Opposite effects of galectin-1 on alternative metabolic pathways of L-arginine in resident, inflammatory, and activated macrophages

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Abstract
Recent evidence has implicated galectins and their carbohydrate ligands as master regulators of the inflammatory response. Galectin-1, a member of this family, has shown specific anti-inflammatory and immunoregulatory effects. To gain insight into the potential mechanisms involved in these effects, we investigated the effects of galectin-1 in L-arginine metabolism of peritoneal rat macrophages. Pretreatment of macrophages with galectin-1 resulted in a dose- and time-dependent inhibition of lipopolysaccharide-induced nitric oxide (NO) production, accompanied by a decrease in inducible nitric oxide synthase (iNOS) expression (the classic pathway of L-arginine). On the other hand, galectin-1 favored the balance toward activation of L-arginase, the alternative metabolic pathway of L-arginine. Inhibition of NO production was not the result of increased macrophage apoptosis because addition of this β-galactoside-binding protein to macrophages under the same experimental conditions did not affect the apoptotic threshold of these cells. To understand how endogenous galectin-1 is regulated in macrophages under inflammatory stress, we finally explored the ultrastructural distribution, expression, and secretion of galectin-1 in resident, inflammatory, and activated macrophages. This study provides an alternative cellular mechanism based on the modulation of L-arginine metabolism to understand the molecular basis of the anti-inflammatory properties displayed by this carbohydrate-binding protein.

Key words: galectins/inflammation/L-arginine metabolism/macrophages/nitric oxide

Introduction

Macrophages (MΦ) are important effector cells involved in antigen presentation and microbicidal and tumoricidal activities (Seljelid and Eskeland, 1993). Early in immune responses, MΦ synthesize bioactive molecules that orchestrate the inflammatory reaction (Nathan, 1987). The production of these mediators in response to several stimuli must be tightly regulated to promote an effective immune response without damaging the host (Morris et al., 1998). Lipopolysaccharides (LPSs) and cytokines including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) activate MΦ through the classic pathway, stimulating the production of many inflammatory mediators and up-regulating the expression of inducible nitric oxide synthase (iNOS). Th2 cytokines and glucocorticoids instead activate MΦ through the alternative pathway, enhancing arginase activity and up-regulating other markers, such as scavenger receptor A and IL-1 antagonist receptor (IL-1ra) (Chang et al., 2000; Morrison and Correll, 2002).

Though arginase and iNOS catalyze the common substrate L-arginine, their products have opposing biological effects. Many of the cytotoxic, microbicidal, and tumoricidal effects of MΦ are associated with the small and short-lived molecule nitric oxide (NO). In contrast, L-arginine metabolized through arginase yields ornithine, a precursor of proline and polyamines involved in cell growth and proliferation (Chang et al., 1998). It has been shown that both iNOS and arginase are coexpressed in LPS-activated MΦ (Benninghoff et al., 1988; Sonoki et al., 1997), suggesting that diversion of L-arginine from the iNOS to the alternative pathway may confer MΦ arginase a regulatory role in NO production (Chang et al., 1998, 2000). Several endogenous compounds already implicated as chemical mediators in inflammatory reactions modulate arginase activity, and most of them also affect NO production (Sonoki et al., 1997).

Galectins are members of an animal lectin family defined by their shared consensus amino acid sequence and affinity for β-galactoside-containing oligosaccharides (Hirabayashi and Kasai, 1993; Barondes et al., 1994; Rabinovich et al., 2002a). They have recently been proposed to play key roles in inflammatory processes (Rabinovich et al., 2002a,b; Liu, 2000; Leffler, 1997; Delbrouck et al., 2002; Sano et al., 2000). Galectin-1 (Gal-1), the first mammalian galectin identified, is secreted as a noncovalently linked homodimer with two ligand-binding sites capable of mediating cell-cell and cell-matrix interactions through recognition of polylactosamine structures on cell surface glycoconjugates (Hirabayashi and Kasai, 1993; Leffler, 1997; Cooper and Barondes, 1999). Gal-1 is widely distributed within the central and peripheral immune systems and is expressed during innate and adaptive immune responses in inflammatory and activated MΦ (Rabinovich et al., 1996, 1998;...
Zúñiga et al., 2001a), thymic epithelial cells (Baum et al., 1995), antigen-primed T cells (Blaser et al., 1998), and activated B cells (Zúñiga et al., 2001b).

