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CD206-positive myeloid cells bind galectin-9 and promote a tumor-supportive microenvironment

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Abstract

In patients with metastatic melanoma, high blood levels of galectin-9 are correlated with worse overall survival and a bias towards a Th2 inflammatory state supportive of tumor growth. Although galectin-9 signaling through TIM3 on T cells has been described, less is known about the interaction of galectin-9 with macrophages. We aimed to determine whether galectin-9 is a binding partner of CD206 on macrophages and whether the result of this interaction is tumor-supportive. It was determined that incubation of CD68+ macrophages with galectin-9 or anti-CD206 blocked target binding and that both CD206 and galectin-9 were detected by immunoprecipitation of cell lysates. CD206 and galectin-9 had a binding affinity of 280 nM. Galectin-9 causes CD206+ macrophages to make significantly more FGF2 and monocyte chemoattractant protein (MCP-1), but less macrophage-derived chemokine (MDC). Galectin-9 had no effect on classical monocyte subsets, but caused expansion of the non-classical populations. Lastly, there was a positive correlation between increasing numbers of CD206 macrophages and galectin-9 expression in tumors, and high levels of CD206 macrophages correlated negatively with melanoma survival. These results indicate that galectin-9 binds CD206 on M2 macrophages, which appear to drive angiogenesis and the production of chemokines that support tumor growth and poor patient prognoses. Targeting this interaction systemically through circulating monocytes may therefore be a novel way to improve local anti-tumor effects by macrophages.

Keywords

CD206; galectin-9; macrophages; monocytes; metastatic melanoma; angiogenesis; tumor microarray

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Introduction

Melanoma is the deadliest form of skin cancer and has been increasing in incidence for the past 30 years, especially in young adults. In 2016, some 87,000 people were diagnosed with melanoma and 10,000 people died of this disease in the United States [1]. Recent advances in tumor immunology have led to the development of numerous immune-activating or -blocking therapies that have greatly improved patient survival [2]. However, these therapies depend on the presence of a functional immune system, which is suppressed in patients with advanced cancer [3–5]. Thus, new methods to reactivate this suppressed systemic immunity could lead to better outcomes for patients with cancer.

Current immune therapies focus on the manipulation of lymphocyte populations, but myeloid cells, such as monocytes and macrophages, are also present in the tumor microenvironment. Monocytes circulate in the blood, while macrophages reside in tissues, and both play an important role in recognizing foreign substances, or tumor cells, destroying them by phagocytosis and by presenting antigens to T cells. Interestingly, the tumor itself is often made up of 5–40% tumor-associated macrophages (TAMs), of which higher densities correlate with worse prognosis [6,7]. TAMs have been defined as having an M2 or “alternatively activated” phenotype, which supports tumor functions by promoting chronic inflammation, proliferation, tissue remodeling, migration and angiogenesis [8]. However, studying macrophages remains challenging due to their cellular plasticity, lack of well-defined surface markers and differences between humans and model organisms [9]. Nonetheless, because TAMs are a major part of the tumor microenvironment and immune system, additional attention should be focused on this population when targeting malignancies.

We have shown previously that galectin-9, a carbohydrate-binding protein, can bias normal macrophages towards a M2 phenotype in a similar manner to interleukin (IL)-4 and IL-13 [10,11]. M2 macrophages were defined by their expression of mannose receptor CD206 and the secretion of MCP-1 and IL-10. Activated CD4+ T helper cells expressing T cell immunoglobulin mucin 3 (TIM3) were found to undergo apoptosis when galectin-9 bound to TIM3 [12]; however, it is not clear how galectin-9 affects myeloid cells. It is clear that galectin-9 signals through an unknown receptor, because cells that do not express TIM3 still undergo apoptosis and secrete cytokines [13,14]. Tumors have been shown to secrete high levels of galectin-9 [10, 15, 16]; however, it appears that other cells with surface galectin-9 expression, including macrophages, will secrete it in response to inflammatory stimulus [17–19]. In metastatic melanoma, galectin-9 is co-localized with the CD68+ macrophage population and soluble forms of galectin-9 in the blood correspond with poor survival [10]. This suggests an undefined interaction between macrophages and galectin-9 that might be manipulable to improve cancer outcomes. Herein, we describe the interactions between human myeloid cells and galectin-9. Because galectin-9 can upregulate CD206 expression on normal donor macrophages [10], we hypothesize that galectin-9 binds CD206 on macrophages to promote a pro-tumor phenotype in the local microenvironment.

Materials and Methods

Flow cytometry

THP-1 (TIB-202) human monocyte cells were purchased from ATCC as mycoplasma-free cell lines, authenticated by STR analysis and cultured according to ATCC instructions. For blocking experiments, THP-1 cells were polarized to M2 macrophages as described [11]. Cells were incubated with 0 or 5 μg recombinant galectin-9 (R&D Systems, Minneapolis, MN) or anti-CD206 (R&D Systems) for 30 minutes and then stained with anti-CD68-PE and anti-CD206-APC or galectin-9 protein conjugated to APC (BD Biosciences, San Jose, CA). Cells were run on a Guava EasyCyte HT (EMD Millipore, Billerica, MA) and analyzed with InCyte Software (EMD Millipore). Imaging flow cytometry was conducted on macrophages incubated with Alexa Fluor 647-conjugated galectin-9 protein and anti-CD206-PE for 30 minutes and cells were run on ImageStream System (EMD Millipore) using IDEAS software for analysis (EMD Millipore).

