

# Expression of galectin-3 in the tumor immune response in colon cancer

Patrick Dumont<sup>1</sup>, Alix Berton<sup>1</sup>, Nathalie Nagy<sup>1</sup>, Flavienne Sandras<sup>1</sup>, Sandrine Tinton<sup>1</sup>, Pieter Demetter<sup>1</sup>, Françoise Mascart<sup>2</sup>, Abdelmounaïm Allaoui<sup>3</sup>, Christine Decaestecker<sup>4</sup> and Isabelle Salmon<sup>1</sup>

The role of tumor-associated macrophages (TAMs) is controversial. Although most studies on different cancer types associate them with a poorer prognosis, interestingly in colon cancer, most articles indicate that TAMs prevent tumor development; patients with high TAMs have better prognosis and survival rate. M1-polarized macrophages produce high level of tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$  or reactive oxygen species, which can effectively kill susceptible tumor cells. In contrast, M2-polarized macrophages can secrete different factors that promote tumor cell growth and survival or favor angiogenesis and tissue invasion. Considering the beneficial role of TAMs in colon cancer, we speculated that they may not display the M2 polarization commonly observed in tumor microenvironment, but rather develop M1 properties. Therefore, we used an *in vitro* model to analyze the effects of supernatants from M1-polarized macrophages on DLD-1 colon cancer cells. Our data indicate that the conditioned medium from LPS-activated macrophages (CM-LAM) contains a high level of granulocyte-macrophage colony-stimulating factor, interleukins-1 $\beta$ , -6, -8 and tumor necrosis factor- $\alpha$ , and that it exerts a marked growth inhibitory activity on DLD-1 cells. Prolonged exposure to CM-LAM results in cell death by apoptosis. Such exposure to CM-LAM leads to the modulation of gal-3 expression: we observed a marked downregulation of gal-3 mRNA and protein expression following CM-LAM treatment. We also describe that the knockdown of gal-3 sensitizes DLD-1 cells to CM-LAM. These data suggest an involvement of gal-3 in the response of colon cancer cells to proinflammatory stimuli, such as the conditioned medium from activated macrophages.

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Several lines of evidence indicate that immune effector cells play an important role in the recognition and destruction of cancer cells. This concept of cancer immunosurveillance is supported by human studies showing that immunodeficiencies predispose patients to the development of cancer and by murine models where inactivation of specific genes coding for components of the immune response results in a higher susceptibility to the development of spontaneous or chemically induced cancers.<sup>1,2</sup> However, other studies are consistent with the view that immune responses may favor the progression of malignancies.<sup>3,4</sup> Persistent immune responses exacerbate the recruitment and activation of immune cells in tumor microenvironments where they can contribute to the selection of nonimmunogenic tumor cell variants as well as to the supply of elements favoring tumor

expansion. Indeed, many cancers arise at sites of infection or chronic inflammation.<sup>5,6</sup>

With regard to the role of macrophages, which usually constitute a significant part of the tumor-infiltrating immune cells, their presence has been associated with good prognosis for patients with colon cancers.<sup>7–10</sup> This somehow constitutes an exception because it is the common view that tumor-associated macrophages (TAMs) promote tumor progression; the majority of studies on different cancer types including breast, prostate, bladder, cervical and kidney cancers have correlated high TAMs density with reduced patient survival.<sup>11,12</sup> Classical morphological analyses of a tumor for the presence of TAMs do not, however, allow for distinction among the types of polarization of these macrophages. Macrophages can produce various agents such as reactive

<sup>1</sup>Laboratory of Pathology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; <sup>2</sup>Laboratory of Vaccinology and Mucosal Immunity, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; <sup>3</sup>Laboratory of Molecular Bacteriology, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium and <sup>4</sup>Laboratory of Toxicology, Institute of Pharmacy, Université Libre de Bruxelles, Brussels, Belgium  
Correspondence: Professor I Salmon, MD, PhD, Laboratory of Pathology, Erasme Hospital, Université Libre de Bruxelles, 808 Lennik Road, Brussels 1070, Belgium.  
E-mail: Isabelle.Salmon@erasme.ulb.ac.be

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oxygen species, nitric oxide, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) that can effectively kill cancer cells, when they have been activated with various stimuli, including lipopolysaccharide (LPS) or interferon- $\gamma$  (IFN- $\gamma$ ), that induce the M1 type of polarization.<sup>12–14</sup> However, TAMs commonly adopt an alternative phenotype, the M2 polarization, as a consequence of factors present in the tumor microenvironment.<sup>12</sup> This M2 polarization is linked to the ability of TAMs to release various factors such as platelet-derived growth factor, fibroblast growth factor-basic, epidermal growth factor, vascular endothelial growth factor, IL-10, transforming growth factor- $\beta$  (TGF- $\beta$ ) and several matrix metalloproteases that can promote tumor cell growth and survival, angiogenesis, tissue invasion and suppression of the immune response.<sup>11,12</sup>

To study the role of infiltrating macrophages on the development of colon cancer, we chose to use a M1 type of model because TAMs appear to behave differentially in colon cancer than in other types of cancer. In this model, the human monocytic leukemia cell line THP-1 is induced to undergo differentiation into macrophages by treatment with phorbol ester, which has been reported to mimic native monocyte-derived macrophages.<sup>15</sup> These macrophages subsequently activated with LPS secrete various cytokines such as IL-1 $\beta$  and TNF- $\alpha$ ,<sup>13,14</sup> allowing the analysis of the effects of supernatants from M1-polarized macrophages on colon cancer cells.

Using this *in vitro* model, we characterized how the supernatants of M1-polarized macrophages modulate the expression and secretion of galectin-3 (gal-3) in colon cancer cells. We previously published that gal-3 is involved in colon cancer development and that the level of gal-3 expression in colorectal tumors is related to their level of aggressiveness.<sup>16–18</sup> Other teams also showed that gal-3 is commonly expressed at a high level by colon cancer cells and that strong expression of gal-3 correlates with disease progression, metastasis and poor survival.<sup>19,20</sup> Gal-3 is a member of the  $\beta$ -galactoside-binding lectin family and is expressed by many cell types including immune cells such as macrophages as well as many cancer cells.<sup>21</sup> Due to its pleiotropic effects on processes, such as cell adhesion, induction of cytokine production, inflammation, chemotaxis, apoptosis, differentiation or regulation of gene expression, gal-3 is involved in many cellular functions that are crucial to cancer progression.<sup>21–25</sup> We hypothesize that gal-3 plays a key role in the various crosstalks that exist between the infiltrating macrophages and the colon cancer cells, and in the regulation of the tumor immune response in colon cancer.

## MATERIALS AND METHODS

### Cell Culture

THP-1 human monocytic leukemia cells (ATCC TIB-202) were maintained at 37°C in a 5% CO<sub>2</sub> humidified atmosphere, in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 100 U/ml

penicillin G, 100  $\mu$ g/ml streptomycin-sulfate and 250 ng/ml amphotericin B. The human colon adenocarcinoma cell line DLD-1 (ATCC CCL-221) was maintained in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin-sulfate and 250 ng/ml amphotericin B. The cell culture media were obtained from Invitrogen.

