Targeting the C-terminus of galectin-9 induces mesothelioma apoptosis and M2 macrophage depletion

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ABSTRACT

Galectin-9 has emerged as a promising biological target for cancer immunotherapy due to its role as a regulator of macrophage and T-cell differentiation. In addition, its expression in tumor cells modulates tumor cell adhesion, metastasis, and apoptosis. Malignant mesothelioma (MM) is an aggressive neoplasm of the mesothelial cells lining the pleural and peritoneal cavities, and in this study, we found that both human MM tissues and mouse MM cells express high levels of galectin-9. Using a novel monoclonal antibody (mAb) (Clone P4D2) that binds the C-terminal carbohydrate recognition domain (CRD) of galectin-9, we demonstrate unique agonistic properties resulting in MM cell apoptosis. Furthermore, the P4D2 mAb reduced tumor-associated macrophages differentiation toward a protumor phenotype. Importantly, these effects exerted by the P4D2 mAb were observed in both human and mouse in vitro experiments and not observed with another antigalectin-9 specific mAb (clone P1D9) that engages the N-terminus CRD of galectin-9. In syngeneic murine models of MM, P4D2 mAb treatment inhibited tumor growth and improved survival, with tumors from P4D2-treated mice exhibited reduced infiltration of tumor-associated M2 macrophages. This was consistent with an increased production of inducible nitric oxide synthase, which is a major enzyme-regulating macrophage inflammatory response to cancer. These data suggest that using an antigalectin 9 mAb with agonistic properties similar to those exerted by galectin-9 may provide a novel multitargeted strategy for the treatment of mesothelioma and possibly other galectin-9 expressing tumors.

Introduction

Galectin-9 is a tandem-repeat-type galectin with two carbohydrate recognition domain (CRD) with distinct physiologic activities, that has recently emerged as a novel candidate for cancer treatment. Both in vitro and in vivo studies showed that recombinant galectin-9 induces apoptosis of tumor cells, such as hematologic malignant cells, melanoma, and gastrointestinal tumors. Studies with immune cells suggest that galectin-9 could also modulate cells of the tumor microenvironment as T cells, B cells, and macrophages, although it is unclear if this modulation leads to an antitumor or protumor effect. MM is a lethal cancer linked to asbestos that is increasing in incidence worldwide. Macrophages were demonstrated to have a crucial role in MM carcinogenesis as well as for its development. Tumor-associated macrophages (TAMs) are abundantly present in the MM microenvironment and play an important role in inducing T-cell suppression. It has been demonstrated that pleural effusions from MM patients induce recruitment of monocytes and influence their differentiation into M2 macrophages. These macrophages promote the development and metastatic capacity of tumors due to the production of protumor factors like the enzyme arginase and a larger M2 component of the total macrophage count is inversely correlated with survival.

The role of galectin-9 in MM remains uncharacterized. In this study, we evaluated the expression of galectin-9 in murine and human MM cells and developed several antigalectin-9 targeted monoclonal antibodies with the goal of modulating the activity of galectin-9 and evaluating the effects on both cancer and immune cells. We provide evidence that immunotherapies utilizing a unique antigalectin 9 mAb exhibiting agonist activity to galectin-9 represents a promising new approach in cancer treatment.

Results

Human MM tumors express galectin-9

Galectin-9 is expressed in several human tumors and has been shown to modulate tumor progression, metastasis, and apoptosis as well as predict cancer patient survival. The expression of galectin-9 in MM remains unknown. Therefore, we performed
immunohistochemistry galectin-9 staining of 16 human MM biopsies and three normal human mesothelial lining samples. Staining analysis indicated that 14 out of 16 MM biopsies showed detectable levels of galectin-9 in the tumor biopsies, ranging from focally to diffusely positive. In contrast, galectin-9 expression was very low to undetectable in the normal mesothelial lining samples (Supplementary Table 1). Galectin-9 staining was localized in both nucleus and cytoplasm of cells (Figure 1).

**Novel antigalectin mAbs bind to both human and mouse galectin-9**

To further evaluate the significance of galectin-9 in MM, we generated a series of antigalectin-9 mAb clones, and evaluated their binding to human and mouse galectin-9. We identified 8 mAbs that bound to human galectin-9, with only P4D2 and P1D9 clones binding to both human and murine galectin-9 (Figure 2a). We evaluated the binding of these two mAb clones to two versions of human galectin-9, with (hGalectin-9M) or without (hG9NC) the linker peptide. Both mAbs showed binding to both versions of galectin-9 (Figure 2b).