For phenotyping, single cell suspensions were obtained from buffy coats from 10 healthy donors collected through the Mayo Clinic blood donor program; only information pertaining to patient age and sex was available. CD14+ cells were isolated by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany) and treated with or without 2 μg/ml galectin-9 for 24 hours. were prepared and stained with the indicated antibodies: anti-CD14, CD16, CD206, CD33, CCR2 and CX3CR1. Antibodies were purchased from BD Biosciences. Flow cytometry data was collected on a Guava EasyCyte HT and analyzed with InCyte Software (EMD Millipore).

Immunoprecipitation

THP-1 monocytes were polarized to M2 macrophages as above. Whole-cell lysates were prepared and were incubated at 4°C rocking for 1 hour with 2 μg/ml recombinant galectin-9 (R&D Systems, Minneapolis, MN) or 1:1 with THP-1 supernatant (high galectin-9). Protein A beads and 5 μl of either anti-galectin-9 or anti-CD206 antibodies (R&D Systems) were added to the lysates and incubated for another hour at 4°C rocking. The mixture was washed and the beads were removed from the protein by centrifugation. Samples were separated by SDS-PAGE and the CD206- or galectin-9-binding proteins detected by immunoblot. Primary antibody working concentrations were 1 μg/ml for each antibody.

Protein binding and quantification

To measure protein-protein binding affinities, 100 μg/ml of biotinylated CD206 (R&D Systems) was bound to a streptavidin probe (Pall ForteBio, Fremont, CA). The binding of galectin-9 protein was measured by light absorbance on the BLItz system (Pall ForteBio) at 347, 174, 87 and 43 nM. Three separate binding experiments were run with association and dissociation times of 120 s. association and dissociation constants were calculated using the BLItz software.

CD14+ monocytes were isolated from normal donor PBMCs following MACS negative selection (Miltenyi). MagPlex Multiplex kits (Millipore, Billerica, MA) were used to determine concentrations of cytokines and angiogenic factors. Following macrophage

polarization, cells were sorted by CD206 expression on a FACSAria II (BD Bioscience). The Angiogenesis MagPlex kit was used to quantify epidermal growth factor (EGF), angiopoietin (Ang)-2, granulocyte colony-stimulating factor (G-CSF), bone morphogenetic protein (BMP)-9, endoglin, endothelin-1, leptin, fibroblast growth factor (FGF)-1, FGF-2, follistatin, IL-8, hepatocyte growth factor (HGF), heparin-binding (HB)-EGF, placental growth factor (PlGF), vascular endothelial growth factor (VEGF)-A, VEGF-C and VEGF-D. The Cytokine/Chemokine MagPlex kit was used to quantify epidermal growth factor (EGF), eotaxin, tumor growth factor (TGF)-α, Flt-3L, granulocyte-macrophage colony-stimulating factor (GM-CSF), fractalkine, interferon (IFN)-α2, IFN-γ, GRO, IL-10, monocyte chemoattractant protein (MCP)-3, IL-12p40, macrophage-derived chemokine (MDC), IL-12p70, IL-13, IL-15, sCD40L, IL-17A, IL-1RA, IL-1α, IL-9, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α and TNFβ. Immunoassays were performed according to manufacturer's instruction. Multiplex assays were measured using Luminex xPONENT technology (Austin, TX). All samples were measured in duplicate, averaged and concentrations were calculated using MILLIPLEX Analyst 5.1 software (Vigene Tech, Carlisle, MA).

Supernatant from THP-1 cells was collected on day 3 of culture and measured for galectin-9 production by ELISA (R&D Systems, Minneapolis, MN) as previously described [10]. Concentration was determined by a 7-point standard curve.

Tumor microarray

Stage IV melanoma tissue arrays were approved by the Mayo Clinic Institutional Review Board. Tissue microarrays were constructed from formalin-fixed paraffin-embedded (FFPE) tissue blocks of 188 routine clinical biopsies collected from 1985–2012. Sections were stained with hematoxylin and eosin (H&E) and tissue adequacy evaluated under conventional optic microscope (Olympus BX53). Immunohistochemistry was performed with mouse monoclonal antibodies against galectin-9 (clone 1G3, ab153673, Abcam, Cambridge, United Kingdom) and CD206 (IgG2B clone # 685645, R&D systems, Minneapolis, MN, United States). Galectin-9 antibodies were diluted 1:500 and CD206 antibodies were diluted 1:200. CD206 macrophage staining was classified from 0 to 3 (0=negative, 3=abundant positive cells) according to the number of positive macrophages in each tumor core. The intensity of tumor cell galectin-9 positivity was classified ranging from 0 (negative) to 3 (intensely positive) and each class denominator was multiplied by the relative percentage of tumor cells in each class, yielding a score between 0 and 600 according to the following formula: H-score = (0 × % cells in class 0) + (1 × % cells in class 1) + (2 × % cells in class 2) + (3 × % cells in class 3).