### Differentiation of THP-1 Cells into Macrophages and LPS Activation

THP-1 cells were differentiated into macrophages by treatment with 100 ng/ml phorbol-12-myristate-13 acetate (PMA; Sigma-Aldrich) for 48 h. The PMA-containing medium was removed and replaced by complete medium. Macrophages were either maintained unstimulated or activated by treatment with 1  $\mu$ g/ml LPS (Sigma-Aldrich). After 24 h, the conditioned media of both unstimulated (CM-UM) and LPS-activated macrophages (CM-LAM) were collected, centrifuged at 10 000 g for 10 min and stored at –20°C until use. To assess the M1 polarization, the concentrations of IL-8, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF- $\alpha$  and IL-1 $\beta$  were determined in the conditioned media by Bio-Plex Cytokine Immunoassays (Bio-Plex suspension array system, Luminex 100; Bio-Rad Laboratories, Nazareth-Eke, Belgium), using the antibodies from Invitrogen according to the manufacturer's instructions.

### MTT Assay

Cells were seeded at 5000 cells per well in 96-well plates and submitted 24 h later to treatments with CM-UM and CM-LAM. At the end of the treatments, cells were incubated with complete RPMI medium containing 0.5  $\mu$ g/ml (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich). After 3 h, we discarded the MTT solution, solubilized the formazan produced by the living cells with a solution of isopropanol/1 M HCl (24:1 v/v) and read the absorbance at 570 nm on a spectrophotometer (Genesis, 10 UV).

### Annexin V Binding Assay

Cells were harvested by trypsinization, resuspended in FBS-supplemented medium to neutralize the trypsin and washed twice with phosphate buffered saline (PBS). Cells were resuspended in ice-cold 1  $\times$  binding buffer (Sigma-Aldrich) at a density of 1  $\times$  10<sup>6</sup> cells per ml. An aliquot of 500  $\mu$ l was stained for 10 min at room temperature with 5  $\mu$ l annexin V (Ann V)-fluorescein isothiocyanate and 10  $\mu$ l propidium iodide (PI; Sigma-Aldrich). The fluorescence was analyzed immediately on an Epics XL-MCL flow cytometer (Beckman Coulter) equipped with a 488 nm argon laser.

### Video Cellular Microscopy

We seeded DLD-1 cells at 60 000 cells per 25 cm<sup>2</sup> flask in RPMI medium containing 10% FBS. After 72 h, cells were

rinsed with PBS and incubated with one of the following medium: (1) RPMI 1640 containing 0.2% FBS; (2) 0.2% FBS RPMI 1640 supplemented with 20% (v/v) CM-UM; (3) 0.2% FBS RPMI 1640 supplemented with 20% (v/v) CM-LAM. The flasks were placed under phase-contrast microscopes coupled to video cameras and filmed for 48 h (1 image every 4 min) as previously described.<sup>26</sup> On the recorded still images from time points 0, 12, 24, 36 and 48 h, we counted the number of cells to quantify the effect of the treatments on cell growth.

### Gal-3 ELISA

DLD-1 cells seeded at  $10^5$  cells per well in 6-well plates were treated 24 h later with CM-UM and CM-LAM. At time points 24, 48 and 72 h, cell culture supernatants were collected and centrifuged at 4°C for 15 min at 1000 g. Gal-3 levels were measured with the Human Galectin-3 ELISA kit from Bender MedSystems, according to the manufacturer's instructions.

### Western Blotting

Cells were scraped and centrifuged at 500 g for 5 min at 4°C. The cell pellets were washed twice with ice-cold PBS and resuspended in ice-cold radio-immunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF) supplemented with protease inhibitors (Protease Inhibitor Cocktail from Roche Diagnostics). The protein content of the lysates was measured by the Bradford method using the Dc Protein Assay kit (Bio-Rad Laboratories). We loaded 50  $\mu\text{g}$  of proteins on SDS-PAGE and the separated proteins were transferred onto Hybond C-extra nitrocellulose membranes (Amersham). Blots were blocked for 30 min in PBS containing 0.1% Tween-20 (PBS-T), 2% nonfat dry milk and 2% bovine serum albumin (BSA). After blocking, the membranes were probed for 1 h with the primary antibody diluted in PBS-T containing 2% nonfat dry milk and 2% BSA. The membranes were washed three times for 20 min with PBS-T and probed with horseradish peroxidase-conjugated secondary antibody used at a 1:10 000 dilution in PBS-T containing 2% nonfat dry milk and 2% BSA. After three washes in PBS-T, the membranes were developed using chemiluminescent substrate (Western Lighting from PerkinElmer) and exposed to Amersham ECL Hyperfilms. The following primary antibodies were used in this study: mouse anti-gal-3 antibody (dilution of 1:1000; Novocastra Laboratories), anti-Lamin A/C mouse ab (dilution of 1:400; Santa Cruz Biotechnology), anti-PARP (19F4) mouse ab (dilution of 1:2000; Cell Signalling) and anti- $\beta$ -actin (AC-15) mouse ab (dilution of 1:5000; Sigma-Aldrich).

### Cell Fractionation

Cells were scraped and centrifuged at 500 g for 10 min (4°C). Cell pellets were washed twice with ice-cold PBS and

resuspended in 10 volumes of hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT) supplemented with protease inhibitors. After allowing the cells to swell on ice for 15 min, the plasma membranes were disrupted by repeated passages through a microtip. The samples were centrifuged at 4000 g for 2 min (4°C). The supernatants (cytoplasmic fraction) were collected and stored. The pellets were resuspended in ice-cold hypertonic buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 20% glycerol) supplemented with protease inhibitors. After incubation on ice for 30 min with frequent vortexing, the samples were centrifuged at 14 000 g for 5 min (4°C). The supernatants (nuclear fraction) were isolated and stored.

