative activation. Peritoneal macrophages were stimulated with IFN-γ (100 U/ml) for 48 h and LPS (100 ng/ml) was added for the last 24 h. Supernatants were assayed for (A) TNF-α, (B) IL-6, and (C) nitrite. The results represent the means ± SEM of three independent experiments. *p < 0.01 compared with WT. E, PBS (1 ml) was administered intratracheally to WT and galectin-3−/− mice and bronchoalveolar lavage fluid was analyzed for YM1 protein expression by Western blot analysis and immunofluorescence staining of YM1 in alveolar macrophages isolated from lavage fluid of WT (left panel) and galectin-3−/− mice (right panel) (scale bar: 5 μm).

**FIGURE 4.** Galectin-3 potentiates IL-4-induced alternative activation and is released from IL-4 treated cells. A, IL-4 stimulates galectin-3 expression and release. Upper panel, Human PBMs and WT mouse BMDMs were incubated for 48 h with 10 ng/ml IL-4 or IL-13. Western blots from cell supernatants were probed for galectin-3. Lower panel, WT BMDMs were treated for 48 h with IL-4 (0–10 ng/ml) in the presence or absence of 100 ng/ml LPS. Western blots from cell supernatants and cell lysates were probed for galectin-3. B, BMDMs were treated with 10 ng/ml IL-4 for 24 h in the presence or absence of 5 μM of galectin-3 inhibitor (bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane) or 10 μM of cynaropicrin, and arginase activity was measured in cell lysates. Results represent the means ± SEM of three independent experiments. *p < 0.01 compared with WT. Inset, Western blot of mannose receptor expression.

**FIGURE 5.** CD98 and galectin-3 expression and release. BMDMs and human peripheral blood monocyte-derived macrophages (PBMs) were treated with IL-4 for 48 h. Western blot analysis shows that IL-4 or IL-13 stimulated the release of galectin-3 into the culture media in both BMDMs and PBMs (Fig. 4A). In contrast, LPS-treated BMDMs showed a marked down-regulation of galectin-3 expression in both cell lysates and supernatants. Thus, classically activated macrophages down-regulate galectin-3 expression and show reduced galectin-3 expression on the cell surface. Bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane is a specific and high-affinity inhibitor of galectin-3 based on N-acetyllactosamine, the best natural ligand for galectin-3, and is a specific and highly selective inhibitor of the carbohydrate recognition domain of galectin-3 (Kᵢ of 60 nM and >20-fold selectivity over other galectins, including galactin-1 (37)). Coincubation with the galectin-3 inhibitor blocked IL-4-mediated arginase activation and mannose receptor expression in BMDMs (Fig. 4B). This suggests that galectin-3 may be mediating its effects at the cell surface via its carbohydrate-recognition domain.

**Figure 4B.** Galectin-3 potentiates IL-4-induced alternative activation and is released from IL-4-treated cells. A, IL-4 stimulates galectin-3 expression and release. Upper panel, Human PBMs and WT mouse BMDMs were incubated for 48 h with 10 ng/ml IL-4 or IL-13. Western blots from cell supernatants were probed for galectin-3. Lower panel, WT BMDMs were treated for 48 h with IL-4 (0–10 ng/ml) in the presence or absence of 100 ng/ml LPS. Western blots from cell supernatants and cell lysates were probed for galectin-3. B, BMDMs were treated with 10 ng/ml IL-4 for 24 h in the presence or absence of 5 μM of galectin-3 inhibitor (bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane) or 10 μM of cynaropicrin, and arginase activity was measured in cell lysates. Results represent the means ± SEM of three independent experiments. *p < 0.01 compared with WT. Inset, Western blot of mannose receptor expression.