One pathologist scored all the cores, and another pathologist confirmed the scoring through random sampling. Both were blinded to the experimental outcome. To calculate survival based on CD206 expression, groups 0 and 1 were combined (low expression) and groups 2 and 3 were combined (high expression) for analysis. For low and high tumor expression of galectin-9 *vs.* survival, the cut-off was defined as a median value of 65.

Statistical analysis

All multiplex assays were run in duplicate and averaged to determine protein concentration and any results having with a coefficient of variation greater than 30% were not considered in the final analysis. Paired *t*-tests were used to compare levels of chemokines, receptors and angiogenic factors after treatment with galectin-9. Significance was defined as a *p*-value <0.05. Survival was defined as the date of biopsy to date of last follow-up or death, and was estimated by Kaplan-Meier curve and compared by log-rank tests in R Studio (RStudio Inc. Boston, Massachusetts). 95% confidence intervals are displayed on the survival graphs.

Results

Galectin-9 strongly interacts with CD206

To understand whether or not galectin-9 associates with CD206 expression, M2 polarized THP-1 macrophages were treated with galectin-9 or anti-CD206 prior to staining for flow cytometry, to determine if the treatment blocks the ability for an anti-CD206 antibody or galectin-9 APC conjugated protein to bind (Figure 1A,B). The untreated macrophages (dark grey) were positive for CD68 and CD206 or galectin-9; however, when binding was blocked with galectin-9 (Figure 1A) or anti-CD206 (Figure 1B) the number of positive cells was decreased (light grey). This suggests that pre-incubation of either CD206 antibodies or galectin-9 protein will interfere with receptor binding. To visualize this interaction on macrophages using imaging flow cytometry, 1 μg of galectin-9 protein labeled with Alexa Fluor 647 and anti-CD206 antibodies were incubated and analyzed by ImageStream. Representative images show that CD206 (yellow) and galectin-9 (red) tend to be co-expressed on M2 polarized macrophages (Figure 1C).

To determine if galectin-9 binds to CD206, immunoprecipitation assays were utilized to pull down proteins bound to both targets, using cell lysates from THP-1 macrophages or M2 polarized macrophages. Lysates were incubated with 1 μg/ml recombinant galectin-9 protein or 1:1 with THP-1 supernatant containing physiologic galectin-9 protein (supplementary material, Figure S1) and immunoprecipitated using antibodies to galectin-9 and CD206. Western blot results demonstrate that CD206 (185 kDa) could be detected in both macrophages and M2 polarized macrophages treated with recombinant galectin-9 and THP-1 supernatant (Figure 1D). Additionally, galectin-9 (34 kDa) was detectable after immunoprecipitation with CD206 in the recombinant galectin-9 samples; however, lysates incubated with THP-1 supernatants showed a much larger band suggestive of a possible doublet conformation in cell supernatant (Figure 1E). To more precisely measure the interaction between galectin-9 and CD206, protein-protein and protein-peptide interactions were determined by BLItz. CD206 was biotinylated and bound to a streptavidin probe. Increasing concentrations of galectin-9 were run against the probe. The average K_D of the galectin-9 and CD206 interaction was measured at 2.8×10^{-7} M, with an on rate of 1.4×10^4 Ms^{-1} and an off rate of 4.0×10^{-3} s^{-1} (Figure 1F). Together, these data indicate that CD206 is a binding target of galectin-9.

Galectin-9 stimulates myeloid cells to secrete angiogenic factors

To understand specifically what galectin-9 does to secretion of angiogenic factors and chemokines of CD206+ M2 macrophages and not to CD14+ monocytes and CD68+CD206− macrophages, cells isolated from normal donors were polarized to macrophages, sorted by CD206 expression and treated with 2 μg/ml galectin-9 overnight. Chemokines and angiogenic factors were measured in supernatants using multiplex bead assays. CD206+ macrophages made more FGF-2 (Figure 2A) and less MDC (Figure 2D) after galectin-9 treatment. On the other hand, CD14+ monocytes increased their secretion of FGF-2 and VEGF on treatment with galectin-9 (Figure 2A, 2C). Specific to the monocyte population, HGF and IP-10 production was decreased in the presence of galectin-9 (Figure 2B, 2F). Interestingly, monocytes, macrophages and M2 polarized macrophages all increased MCP-1 secretion in the presence of galectin-9 (Figure 2E). The data indicate that based on their level of differentiation, myeloid cells secrete altered angiogenic factors and chemokines in response to galectin-9; however, these effects are not fully dependent on galectin-9.

Changes in monocyte subsets after galectin-9 treatment

It was previously shown that macrophages treated with galectin-9 upregulate CD206 expression [10]; therefore, we aimed to understand the ability of galectin-9 to modulate monocyte subsets. Differences in receptor expression on monocytes treated for 24 hours with and without galectin-9 were measured by flow cytometry. Classical monocytes (CD14+CD16−) were not changed in response to galectin-9 addition; however, CD14+CD16+ non-classical monocytes did expand in the presence of galectin-9 (Figure 3A). To further understand this difference, we measured recruitment receptors to inflamed (CCR2+) or non-inflamed (CX3CR1+) tissue. MCP-1 production was increased with galectin-9, yet its receptor, CCR2, was slightly decreased after treatment (Figure 3B). Most interestingly, CD14+CD16+CX3CR1 cells were significantly upregulated following treatment with galectin-9 (Figure 3C). This suggests that monocytes in a high galectin-9 environment take on a non-classical role in immunity. All CD14+ monocytes increase surface expression of CD206 after incubation with galectin-9 (Figure 3D).