### Total RNA Purification, cDNA Synthesis and Quantitative Real-Time PCR

Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions, digested with DNase I and purified on columns using the RNeasy Mini kit from Qiagen (Hilden, Germany). RNA integrity was confirmed by agarose gel electrophoresis. Total RNA (1  $\mu\text{g}$ ) was reverse transcribed using oligo-dT primers (0.5  $\mu\text{g}$ ) and Superscript II Reverse Transcriptase (Invitrogen), in a final volume of 20  $\mu\text{l}$  containing 1 mM dNTP, 10 mM DTT and first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM  $\text{MgCl}_2$ ). After 50 min of incubation at 42°C, the reactions were inactivated for 15 min at 70°C. The cDNAs were purified with the High Pure PCR Product Purification kit (Roche Diagnostics) according to the manufacturer's instructions. In each cDNA sample, we measured the expression of gal-3 as well as that of two housekeeping genes, the transcription factor SP1 and TATA box-binding protein (TBP). The analysis of gal-3, SP1 and TBP expression in the different cDNA samples was performed by quantitative real-time PCR with the following primers: gal-3 sense, 5'-CAA TACAAAGCTGGATAATAACTGG-3' and gal-3 antisense, 5'-GATTGTACTGCAACAAGTGAG-3' (amplicon of 149 bp); SP1 sense, 5'-CCGCTCCCAACTTACAGAAC-3' and SP1 antisense, 5'-ATGATGTTGCCCTCCACTTCC-3' (amplicon of 223 bp); TBP sense, 5'-TTCGGAGAGTTCTGGGATTG-3' and TBP antisense, 5'-AATCAGTGCCGTGGTTCGT-3' (amplicon of 175 bp). We used 20 ng of cDNAs of each sample for analysis. Briefly, in a reaction, 2  $\mu\text{l}$  of cDNA (20 ng) were mixed with the primers (each at 0.5  $\mu\text{M}$ ) and the Lightcycler FastStart DNA Master SYBRGreen I reagents (Roche Diagnostics) in a 20  $\mu\text{l}$  final volume. Reactions were carried out in a Lightcycler thermocycler instrument (Roche Diagnostics). In addition to the samples, each run included a standard curve for the gene analyzed. These standard curves consisted of PCRs performed on serial dilutions of a purified DNA fragment as substrate. All primers were from Eurogentec (Seraing, Belgium). Gal-3 mRNA expression was normalized by the geometric mean of the quantifications obtained for the housekeeping genes. The results are expressed as percentage of the control (0 h).

### Generation of Stable Cell Lines Knockdown for Gal-3

The pSUPER and pSUPER-shGal3-551 vectors were a generous gift from Silvia Soddu (Regina Elena Cancer Institute, Rome, Italy). The pSUPER-shGal3-551 vector was previously shown to downregulate gal-3 expression.<sup>27</sup> It contains the following sequence (conserved motif, underlined): GATCCC CCAACAGGAGAGTCATTGTTTCAAGAGAAACAATGAC TCTCCTGTGTTTTGGAAA-3' (Gal3-551, sense) and 5'-AGCTTTTCCAAAAACAACAGGAGAGTCATTGTTTCTCT TGAAAACAATGACTCTCCTGTTGGGG-3' (Gal3-551, anti-sense). To create pSUPER-shScr, the following oligonucleotides were annealed and cloned into pSUPER (between the *Bgl*II and *Xho*I sites): 5'-GATCCCCGTTCAATTCAACGTA GATGTTCAAGAGACATCTACGTTGAATTGAACTTTTTGG AAC-3' and 5'-TCGAGTTCCAAAAAGTTCAATTCAACG TAGATGTCTCTTGAACATCTACGTTGAATTGAACGGG-3'. These oligonucleotides contain a sequence (underlined) that is a randomization of base pair 943–961 of the *LGALS3* (gal-3) mRNA. The pSUPER-shScr plasmid was sequenced to confirm correct insertion of the oligonucleotides. The empty pSUPER as well as the pSUPER-shScr and pSUPER-shGal3-551 vectors were co-transfected (at 10:1 ratio) with the pPUR vector (BD Biosciences Clontech) that carries a selectable puromycin-resistance marker.<sup>28</sup> The Fugene 6 transfection reagent (Roche Diagnostics) was used. Selection started at 48 h after transfections under 2 µg/ml puromycin. Independent clones were expanded and screened for gal-3 protein and mRNA expression. Out of 40 clones transfected with the pSUPER-shGal3-551 vector, 3 displayed a marked decrease of gal-3 mRNA expression (above 80%), 2 of which, named shGal3-C11 and -C12, were subsequently used in our studies. Stable puromycin-resistant derivatives transfected with the empty pSUPER and the pSUPER-shScr vector were also cloned and had unchanged gal-3 protein level.

### Statistical Analysis

The Mann–Whitney and the Kruskal–Wallis tests were used to compare independent groups of numerical data (for two groups and more than two groups, respectively). When the Kruskal–Wallis test was significant, *post hoc* tests (Dunn procedure) were used to compare the group pairs of interest in avoiding multiple comparison effects.

## RESULTS

### The Conditioned Medium of LPS-Activated Macrophages Contains High Level of M1 Type Cytokines

To analyze the role of infiltrating macrophages on the development of colon cancer, we used an *in vitro* model in which monocytic THP-1 cells previously differentiated into macrophages with PMA are activated by a 24 h treatment with LPS. Table 1 indicates that CM-LAM contains elevated amounts of GM-CSF, IL-6, IL-8, TNF-α and IL-β as compared to CM-UM. This is in agreement with data reported by others showing that LPS causes the M1 polarization

of macrophages and the release of proinflammatory cytokines.<sup>13,14</sup>

### The Conditioned Medium of LPS-Activated Macrophages Exerts Growth-Inhibitory and Death-Inducing Activities on Colon Adenocarcinoma DLD-1 Cells

The effect of CM-LAM on cell growth and viability of DLD-1 cells was first analyzed by video cellular microscopy (Figure 1). The recorded films highlight the marked morphological changes as well as the growth decrease occurring in the presence of 20% CM-LAM, as compared to untreated controls or cells cultivated in the presence of 20% CM-UM. Such changes are already observed after 12 h of treatment (Figure 1a). Although DLD-1 control cells typically grow in small colonies or clusters, cells cultivated in the presence of 20% CM-LAM detach from each other and only remain connected through multiple spindle-like membrane protrusions. At longer time points, such spindle connections disappear and the isolated cells became round, suggesting apoptotic cell death (Figure 1a). Based on the films images corresponding to time points 0, 12, 24, 36 and 48 h, we evaluated the effects of the different treatments on cell growth by counting the number of cells. The number of cells increases linearly in the control condition. By comparison, the growth rate of DLD-1 cells treated with 20% CM-UM significantly decreases ( $P=0.003$ ; Figure 1b). Nonetheless, treatment of DLD-1 cells with 20% CM-LAM not only drastically inhibits cell growth but strongly impairs cell survival. As compared to time 0 h, the addition of CM-LAM led to a dramatic decrease in the number of cells that reaches 95% at 48 h of treatment ( $P<0.001$ ; Figure 1b).

