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Regulation of Alternative Macrophage Activation by Galectin-3¹

Alison C. MacKinnon,^{2*} Sarah L. Farnworth,^{2*} Philip S. Hodgkinson,* Neil C. Henderson,* Kirsten M. Atkinson,* Hakon Leffler,[†] Ulf J. Nilsson,[‡] Christopher Haslett,* Stuart J. Forbes,* and Tariq Sethi^{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-methoxybenz-amido)- 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)⁴

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-4R α) induces heterodimerization of the γ -chain (type I receptor) or the IL-13R α 1 (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 p70^{S6} 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, atherosclerotic 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

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⁴ Abbreviations used in this paper: NOS2, NO synthase 2; BMDM, bone marrow-derived macrophage; IL-4R α , IL-4 receptor α -chain; PBM, peripheral blood monocyte-derived macrophage; PBS, phosphate-buffered saline; PI(3,4,5)P₃, phosphatidylinositol-3,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; NOS2, nitric oxide synthetase 2; MGC, multinucleated giant cell; WT, wild type.

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 β_1 integrins, and cross-linking CD98 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)- 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). Cells were incubated for 48 h in complete media before addition of IL-4 or 4F2. Initial experiments were conducted using four duplexes directed against human CD98 (SLC3A2) as supplied in CD98 SMARTpool (Dharmacon, catalog no. M-003542). All additional experiments used siRNA duplexes (Dharmacon) directed to the target sequence AGAATG GTCTGGTGAAGA. Initial experiments to target galectin-3 used siRNA duplexes directed against the target sequences: 1) GAAGAAAGACAGTC GGTTC, 2) GCAATACAAAGCTGGATAA, 3) GTACAATCATCGGG TTTAAA, 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 cytometric 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 (4×10^6 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'-AGAGGGAAATCGTGCGTG AC-3', reverse 5'-CAATAGTGATGACCTGGCCGT-3'; mouse FIZZ1: forward 5'-TACTTGCAACTGCCTGTGCTTACT-3', reverse 5'-TATC AAAGCTGGGTTCTCCACCTC-3'; mouse Ym1: forward 5'-TCTCTA CTCCTCAGAACCGTCAGA-3', reverse 5'-GATGTTTGTCTTAGGAG GGCTTC-3'; mouse arginase-1: forward 5'-TTGGGTGGATGCTCACAC TG-3', reverse 5'-TTGCCATGCAGATTCCTCC-3'; mouse mannose receptor: forward 5'-CATGAGGCTTCTCCTGCTTCT-3', reverse 5'-TT CGCTCTGAACCTGAGATGG-3'; mouse NOS2: forward 5'-CAGCTG GGCTGTACAAACCTT-3', reverse 5'-CATTGGAAGTGAAGCGTTT CG-3'; mouse IL-4R α : forward 5'-ACCTGAGAACAGCGGAGGC-3', reverse 5'-TCGGAAAACAGGTTCTCAGTGAG-3'; human β -actin: forward 5'-CATCACCAATTGGCAATGAGC-3', reverse 5'-CGATCCACAC GGAGTACTTG-3'; human mannose receptor: forward 5'-GCCAAATG ACGAATTGTGGA-3', reverse 5'-CACGAAGCCATTGGTAAACG-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-PKB and anti-pPKB (S473) (Invitrogen: BioSource), rabbit anti-STAT6 and anti-pSTAT6 (T641) (Cell Signaling Technology), rabbit anti-mouse IL-4R α (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 μ g goat polyclonal anti-CD98 (sc-7095, Santa Cruz Biotechnology) or 2 μ g 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 \pm 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

