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Endogenous Galectin-1 Exerts Tonic Inhibition on Experimental Arthritis

Asif J. Iqbal,*1 Dianne Cooper,*1 Alexander Vugler,† Beatrice R. Gittens,* Adrian Moore,† and Mauro Perretti*†

Little is known about the role(s) of endogenous galectin-1 (Gal-1) in arthritis. In this study we queried whether antiarthritic functions for this effector of endogenous anti-inflammation could be unveiled by studying collagen-induced arthritis in Gal-1−/− mice. Gal-1−/− and C57BL/6J [wild-type (WT)] mice received an immunization of chicken type II collagen (CII) in CFA followed by a booster on day 21, which consisted of CII in IFA. Animals were monitored for signs of arthritis from day 14 onward. Clinical and histological signs of arthritis were recorded, and humoral and cellular immune responses against CII were analyzed. A distinct disease penetrance was apparent, with ∼70% of Gal-1−/− mice developing arthritis compared with ∼50% in WT animals. Gal-1−/− mice also exhibited an accelerated disease onset and more severe arthritis characterized by significantly elevated clinical scores. Postmortem analyses (day 42) revealed higher levels of IgG1 and IgG2b anti-CII Ig isotypes in the serum of Gal-1 null animals compared with WT. Finally, T cell responses following ex vivo stimulation with CII revealed a greater degree of proliferation in T cells of Gal-1−/− mice compared with WT, which was associated with increased production of IL-17 and IL-22. These data suggest the novel idea that endogenous Gal-1 is an inhibitory factor in the development of arthritis affecting disease severity. We have also highlighted the importance of endogenous Gal-1 in regulating T cell reactivity during experimental arthritis.


Despite successes achieved by the introduction of biologics for the management of rheumatoid arthritis (RA), up to a third of patients fail to respond adequately to anti-TNF therapy (1, 2). The need is apparent for novel pharmacological or immunoregulatory approaches, with novel molecules to be used, perhaps, in association with current therapies. Galectins may provide a novel opportunity in the development of anti-RA therapeutics, with profound immunoregulatory effects for members of this family of glycanc-binding proteins identified (3).

Among the 15 galectins, our own work has focused on galectin-1 (Gal-1), starting from the observation that treatment of mice with small doses of this protein elicits potent inhibitory effects on the process of leukocyte recruitment in inflammation (4, 5). These data have been extended from mouse to human systems, indicating the potential translational relevance of this line of research (6, 7).

More recently we have complemented these pharmacological analyses by addressing the pathophysiological role of endogenous Gal-1, using the Gal-1−/− mouse (8) in a model of paw edema (9). Against expectations, edema was significantly diminished in the absence of endogenous Gal-1 (9), a finding that highlights the importance of studying the role of endogenous galectins in experimental models of disease.

Limited information exists regarding Gal-1 and arthritis. In a seminal study (10), delivery of Gal-1 via secretion by syngeneic fibroblasts reduced the clinical severity of collagen-induced arthritis (CIA). Such marked macroscopic effect was associated with induction of apoptosis in Th1 cells. This conclusion was corroborated by the observation that local intra-articular delivery of a lentiviral vector overexpressing Gal-1 alleviated clinical CIA in rats (11). Although these two studies indicate potential for the pharmacological administration of Gal-1, they shed little light on the potential properties of the endogenous protein in the processes characteristic of experimental CIA. In the current study we addressed this issue by monitoring Gal-1 expression in arthritic joints of DBA/1 mice and subsequently tested the Gal-1−/− mouse in a protocol adapted to the C57BL/6J strain. We show that absence of endogenous Gal-1 results in an exacerbation of disease, in terms of both incidence and clinical severity.

Materials and Methods

Animals

Male DBA/1 mice (Harlan, Oxfordshire, U.K.) and female C57BL/6J (WT) and Gal-1−/− mice (Charles River, Kent, U.K.) were used at 12–15 wk old under Home Office UK regulations. Original breeding pairs of Gal-1−/− (Lgals1l null) animals on a C57BL/6J background were provided by the Consortium for Functional Glycomics (www.functionalglycomics.org). These mice were verified as being 88% C57BL/6J by a 384–single-nucleotide polymorphism panel analysis performed by Charles River (Wilmington, MA).