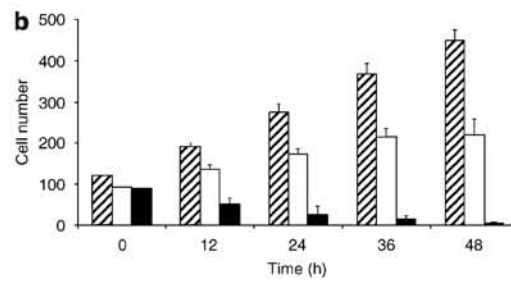
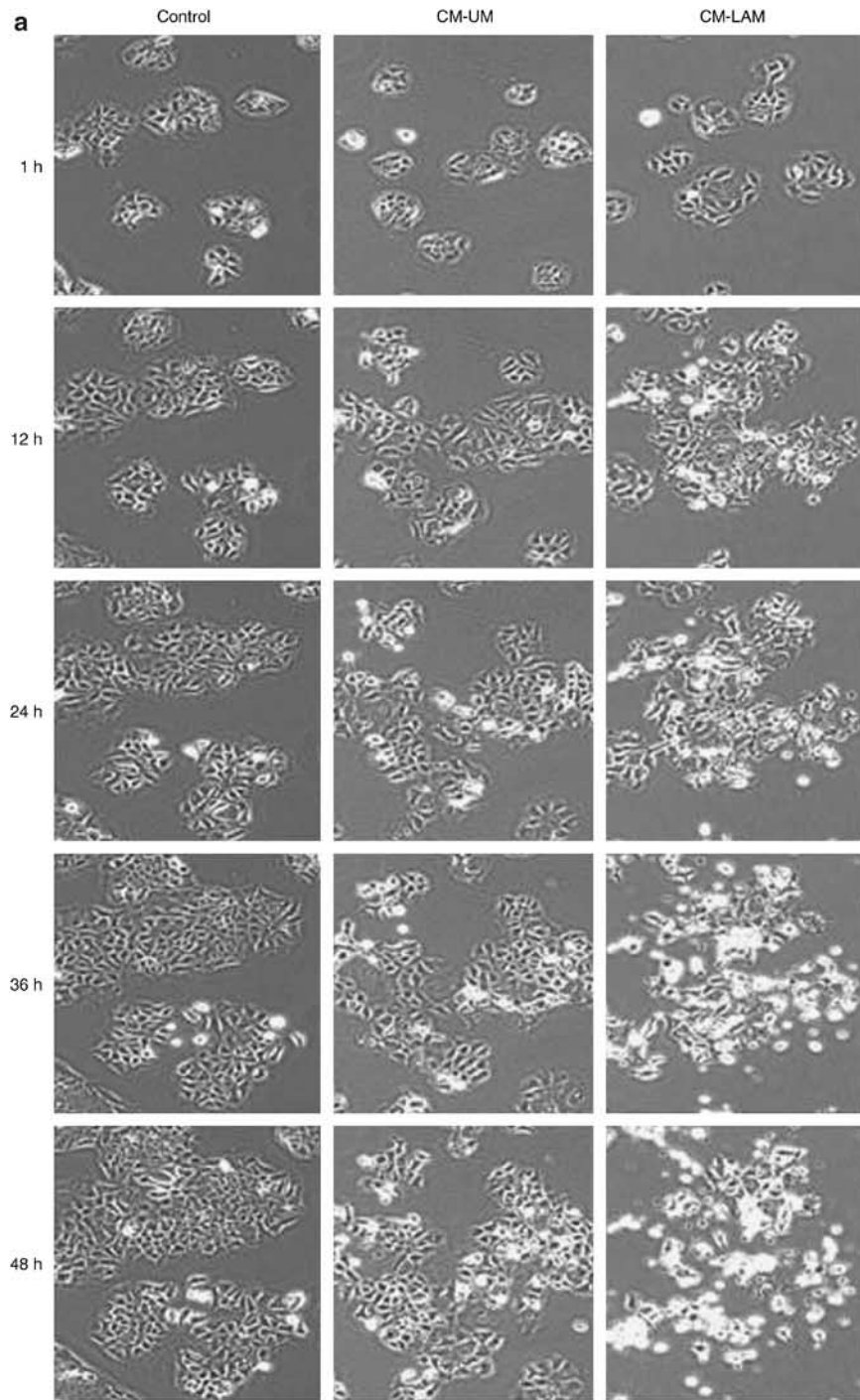
### The Conditioned Medium of LPS-Activated Macrophages Causes Apoptotic Cell Death

To determine whether cell death occurred by apoptosis, we used the well-described Ann V binding assay coupled to a flow cytometry analysis which allows the distinction to be made between early (Ann V-positive/PI-negative cells) and late (Ann V-positive/PI-positive cells) apoptosis. Only a

**Table 1 Validation of M1 response in LPS-activated macrophages**

	Unstimulated	LPS-activated
GM-CSF	ND	0.3
IL-1β	0.008	7.1
IL-6	0.01	43.4
IL-8	1.03	365.6
TNF-α	ND	167.5

Monocytic THP-1 cells previously differentiated into macrophages by treatment with PMA for 48 h were activated with 1 µg/ml LPS. At 24 h, the conditioned media were assayed for the presence of GM-CSF, IL-1β, IL-6, IL-8 and TNF-α (ND: not determined). Concentrations are in ng/ml.



slight increase in the number of apoptotic cells was noted in the presence of 20% (v/v) CM-UM (Figure 2a). In contrast, marked apoptosis was observed when DLD-1 cells were incubated for 48 h in the presence of 20% CM-LAM. By comparison to cells cultivated in 0.2% FBS medium, the percentage of early apoptotic cells increases by 11-fold in cultures exposed to 20% CM-LAM ( $P=0.003$ ; Figure 2a). Apoptotic cell death was confirmed by analyzing the caspase-dependent cleavage of PARP. The cleaved form (p85-PARP) was clearly detectable after a 24 h incubation in the presence of 20% CM-LAM and its abundance further increases at later time points (48 and 72 h), indicating apoptosis (Figure 2b).

### Decreased Expression of Gal-3 in DLD-1 Cells Treated with the Conditioned Medium of LPS-Activated Macrophages

As described in the introduction, several articles report an association between gal-3 expression and the malignant properties of different kinds of cancer, including colon cancer,<sup>16–22</sup> and this could be at least in part due to the antiapoptotic activities of this protein.<sup>29</sup> As DLD-1 cells express a high level of gal-3, we investigated, at both the protein and mRNA level, whether the expression of gal-3 was modified following treatment with CM-LAM. Short treatments of 3 or 7 h had no effect on gal-3 mRNA expression. However, at 24 h, a marked decrease of gal-3 mRNA was observed in cells treated with 20% CM-LAM, as compared to the time point 0 h ( $P=0.004$ ) or to cells incubated with CM-UM ( $P=0.002$ ; Figure 3a). This difference persists at 48 and 72 h (Figure 3a). To analyze whether the decrease of gal-3 mRNA was associated with a decrease at the protein level, total protein lysates were prepared from DLD-1 cells treated with 20% CM-UM or CM-LAM. No change in gal-3 expression was observed following treatment with CM-UM. In contrast, the expression of gal-3 was decreased after 24 h of incubation with 20% CM-LAM as well as at later time points (48 and 72 h; Figure 3b).

