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Regulation of Alternative Macrophage Activation by Galectin-3


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.

Macrophages display broad phenotypic heterogeneity depending on their microenvironment (1–3). The initial inflammatory response is predominantly mediated by classically activated (M1-polarized) macrophages, which eradicate invading organisms and tumor cells (4, 5). The proinflammatory and cytotoxic activities of classically activated M1 macrophages are enhanced in the presence of microbial agents and/or Th1 cytokines such as IFN-γ or IL-12 (6, 7). Classical activation by IFN-γ in particular is associated with NO synthase 2 (NOS2) expression and the production of large amounts of NO and proinflammatory cytokines, including TNF-α and IL-6 (5, 8, 9). In contrast, the resolution phase of inflammation is driven by alternatively activated (M2-polarized) macrophages. Macrophages undergo alternative activation when stimulated with the Th2 cytokines IL-4 or IL-13 (1, 8, 10). Treatment of murine macrophages with IL-4 or IL-13 causes up-regulation of mannose receptor (11), arginase (10), Ym1 (chitinase-like lectin) (12, 13), and FIZZ1 (resistin-like secreted protein) expression (14, 15). The binding of IL-4 to the IL-4 receptor α-chain (IL-4Rα) induces heterodimerization of the γ-chain (type I receptor) or the IL-13Rα1 (type II receptor) (16). This dimerization activates the JAK family of tyrosine kinases, leading to phosphorylation of the receptor cytoplasmic tails and exposure of docking sites for STATs, primarily STAT6 and members of the insulin receptor substrate-1 family (16–19). Phosphorylated STAT6 migrates to the nucleus and initiates transcription of a number of target genes. Phosphorylated insulin receptor substrate-1 family binds to the p85 subunit of PI3K and Grb2. Activation of PI3K and downstream effectors protein kinase B (PKB) and p70S6 kinase are important mediators of proliferative and survival signaling and regulation of gene expression by IL-4 (16).

Alternative (M2) macrophage activation has been implicated in diverse disease pathologies in the host response to parasitic infection, asthma, wound repair, fibrosis in granulomatous diseases, athromatous plaques, and tumor-associated macrophages (14, 15, 20–22). Inhibitors of alternative 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, by...
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 cyto metric 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)-β-D-galactopyranosyl) sulfane 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 10% 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 100 ng/ml PMA for 24 h, washed, and transfected with 100 pmol siRNA duplexes using Oligofectamine (Invitrogen: BioSource), rabbit anti-STAT6 and anti-pSTAT6 (T641), mouse anti-IL-1Ra: forward 5′-CATTGGAAGTGAAGCGTTT-3′; mouse NOS2: forward 5′-GCCGTCTGAACTGAGATGG-3′; mouse arginase-1: forward 5′-GATGTTTGTCCTTAGGAG-3′, reverse 5′-AAAGCTGGGTTCTCCACCTC-3′; mouse IL-4R: forward 5′-ACCTGAGAACCGCCAGG-3′, reverse 5′-TCGGAAACAGGTCTCAGTGAG-3′; human β-actin: forward 5′-CATCACATTGCCGAATGCG-3′, reverse 5′-CGATCCACGGAGTCCG-3′; mouse mannose receptor: forward 5′-GCCAAATGACGAATTGGA-3′, reverse 5′-GCAAGAGCGTATTGAGA-3′. Tissue culture reagents were purchased from Life Technologies. Cytokines and recombinant mouse and human galectin-3 were purchased from R&D Systems and PeproTech.

Cytokine/nitrite analysis

Total RNA from BMDMs or THP-1 cells (4×10⁶ cells/sample) was prepared using RNeasy kits (Qiagen) and reverse transcribed into cDNA by the touch-down method (26). 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 quantitation method (Applied Biosystems). The following primers pairs were used: mouse β-actin: forward 5′-AGAGGGAAATCTGCTGGTCG-3′, reverse 5′-CAATTGAGACCTGGCCTG-3′; mouse ferritin light chain (ferritin-L): forward 5′-TATTGTGACGATGTTCTAC-3′, reverse 5′-TATC-AAAGCTGGTTTCCACCTC-3′; mouse Ym1: forward 5′-TCCTCACTCCAGAACCTGAG-3′, reverse 5′-GATGTTGTCCTCGTCCAGAG-3′; mouse arginase-1: forward 5′-TTGGTGTTGCTGCACAC-3′, reverse 5′-TTGCGCATGACATCC-3′; mouse mannose receptor: forward 5′-CATGAGGCTTCTCCTGCTTCT-3′, reverse 5′-TTGCCGCTCTGAACTGAGATGG-3′; mouse NOS2: forward 5′-CAGCTGGTCGTACACACCTT-3′, reverse 5′-CTCGGAATAAGGGTGACCT-3′; mouse β-actin: forward 5′-ACCTGAGAACCGCCAGGACG-3′, reverse 5′-CGATCCACGGAGTCCG-3′; human mannose receptor: forward 5′-GCCAAATGACGAATTGGA-3′, reverse 5′-GCAAGAGCGTATTGAGA-3′.