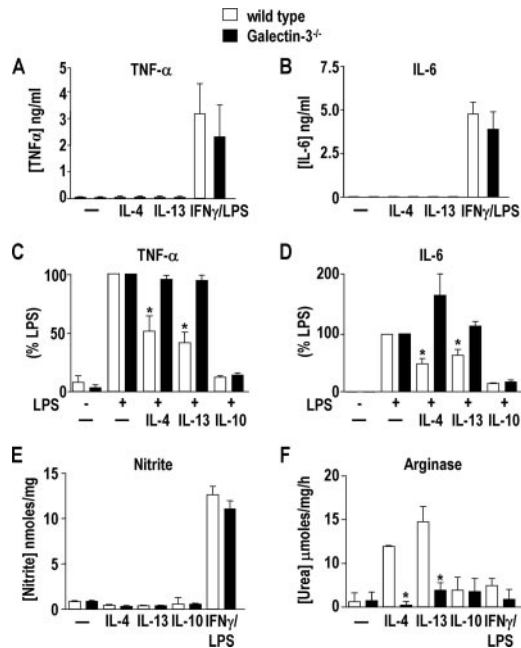


FIGURE 1. Inhibition of LPS-induced cytokine release by IL-4 and IL-13 is abrogated in galectin-3^{-/-} BMDMs. WT (open bars) or galectin-3^{-/-} BMDMs (filled bars) were cultured in the presence of IL-4, IL-13, IL-10 (10 ng/ml), or IFN- γ (100 U/ml) for 48 h. Where indicated, LPS (100 ng/ml) was added for the last 24 h. Supernatants were harvested and analyzed for TNF- α (A and C) and IL-6 (B and D) by cytometric bead array. The results represent the means \pm SEM of three to four independent experiments. *, $p < 0.01$ compared with LPS alone. Nitrite release (E) and arginase (F) activity were measured as described in *Materials and Methods*. The results represent the means \pm SEM of three independent experiments. *, $p < 0.01$ compared with WT.

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-4R α , in WT and galectin-3^{-/-} macrophages (Fig. 2B). Western blots showed equal protein expression of IL-4R α . 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).

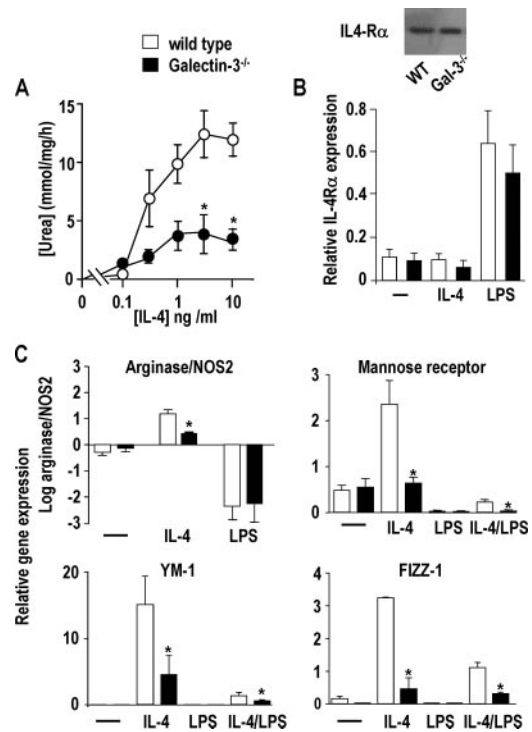


FIGURE 2. IL-4- and IL-13-induced alternative activation is reduced in galectin-3^{-/-} BMDMs. A, WT (○) or galectin-3^{-/-} (●) BMDMs were treated with IL-4 (0–10 ng/ml) for 24 h and arginase activity was determined. The results represent the means \pm SEM of three independent experiments. *, $p < 0.01$ compared with WT. B, WT (open bars) and galectin-3^{-/-} (filled bars) BMDMs were treated with 10 ng/ml IL-4 or 100 ng/ml LPS for 24 h and total RNA was extracted for real-time RT-PCR analysis. The results represent mean relative IL-4R α gene expression compared with β -actin \pm SEM of three independent experiments. *Inset*, Western analysis of IL-4R α in whole-cell lysates from WT and galectin-3^{-/-} macrophages (C) WT (open bars) and galectin-3^{-/-} (filled bars) BMDMs were treated with 10 ng/ml IL-4 \pm 100 ng/ml LPS for 48 h and total RNA was extracted for real-time RT-PCR analysis. The results represent log(arginase/NOS2 expression ratio) and relative mannose receptor, Ym1, and FIZZ1 expression compared with β -actin. The results represent the means \pm SEM of three experiments. *, $p < 0.01$ compared with WT.