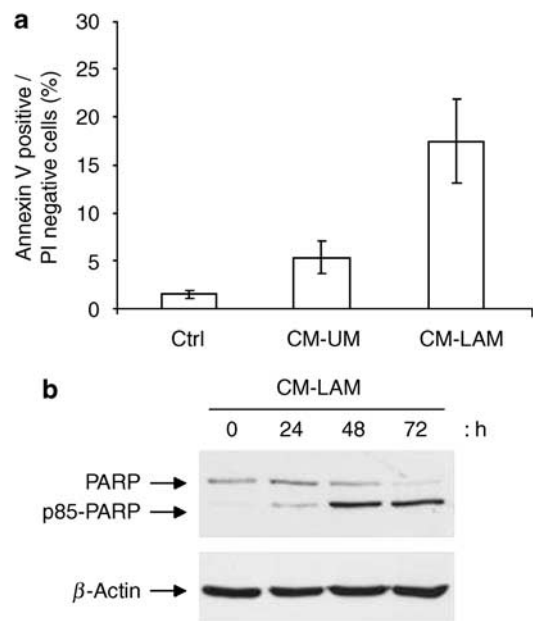
### Determination of IC<sub>50</sub> of the Conditioned Medium from LPS-Activated Macrophages

IC<sub>50</sub> (concentration inhibiting the overall growth by 50%) was determined by treating DLD-1 cells for 72 h with various concentrations (v/v) of CM-UM or CM-LAM in 0.2% or in 10% FBS culture medium (Figure 4). We found that on DLD-1 cells, CM-LAM has an IC<sub>50</sub> of 1.6% in 0.2% FBS culture medium (Figure 4a). It should also be pointed out that, as shown in Figure 1b, CM-UM displays a growth inhibitory activity, although minimal as compared to CM-LAM

(Figure 4a). In 10% FBS culture medium, a higher dose of CM-LAM was required to achieve a similar growth inhibition. Indeed, in this culture condition, CM-LAM has an IC<sub>50</sub> of 20% (Figure 4b).

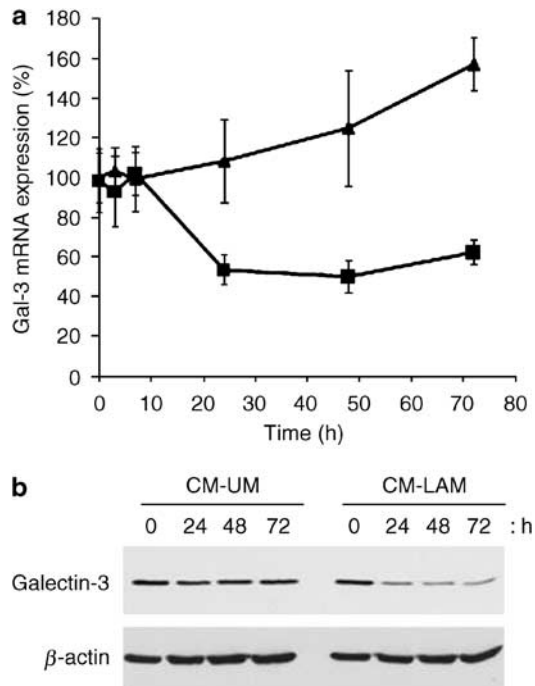
### The Conditioned Medium of LPS-Activated Macrophages Modulates the Localization of Gal-3

Gal-3 exerts different effects on cell survival and proliferation depending on its subcellular localization. For instance, its antiapoptotic activities are commonly linked to its presence in the cytoplasm.<sup>29,30</sup> In addition, extracellular gal-3 in the tumor microenvironment can markedly influence the tumor-associated immune response because the protein is proinflammatory.<sup>23,24</sup> Using the IC<sub>50</sub> determined above (20% CM-LAM in 10% FBS culture medium, Figure 4b), we tested whether the secretion or subcellular localization of gal-3 was modified in response to incubation with CM-LAM. We first verified that CM-LAM, at the IC<sub>50</sub> concentration, causes a decrease in gal-3 expression. Following incubation with 20% CM-LAM, gal-3 mRNA expression was downregulated at



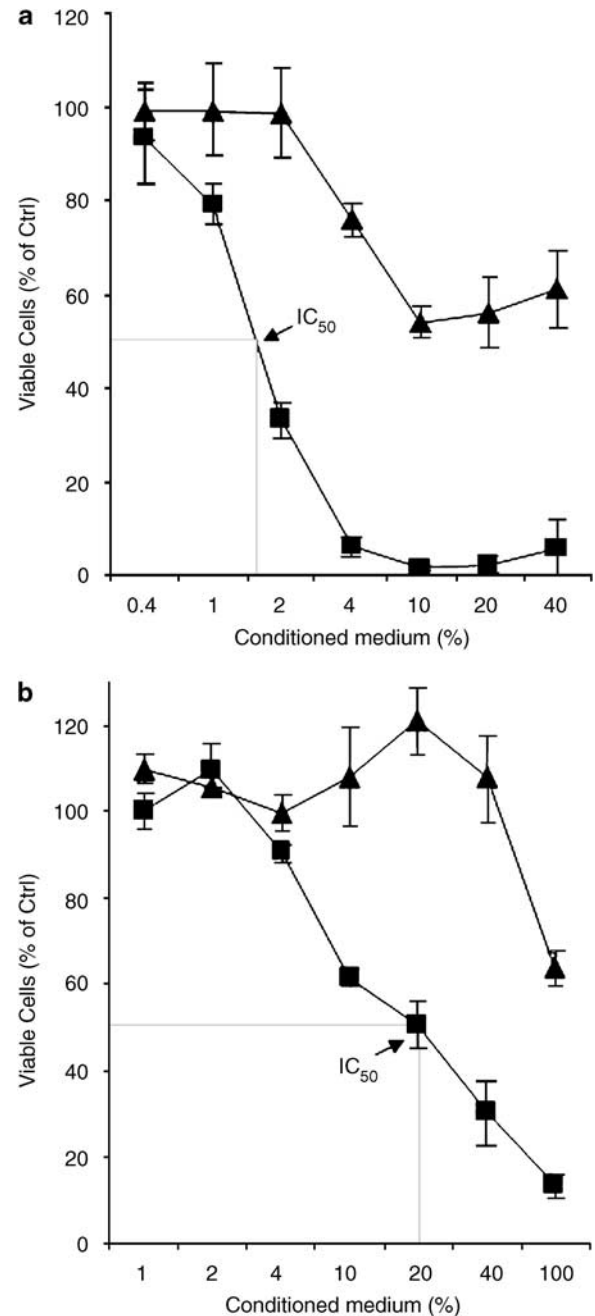
**Figure 2** CM-LAM induces apoptosis. **(a)** Flow cytometry analysis of annexin V reactive cells. DLD-1 cells were incubated for 48 h in culture medium containing 0.2% FBS and the same medium supplemented with 20% (v/v) of either CM-UM or CM-LAM. The graph shows the percentages of early apoptosis (cells positive for annexin V and negative for PI). Data are mean of quadruplicates  $\pm$  s.d. **(b)** Western blot analysis of the cleavage of PARP in DLD-1 cells exposed for 0, 24, 48 and 72 h to 20% CM-LAM in 0.2% FBS culture medium.

**Figure 1** Effect of CM-LAM on DLD-1 cell growth and viability, using video cellular microscopy. **(a)** Recorded still images obtained by video cellular microscopy of time points 1, 12, 24, 36 and 48 h. Cells were cultured for 48 h in 0.2% FBS culture medium (control) and the same medium supplemented with 20% (v/v) CM-UM or CM-LAM. Pictures are representative of four individual experiments. **(b)** Global growth was assessed by counting the number of morphologically viable cells in the different conditions: control cells (dashed columns), cells incubated with CM-UM (white columns), cells treated with CM-LAM (black columns). Countings were based on the still images corresponding to time points 0, 12, 24, 36 and 48 h. Data are representative of four individual experiments (mean  $\pm$  s.d.).