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was visualized at ~30 kDa (lane 2). This was not seen following immunoprecipitation with control IgG Ab (lane 4) and confirms that galectin-3 associates with CD98 in differentiated THP-1 cells. Moreover, incubation of THP-1 cells for 1 h with the galectin-3 inhibitor (5 μM) before lysis blocked co-immunoprecipitation of galectin-3 with CD98. We then sought to determine whether inhibition of galectin-3 or CD98 expression could inhibit alternative activation. Four siRNA duplexes directed against human CD98 or galectin-3 were tested in THP-1 cells. All four galectin-3 siRNA duplexes caused an ~90% knockdown of galectin-3 protein expression, and CD98 siRNA duplexes 1 and 3 reduced CD98 protein expression by >95% (data not shown). In subsequent experiments, galectin-3 duplex 1 and CD98 duplex 1 were used and compared with a nontargeting control duplex. Results were confirmed with galectin-3 duplex 4 and CD98 duplex 3 in all experiments (data not shown). siRNA-targeted inhibition of galectin-3 or CD98 expression blocked IL-4-stimulated increase in mannose receptor expression as judged by Western blot analysis and real-time RT-PCR (Fig. 5, C and D). Inhibition of galectin-3 function either by siRNA or with the galectin-3 inhibitor, and inhibition of CD98 expression in THP-1 cells did not affect TNF-α release in response to IFN-γ/LPS (Fig. 5E). As a whole, our data suggest that alternative macrophage activation by IL-4 is driven by a galectin-3 feedback mechanism, which activates CD98.

Galectin-3-mediated alternative activation involves CD98 and activation of PI3K

Our previous work has shown that CD98 stimulates PI3K activation (35, 36). siRNA-targeted inhibition of CD98 expression or...
inhibition of galectin-3 carbohydrate binding by the galectin-3 inhibitor both blocked IL-4 and galectin-3-stimulated PI3K activation as measured by phosphorylation of PKB phosphorylation in THP-1 cells (Fig. 6, A and C). Cross-linking CD98 with the mAb 4F2 stimulated a sustained PI3K activation (evident at 20 min and maintained at 24 h) and increased mannose receptor expression to a similar level as IL-4 in THP-1 cells (Fig. 6B). Blocking PI3K activity with LY294002 prevented IL-4-, galectin-3-, and 4F2-mediated PKB phosphorylation and mannose receptor expression (Fig. 6B). LY294002 blocked IL-4-stimulated arginase activation in BMDMs to a level observed in galectin-3 inhibitor depletion or treatment with a specific galectin-3 inhibitor blocked IL-4-mediated alternative macrophage activation as measured by arginase activation and alternative marker expression. In contrast, classical activation induced by IFN-γ and LPS and inactivation with IL-10 was not affected by galectin-3 deletion.

Galectin-3 binds N-acetyllactosamine residues on surface glycoproteins (44, 45). In addition to the carbohydrate-binding domain, galectin-3 contains a proline- and glycine-rich N terminal domain (ND) through which it is able to form oligomers. It has therefore been suggested that galectin-3 can prolong receptor signaling by restricting receptor movement within the membrane and by delaying removal by constitutive endocytosis (45). Intracellularly, galectin-3 interacts with several unglycosylated molecules through protein–protein interactions (44, 46). Bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane is a high-affinity inhibitor of galectin-3 carbohydrate binding based on N-acetyllactosamine (37). The finding that this galectin-3 inhibitor blocked alternative macrophage activation suggests that the carbohydrate recognition domain of galectin-3 is responsible for these effects and supports an extracellular mechanism of action.

Discussion

Our present data demonstrate that IL-4 stimulates a galectin-3 feedback loop that causes sustained PI3K activation via activation of CD98, and that this is a key mechanism required for activation of an alternative macrophage phenotype. Alternative macrophage activation is specifically defined as the unique phenotype that is associated with exposure to IL-4/IL-13 and is characterized by the up-regulation of a number of genes such as mannose receptor, as well as a unique pattern of functions that is distinct from those induced by IFN-γ (classical activation) or IL-10 (true inactivation) (1). We have shown in macrophages that siRNA-targeted depletion of galectin-3 or treatment with a specific galectin-3 inhibitor blocked IL-4-mediated alternative macrophage activation as measured by arginase activation and alternative marker expression. In contrast, classical activation induced by IFN-γ/LPS and inactivation with IL-10 was not affected by galectin-3 deletion.

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We have shown that alternative activation of macrophages with IL-4 and IL-13 stimulates galectin-3 expression and release and, conversely, classical activation with IFN-γ/LPS inhibits galectin-3 expression. Previous work has also shown that activation of peripheral macrophages with IFN-γ and LPS reduces galectin-3 surface expression (47). Thus, up-regulation of galectin-3 expression is a feature of the alternative macrophage phenotype, and release of galectin-3 by alternatively activated macrophages sustains the alternative macrophage phenotype and may also contribute to some of the functions of alternatively activated macrophages in vivo. Galectin-3 has been shown to be a specific and highly up-regulated marker of myocardial macrophages in failing hearts (27), suggesting that increased galectin-3 expression by macrophages has a potentially important pathophysiological role in cardiac dysfunction.