Immunofluorescence

Alveolar macrophages were fixed in 3% paraformaldehyde and stained with rabbit anti-Ym1 (Prof. 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 A31A2 (Alexis Biochemicals), goat polyclonal anti-CD98 (sc-7095, Santa Cruz Biotechnology), rabbit polyclonal anti-CD98 (sc-7095, Santa Cruz Biotechnology), rabbit anti-PKB and anti-pPKB (S473) (Upstate; BioSource), rabbit anti-STAT6 and anti-pSTAT6 (T641) (Cell Signaling Technology), rabbit anti-mouse IL-4R (sc-686, Santa Cruz Biotechnology), and rabbit polyclonal anti Ym1 (from Professor J. Allen).

CD98 was immunoprecipitated from THP-1 cells using a 0.5 mg protein mixture 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 galecin-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 galecin-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 galecin-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 galecin-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 galecin-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 galecin-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 galecin-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 galecin-3−/− BMDMs (Fig. 2C).
and galectin-3 (42) was added for the last 24 h. Supernatants were assayed for (A) TNF-α, (B) IL-6, and (C) nitrite. Cell lysates were assayed for arginase activity (D). The results represent the means ± SEM of three 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).

mice (Fig. 3E). These data suggest that both in vitro- and in vivo-differentiated galectin-3−/− macrophages have a specific defect in IL-4/IL-13-stimulated alternative activation.

An IL-4-mediated galectin-3 autocrine loop promotes alternative macrophage activation and is blocked by pharmacological inhibition of galectin-3 and CD98

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-acetylactosamine, the best natural ligand for galectin-3, (42) 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 galactin, 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 do-

IL-4-stimulated alternative macrophage activation is dependent on expression of galectin-3 and CD98

We used the human monocyctic cell line THP-1 to establish whether galectin-3-dependent alternative activation was mediated via CD98. THP-1 cells were differentiated into a macrophage phenotype by treatment with 100 ng/ml PMA for 24 h. As shown previously, differentiation of THP-1 cells with PMA caused a significant increase in expression of galectin-3 (Fig. 5A). This was paralleled with an increase in expression of CD98. To confirm a direct association of galectin-3 with CD98, CD98 was immunoprecipitated from THP-1 whole-cell lysates using a goat polyclonal anti-galectin-3 Ab and was compared with normal goat IgG as control. Western blots were probed for galectin-3 using a mouse monoclonal anti-galectin-3 (A3A12). Fig. 5B shows that upon immunoprecipitation of CD98 Ab and Western blotting for galectin-3, a band...
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 galectin-3 (duplex 1), human CD98 (duplex 1), or control duplex as described in Materials and Methods. Forty-eight-hour posttransfection THP-1 cells were incubated with 10 ng/ml IL-4 for 24 h. Total-cell RNA was extracted and mannose receptor gene expression was determined by real-time RT-PCR. Results are expressed as relative gene expression compared with β-actin and represent the means ± SEM of three independent experiments. E, THP-1 cells differentiated and transfected with siRNA as in C. Forty-eight-hour posttransfection THP-1 cells were incubated with 10 ng/ml IL-4 for 24 h. Total-cell RNA was extracted and mannose receptor gene expression was determined by real-time RT-PCR. Results are expressed as relative gene expression compared with β-actin and represent the means ± SEM of three independent experiments. F, Dose response to IL-4 in the presence of galectin-3 inhibitor. THP-1 cells were treated with 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). Western blots of cell lysates were probed with pPKB, total PKB, pSTAT6, mannose receptor (MR), and β-actin as indicated. C, Dose response to IL-4 by galectin-3 inhibitor. THP-1 cells were treated with 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). Western blots of cell lysates were probed with pPKB and β-actin as indicated. D, WT BMDMs were treated with IL-4 (0.1–100 ng/ml) in the absence (●) or presence of 5 μM of galectin-3 inhibitor (bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane) (□) or 10 μM of LY294002 (○) for 24 h. Arginase activity was measured from cell lysates as described in Materials and Methods. Results represent the means ± SEM of three experiments.