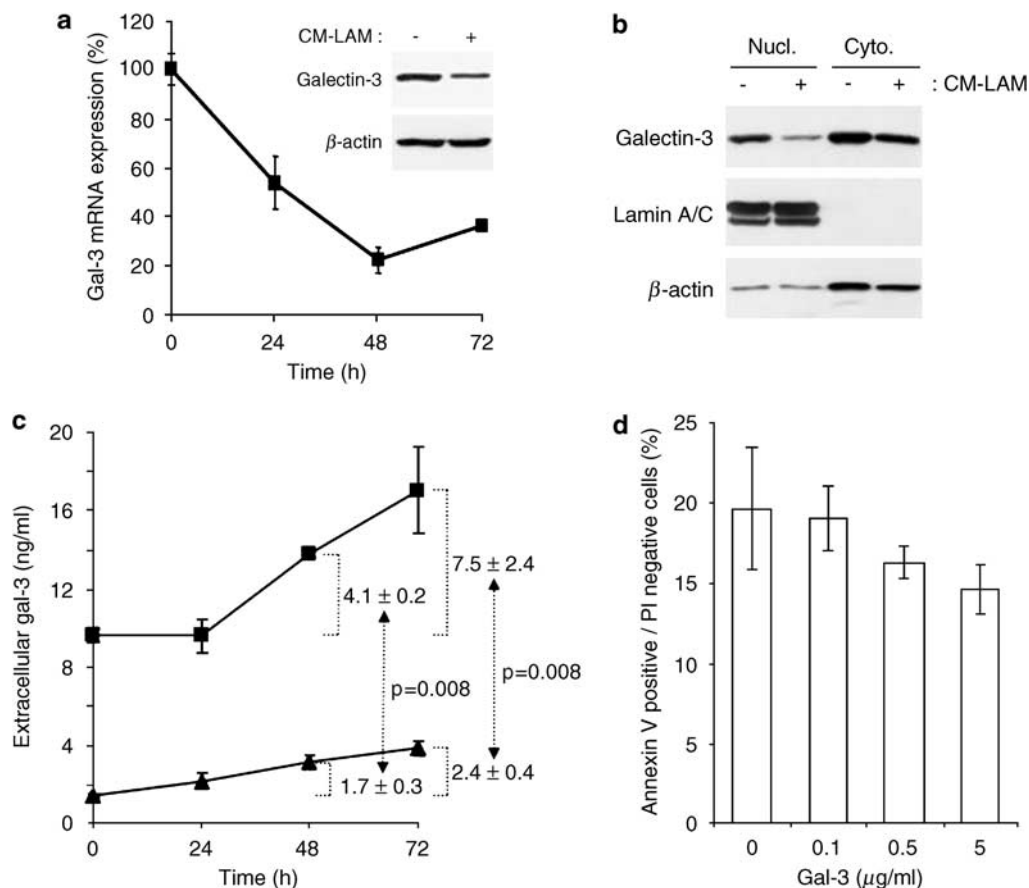
**Figure 3** Decreased expression of gal-3 mRNA and protein after treatment with CM-LAM. **(a)** Effect of CM-LAM on gal-3 mRNA expression measured by real-time quantitative PCR. DLD-1 cells were incubated for 0, 3, 7, 24, 48 and 72 h in 0.2% FBS culture medium supplemented with 20% (v/v) CM-UM (triangles) or CM-LAM (squares). Gal-3 mRNA level is expressed as percentage of the control (0 h). Data are mean of sextuplicates  $\pm$  s.d. **(b)** Western blot showing the gal-3 protein expression in DLD-1 cells incubated for 0, 24, 48 and 72 h in 0.2% FBS culture medium supplemented with 20% (v/v) of either CM-UM or CM-LAM. Representative data of three experiments are shown.

24 h or later time points ( $P=0.003$ ; Figure 5a). Similarly, gal-3 protein level was decreased following a 72 h incubation in the presence of CM-LAM (Figure 5a). To assess gal-3 subcellular localization, we performed a cell fractionation in cells treated or not treated for 72 h with CM-LAM. The level of gal-3 was determined in both the nuclear and cytoplasmic fraction. The integrity of the fractions was monitored by performing a western blot for lamin A/C, a nuclear protein (Figure 5b). As compared to untreated control cells, a sharp decrease in nuclear gal-3 was observed. A decrease in cytoplasmic gal-3 was also noted, although modest as compared to that in the nuclear fraction (Figure 5b). These data can suggest either a differential degradation rate between the two compartments or an imbalance in the nuclear/cytoplasmic shuttling of gal-3. The gal-3 extracellular level was assessed by an ELISA. A higher secretion of extracellular gal-3 was observed in DLD-1 cells treated with 20% CM-LAM as compared to cells incubated in the presence of 20% CM-UM (Figure 5c). The concentration of gal-3 secreted by the DLD-1 cells was obtained by subtracting the gal-3 level recorded at 0 h (gal-3 secreted by the macrophages and already present in 20% CM-LAM and CM-UM prior the incubation) from the



**Figure 4**  $IC_{50}$  determination of CM-LAM. DLD-1 cells were incubated for 72 h in culture medium containing different concentrations of CM-UM (triangles) or CM-LAM (squares). Incubations were performed in **(a)** 0.2% FBS culture medium and **(b)** 10% FBS culture medium. Viability was assessed by MTT assay. Results are expressed as percentage of the control (0% conditioned media). Data are mean of quadruplicates  $\pm$  s.d.

gal-3 concentration at 48 or 72 h. As shown in Figure 5c, CM-LAM significantly increases the release of gal-3 by 2.4-fold at 48 h ( $P=0.008$ ) and 3.1-fold at 72 h ( $P=0.008$ ), as compared to CM-UM (Figure 5c). We next tested whether extracellular gal-3 modulates the sensitivity to CM-LAM. DLD-1 cells were incubated for 72 h with 1.6% CM-LAM