Collagen-induced arthritis

Initial experiments were performed to assess galectin expression in the classical model of CIA performed in the DBA/1 strain, as previously described (12). Briefly, mice were immunized with 100 μg chicken type II collagen (CII; MD Bioproducts, Zurich, Switzerland) in CFA followed by

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Abbreviations used in this article: CIA, collagen-induced arthritis; CII, type II collagen; EAE, experimental autoimmune encephalomyelitis; Gal-1, galectin-1; L.N., lymph node; SAA, serum amyloid A; Treg, regulatory T cells; WT, wild-type.
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a booster injection using IFA on day 14. Mice were examined daily for signs of arthritis. CIA was induced in Gal-1$^{-/-}$ and WT mice (C57BL/6j background), as recently described (13). Briefly, mice were injected with 200 μg CII in CFA followed by a booster injection on day 21 in IFA. Mice were scored daily from day 14 for clinical signs of arthritis (0–3 scale, taking into account swelling of the wrist/ankle, pad, and digits); edema was assessed by plethysmometry. Serum and draining lymph nodes (LNs) were collected from mice at early (day 26, coinciding with disease onset in the majority of animals) and late (day 41, established arthritis in both groups) time-points for further analyses.

**Real-time PCR**

RNA was extracted from homogenized joints using the RNeasy Plus Mini Kit (Qiagen, Sussex, U.K.). Real-time PCR was performed using primers from a commercial source (Qiagen). Ct values were normalized to endogenous Gapdh and data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

**Cell culture**

Published protocols (13) were used to analyze T cell responses in mice subjected to CIA. Draining inguinal LN cells were collected at days 26 and 41 and stimulated with CII (50 μg/ml) or anti-CD3 (100 ng/ml; eBioscience, Wembley, U.K.) for 48 h. IL-17, IL-22, and IFN-γ levels were measured by ELISA, following the manufacturer’s guidelines (R&D Systems, Oxford, U.K.), and cell proliferation was assessed by $[^{3}H]$ thymidine incorporation, as previously described (14).

**ELISA**

Serum IgG levels were detected with anti-mouse IgG1 and/or IgG2b HRP-conjugated Abs diluted 1:5000 (AbD Serotec, Abingdon, U.K.). Serum amyloid A (SAA) levels were measured by ELISA according to the manufacturer’s guidelines (Tridelta Development).

**Histology**

For histological study, 5-μm sections of paws and joints were stained with H&E or safranin O/fast green (Sigma-Aldrich). Specific immunostaining was performed for Gal-1, using polyclonal goat-anti-mouse Gal-1 (R&D Systems). A minimum of four sections per animal were evaluated.

**Flow cytometry**

Freshly harvested draining LN cells were stained with FITC-conjugated rat-anti-mouse CD4, PE-conjugated rat-anti-mouse CD8, PE-Cy5-conjugated rat-anti-mouse CD44, APC-conjugated rat-anti-mouse CD62L, and isotype controls (eBR2a). Cell populations were analyzed by a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Oxford, U.K.).

**Statistical analysis**

Data are expressed as mean ± SEM and analyzed using one-way ANOVA, t test, or area under the curve followed by the Student t test. A p value ≤ 0.05 was considered significant.

**FIGURE 1.** Selected mediator expression in arthritic joints of DBA/1 mice. Male DBA/1 mice were induced with CIA, and quantitative real-time PCR was performed on cDNA from ankle and wrist joints collected across the course of the disease. (A) Saa, (B) Tnf, (C) Il6, (D) Il17, (E) Il10, and (F) Tgfb1. Data are expressed as mean ± SEM (n = 6). *p < 0.05 versus control (control joints were taken from nonimmunized mice).

**FIGURE 2.** Gal-1 expression in arthritic joints of DBA/1 mice. Male DBA/1 mice were induced with CIA, and quantitative real-time PCR was performed on cDNA from ankle and wrist joints collected across the course of the disease. (A) Lgals1 (Gal-1), Lgals3 (Gal-3), and Lgals9 (Gal-9) expression. Data are expressed as mean ± SEM (n = 6). (B-G) Gal-1 expression in cartilage and bone at day 28. (B) Lgals1 (Gal-1) cells/synoviocytes at sites of invasion. (C) Cartilage; Ct, connective tissue; EB, endochondral bone; JC, joint cavity/space; MC, marrow cavity; Oc, osteoclast; PB, periosteum.
Results
Characterization of endogenous galectin expression in arthritic joints

In DBA/1 mice, clinical signs of arthritis were first observed by day 28 (mean clinical score, 0.86 ± 0.26), which correlated with peak expression of Saa in ankle and wrist joints (Fig. 1A). By day 40, 80% of mice displayed signs of arthritis (mean clinical score, 3.47 ± 0.65).