Our results show that IL-4 stimulates a galectin-3 autocrine loop with increased expression and secretion of galectin-3 that binds to and cross-links CD98 on macrophages. Blocking this binding prevents alternative macrophage activation and PI3K activation by

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FIGURE 7. Inhibition of STAT6 blocks alternative activation but not galectin-3 expression. A, THP-1 cells were transfected with siRNA against STAT6 or control duplex and stimulated with 10 ng/ml IL-4 or 10 μg/ml galectin-3 for 24 h. Blots from cell lysates were probed for STAT6, pSTAT6, mannose receptor (MR), pPKB, or β-actin as indicated. B, THP-1 cells were transfected with siRNA against STAT6 or control duplex and stimulated with IL-4 for 24 h. Blots from cell lysates were probed for STAT6, mannose receptor (MR), galectin-3, or β-actin as indicated. C, Quantitation of mannose receptor expression by optical densitometry of Western blots by Image J. THP-1 cells were treated with siRNA to CD98 or STAT6, or they were treated with 10 μM of LY294002 or 5 μM of galectin-3 inhibitor in the presence or absence of 10 ng/ml IL-4 as described above. Results represent the means ± SEM of three to four independent experiments.
IL-4 and galectin-3. We have previously shown that CD98 stimulates PI3K activation via an association with \( \beta_1 \)-integrins (35, 36). Our work shows that activation of CD98 is associated with cellular activation and transformation and is dependent on functional \( \beta_1 \)-integrin expression and focal adhesion kinase phosphorylation, causing elevation of intracellular phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P\(_3\)) and PKB activation (35, 36). In the present study, we suggest that CD98-mediated PI3K activation is a key mediator of alternative macrophage activation. The role of CD98 was evaluated in experiments where CD98 function was blocked using two strategies: inhibition of CD98 expression with siRNA and by blocking CD98 function using the sesquiterpene lactone cynaropicrin. At the concentrations of cynaropicrin used in this study no acute cytotoxic effects were noticed as determined by acridine orange/propidium iodide staining. This is in keeping with previous studies with cynaropicrin on macrophages that showed no cytotoxicity at 1–10 \( \mu \)M concentrations (43). Inhibition of CD98/\( \beta_1 \)-integrin function and blocking PI3K activation with LY294002 inhibited IL-4-, galectin-3-, and 4F2-stimulated alternative macrophage activation, therefore demonstrating that activation of PI3K is a key mediator of alternative activation downstream of CD98. Cynaropicrin has been shown to inhibit CD98 and \( \beta_1 \)-integrin-mediated responses in macrophages. This ability to inhibit integrin function in the absence of activating ligand (e.g., CD98/IL-4) may explain its ability to block basal arginase activity and mannose receptor expression. We would hypothesize that CD98 through its association with \( \beta_1 \)-integrins may be tonically driving PI3K activation, which leads to a basal expression of mannose receptor. This expression can be further enhanced by activation of CD98 by galectin-3. The galectin-3 inhibitor bis-(3-deoxy-3-(3-methoxybenzamido)-\( \beta \)-D-galactopyranosyl)sulfane only inhibits this elevated response. This hypothesis is strengthened by our observations that cynaropicrin blocks both basal and IL-4-stimulated arginase activity in WT macrophages, whereas the galectin-3 inhibitor only blocks the galectin-3-augmented response and inhibits arginase activity to a level similar to that observed in galectin-3\(^{-/-}\) macrophages (Fig. 4B).