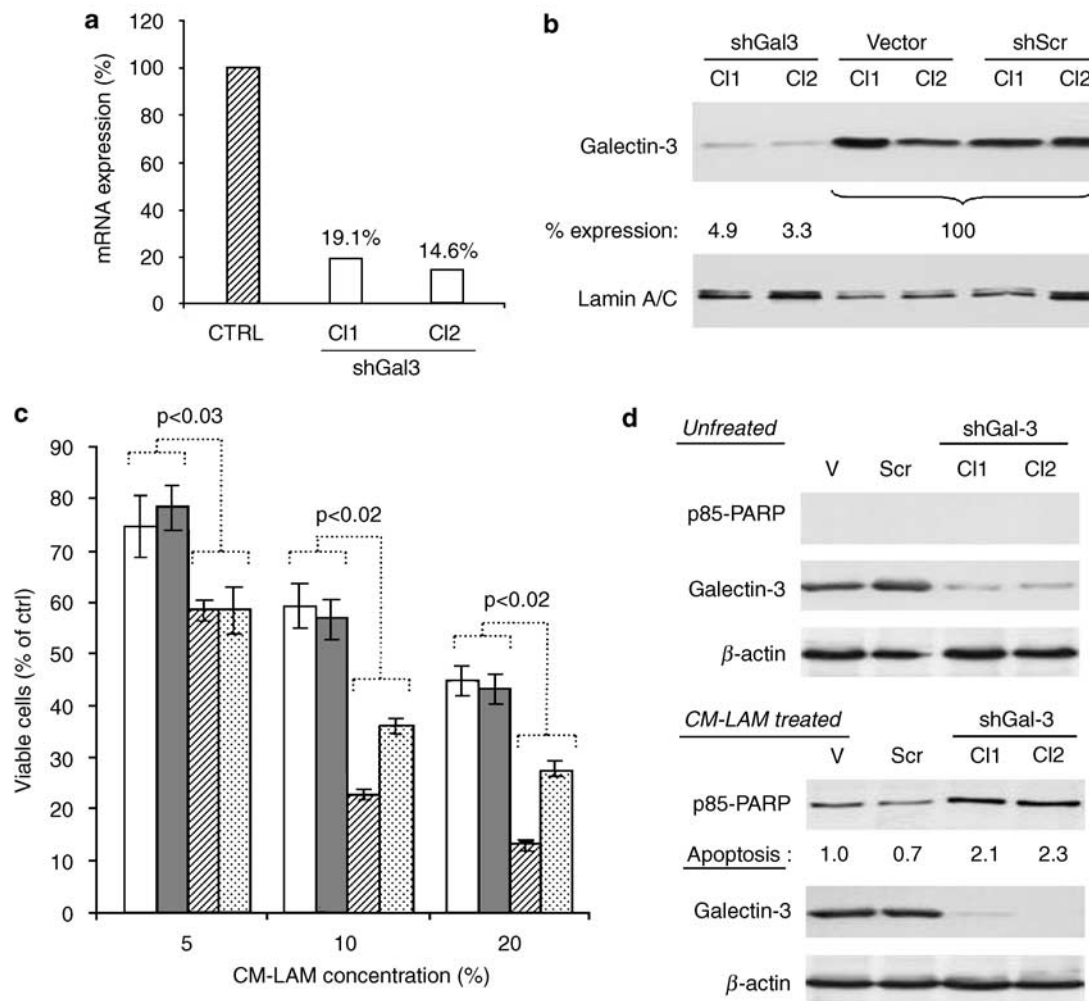
**Figure 5** Analysis of the intracellular localization and the secretion of gal-3 in DLD-1 cells treated with CM-LAM. (a) Expression of gal-3 mRNA and protein following exposure of DLD-1 cells to 20% v/v CM-LAM in 10% FBS culture medium. Gal-3 mRNA level was measured by real-time quantitative PCR and is expressed as percentage of the control (0 h). Data are mean of quadruplicates  $\pm$  s.d. Gal-3 protein level was assessed after a 72 h incubation in 10% FBS culture medium or the same medium supplemented with 20% CM-LAM. (b) Effect of CM-LAM on the intracellular localization of gal-3. A cell fractionation was performed, as described in the 'Materials and Methods', in DLD-1 cells exposed for 72 h to 10% FBS culture medium or the same medium supplemented with 20% CM-LAM. Lamin A/C, a nuclear protein, is used as control for the integrity of the fractions.  $\beta$ -Actin is localized in both the nucleus and the cytoplasm. Data shown are representative of three experiments. (c) Secretion of gal-3 by DLD-1 cells in the presence of CM-LAM and CM-UM. Squares represent 20% v/v CM-LAM in 10% FBS culture medium; triangles, 20% v/v CM-UM in 10% FBS culture medium. The gal-3 concentration at 0 h is the gal-3 level present in the diluted conditioned media just prior the beginning of the incubation. Concentrations are in ng/ml. Data are mean of quintuplicates  $\pm$  s.d. (d) Effect of the extracellular gal-3 level on the sensitivity to CM-LAM. DLD-1 cells were incubated in 0.2% FBS culture medium supplemented with 1.6% (v/v) CM-LAM and different doses of human recombinant gal-3 (0, 0.1, 0.5 and 5  $\mu$ g/ml). After 72 h of treatment, cells were harvested, labeled with annexin V-FITC (Ann V) and propidium iodide (PI) and analyzed by flow cytometry. The graph shows the percentages of early apoptosis (cells positive for annexin V and negative for PI). Data are mean of triplicates  $\pm$  s.d.

in 0.2% FBS culture medium ( $IC_{50}$  concentration described in Figure 4a) supplemented with increasing amounts (0, 0.1, 0.5 and 5  $\mu$ g/ml) of human recombinant gal-3 (Figure 5d). A decrease in the percentage of apoptotic cells was observed in the presence of extracellular gal-3 at concentrations of 0.5 and 5  $\mu$ g/ml (Figure 5d). It must be stressed that the protective effect is only observed at concentrations much higher than those actually released by DLD-1 cells (Figure 5c).

#### The Knockdown of Gal-3 Sensitizes DLD-1 Cells to the Conditioned Medium of LPS-Activated Macrophages

As CM-LAM induced a significant decrease of the intracellular gal-3 protein level, we examined how such a decrease can affect the sensitivity to the proinflammatory/

apoptotic stimulus. To directly test that, we interfered with gal-3 expression by stably transfecting DLD-1 cells with the pSUPER-shGal3-551 vector, as described in 'Materials and Methods'. At the end of the selection process, we obtained stable clones displaying a greater than 80% decrease of gal-3 mRNA expression (Figure 6a). Knockdown clones shGal3-C11 and -C12 were characterized for gal-3 protein expression. As compared to clones transfected with the empty pSUPER or the pSUPER-shScr vector, a 95% decrease of gal-3 protein level was observed (Figure 6b). To test how gal-3 knockdown affects the sensitivity to CM-LAM, we performed MTT assays using two control clones (pSUPER-C11 and pSUPER-shScr-C11) and the two gal-3 knockdown clones. After a 72 h exposure to 5, 10 or 20% CM-LAM (in 10% FBS culture