Research over the past few years, using different experimental models of chronic inflammation and autoimmunity, revealed that Gal-1 has specific immunosuppressive and anti-inflammatory effects (Rabinovich et al., 1999b, 2000b; Offner et al., 1990; Santucci et al., 2000; Tsuchiyama et al., 2000). Using gene and protein therapy strategies, we have demonstrated that Gal-1 ameliorates chronic inflammation and suppresses the autoimmune response in a murine model of rheumatoid arthritis (Rabinovich et al., 1999b). Investigation of the molecular mechanisms involved in these immunoregulatory properties revealed that Gal-1 skewes the balance toward a type-2-polarized immune response (Rabinovich et al., 1999b), blocks proinflammatory cytokine secretion from activated T cells (Rabinovich et al., 1999a), inhibits T-cell adhesion to extracellular matrix glycoproteins (Rabinovich et al., 1999a), induces partial TCR-ι-chain phosphorylation and dysregulation of the phosphotyrosine kinase Lyn (Chung et al., 2000; Fouillit et al., 2000), and induces T-cell apoptosis at high inflammatory concentrations (Perillo et al., 1995; Pace et al., 1999; Rabinovich et al., 1999b, 2000a,c). Moreover, recent work from our laboratory also indicates that Gal-1 has also a role in acute inflammation and inhibits both soluble and cellular components of the early inflammatory response (Rabinovich et al., 2000b).

Prompted by the powerful anti-inflammatory effects of Gal-1 and its presence in peritoneal Mφ (Rabinovich et al., 1996, 1998), in the present study we extend our previous findings and evaluate the effect of exogenous Gal-1 on the metabolic pathways of L-arginine in resident, inflammatory, and activated rat Mφ. In addition, the regulated secretion as well as the ultrastructural distribution of endogenous Gal-1 were assessed in these inflammatory cells. Our results show that Gal-1 down-modulates the classic metabolic pathway of L-arginine in activated rat Mφ by inhibiting NO production and iNOS expression, and it enhances in a similar magnitude arginase activity (the alternative metabolic pathway of L-arginine). Moreover, the ultrastructural distribution and regulated secretion of endogenous Gal-1 provides different patterns for resident, inflammatory, and activated Mφ. Our work strengthens the role of Gal-1 in inflammatory responses and provides an alternative mechanism to understand its autocrine or paracrine anti-inflammatory and immunoregulatory effects.

Results

Modulation of NO production, iNOS expression, and arginase activity by exogenous Gal-1

To better understand the molecular basis of the anti-inflammatory activity of Gal-1, we explored the effects of this β-galactoside-binding protein on NO production in Mφ preincubated for 2 h with Gal-1 (2–8 μg/ml) and then challenged with LPS for 48 h (Figure 1A). NO release was inhibited 16% at 2 μg/ml, 30% at 4 μg/ml, and 27% at 8 μg/ml (p < 0.05 Mφ 2–8 μg/ml Gal-1 + LPS versus Mφ LPS alone). Interestingly, the maximal inhibition obtained with 4 μg/ml lectin was comparable with the effect of aminoguanidine, a specific NO inhibitor (~40%). When added alone, Gal-1 (2–8 μg/ml) had no effect on NO production in Mφ. Besides, no changes in NO production were observed in Mφ treated simultaneously with LPS and increasing concentrations of Gal-1 (Figure 1B). Pretreatment of Mφ with the optimal dose of Gal-1 (4 μg/ml) for 0–120 min showed a time-dependent inhibition of NO production, with the maximal inhibition achieved after 120 min of pretreatment (Figure 1C). The inhibitory effect was blocked ~50% with 30 mM lactose, when Gal-1 was used at 4 or 8 μg/ml (data not shown). Moreover, a synergic inhibitory effect of aminoguanidine and Gal-1 was not found (Figure 1A), suggesting that these agents suppress NO production through different independent mechanisms.