CD206 macrophages correlate with tumor galectin-9 expression and poor prognosis

Table 1 summarizes the characteristics of 188 TMA patients, of which 8 cases were excluded due to insufficient tissue for evaluation. CD206 staining was given a score of 0 (n=3), 1 (n=81), 2 (n=76) or 3 (n=20). There was a significant positive correlation between galectin-9 tumor cell expression (H-score; Figure 4A) and the CD206+ macrophage tumor infiltration levels (classes 0–3; Figure 4B); (Spearman's correlation co-efficient ρ=0.244, p=0.001; Table 2). Additionally, the independent samples Kruskal-Wallis test was used to look for differences in the galectin-9 H-score among the four tumor subsets classified by CD206+ macrophage tumor infiltration level (classes 0–3). Statistically significant differences were identified (p=0.003), with average H-score levels being higher with higher levels of CD206+ macrophages (Figure 4C). Due to low numbers in CD206 TMA groups 0 and 3, groups 0 and 1 were combined into "low" CD206 expression, and groups 2 and 3 into "high". Patients with high levels of CD206+ tumor core macrophages had a worse prognosis than those with low CD206 infiltration (p=0.019; Figure 4D). Using the median H-score as

the cutoff, 84 tumors were classified as low galectin-9 (H-score<65) and 96 as high galectin-9 (H-score≥65). In contrast to CD206 results, galectin-9 tumor expression did not affect stage IV melanoma survival (p=0.3; Figure 4E).

Discussion

Macrophages are crucial for innate immune defense against pathogens and maintaining homeostasis. CD206 is a marker of M2 (alternatively activated) macrophages, which have important roles in wound healing in response to tissue damage or chronic infection and in promoting tumor progression [20,21]. Tumor-associated macrophages (TAMs), which are modulated by signals from the tumor, can shut down anti-tumor immunity, promote blood vessel formation and initiate metastasis [22]. Although the TAM phenotype can vary between tumor types, CD206 is not expressed on M1 classical macrophages, making it useful for distinguishing M2 TAMs [8, 20]. Carbohydrate-binding protein galectin-9 has become an attractive target for cancer therapeutics, due to its ability to adversely regulate anti-tumor immune responses and promote tolerance [12, 23], but the extent of galectin-9 binding partners and signaling remains unclear. We showed previously that galectin-9 induced a M2 or tumor-promoting phenotype in healthy macrophages [10]. Galectin-9 has been shown to interact with lymphoid cells via TIM3 [12], CD44 [24], protein disulfide isomerase (PDI) [25] and 4–1BB [26]. The current study indicates that galectin-9 binds to CD206 on macrophages, changing the phenotype to promote tumor survival. Recently, dectin-1 on macrophages in pancreatic ductal adenocarcinoma (PDA) was shown to bind galectin-9, but inhibiting this interaction would result in a strong anti-tumor response [23]. The latter mouse model of PDA also demonstrated a decrease in tumor infiltration of CD206+ macrophages when dectin-1 signaling was removed. Therefore, due to the plasticity of macrophages, dectin-1 and CD206 could share a redundant function in myeloid cells, which should be addressed in future studies.

Using both endogenous (from THP-1 supernatant) and exogenous galectin-9, we have shown that not only does galectin-9 interfere with CD206 binding on M2 polarized cells, but that they bind directly, as indicated by immunoprecipitation. An increased molecular weight of bound THP-1-secreted galectin-9, compared to recombinant, might be due to galectin-9 dimer formation in solution, as demonstrated by crystal structure [27].

Because TAMs play a crucial role in angiogenesis and trafficking, we sought to define differences in secretion of angiogenic factors and chemokines by monocytes, macrophages and M2 polarized macrophages after treatment with galectin-9. M2 biased CD206+ macrophages secreted higher levels of monocyte chemoattractant protein (MCP)-1 and fibroblast growth factor (FGF)-2. In esophageal cancer, overexpression of FGF-2 in the tumor microenvironment was correlated with a more aggressive phenotype and higher numbers of TAMs [28]. Additionally, high levels of MCP-1 correlated with increased accumulation of TAMs [29]. Thus, galectin-9 may help orchestrate this pro-tumor phenotype through its interaction with M2 TAMs. Conversely, M2 polarized macrophages secreted less macrophage derived chemokine (MDC) after treatment with galectin-9. High expression of MDC has been associated with longer survival and lower recurrence risk in lung cancer

patients [30]. Our data suggest that this positive effect may be mitigated by galectin-9 through M2 macrophages.