The mRNA for the proinflammatory cytokines Tnf, Il6, and Il17a was observed as early as day 6, peaking at day 28 (Fig. 1B–D). Anti-inflammatory cytokines Il10 and Tgfb were also monitored: Il-10 mRNA levels were downregulated (up to day 15) and then returned to basal values (day 28; Fig. 1E), whereas Tgfb mRNA expression peaked at day 28 (Fig. 1F). Analysis of the same cytokines in C57BL/6J and Gal-1−/− mice revealed upregulation of Saa, Il6, Il10, Tgfb, and Il4 at day 42, with only Saa levels significantly different between genotypes (Supplemental Fig. 1).

The mRNA for Gal-1 and Gal-9 was strongly modulated, with peak expression correlating with clinical onset of disease (day 28) and remaining elevated up to day 40 (peak clinical disease) (Fig. 2A). Gal-3 mRNA expression was not modulated throughout this time-course (Fig. 2A).

Given the strong degree of modulation of Gal-1 and Gal-9 at the mRNA level, expression was monitored by immunohistochemistry in joints with maximal clinical score. Gal-1 expression was intense in the synovial lining and throughout the cellular infiltrate (Fig. 2B–G). Concentrated staining was also observed in osteoclasts (Fig. 2D) and chondrocytes located within the proliferative zone of the cartilage (Fig. 2F, 2G). Gal-1 positive infiltrating leukocytes could also be observed (Fig. 2H, 2I). Gal-9 staining appeared to be restricted to fibroblast-like synoviocytes and osteoblasts (data not shown). Collectively, these initial analyses prompted us to test the potential functional relevance of endogenous Gal-1.

Absence of endogenous Gal-1 leads to increased disease incidence and severity

A significantly larger proportion Gal-1−/− mice (69%) developed arthritis, compared with WT mice (53%) (Fig. 3A, 3B). The mean day of onset was also earlier in Gal-1−/− mice. In terms of clinical manifestations, Gal-1−/− mice displayed enhanced disease severity (Fig. 3C, 3D), with a mean clinical score of 4.57 ± 1.28 compared with 2.89 ± 0.93 in WT mice. Edema was mildly elevated in Gal-1−/− mice compared with WT mice (Fig. 3E, 3F). Histological analysis of joints taken at days 31 and 42 from both WT and Gal-1−/− mice revealed a modest elevation in score in Gal-1−/− mice that was not statistically significant (data not shown).

FIGURE 3. Increased incidence and severity of CIA in Gal-1−/− mice. Female WT (C57BL6/J) and Gal-1−/− mice were induced with CIA and monitored daily for signs of arthritis. (A) Incidence, (C) clinical score (0–3 scale, taking into account swelling of the wrist/ankle, pad, and digits; maximum score, 12 per mouse), and (E) level of edema. Area under the curve analyses for (B) incidence, (D) clinical score, and (F) edema. Data are expressed as mean ± SEM (n = 15 WT; n = 13 Gal-1−/−). *p < 0.05, **p < 0.01 versus WT.

FIGURE 4. Circulating levels of SAA and anti-CII Abs in WT and Gal-1−/− mice during CIA. Serum was collected at disease onset (day 26) and during established disease (day 42) as well as from naive (n = 2) WT mice at day 42. Levels of (A) SAA, (B) IgG1, and (C) IgG2b were detected by ELISA. Data are expressed as mean ± SEM (day 26, n = 3; day 42, n = 10 WT and 7 Gal-1−/−). *p < 0.05, **p < 0.01, ***p < 0.001 versus WT (day 42).
Elevated SAA and anticollagen IgG levels in Gal-1\(^{-/}\) mice

Elevated levels of SAA were observed in both groups by day 26 (disease onset; Fig. 4A). Of interest, on day 42, SAA levels in Gal-1\(^{-/}\) mice increased further and were significantly higher than those observed in WT animals. Coupled with the clinical data, this result suggests that absence of endogenous Gal-1 may promote joint inflammation during the progression/active phase of the disease.