**Differential binding of antigalectin mAbs to the C-terminal (P4D2) and N-terminal (P1D9) CRD of galectin-9**

Galectin-9 contains two CRDs in the N- and C-terminal regions (N-CRD and C-CRD, respectively) that display different activities, with the former involved in the regulation of innate immune cells and the latter more effective in inducing T-cell apoptosis. To identify the CRD recognized by each mAb P4D2 and P1D9, we generated two fusion proteins, hG9G8 and hG8G9, which has one of the CRD from galectin-9 substituted with the CRD from galectin-8. The fusion protein hG9G8 comprises the N-terminal CRD from human galectin-9, but has the C-terminal CRD of galectin-8, while hG8G9 includes the N-terminal from human galectin-8 and the C-terminal from galectin-9. ELISA plates were coated with these two fusion proteins, and binding of either of the two (P4D2 or P1D9) mAbs evaluated. In these experiments, P4D2 showed strong binding with hG8G9, containing the C-terminal region of galectin-9. Binding of the P4D2 clone to hG9G8 was significantly reduced compared with binding to hG8G9. In contrast, the P1D9 mAb showed stronger binding with hG9G8 when compared with hG8G9 (Figure 2c). To further characterize the interaction between the mAbs and galectin-9, we sequenced the variable domain (Fv) of the P4D2 clone and used this information to design a digital model of this mAb. We then performed simulation modeling between the digital prototype of P4D2 clone and the crystal-structure of galectin-9 using SAbPred. This analysis confirmed binding of P4D2 mAb to the C-terminal of galectin-9. The galectin-9 amino acids involved in the interaction with the Fv of P4D2 mAb that were identified with SAbPred are listed (Figure 2d).

**P4D2 has agonistic properties and induces apoptosis of MM cells**

It has been demonstrated that endogenous galectin-9, by binding carbohydrates on the cell membrane, forms two- or three-dimensional lattice structures that perform important functions including organizing cell membrane domains, determining thresholds of cell signaling and regulating receptor turnover on the cell surface. Treatment with recombinant galectin-9 has been demonstrated to interact with these lattice structures and promote apoptosis in different types of tumor cells. In our

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**Figure 1.** Profiling of galectin-9 tissue expression in MM tumors. (a–c) Galectin-9 staining on three representative MM samples; (d) Gal9 staining on a representative normal mesothelial lining. Original magnification 200×.
preliminary experiments, we observed that both human and mouse MM cells express surface galectin-9 (Supplementary Figure 1a–c) and therefore investigated if P4D2 and P1D9 mAbs could mimic the apoptotic effect induced by recombinant galectin-9. We assayed two human MM cell lines (Mill and ROB) and two mouse MM cell lines (CRH5 and EOH6). In Mill cells, we observed reduced viability during P4D2 treatment compared with untreated controls at day 2, 3, 4, and 5. hG9NC treatment also reduced cell viability of Mill cells from day 2 until the end of the assay (day 5) (Figure 3a). Both P4D2 and hG9NC reduced viability significantly of ROB cells, from day 2 until the end of the assay (day 5). In CRH5 cells, P4D2 significantly reduced cell viability for the entire duration of the culture from day 1 to day 5. Mouse galectin-9 (mG9NC) also decreased cell viability but only at day 5. In EOH6 cells, both P4D2 and mG9NC reduced viability at day 3 through the end of the assay. We also evaluated MM cell viability following combined treatment using recombinant galectin-9 and P4D2. In these assays, we observed decreased cell viability using the two agents in combination compared with the single treatments. Importantly, we did not observe reduced MM cell viability following treatment with other lectins such as galectin 3 or erythrina cristagalli (Supplementary Figure 2a,b).