Since it is clear that macrophages are sensitive to galectin-9, we wanted to understand if their precursor CD14+ monocyte cell is also influenced and if it is subtype-specific. Classical monocytes (CD14+CD16−) promote inflammation; conversely, non-classical monocytes (CD14+ and CD16+) have an important role in wound healing [31]. Galectin-9 did not influence classical monocytes, but did expand the non-classical population. Galectin-9 effectively induces MCP-1 secretion; however, its receptor, CCR2, was only slightly decreased on CD14+CD16− monocytes in response to galectin-9. CD14+CD16−CCR2+ monocytes make up the majority of blood monocytes which traffic to sites of tissue damage or infection and produce inflammatory cytokines [32]. Therefore, galectin-9 does not promote the inflammatory phenotype of monocytes that would target a tumor. Conversely, CX3CR1 is highly expressed on CD14+CD16+ non-classical monocytes, a long-lived population that becomes a resident cell in non-inflamed tissue [33]. Monocyte expression of CX3CR1 is believed to have important anti-apoptotic effects and has been show to correlate with worse survival in ovarian cancer [34, 35]. Here, galectin-9 upregulates CX3CR1 on monocytes, possibly as a mechanism to promote tumor growth by supporting monocytes without inflammatory properties. The data suggest that conditions with high levels of galectin-9, such as cancer, can promote circulating monocytes to differentiate into non-classical monocytes that support tumor survival.

Tissue microarrays revealed a positive correlation between the number of CD206+ macrophages and galectin-9 tumor expression. For assessing galectin-9 tumor cell immunopositivity, most previous studies have used a semi-quantitative approach, mainly due to the heterogeneity of positivity that can be observed [16, 36–38]. In this context we used the H-score as applied by Liu et al. [38], but also established by other studies [39–40]. In our stage IV melanoma TMA cohort, survival was negatively correlated with high infiltration of CD206+ macrophages. Others have demonstrated a worse prognosis in renal cell carcinoma and hepatocellular carcinoma patients with increasing density of TAMs [41, 42]. Galectin-9 tumor expression did not affect stage IV melanoma survival in our cohort, while others have demonstrated that galectin-9 expression is associated with an improved prognosis [43,44]. Discrepancies could be due to tumor heterogeneity during sampling or variations in scoring.

Although current immunotherapies have focused on targeting T or B cell populations, modulating TAMs may be an effective way to improve cancer outcomes, especially since most cells in the tumor microenvironment are macrophages. For example, blocking MARCO, a pattern recognition receptor on TAMs, reprogrammed TAMs in breast, colon and melanoma models to become pro-inflammatory and initiate anti-tumor immune responses [45]. Another approach is to reduce the number of TAMs in the tumor microenvironment. A legumain DNA vaccine [46] resulted in a robust cytotoxic T cell response against TAMs and suppressed tumor growth and metastasis in murine models of multiple tumor types. Lastly, treating hepatocellular carcinoma cells with gadolinium chloride was shown to down-regulate CD206 expression on M2 macrophages, effectively inhibiting disease progression in mice [47]. Taken together, there is growing evidence that

modifying the macrophage population can effectively target tumors and improve cancer outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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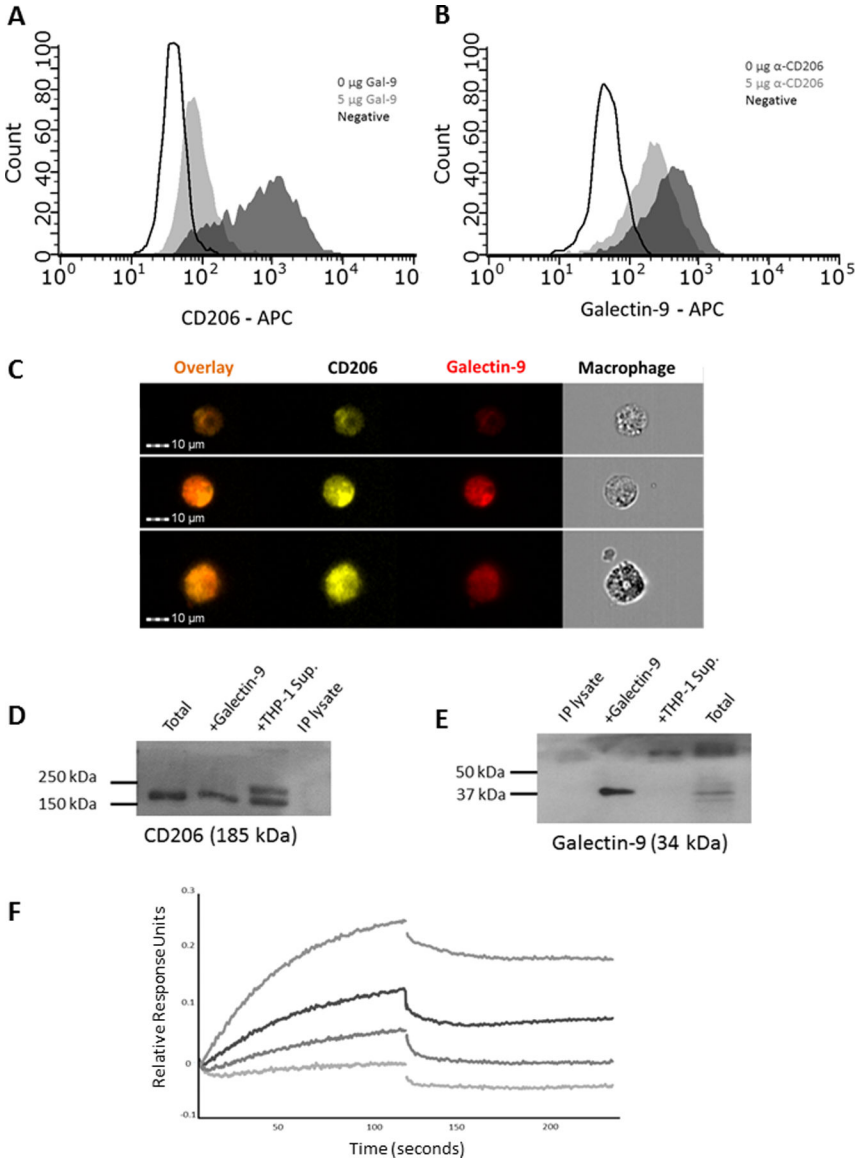

Figure 1. Galectin-9 binds to CD206 on M2 polarized macrophages.