Our results suggest that the effect of galectin-3 is not due to an increase in expression of IL-4 receptors, as analysis of transcript expression and protein expression in whole-cell lysates from WT and galectin-3\(^{-/-}\) macrophages showed no difference in IL-4R\(\alpha\). However, galectin-3 may affect receptor cycling and expression at the cell surface (45). IL-4, but not 4F2 or galectin-3, increased STAT6 phosphorylation, and this was not inhibited by LY294002. However, IL-4- and galectin-3-stimulated alternative macrophage activation was blocked with siRNA to STAT6, suggesting that while STAT6 activation is permissive, it is not sufficient to drive alternative macrophage activation. Additionally, blocking STAT6 with siRNA did not inhibit basal or IL-4-induced galectin-3 expression. This would suggest that galectin-3 secretion is not STAT6 dependent and may be controlled by an independent pathway. Taken together, our work suggests that PI3K is the main pathway driving alternative activation in macrophages but that some STAT6 activity is required. The precise role of STAT6 in alternative activation requires further study. A number of studies suggest that PI3K activation may be the final common pathway to alternative (M2) macrophage activation. The PI3K/PKB pathway in macrophages negatively regulates NOS2 expression (48) and suppresses LPS-induced inflammation in endotoxemic mice (49). Constitutive elevation of PI3K and PI(3,4,5)P\(_3\) in SHIP\(^{-/-}\) mice produces M2 skewing (50). These mice have high Ym1 concentrations in the lung, which may represent an exaggerated manifestation of immune tolerance and healing, which contribute to chronic lung inflammation and fibrosis.

Our results provide a mechanistic link between IL-4, galectin-3, and CD98 in driving alternative macrophage activation. These results may also be relevant to the formation of multineucleated giant cells (MGCs), a phenotype induced by IL-4 or IL-13 and associated with chronic inflammatory and fibrotic diseases (30, 31). IL-4-induced MGC formation is dependent on \( \beta_1 \)- and \( \beta_2 \)-integrin expression and is inhibited by function-blocking Abs and by inhibitors of PI3K (51). Galectin-3 promotes monocyte–monocyte interactions that ultimately lead to MGC formation. Furthermore, cross-linking CD98 with anti-CD98 mAbs promotes polykaryon formation (52). Therefore, modulation of galectin-3 expression and interaction with CD98/integrins during macrophage differentiation may be important in the regulation of macrophage plasticity and control of polykaryon formation in chronic inflammatory diseases.

Macrophages are involved in all stages of inflammatory process including fibrosis, tissue repair, and healing (53, 54). In progressive inflammatory injury, macrophage depletion results in amelioration of fibrosis. By contrast, depletion during recovery results in a failure of resolution, with persistence of cellular and matrix components of the fibrotic response occurring. Thus, macrophages play distinct roles in injury and repair, highlighting that macrophages may be both pathogenic and beneficial depending on the timing and injury. A number of studies provide evidence for the association between alternative macrophage activation and enhanced fibrosis (10, 14, 15, 55, 56). IL-4/IL-13-activated macrophages upregulate several genes involved in the mechanisms of fibrosis (21, 57, 58) and they stimulate production of fibronectin and other matrix proteins (14, 59, 60). Mice deficient in IL-4 and IL-13 or their common receptor IL-4R\(\alpha\) show inhibition of alternative macrophage activation correlating with decreased lung fibrosis (58). However, macrophage/neutrophil specific knockout of the IL-4/IL-13 common receptor, IL-4R\(\alpha\), does not prevent collagen deposition and hepatic fibrosis in Schistosoma mansoni-infected mice. In this model, it was suggested that alternative macrophage activation down-regulates Th1 responses and that blocking alternative macrophage activation enhances S. mansoni egg-induced inflammation with increased mortality due to Gram-negative septicemia (14).

Our previous work has shown that galectin-3-deficient mice have a reduced fibrotic phenotype (32), and other studies have associated galectin-3 expression with a worse outcome in myocardial fibrosis (27). However, in studies of diabetic nephropathy (61) and in asthma (62), galectin-3 expression is associated with a decrease in tissue fibrosis. The divergent function of galectin-3 in these disease models may reflect the dualistic function of macrophages.

Given the role that alternatively activated macrophages play in diverse disease pathologies, modulation of macrophage phenotype may provide a novel approach to therapy. Inhibition of alternative (M2) macrophage activation may restrict fibrosis in granulomatous diseases (10) and enhance host immunity against cancers (22). In disease states where alternatively activated macrophages limit tissue injury or promote repair, it might be helpful to augment their activity, for example, in stabilizing atherosclerotic plaques. Finally, our study provides insight into the mechanisms regulating alternative macrophage activation and may provide more clearly defined therapeutic targets in a wide range of human diseases. Targeting the galectin-3/CD98/PI3K pathway with specific inhibitors such as bis-(3-deoxy-3-(3-methoxybenzamido)-\( \beta \)-D-galactopyranosyl)sulfane or cynaropicrin may represent a novel therapeutic target for manipulating macrophage phenotype in the treatment of cancer, chronic inflammation, and fibrosis.