As shown by western blot analysis (Figure 1D), iNOS protein (~130 kDa) was induced after 48 h stimulation with 1 μg/ml LPS (lane 4). When Mφ were pretreated for 2 h with 4 μg Gal-1 and then stimulated for 48 h with LPS, a marked decrease in iNOS expression was detected (lane 1). No changes in the levels of the enzyme were observed when Mφ were exposed to Gal-1 in the absence of LPS (lane 2). Lysates from untreated Mφ were used as controls (lane 3). Because Gal-1 has been shown to induce T cell apoptosis, we evaluated whether the inhibitory effects of this lectin could be related to a modulation of Mφ survival. Resident Mφ were cultured for 2 h with 4 or 8 μg/ml Gal-1 under similar conditions and processed after activation for apoptotic cell detection. Ladder-type DNA fragmentation was not observed in genomic DNA extracted from Gal-1-treated (8 μg/ml) versus nontreated Mφ (Figure 1E, lane 2 versus lane 1). As a positive control, spleen T cells were incubated with the same concentration of Gal-1 (lane 3). Absence of apoptosis was confirmed by analyzing the levels of subdiploid DNA content in Mφ cultured for 2 h in the presence of Gal-1 (data not shown). Considered together, our results indicate that pretreatment with Gal-1 inhibits NO production and iNOS expression (the classic metabolic pathway of L-arginine) in LPS-stimulated Mφ in a manner independent of its proapoptotic properties.

Regulation of L-arginine metabolism in cells that possess both iNOS and arginase activities is poorly understood, and it has been shown that LPS stimulates in moderate levels both pathways (Benninghoff et al., 1988). On the other hand, arginase is the predominant pathway of arginine metabolism under strenuous metabolic conditions (Albina et al., 1991, 1995). To gain a more complete picture of the role of Gal-1 in Mφ arginine metabolism, we evaluated the effects of Gal-1 on resident or inflammatory Mφ pretreated with 4 μg/ml Gal-1 for 2 h and stimulated in vitro with LPS or LPS-IFN-γ. After 48 h supernatants were sampled for NO production, and cell lysates were evaluated for arginase activity by measuring the levels of urea (Figure 2). We determined NO2⁻ and urea to assess iNOS and arginase activities because changes in these end products are indicative of the activity of the respective enzymes or the amount of L-arginine substrate available. Interestingly, both in resident and inflammatory Mφ, Gal-1 exhibited a dual effect; although inhibited ~30% the NO release induced by LPS or LPS-IFN-γ (Figure 2A and B), this lectin enhanced arginase
activity in a similar proportion (Figure 2A and B). Thus, Gal-1 reciprocally modulates L-arginine metabolism in peritoneal Mφ by inhibiting the classical pathway of NO production and favoring the alternative pathway mediated by arginase.

Intracellular expression and ultrastructural distribution of endogenous Gal-1 in resident, inflammatory, and activated Mφ