(A) Pre-incubation with galectin-9 (light grey) decreases the ability of anti-CD206 to bind CD68+CD206+ macrophages. (B) Pre-incubation with anti-CD206 antibody (light grey) decreases the ability of galectin-9 protein to bind CD68+CD206+ macrophages. (C) Co-localization of CD206 and galectin-9 on human M2 macrophages. (D) Western blot of CD206 after galectin-9 immunoprecipitation using M2 polarized THP-1 cells. (E) Western blot of galectin-9 after CD206 immunoprecipitation using M2 polarized THP-1 cells. (F) Protein-protein binding by Blitz indicates a dissociation constant of 2.8×10^{-7} M. Data are representative of four different experiments.

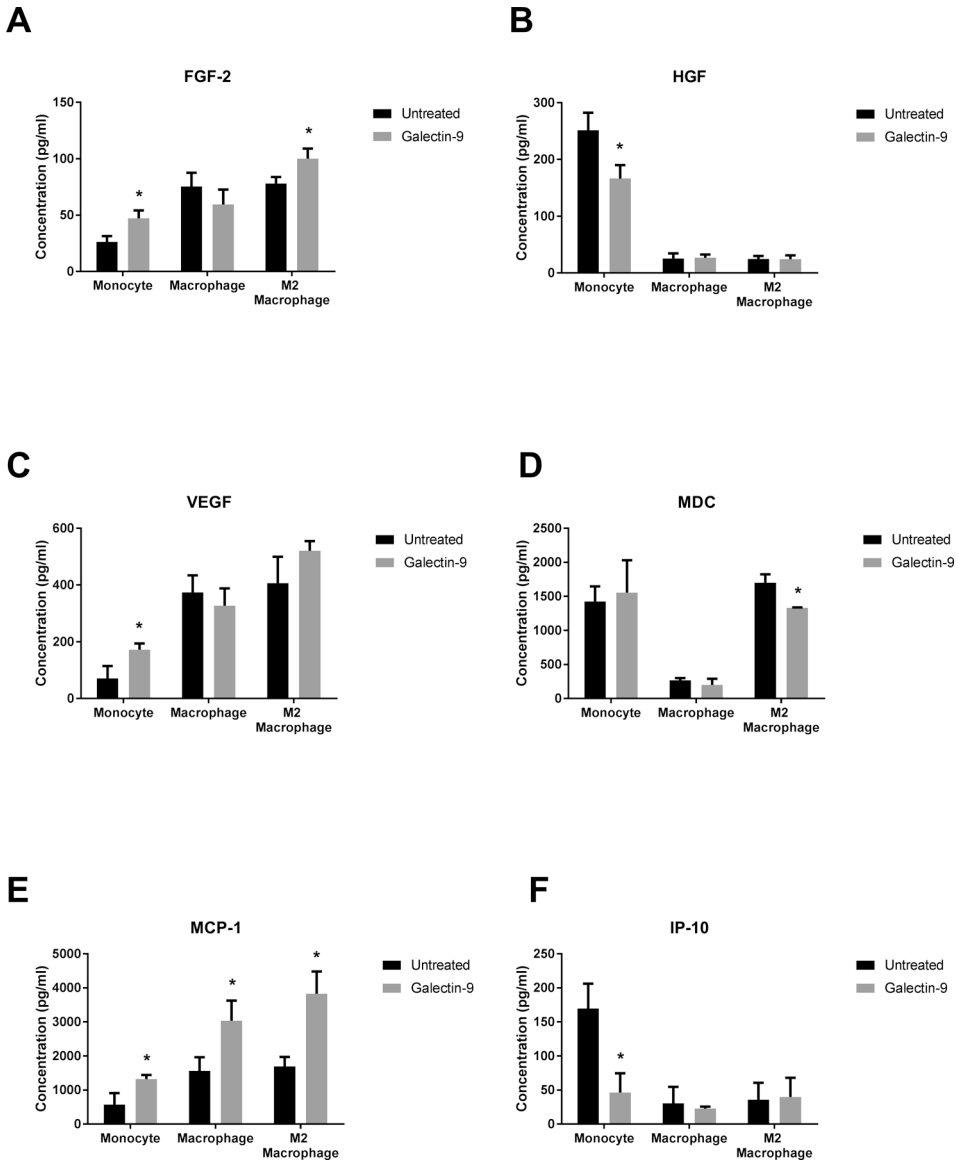

Figure 2. Galectin-9 increases tumor-promoting factors and decreases tumor-suppressive factors in human myeloid cells between stages of differentiation.