**Figure 6** The knockdown of gal-3 sensitizes DLD-1 cells to CM-LAM. (a) Gal-3 mRNA expression of knockdown clones, shGal3-C11 and -C12. These clones were stably transfected with the vector pSUPER-shGal3-(551) as described in ‘Materials and Methods’. Gal-3 mRNA level was measured by real-time quantitative PCR. The control (CTRL, 100%) is the mean gal-3 mRNA expression of six control clones, three transfected with the empty vector and three transfected with pSUPER-shScr. Data are representative of two experiments. (b) Western blot of gal-3 protein level in clones shGal3-C11 and -C12, by comparison to clones transfected with the empty pSUPER vector (Vector) or the pSUPER-shScr vector (shScr). The residual gal-3 expression in the knockdown clones was assessed by a densitometry analysis of the gal-3 bands, and normalization to the loading control (lamin A/C). The value of 100% expression is given by the mean of the four control clones. (c) MTT assay showing the enhanced sensitivity to CM-LAM in gal-3 knockdown cells. Cells were incubated for 72 h in 10% FBS culture medium containing 0, 5, 10 or 20% CM-LAM. White columns indicate empty vector (Cl1); gray columns, shScr vector (Cl1); hatched columns, shGal3-C11; dotted columns, shGal3-C12. Results are expressed as percentage of the control (0% CM-LAM). Data are mean of sextuplicates  $\pm$  s.d. The displayed *P*-values result from the *post hoc* test and are the maximum *P*-values obtained for the different pairs involved in the brackets. (d) Western blot of the cleavage of PARP in untreated cells (top) or cells treated for 16 h with 20% CM-LAM in 10% FBS culture medium (bottom). The cleavage of PARP was analyzed in the gal-3 knockdown clones, shGal3-C11 and -C12, as well as two control clones—V, empty vector (Cl1) and Scr, shScr vector (Cl1).

medium), we observed an increased sensitivity of the knockdown cells to CM-LAM, as compared to the control cells (Figure 6c). The enhanced sensitivity was confirmed by a subsequent western blot analysis for the cleavage of PARP, a marker of apoptosis, after a 16 h exposure to 20% CM-LAM. In untreated cells, no PARP cleavage was detected (Figure 6d, top). Upon exposure to CM-LAM, PARP cleavage was clearly observed in the four clones. In addition, the cleavage of PARP was more abundant in cells knockdown for gal-3, as compared to control cells (Figure 6d, bottom). A densitometry

analysis of the bands indicated a greater than twofold enhancement of the cleavage of PARP in the knockdown cells.

**DISCUSSION**

Macrophages constitute a significant part of the tumor-infiltrating immune cells and the increasing literature on the subject suggests that they can considerably affect the course of the disease.<sup>11,12</sup> In most clinical studies, it has been observed that a high infiltration of the so-called TAMs correlate with a poor prognosis.<sup>11,12</sup> High TAM density is often

associated with a high vessel density, enhanced tumor invasiveness and metastasis occurrence, suggesting that they may behave as tumor promoters.<sup>11,12</sup> Interestingly, in colon cancers, most articles suggest that TAMs prevent tumor development because patients with high TAMs have better prognosis and survival rate.<sup>7–9</sup> This was evidenced recently by Forssell *et al*<sup>10</sup> who performed a large study on 446 colorectal cancer specimens. Considering these findings, it is tempting to speculate that TAMs in colon cancer may not display a clear M2 polarization. As developed by Sica *et al*,<sup>12</sup> the distinction between M1 and M2 macrophages does not encompass the entire spectrum of states that macrophages can express but rather represents two extremes. For instance, THP-1 monocytes that have been differentiated into macrophages with vitamin D<sub>3</sub> are poor TNF- $\alpha$  producers but are able to generate substantial amount of superoxide anion,<sup>31</sup> thus displaying both M1 and M2 characteristics.

Here we used an *in vitro* model to analyze the effects of culture supernatants from M1-polarized macrophages on colon cancer cells. In accordance with the literature, we found that activation of differentiated THP-1 cells with LPS led to the release of proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ .<sup>13,14</sup> When applied to DLD-1 colon adenocarcinoma cells, the culture supernatants obtained from these LPS-activated macrophages displayed a strong growth inhibitory activity and caused extensive cell death. Cell death occurred, at least partly, by apoptosis as evidenced by Ann V binding assays and PARP cleavage, in accordance with the high level of cytokines contained in CM-LAM known to be proapoptotic to susceptible tumor cells.<sup>32,33</sup>

DLD-1 colon adenocarcinoma cells express a high level of gal-3 in both their cytoplasm and nuclei. We found that CM-LAM induces a marked decrease of the expression of gal-3 in DLD-1 cells at both the mRNA and protein levels. Interestingly, cell fractionation indicated that the decrease in gal-3 protein was more pronounced in the nucleus as compared to the cytoplasm, suggesting either an imbalance of the degradation rate between the two compartments or alternatively a modification in the nuclear/cytoplasmic shuttling of the protein. The effect of cytokines or inflammatory stimuli on gal-3 expression is a matter of controversy and likely depends on the experimental model used or the cell line studied. For instance, the expression of gal-3 is downregulated in the intestinal epithelia of Crohn's disease patients.<sup>34</sup> Furthermore, TNF- $\alpha$  decreases the level of gal-3 mRNA in the human intestinal epithelial cell line HCT-8.<sup>34</sup> In contrast, proteome analysis of isolated Langerhans rat islets exposed to IL-1 $\beta$  identified gal-3 as the most upregulated protein.<sup>35</sup> Upregulation of gal-3 mRNA expression in human islets treated with IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$  or a mix of these cytokines was also reported.<sup>35</sup> We report that knocking down gal-3 in DLD-1 cells results in a sensitization to CM-LAM, as evidenced by MTT assays and PARP cleavage. This is in accordance with different articles describing the antiapoptotic activity of gal-3, in response to a wide range of apoptotic

insults such as staurosporine, anti-fas antibodies, nitric oxide, radiation, anoikis and TNF- $\alpha$ .<sup>29,30</sup>

Last, we found that DLD-1 cells treated with CM-LAM secrete an increased amount of gal-3. Although, the protective effect of extracellular gal-3 during CM-LAM treatment was only observed at very high concentrations (more than 60-fold higher than what is actually released by DLD-1 cells), this observation remains interesting with regard to the involvement of gal-3 in the tumor-associated immune response. Gal-3 has been shown to be chemoattractive for monocytes and macrophages and is able to trigger an oxidative burst in macrophages.<sup>36</sup> Nishi *et al*<sup>37</sup> recently demonstrated that PMA-differentiated THP-1 cells incubated with recombinant gal-3 release TNF- $\alpha$  and IL-8 (but not IL-4, IL-13 or TGF- $\beta$ ) at levels similar to what LPS induces. It therefore appears that *in vivo* an amplification loop could take place in which activated macrophages would, through the release of proinflammatory cytokines, induce the secretion of gal-3 by colon cancer cells. This secreted gal-3 would in turn stimulate the release of different factors by macrophages as well as recruit more macrophages because it is chemoattractive for this type of cells.

To conclude, the role of TAMs in tumorigenesis is complex because their behavior is modulated by the tumor microenvironment itself. Various factors are involved in the crosstalks that exist between TAMs and cancer cells and this report emphasizes that gal-3 is involved in this process. Indeed activated macrophages are able to modulate both the expression and the secretion of gal-3 by colon cancer cells. It will be crucial to determine the phenotype of TAMs in colon cancer, with regard to the M1 and M2 types of polarization, because this will allow the development of *in vitro* models that are closer to the reality of the disease and will help to better understand why high TAMs densities are associated with a good prognosis in colon cancer.

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#### DISCLOSURE/DUALITY OF INTEREST

None.

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