We have previously shown that Gal-1 is present in rat peritoneal Mφ, and several stimuli are able to modulate expression of this endogenous lectin (Rabinovich et al., 1996, 1998). Because Gal-1 could be acting in an autocrine manner to control the Mφ inflammatory activity, we extended our previous findings (Rabinovich et al., 1996) and investigated the ultrastructural distribution of this β-galactoside-binding lectin by immunocytochemical studies of resident, inflammatory, and activated Mφ using the specific anti-Gal-1 antibody. Analysis by transmission electron microscopy revealed remarkable differences in immunogold staining intensity and ultrastructural distribution of Gal-1 in resident, inflammatory and activated Mφ. Figure 3A shows the ultrastructural morphology of a normal peritoneal rat Mφ. The nucleus is irregular in shape with condensed chromatin next to the nuclear...
membrane. As expected, normal resident Mφ showed scarce gold labeling (Figure 3B). On the other hand, inflammatory and activated Mφ exposed to different stimuli showed a round or slightly oval nuclei and contained predominantly euchromatin (Figure 3C and D). Gold labeling was found mainly in the cytoplasm but also in the nuclei of in vivo elicited-inflammatory Mφ or in vitro activated Mφ (Figure 3C and D, respectively). The gold particles were deposited on secretory granules. This localization was a prominent feature of phorbol 12-myristate 13-acetate (PMA)- (Figure 3D) or fMLP-activated Mφ (not shown). Moreover, few gold particles were localized in the cytoplasm adjacent to the periphery of the granules and over the nucleus. Substitution of the primary antibody with preimmune rabbit IgG or preadsorption of the antibody with recombinant Gal-1 resulted in little or no staining (Figure 3D, inset).

We evaluated the surface expression of Gal-1 by fluorescence-assisted cell sorting (FACS) analysis on ED1+ Mφ, and we observed that the percentage of positive fluorescence correlated with the activation status of the cells (Rabinovich et al., 1998). As summarized in Figure 3E, 14.3% of double-positive cells was found in resident peritoneal Mφ, and the percentage increased to 18.4% in inflammatory Mφ and to 55.6 and 68.2% in PMA- and fMLP-activated Mφ. The differences found in the expression and ultrastructural distribution of Gal-1 associated to the activation state of Mφ prompted us to examine the effects of classical proinflammatory stimuli on the secretion of this β-galactoside-binding protein. Resident peritoneal Mφ were cultured in medium alone or in the presence of PMA or recombinant TNF-α for 2 h. After incubation, cell-free supernatants were collected and analyzed for Gal-1 detection by western blot analysis (Figure 3F). As expected, Gal-1 secretion was markedly enhanced in PMA-stimulated Mφ (lane 3, Act), compared with minimal secretion in resident cells (lane 2, Res). The densitometric profile shows that TNF-α treatment up-regulated Gal-1 secretion to intermediate levels after 2 h of incubation (lane 4, TNF-α), compared to resident Mφ (lane 2), suggesting that the secretion of Gal-1 is an early event in the context of a proinflammatory cytokine milieu. Considered together, these results suggest that after chemical or inflammatory stimulation endogenous Gal-1 expression increases significantly and localizes in the cytoplasm of activated Mφ. Shortly after accumulation in intracellular compartments, the lectin is released to the extracellular medium to exert autocrine or paracrine functions.

Discussion

The present study documents an alternative cellular mechanism to explain the anti-inflammatory properties of Gal-1 and provides new clues into the understanding of the relationship between subcellular localization, secretion, and function of this family of β-galactoside-binding proteins.

To gain insight into the cellular and molecular mechanisms implicated in the anti-inflammatory properties of Gal-1, we evaluated the role of this lectin on L-arginine metabolism in resident and inflammatory Mφ. We determined NO and urea to assess iNOS and arginase activities because changes in these end products are indicative of the activity of the respective enzymes or the amount of L-arginine substrate available. Under our experimental conditions, Gal-1 inhibited the classical pathway of NO production and iNOS expression, increased arginase activity, and favored the alternative route of L-arginine metabolism.

The regulation of L-arginine metabolism in tissues, such as Mφ, that possess both arginase and iNOS activity is poorly understood. The NO production for LPS-activated
Mφ is dependent on the replenishment of intracellular arginine from the extracellular medium up to 0.5 mM (Chang et al., 1998). The competition between arginase and iNOS is more pronounced when L-arginine availability is compromised, that is, approximately below the physiological level (0.1 mM). It appears that the competition between arginase and iNOS can be overcome if L-arginine availability is high enough for iNOS to fully exert its function (Chang et al., 1998).