We next investigated the degree of apoptosis induced by the antigalectin-9 mAb treatment of MM cells using a combination of Propidium Iodide (PI) + Annexin V stain. In Mill cells, hG9NC and P4D2 induced higher percentages of PI<sup>+</sup>Annexin V<sup>+</sup> cells representing late apoptotic cells, compared with untreated MM cells. Also, an increase in early apoptotic cells (PI<sup>−</sup>/Annexin V<sup>+</sup>) was detected with P4D2 (Figure 3b). In ROB cells, we observed an increase in both early and late apoptotic cells for P4D2, while hG9NC treatment induced only an increase in late apoptotic cells. In CRH5 cells, both P4D2 and mG9NC induced higher percentages of late apoptotic cells compared with untreated controls. In these cells, a significant increase in the numbers of early apoptotic cells was assessed also for both treatments (P4D2 and mG9NC) when compared with controls. In EOH6 cells, P4D2 was the only treatment that induced higher percentages of both early and late apoptotic cells when compared with untreated controls.

**P4D2 mAb modulates human monocyte differentiation with reduced formation of CCR5<sup>+</sup> tumor macrophages**

Next, we investigated whether the antigalectin-9 mAbs would alter monocyte differentiation or reduce formation of protumor...
myeloid cells. Human blood-derived monocytes (hBDM), which express galetin-9 (Supplementary Figure 3) were differentiated with human AB serum in the presence of either P4D2 or P1D9 and compared to those differentiated in the presence of hG9NC. After 1 week of culture, cell surface levels of CD68 and CCR5 were assessed. CD68 is a marker highly expressed by cells of the monocyte lineage, including circulating and tissue macrophages, while CCR5 increases during monocyte-macrophage differentiation and is responsible for the recruitment of TAMs in the tumor microenvironment. We observed a significant reduction in CD68+CCR5+ mature macrophages after treatment with either P4D2 or hG9NC, compared with control cells differentiated in AB serum without any treatment. Interestingly, the number of live cells significantly increased with P4D2, suggesting that this mAb acts by blocking monocyte differentiation rather than killing mature macrophages (Supplementary Figure 4). In contrast, P1D9 displayed the same percentage of CD68+CCR5+ cells compared to controls (Figure 4a,b). We also induced monocyte-macrophage differentiation using supernatants from either untreated human ROB MM cells or treated with P4D2. In these assays, the number of CD68+ and CCR5+ cells was sharply reduced when the supernatant from P4D2-treated ROB MM cells was used, compared to controls cultured with supernatant of untreated ROB MM cells (Figure 4d,e).

Real-time PCR was used to assess mRNA levels for CD68 and the macrophage receptor with collagenous structure (MARCO) in cells differentiated with supernatants from human ROB MM cells untreated or treated with P4D2 (Figure 4c). In these experiments, the number of CD68+ and CCR5+ cells was sharply reduced when the supernatant from P4D2-treated ROB MM cells was used, compared to controls cultured with supernatant of untreated ROB MM cells (Figure 4d,e).

Figure 3. P4D2 mAb exerts agonist effects in inducing apoptosis in MM cells. (a) Human MM cells (ROB and Mill) were treated with P4D2 or P1D9 mAbs, and viability assessed with an MTT assay. Controls included the human stable recombinant galectin-9 (hG9NC) and no treatment (Ctrl). Differences between Ctrl and P4D2 as well as Ctrl and hG9NC were statistically significant with P ≤ 0.01; n = 3 (*). A viability assay was used to evaluate the effects of P4D2 and P1D9 mAbs on mouse MM cells (CRH5 and EOH6). Mouse stable recombinant galectin-9 (mG9NC) was included for comparison. Differences between Ctrl and P4D2 as well as Ctrl and mG9NC were statistical significant with P ≤ 0.01; n = 3 (*). (b) Analysis of apoptosis for human (ROB and Mill) and mouse (CRH5 and EOH6) MM cells after P4D2 or recombinant galectin-9 (hG9NC or mG9NC) treatment as evaluated by flow cytometry. Percentages of PI-Annexin V+ (early apoptotic) and PI+Annexin V+ (late apoptotic) cells are shown. Statistically significant differences between treatment and no treatment (Ctrl) were assessed with two-way ANOVA followed by the Bonferroni test and indicated with *, P ≤ 0.01, n = 3.
**P4D2 shifts mouse monocyte differentiation toward an M1 phenotype**