(A) Secretion of FGF-2 after incubation with galectin-9. (B) Concentration of HGF in the supernatant of cells treated with galectin-9. (C) VEGF concentration in culture media after galectin-9. (D) Secretion of MDC after galectin-9 incubation. (E) Culture media concentrations of MCP-1 after galectin-9 treatment. (F) Media concentration of IP-10 after galectin-9 treatment. Data presented as average ± SD; * indicates $p < 0.05$. Black: untreated, grey: 2 μg/ml galectin-9; $n = 6$.

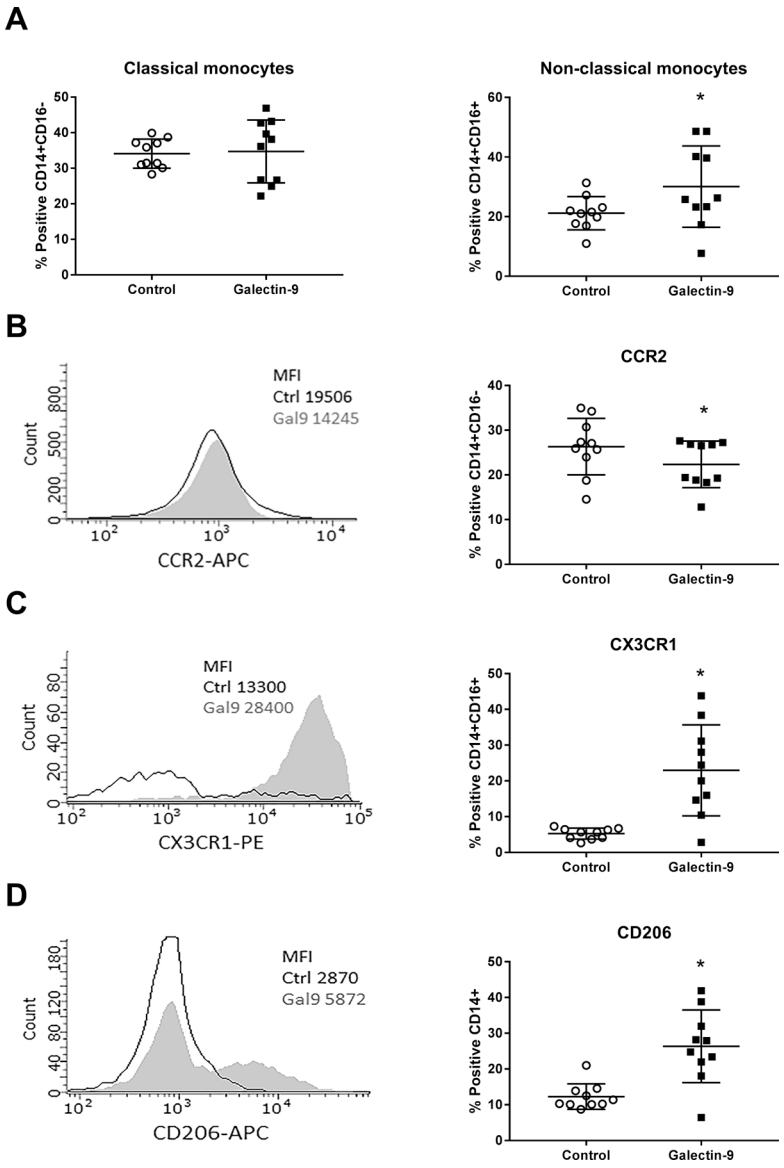

Figure 3. Non-classical monocytes expand in the presence of galectin-9.

(A) Changes in classical and non-classical monocyte subtypes after galectin-9 treatment. (B) CCR2 receptor is slightly downregulated on classical monocytes after galectin-9. (C) Galectin-9 significantly increased CD14+CD16+CX3CR1 populations. (D) CD206 is expressed at a higher level after monocytes are treated with galectin-9. Data presented as mean fluorescence intensities (MFI) or percentage of positive cells ± s.d. * indicates $p < 0.05$. White: untreated, grey: 2 μg/ml galectin-9; n=10.