The extent of inhibition of NO production induced by Gal-1 (~30% at 4 μg/ml) is only slightly inferior to that shown by aminoguanidine, a representative iNOS inhibitor (~40%) (Figure 1A) and other novel synthetic inhibitors of iNOS, such as 1,3-(2H,4H)-isoquinoliniedione (FR038470) (Kita et al., 2002). However, Gal-1 at this concentration was able to induce a marked inhibition of iNOS expression (Figure 1D). This discrepancy could be easily explained by the fact that western blot analysis indicates enzyme expression and not enzyme activity. Hence, it is likely that residual iNOS expressed by LPS-stimulated macrophages in the presence of Gal-1 (Figure 1D, lane 1) is highly active and sufficient to produce a considerable amount of the final product (NO), as has been reported by previous studies (Mattace Raso et al., 1999).

There are two isoforms of vertebrate arginase, both of which catalyze the conversion of arginine to ornithine and urea, but with differences on subcellular localization, tissue distribution, and certain enzymatic properties (Louis et al., 1998; Mori and Gotoh, 2000). Sonoki et al. (1997) reported that iNOS and arginase I are coinduced by LPS in cultured rat peritoneal Mφ and that arginase I induction is slower. Several endogenous compounds already implicated as chemical mediators in inflammatory reactions have been shown to modulate arginase activity (Waddington et al., 1998), and most of these compounds also affect NO production. A 30-fold greater level of arginase activity was
obtained in RAW 264.7 cells treated with 8-bromo-cAMP plus LPS compared with LPS alone. However, the much higher level of arginase expression did not diminish cellular NO production in this case (Morris et al., 1998), suggesting that these metabolic pathways are not always opposite.

We have previously found that Gal-1 skews the balance from a Th1- to a Th2-polarized immune response (Rabinovich et al., 1999b). Because the iNOS/arginase balance in murine MΦ has been proposed to be competitively regulated in the context of Th1 versus Th2-driven immune reactions (Munder et al., 1998), we hypothesized that Gal-1 could oppositely regulate iNOS and arginase activities. Interestingly, and confirming our hypothesis, Gal-1 was able to down-regulate iNOS activity and NO production and induced an up-regulation of arginase activity.

The present study provides a novel control point of Gal-1 during the inflammatory response. Because iNOS expression and NO overproduction in the inflamed joint is one of the most consistent findings in experimental or human rheumatoid arthritis (Kolb and Kolb-Bachofen, 1998), inhibition of these endogenous mediators by Gal-1 might be an alternative molecular mechanism to explain the therapeutic effects of this protein in rheumatoid arthritis (Rabinovich et al., 1999b). Because Gal-1 has been shown to induce T-cell apoptosis (Rabinovich et al., 1998, 2000a; Perillo et al., 1995), we finally evaluated whether inhibition of NO production could be related to a modulation of MΦ survival. A toxic or apoptotic effect of Gal-1 was ruled out at the times and concentrations tested.

Furthermore, the release of NO and other reactive nitrogen intermediates is also an important part of MΦ effector functions against a variety of pathogenic microorganisms, and its role in the immunopathology of several infectious disease is well documented. In this sense, we have recently proposed a biphasic modulation of the MΦ microbicidal activity against Trypanosoma cruzi trypomastigotes by increasing concentrations of Gal-1 (Zúñiga et al., 2001a).