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 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^{-/-}

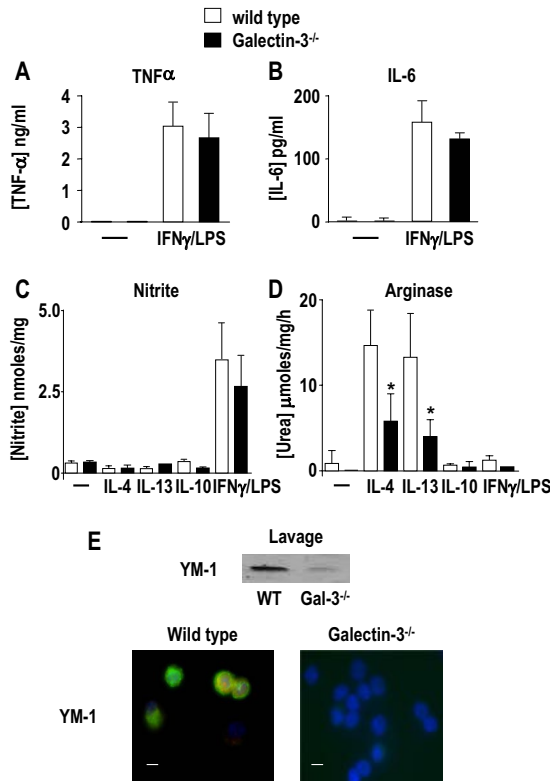


FIGURE 3. Galectin 3^{-/-} peritoneal and alveolar macrophages display normal classical activation but impaired alternative activation. Peritoneal macrophages were stimulated with IFN- γ (100 U/ml) for 48 h and LPS (100 ng/ml) was added for the last 24 h. Supernatants were assayed for (A) TNF- α , (B) IL-6, and (C) nitrite. Cell lysates were assayed for arginase activity (D). The results represent the means \pm 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 μ m).

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-acetylglucosamine, the best natural ligand for galectin-3, (42) and is a specific and highly selective inhibitor of the carbohydrate recognition domain of galectin-3 (K_d of 60 nM and >20-fold selectivity over other galectins, including galectin-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-

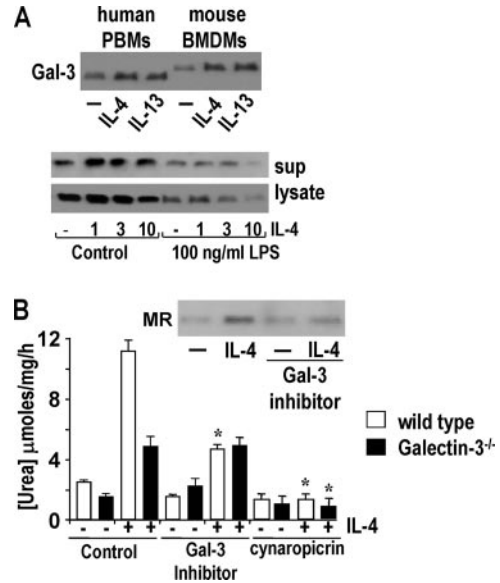


FIGURE 4. Galectin-3 potentiates IL-4-induced alternative activation and is released from IL-4 treated cells. A, IL-4 stimulates galectin-3 expression and release. Upper panel, Human PBMs and WT mouse BMDMs were incubated for 48 h with 10 ng/ml IL-4 or IL-13. Western blots from cell supernatants were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

main. CD98 is a well-characterized macrophage receptor for galectin-3 (34, 42). CD98 has been shown to promote PI3K activation, which is dependent on association with β_1 -integrins and β_1 -integrin expression (35, 36). We therefore sought to establish whether activation of CD98 by galectin-3 may mediate its effects on alternative activation. Cynaropicrin is a sesquiterpene lactone that inhibits CD98 and β_1 -integrin-induced homotypic aggregation and down-regulates β_1 -integrin expression on macrophages (43). Cynaropicrin inhibited arginase activation in galectin-3^{-/-} and WT BMDMs stimulated by IL-4 down to basal levels (Fig. 4B), suggesting that the effects of galectin-3 may be mediated via this surface receptor. Neither cynaropicrin or bis-(3-deoxy-3-(3-methoxybenzamido)- β -D-galactopyranosyl) sulfane affected macrophage number or viability as judged by cell counts, morphology, and trypan blue exclusion (data not shown).