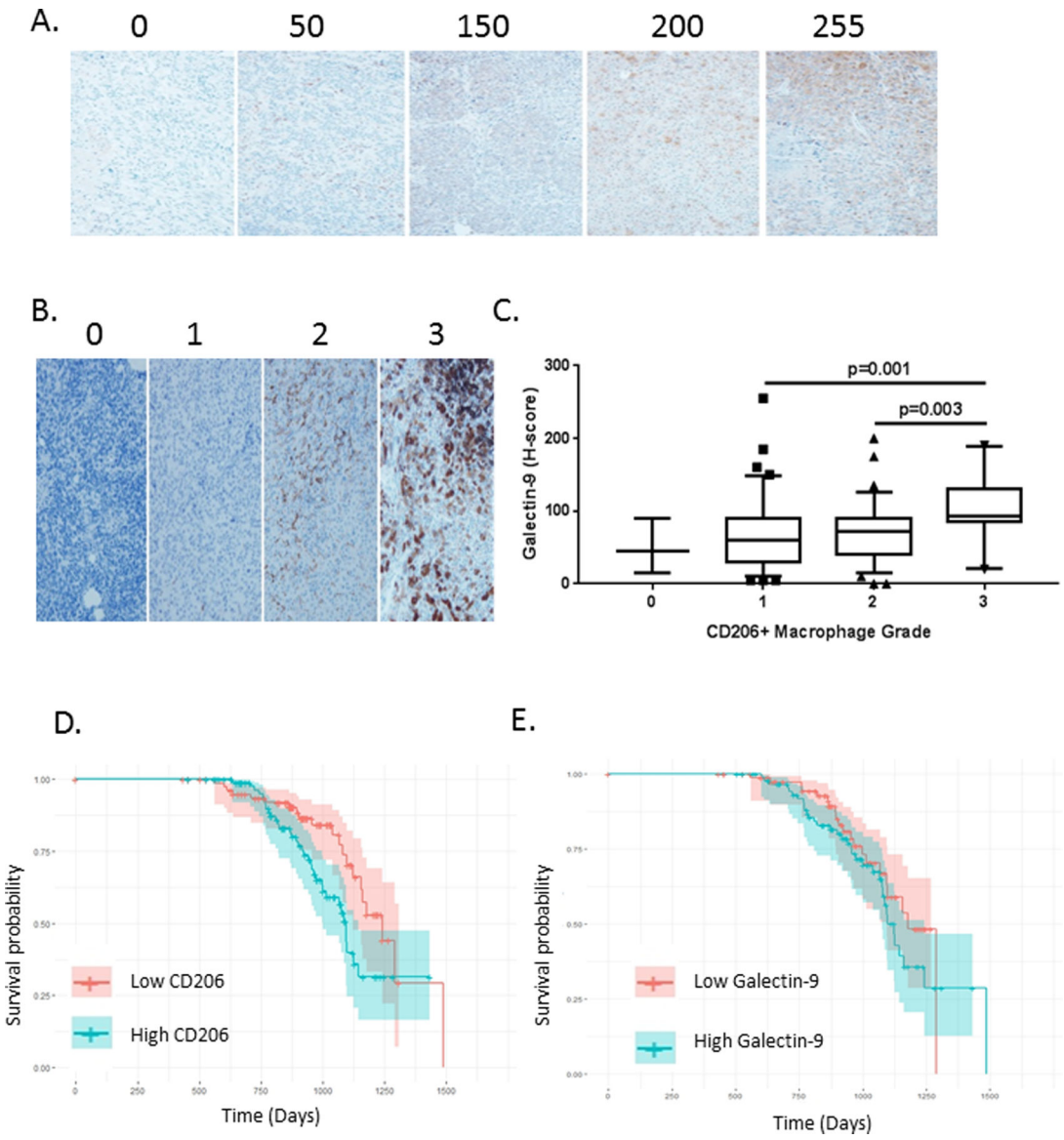

Figure 4: Positive correlation between tumor galectin-9 expression and CD206 macrophage infiltration in metastatic melanoma (*n*=180).

(A) Examples of galectin-9 tumor cell expression as assessed by H-score. (B) Representative groups with different levels of intratumoral CD206+ macrophage staining: 0 = negative (*n* = 3), 1 = minimal (n = 81), 2 = moderate (n = 76), 3 = dense (n = 20). (C) Tumors highly positive for galectin-9 are associated with highest staining of CD206. (D) Kaplan-Meier curve of high (groups 2–3) versus low (groups 0–1) CD206 expression with confidence intervals ($p = 0.019$). (E) Kaplan-Meier curve of high (≥65) versus low (<65) galectin-9 tumor expression with confidence intervals ($p = 0.3$).

Table 1.

Stage IV tumor microarray patient characteristics

Sex (count)	
Male	120
Female	68
Age (years)	
Mean (range)	57 (22–93)
Lactate dehydrogenase (LDH)	
Mean (range)	212 (103–2404 U/L)
Primary Site	
Head	22%
Lower Extremity	15%
Upper Extremity	11%
Trunk	30%
Other	5%
Unknown	17%

Table 2:

Tumor Microarray Statistics

Spearman's rho		Galectin-9 granular +	CD206+ macrophages	Galectin-9 H-score	CD68+ macrophages	Galectin-9 macrophages
Galectin-9 granular perinuclear positivity	Correlation coefficient	1.000	0.006	−0.020	−0.089	−0.143
	Sig. (2-tailed)		0.933	0.794	0.232	0.056
	N	180	180	180	180	180
CD206+ macrophages	Correlation coefficient	0.006	1.000	0.244**	0.434**	0.465**
	Sig. (2-tailed)	0.933	.	0.001	0.000	0.000
	N	180	180	180	180	180
Galectin-9 tumor cell H-score	Correlation coefficient	−0.020	***0.244***	1.000	0.324**	0.373**
	Sig. (2-tailed)	0.794	***0.001***	.	0.000	0.000
	N	180	180	180	180	180
CD68+ macrophages	Correlation coefficient	−0.089	***0.434***	***0.324***	1.000	0.705**
	Sig. (2-tailed)	0.232	***0.000***	***0.000***	.	0.000
	N	180	180	180	180	180
Galectin-9+ macrophages	Correlation coefficient	−0.143	***0.465***	***0.373***	***0.705***	1.000
	Sig. (2-tailed)	0.056	***0.000***	***0.000***	***0.000***	.
	N	180	180	180	180	180