In addition to the role of exogenous Gal-1 in L-arginine metabolism, in the present study we provide evidence of the regulated intracellular expression and secretion of the endogenous β-galactoside-binding protein in resident, inflammatory (in vivo–elicited), and activated (in vitro) MΦ. Galectin-1 was found to be differentially regulated in the different subpopulations of MΦ and found to be mainly localized in the cytoplasmic compartment at the level of secretory granules in inflammatory and activated MΦ. The increased gold labeling in activated MΦ is in agreement with the increased total and surface expression of this protein found in MΦ stimulated with phorbol esters (PMA), formylated peptides (fMLP), and LPS (Rabinovich et al., 1996, 1998). Here we extended these findings showing that Gal-1 is rapidly secreted following stimulation with PMA or TNF-α, powerful proinflammatory and activating agents. The absence of a secretion signal peptide in Gal-1 leaves unclear the route of entry of this protein into the secretory granules. As has been previously reported (Cooper and Barondes, 1990; Mehul and Hughes, 1997; Sato and Hughes, 1994), most galectins are synthesized in free ribosomes in the cytoplasm and are secreted by a novel apocrine mechanism, in which the translated protein becomes concentrated at the level of plasma membrane evaginations prior to secretion and are further externalized to form galectin-enriched extracellular vesicles, a kind of infrequent mechanism of secretion also used by many cytokines and growth factors. In fact, it has been observed that substances, such as brefeldin A, that block the progression through the endoplasmic reticulum–Golgi pathway, do not affect secretion of galectins (Sato et al., 1993).

Similarly to our findings, Craig et al. (1995) found that Gal-3 is localized in secretory granules of mast cells and basophiles and suggested that the lectin is released when these cells are activated to degranulate (Frigeri and Liu, 1992). To reconcile the lack of a signal peptide with the striking ultrastructural localization of Gal-3, it has been suggested that this protein might be secreted by activation stimuli, but then it binds to cell surface glycoconjugates and is incorporated into secretory granules (Craig et al., 1995; Frigeri and Liu, 1992).

The rapid secretion of Gal-1 induced by TNF-α suggests that this anti-inflammatory β-galactoside-binding protein could be released during an inflammatory episode to achieve homeostasis by autocrine or paracrine mechanisms. Because TNF-α is the major proinflammatory cytokine found in the rheumatoid synovia, its inhibition might be also associated with the anti-inflammatory properties of Gal-1 observed in the collagen-induced arthritis model (Rabinovich et al., 1999b). Accordingly, we found that Gal-1 suppresses TNF-α and IFN-γ production from activated T cells (Rabinovich et al., 1999a,b, 2002c).

In contrast to the anti-inflammatory effects shown by Gal-1, a positive role on cell growth regulation and inflammation has been assigned to Gal-3 (Hsu et al., 2000; Colnot et al., 1998; Yamaoka et al., 1995; Karlsson et al., 1998). In this sense, one might speculate that a cross-regulation exists between these two closely related members of the same family of endogenous lectins to control the initiation and termination of the inflammatory response. This cross-regulation has been well studied in the context of the inflammatory response in juvenile rheumatoid arthritis (JIA). We have shown that down-regulated apoptosis in patients with polyarticular JIA and increased proliferation in patients with pauciarticular JIA can be partly explained by an imbalance between Gal-1 and Gal-3 expression in the rheumatoid synovia (Harjacek et al., 2001). However, it seems unlikely that all the biological functions of Gal-1 and Gal-3 may be restricted under this paradigm. In this sense, Almkvist et al. (2002) have challenged this paradigm and recently shown that Gal-1 plays also a role in the activation of neutrophil NADPH oxidase and the neutrophil respiratory burst. In this sense, here we show that Gal-1 inhibits iNOS expression but up-regulates arginase activity in the same cell type.