IL-4-stimulated alternative macrophage activation is dependent on expression of galectin-3 and CD98 and activation of PI3K

We used the human monocytic 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-CD98 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

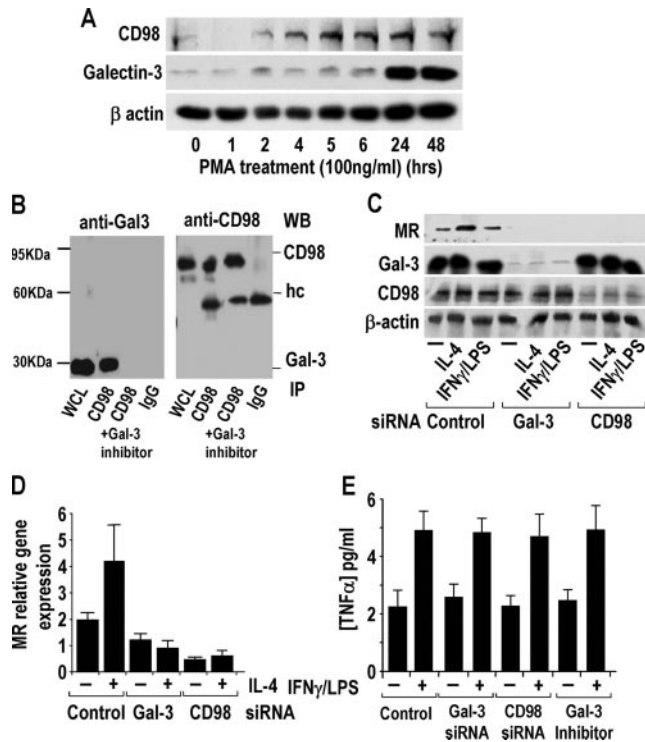


FIGURE 5. Alternative activation of human macrophages requires galectin-3 and CD98. *A*, THP-1 cells were differentiated with 100 ng/ml PMA, and lysates were prepared for Western analysis at the time points indicated. Blots were probed for CD98, galectin-3, and β -actin. *B*, CD98 and galectin-3 co-immunoprecipitate from THP-1 cell lysates. Normalized protein aliquots from lysates prepared from control THP-1 cells and THP-1 cells treated for 1 h with 5 μ M of galectin-3 inhibitor were immunoprecipitated with 2 μ g of goat anti-human CD98 and captured by protein-A/G sepharose. Washed immunoprecipitates and whole-cell lysates (WCL) were blotted for galectin-3 and CD98. hc indicates immunoprecipitating Ab H chain. *C*, THP-1 cells differentiated for 24 h were transfected with individual siRNA duplexes to 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 or 100 U/ml IFN- γ /100 ng/ml LPS for 24 h. Cells lysates were resolved on 12% SDS-PAGE gels. Blots were probed for mannose receptor (MR), galectin-3 (Gal-3), CD98, and β -actin. Representative blots from three independent experiments are shown. *D*, THP-1 cells were 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 \pm SEM of three independent experiments. *E*, THP-1 cells differentiated and transfected with siRNA as in *C* were incubated with 100 U/ml IFN- γ /100 ng/ml LPS in the presence or absence of galectin-3 inhibitor (5 μ M) for 24 h. TNF- α was measured by ELISA. Results represent the means \pm SEM of three experiments.

was visualized at \sim 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 μ M) 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 \sim 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 exper-