Collagen-specific IgG levels serve as markers of Th1/Th17 and Th2 responses, with, respectively, IgG1 indicative of a Th2 response, IgG2a and b of a Th1 response, whereas Th17 cells have been shown to increase class switch recombination to IgG1, IgG2a, IgG2b, and IgG3 (15). IgG2a levels were not measured, as the gene encoding this isotype is deleted in C57BL/6 mice (16). IgG1 levels in Gal-1\(^{-/}\) mice increased across the time-course and were significantly elevated at day 42 (Fig. 4B). IgG2b increased in both genotypes over the course of the disease, with significantly higher levels detectable at day 42 in Gal-1\(^{-/}\) mice (Fig. 4C).

Arthritic Gal-1\(^{-/}\) mice show increased T cell effector responses, proliferation, and skewing toward a Th17 phenotype

In response to stimulation with CII, Gal-1\(^{-/}\) LN cells exhibited a significantly higher proliferative response than did WT cells (Fig. 5A, 5B). Furthermore, CII stimulation led to significantly higher levels of IL-17 and IL-22 being produced by Gal-1\(^{-/}\) cells compared with WT cells at both onset and established stages of disease, days 26 and 42, respectively (Fig. 5D, 5E). Comparable levels of IFN-\(\gamma\) were recovered from both WT and Gal-1\(^{-/}\) cells harvested at day 26 (data not shown).

At day 42, the percentage of CD4\(^+\) and CD8\(^+\) T cells in draining LNs was proportional in WT mice, in contrast to Gal-1\(^{-/}\) mice, in which double the percentage of CD4\(^+\) T cells was found (Fig. 6A, 6B). In terms of T cell counts, Gal-1\(^{-/}\) mice displayed double values of CD4\(^+\) T cells, compared with WT cells (Fig. 6E). Similar numbers of CD8\(^+\) T cells were observed in both strains (data not shown). Additional staining was carried out on the CD4\(^+\) population for CD44 and CD62L (Fig. 6C, 6D) to identify the percentage of effector memory T cells. Fig. 6F shows that the percentage of effector memory CD4\(^+\) T cells (CD44\(^+\)CD62L\(^{lo}\)) was significantly increased in Gal-1\(^{-/}\) mice compared with WT mice.

**Discussion**

The effects of galectins are complex and vary depending on their cellular localization; it is therefore important to consider the actions of both the endogenous and the exogenous protein to fully appreciate their biology. This study demonstrates, to our knowledge, for the first time in a model of CIA, that absence of endogenous Gal-1 leads to enhanced susceptibility and disease severity, which is in part associated with exacerbated T cell expansion and skewing toward a Th17 profile.

The first set of results reported mRNA expression of several cytokines in joints taken from DBA/1 mice during the course of CIA. As expected, levels of the proinflammatory cytokines IL-6, TNF-\(\alpha\), and IL-17 were elevated, all of which have been implicated in the pathogenesis of both RA and CIA (17–20). Of note, endogenous IL-10 mRNA levels remained below control threshold, indicating a downmodulation in gene expression. We propose that during onset and active phases of CIA the immunosuppressive actions of IL-10 are overrun by the plethora of proinflammatory cytokines present in the joint.

In RA, galectins display a broad pattern of expression within the synovium (21, 22). However, to the best of our knowledge, this is...
the first study to investigate Gal-1 expression over the course of CIA in mice. In line with other inflammatory models such as experimental autoimmune encephalomyelitis (EAE) (23), Gal-1 mRNA expression peaked at the clinical onset of disease and remained elevated during disease progression, which may be indicative of a proresolutory role for the protein and may correlate with an increased expression in activated T cells recruited to the joint. Immunohistochemistry of arthritic joints indicated strong colocalization between Gal-1 and infiltrating synovial cells, with a strong degree of immunoreactivity also in chondrocytes, a result in line with the recent description of Gal-1 as a novel factor in chondrocyte growth, cartilage formation, and maturation (24). Although numerous Gal-1–positive leukocytes were observed in the synovium, it is not clear whether these cells express Gal-1 or have taken it up from the extracellular environment. Peripheral blood cells express little or no Gal-1, whereas activated T cells upregulate Gal-1 over a period of days (25). Recent evidence has shown that another galectin (Gal-3) is endocytosed by macrophage-like cells (26), whereas neutrophils recovered from bronchoalveolar lavage fluid of *Streptococcus pneumoniae*–infected mice have increased levels of Gal-3 bound extracellularly (27).