In conclusion, the present study reports an additional role for Gal-1 in inflammation and provides an alternative cellular mechanism to understand the powerful immunoregulatory effects of this carbohydrate-binding protein in experimental models in vivo. Careful examination of the biochemical pathways and molecular interactions will help delineate novel therapeutic strategies in chronic inflammatory processes, autoimmunity, cancer, and infections using endogenous nontoxic sugar-binding proteins.
Materials and methods

Reagents

Hank’s balanced salt solution, RPMI 1640, PMA, fMLP, LPS from Escherichia coli serotype 011:B4, recombinant TNF-α, protease inhibitors, lactose, thiodigalactoside, horseradish peroxidase–conjugated goat anti-rabbit IgG, and phycoeritrin-conjugated goat anti-rabbit IgG, aminoguanidine, DNase-free RNase A, and protease K were from Sigma (St. Louis, MO). fluorescein isothiocyanate–labeled ED1+ mononclonal antibody was from Serotec (Oxford, UK). Fetal calf serum and L-glutamine were from Life Technologies (Paisley, UK). All other chemical reagents were commercially available analytical grade.

Animals

Eight- to 12-week-old female Wistar rats (average weight 250 g) were used in this study. Animals were housed and cared for at the Animal Resource Facilities in accordance with institutional guidelines.

Cell preparation and stimulation procedures

Macrophages were purified from peritoneal cells by plastic adherence as previously described (Rabinovich et al., 1996, 1998). The resultant Mφ monolayer showed >90% purity according to morphologic analysis and ED1 immunoreactivity. Mφ from untreated animals were used as resident Mφ. Inflammatory Mφ were obtained from animals injected intraperitoneally with 3 ml sterile 10% proteose peptone 3 days before cell collection. Activated Mφ were obtained by in vitro treatment of resident Mφ plus 1 μg/ml PMA or 200 nM fMLP as previously described (Rabinovich et al., 1996) or inflammatory Mφ plus 1 μg/ml LPS or 1 μg/ml LPS plus 25 ng/ml IFN-γ. Cell viability assessed by the trypan blue exclusion test was greater than 93%.

Gal-1 purification and anti-Gal-1 antibody preparation

Gal-1 was purified essentially as described (Rabinovich et al., 1998) from cell extracts or serum-free supernatants obtained from PMA- or fMLP-activated rat Mφ. The purified protein was stored in 1 mM dithiothreitol at −70°C and used in all procedures in medium or phosphate buffered saline (PBS) containing 1.0 mM dithiothreitol. The polyclonal anti-Gal-1 antibody was obtained as described (Rabinovich et al., 1998).

Western blot analysis of iNOS expression

To evaluate iNOS expression, peritoneal Mφ were pre-incubated with Gal-1 (4 μg/ml) and further stimulated with LPS (1 μg/ml) as described. Cells were washed twice with ice-cold PBS, resuspended in 200 μl lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM ethylenediamine tetra-acetic acid (EDTA) and a protease inhibitor cocktail (Sigma) and left on ice for 30 min. The solution was then centrifuged at 4°C at 10,000 × g, and the resultant cell lystate was mixed 1:1 with 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. Equal amounts of protein (15 μg/lane) were fractionated in 10% SDS–PAGE, and proteins were electrotransferred onto nitrocellulose membranes. The anti-iNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted to 2 μg/ml and incubated overnight. Immunodetection was performed with the enhanced chemiluminescence kit as described. The immunoreactive protein bands were analyzed with a Fotodyne Image Analyzer (Fotodyne, Hartland, WI).

Determination of arginase activity

Arginase activity was measured as previously reported (Corraliza et al., 1994). Cell lysates were mixed with 10 mM MnCl₂, and the enzyme was activated by heating for 10 min at 56°C. L-arginine hydrolysis was conducted by incubating 25 μl of the activated lysate with 25 μl of 0.5 M L-arginine, pH 9.7, at 37°C for 60 min. The reaction was stopped using 400 μl H₂SO₄ (96%/H₃PO₄ (85%)/H₂O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after addition of 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95°C for 30 min. A calibration curve was prepared with increasing amounts of urea ranging from 1.5 to 30 μg. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol urea per min.