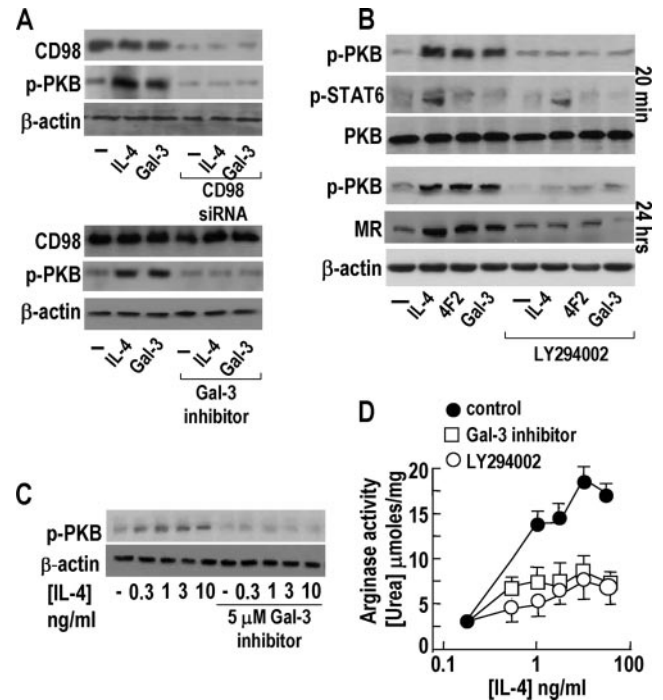


FIGURE 6. Inhibition of PI3K blocks alternative activation. *A*, THP-1 cells were transfected with siRNA against CD98 or control duplex and stimulated with 10 ng/ml IL-4 or 10 μ g/ml galectin-3 for 24 h in the presence or absence of 5 μ M of galectin-3 inhibitor (bis-(3-deoxy-3-(3-methoxybenzamido)- 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 μ M of LY294002 20 min before addition of 10 ng/ml IL-4, 10 μ g/ml 4F2, or 10 μ g/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 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 μ M 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 (\bullet) or presence of 5 μ M of galectin-3 inhibitor (bis-(3-deoxy-3-(3-methoxybenzamido)- β -D-galactopyranosyl) sulfane) (\square) or 10 μ M of LY294002 (\circ) for 24 h. Arginase activity was measured from cell lysates as described in *Materials and Methods*. Results represent the means \pm SEM of three experiments.

iments, 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. 5*E*). 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

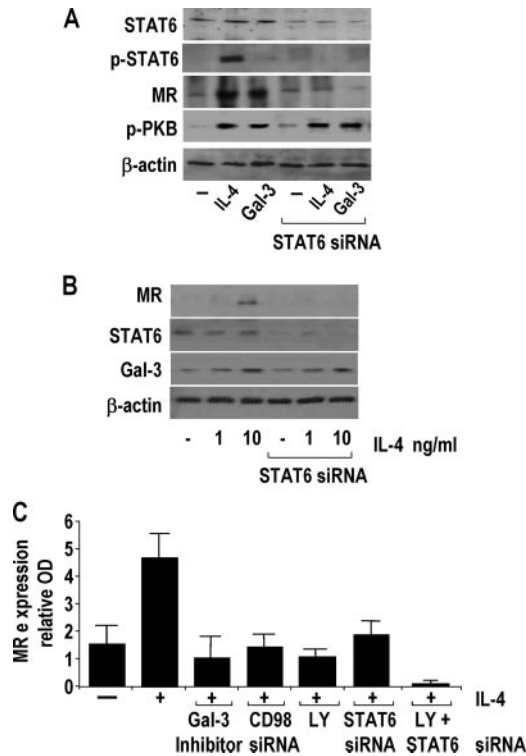


FIGURE 7. Inhibition of STAT6 blocks alternative activation but not galectin-3 expression. *A*, THP-1 cells were transfected with siRNA against STAT6 or control duplex and stimulated with 10 ng/ml IL-4 or 10 μ g/ml galectin-3 for 24 h. Blots from cell lysates were probed for STAT6, pSTAT6, mannose receptor (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 μ M of LY294002 or 5 μ M of galectin-3 inhibitor in the presence or absence of 10 ng/ml IL-4 as described above. Results represent the means \pm SEM of three to four independent experiments.

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. 6*B*). Blocking PI3K activity with LY294002 prevented IL-4-, galectin-3-, and 4F2-mediated PKB phosphorylation and mannose receptor expression (Fig. 6*B*). 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 μ M of galectin-3 inhibitor (Fig. 6*D*). IL-4, but not 4F2 or galectin-3, increased STAT6 phosphorylation, and this was not inhibited by LY294002 (Fig. 6*B*). However, targeted deletion of STAT6 with siRNA blocked IL-4 and galectin-3-mediated mannose receptor expression but not PKB activation (Fig. 7*A*) and did not affect basal or IL-4-induced galectin-3 expression (Fig. 7*B*). Therefore, inhibition of galectin-3 binding with bis-(3-deoxy-3-(3-methoxybenzamido)- β -D-galactopyranosyl) sulfane, inhibition 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. 7*C*). However, inhibition of both PI3K (LY294002) and STAT6 activation (siRNA) completely abolishes IL-4- and galectin-3-induced mannose receptor expression (Fig. 7*C*). 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 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*-acetylglucosamine 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 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*-acetylglucosamine (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 β_1 -integrins (35, 36). Our work shows that activation of CD98 is associated with cellular activation and transformation and is dependent on functional β_1 -integrin expression and focal adhesion kinase phosphorylation, causing elevation of intracellular phosphatidylinositol-3,4,5-triphosphate (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 μ M concentrations (43). Inhibition of CD98/ β_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 β_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 β_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)- 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-4R α . 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)P₃ 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 multinucleated 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 β_1 - and β_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 up-regulate 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 α 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 α , 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

The authors have no financial conflicts of interest.

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