The action of Gal-1 in models of inflammation signals a dominant anti-inflammatory role for this protein (3, 5). Recent data suggest several mechanisms through which Gal-1 may be protective in chronic autoimmune diseases, including RA and CIA. These would include induction of apoptosis of Th1 and Th17 effector cells (23), antagonism of T cell activation and proliferation (28–30), enhanced immune tolerance through positive effects on regulatory T cell (Treg) development and function (31, 32), and induction of IL-10 synthesis in activated Th cells (33, 34).

The significant increase in proliferation observed in Gal-1−/− cells in response to the specific Ag CII supports an antiproliferative role for Gal-1 in response to Ag stimulation (28). Further characterization of CD4+ T cells recovered at day 42 revealed a greater proportion of CD4+ effector memory T cells in the LNs of Gal-1−/− mice compared with WT mice. Clearly, absence of endogenous Gal-1 appears to have a significant impact on the regulation of T cell responses, which could, in part, explain the disease profile observed in Gal-1−/− mice.

IL-4–secreting Th2 cells have been demonstrated to support IgG1 production by plasma cells, and IL-17 in combination with IL-21 generated by Th17 cells supports class switching and production of IgG1 and IgG2b isotypes (15, 35, 36). Levels of both IgG1 and IgG2b were elevated in Gal-1−/− mice, suggesting the establishment of a dominant Th17 immune response. This idea was further supported by increased production of IL-17 and IL-22 by draining LN cells of Gal-1−/− mice in response to CII. Th17 cells induce tissue inflammation and drive disease pathogenesis in a variety of experimental autoimmune diseases, including EAE (37) and CIA (19, 38) as well as RA (39) and multiple sclerosis.
(40) in humans. A role for IL-22 in driving CIA in C57BL/6 mice has also been demonstrated (41). Strong evidence in the literature demonstrates a role for Gal-1 in dampening Th1- and Th17-mediated responses and skewing toward a Th2 profile. An elegant study by Toscano et al. (23) showed that Th1 and Th17 cells express a specific repertoire of cell surface glycanics, which increases their susceptibility to Gal-1–induced cell death; in contrast, Th2 cells are protected owing to differential α2-6 sialylation of cell surface glycoproteins. This finding could explain the bias toward a Th2 phenotype following treatment with Gal-1. Furthermore, increased Ag-specific Th1 and Th17 responses coupled with enhanced susceptibility to EAE were observed in Gal-1−/− mice, further supporting a role for endogenous Gal-1 in dampening Th1- and Th17-mediated diseases (22). Recently, a Gal-1 chimeric molecule was shown to induce apoptosis of Th1 and Th17 cells obtained from the synovial fluid of RA patients, further highlighting the therapeutic potential of this protein (34).

The importance of Tregs in mediating peripheral tolerance is well established, and both mouse and human Tregs can produce IL-10. IL-10 production has also been shown to be reduced in draining LNs from Gal-1−/− mice (41). Strong evidence in the literature demonstrates a role for Gal-1 in dampening Th1- and Th17-mediated responses and skewing toward a Th2 profile. An elegant study by Toscano et al. (23) showed that Th1 and Th17 cells express a specific repertoire of cell surface glycanics, which increases their susceptibility to Gal-1–induced cell death; in contrast, Th2 cells are protected owing to differential α2-6 sialylation of cell surface glycoproteins. This finding could explain the bias toward a Th2 phenotype following treatment with Gal-1. Furthermore, increased Ag-specific Th1 and Th17 responses coupled with enhanced susceptibility to EAE were observed in Gal-1−/− mice, further supporting a role for endogenous Gal-1 in dampening Th1- and Th17-mediated diseases (22). Recently, a Gal-1 chimeric molecule was shown to induce apoptosis of Th1 and Th17 cells obtained from the synovial fluid of RA patients, further highlighting the therapeutic potential of this protein (34).

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Supplementary Figure 1

**Selected mediator expression in arthritic joints of C57BL6/J and Gal-1⁻/⁻ mice.**

Female C57BL6/J and Gal-1⁻/⁻ mice were induced with CIA and quantitative real-time PCR was performed on cDNA from ankle joints collected at days . (A) *Saa*, (B) *Il17*, (C) *Tnf*, (D) *Il10*, (E) *Il6*, (F) *Tgf*⁻ and (G) *Il4*. Data are expressed as mean ± SEM (n= 6-8/group) *P<0.05 vs. control (control joints were taken from non-immunized mice).