Apoptosis assays

Macrophage susceptibility to Gal-1-induced apoptosis was analyzed after different treatments by the DNA fragmentation assay and propidium iodide staining as previously described (Rabinovich et al., 1998, 1999b). For DNA fragmentation, cells were harvested, washed with TNE buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, pH 8) and lysed by the addition of 0.5% SDS. Cell lysates were incubated at 56°C for 3 h in the presence of 100 μg/ml proteinase K. After digestion, DNA was purified by successive phenol-chloroform extractions, and the resultant aqueous phase was mixed with 3 M sodium acetate (pH 5.2) and absolute ethanol. The mixture was incubated at −20°C overnight, and the ethanol-precipitated DNA was washed with 70% (v/v) ethanol. The purified DNA was resuspended in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.5), and treated with 5 μl of 1 mg/ml DNase-free RNase A for 1 h. Samples were finally resuspended in loading buffer and resolved on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide. Analysis of subdiploid nuclei was assessed by propidium
iodide staining essentially as described (Rabinovich et al., 1999b).

**FACS analysis**

The presence of Gal-1 on resident, inflammatory, or activated MΦ was determined by double-staining FACS analysis. Cells were subsequently incubated with rabbit anti-Gal-1 and phycoerythrin-goat anti rabbit polyclonal antibody and with fluorescein isothiocyanate–ED1 monoclonal antibody, as previously described (Rabinovich et al., 1996), and analyzed for relative fluorescence intensity on a FACStart-Plus instrument (Becton Dickinson, Mountain View, CA).

**Ultrastructural immunocytochemistry and electron microscopy**

For ultrastructural studies, resident, inflammatory, or activated MΦ were fixed in 1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, for 1 h at 4°C, dehydrated in increasing concentrations of ethanol solutions up to 90%, and embedded in LR White (London Resin Company, Hampshire, UK). Thin sections were cut in a Porter-Blum MT-1 Sorvall microtome, mounted on 250 mesh nickel grids, and etched with 10% (v/v) hydrogen peroxide for 7 min. For Gal-1 detection, grids were incubated with the rabbit anti-Gal-1 IgG diluted 1:700 in PBS–bovine serum albumin at 4°C, dehydrated in aqueous (1%) uranyl acetate, examined and photographed using a Siemens Elmiskop 101 electron microscope. For control purposes, the primary IgG polyclonal antibody was preabsorbed with macrophage Gal-1 or replaced with a rabbit preimmune serum.

**Analysis of Gal-1 secretion**

To analyze Gal-1 secretion, resident peritoneal MΦ were stimulated in vitro with PMA (1 µg/ml) for 2 h or with recombinant TNF-α (1000 U/ml) for 2 h at 37°C in 5% CO2. Serum-free supernatants were collected, centrifuged at 1000 × g for 5 min to discard cell debris, and stored frozen at −70°C until use. Protein concentration was estimated by using the micro-BCA Protein Assay reagent kit (Pierce, Rockford, IL). SDS-PAGE was performed as described (Rabinovich et al., 1999b). After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed subsequently with a 1:1000 dilution of the rabbit anti-Gal 1 polyclonal antibody and with a 1:3000 dilution of a horseradish peroxidase–goat anti-rabbit IgG. The immunoreactive protein bands were developed using enhanced chemiluminescence detection followed by exposure for 3–5 min to Amersham Hyperfilm (Uppsala, Sweden). Recombinant Gal-1 (rGal-1, 1 µg) was used as a positive control for western blot detection. The immunoreactive protein bands were analyzed with a Fotodyne Image Analyzer. Results were expressed as relative densitometric values by means of the Image Quan Software.

**Statistical analysis**

Statistical significance and differences between groups were determined by analysis of variance and Bonferroni test. Each point represents the mean ± SD of at least six independent determinations.

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**Abbreviations**

EDTA, ethylenediamine tetra-acetic acid; FACS, fluorescence-assisted cell sorting; fMLP, N-formyl-Met-Leu-Phe; Gal-1, galectin-1; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JIA, juvenile rheumatoid arthritis; LPS, lipopolysaccharide; MΦ, macrophages; NO, nitric oxide; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor.

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