FIGURE 6. Inhibition of PI3K blocks alternative activation. A, THP-1 cells were transfected with siRNA against CD98 or control duplex and stimulated with 10 ng/ml IL-4 or 10 μg/ml galectin-3 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). Western blots of cell lysates were probed with CD98, pPKB, and β-actin as indicated (bottom panel). B, Differentiated THP-1 cells were treated with 10 μM of LY294002 20 min before addition of 10 ng/ml IL-4, 10 μg/ml 4F2, or 10 μg/ml human recombinant galectin-3 for 20 min (top panel) or 24 h (bottom) as indicated. Western blots of cell lysates were probed with pPKB, total PKB, pSTAT6, mannose receptor (MR), and β-actin as indicated. C, Dose response to IL-4 by galectin-3 inhibitor. THP-1 cells were treated with 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). Western blots of cell lysates were probed with pPKB and β-actin as indicated. D, WT BMDMs were treated with IL-4 (0.1–100 ng/ml) in the absence (●) or presence of 5 μM of galectin-3 inhibitor (bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane) (□) or 10 μM of LY294002 (○) for 24 h. Arginase activity was measured from cell lysates as described in Materials and Methods. Results represent the means ± SEM of three experiments.

Regulation of Alternative Macrophage Activation by Galectin-3

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−/− BMDMs or WT BMDMs treated for 24 h with 5 μM of galectin-3 inhibitor (Fig. 6D). IL-4, but not 4F2 or galectin-3, increased STAT6 phosphorylation, and this was not inhibited by LY294002 (Fig. 6B). However, targeted deletion of STAT6 with siRNA blocked IL-4 and galectin-3-mediated mannose receptor expression but not PKB activation (Fig. 7A) and did not affect basal or IL-4-induced galectin-3 expression (Fig. 7B). Therefore, inhibition of galectin-3 binding with bis-(3-deoxy-3-(3-methoxybenzamido)-β-D-galactopyranosyl) sulfane, an inhibitor of CD98 and STAT6 with siRNA, and inhibition of PI3K with LY294002 all block IL-4-stimulated alternative macrophage activation as judged by mannose receptor expression back to basal levels (Fig. 7C). However, inhibition of both PI3K (LY294002) and STAT6 activation (siRNA) completely abolishes IL-4- and galectin-3-induced mannose receptor expression (Fig. 7C). These treatments did not affect cell viability as judged by trypan blue staining (results not shown). This suggests that STAT6 and PI3K are independent signaling pathways and that STAT6 activation is not sufficient for alternative macrophage activation. However, robust sustained activation of the PI3K pathway by galectin-3 binding to CD98 drives alternative macrophage activation, but some basal STAT6 activity is necessary.

**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 macrophage marker expression. In contrast, classical activation induced by IFN-γ/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.

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 peritoneal 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
IL-4 and galectin-3. We have previously shown that CD98 stimulates PI3K activation via an association with β1-integrins (35, 36). Our work shows that activation of CD98 is associated with cellular activation and transformation and is dependent on functional β1-integrin expression and focal adhesion kinase phosphorylation, causing elevation of intracellular phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) 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 μM concentrations (43). Inhibition of CD98/β1-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 β1-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 β1-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)-β-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-4Rα. 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 activation in macrophages but that several 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 up-regulate several genes involved in the mechanisms of fibrosis (21, 57, 58) and they stimulate production of fibroconnectin and other matrix proteins (14, 59, 60). Mice deficient in IL-4 and IL-13 or their common receptor IL-4Rα show inhibition of alternative macrophage activation correlated with decreased lung fibrosis (58). However, macrophage/neutrophil specific knockout of the IL-4/IL-13 common receptor, IL-4Rα, 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)-β-D-galactopyranosyl)sulfane or cynaropicrin may represent a novel therapeutic target for manipulating macrophage phenotype in the treatment of cancer, chronic inflammation, and fibrosis.
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Disclosures
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