We demonstrated above that P4D2 hinders human monocyte maturation to macrophages with tumor-promoting characteristics, and next investigated mouse bone marrow-derived monocytes (mBMMs). In these assays, we used four different conditions to induce mBMM differentiation to macrophages: (1) M-CSF; (2) M-CSF plus P4D2; (3) supernatants from mouse MM CRH5 cells; and (4) supernatants from CRH5 cells treated with the P4D2. Flow cytometry was used to measure F480⁺ cells as a marker that identifies mouse macrophages. Results showed a significant increase of F480⁺ macrophages in cultures using the supernatant from P4D2-treated MM cells when compared with cells cultured with the supernatant from untreated MM cells. No differences were observed between cells differentiated with M-CSF with or without P4D2 (Figure 5a,b). We further characterized F480⁺ cells using markers for M1 antitumor (CD38⁺) and M2 protumor (Egr2⁺) macrophages. With both M-CSF and CRH5 media used as maturation stimuli, we observed a significant increase in F480⁺CD38⁺ macrophages when P4D2 was employed. Regarding F480⁺Egr2⁺ macrophages, we observed a complete lack of differentiation into these cells in the presence of P4D2, either when mBMMs were differentiated with M-CSF or CRH5 media (Figure 5c,d). When we calculated M1 (F480⁺CD38⁺) and M2 (F480⁺Egr2⁺) ratios, we observed an increase toward the M1 phenotype for cells treated with P4D2 mAb and M-CSF compared with cells treated with M-CSF, and for mBMMs cultured with the supernatant from P4D2-treated MM cells compared with those cultured with supernatant from untreated CRH5 MM cells (Figure 5e). For all of these conditions, we did not detect differences in the number of live cells (Figure 5f). We also performed experiments to evaluate the effects of P4D2 during mBMM differentiation induced with higher dosages of M-CSF, to mimic the tumor microenvironment in which higher concentrations of this cytokine were found compared with normal tissues. As observed for lower doses of M-CSF, P4D2 strongly reduced the percentages of F480⁺ cells, but the number of live cells was also dramatically reduced (Supplementary Figure 9). Surprisingly, mG9NC also decreased the number of F480⁺ cells, but viability was not altered, even at high concentrations of M-CSF. In vivo P4D2 treatment hinders tumor growth and improves survival in MM animal models

The in vitro findings with murine MM cells and macrophage differentiation prompted us to assess the potential antitumor effect of P4D2 in animal models of MM. BALB/c mice were inoculated subcutaneous (s.c.) with either CRH5 or EOH6 MM cells. When tumors reached 3–4 mm in diameter, mice were i.p. injected with 400 μg of P4D2 mAb followed by another injection of the same dose 7 d later. Control mice were left untreated or injected with P1D9. Treatment with P4D2 resulted in reduced tumor growth compared to controls for both MM cells (Figures 6a and Supplementary Figure 10). Survival analyses revealed that P4D2 injected mice, carrying CRH5 tumors, also exhibited prolonged median survival compared with untreated controls and P1D9-treated mice (Figure 6b). Since MM develops from mesothelial cells lining internal body cavities, we developed a clinically relevant peritoneal MM model for testing the therapeutic efficacy of P4D2. In these experiments, we injected CRH5 MM cells into the peritoneum of two groups of BALB/c mice. Seven days later, when
Figure 5. P4D2 mAb shifts mouse monocyte differentiation toward an M1 phenotype. Mouse bone marrow-derived monocytes were differentiated to mature macrophages using either M-CSF or supernatant from mouse MM CRH5 cells. During M-CSF-driven differentiation, cells were also treated with P4D2 mAb (M-CSF + P4D2) while controls only received m-CSF. Differentiation with MM supernatant was instead performed using media from CRH5 mouse MM cells untreated (conditioned media) or treated for 24 h with P4D2 mAb (conditioned media +P4D2). (a and b) The left panels contain representative flow cytometry images showing the effects of P4D2 mAb on F480+ cells. On the right, percentages of F480+ macrophages are shown for the different treatments. Differences between CRH5 media compared to media from P4D2 mAb-treated CRH5 were evaluated using one-tailed paired Student’s t test (n = 3/group) and indicated with *P ≤ 0.001. (c and d) Phenotype of the differentiated macrophages was also investigated using markers for M1 (CD38hi) and 2 (Egr2+). On the top left, representative flow cytometry images show the effects of P4D2 mAb on F480+ CD38hi M1. On the top right, percentages of F480+ CD38hi cells are indicated for the different treatments. On the bottom left, representative flow cytometry images show the effects of P4D2 mAb on F480+ Egr2+ M2. On the bottom right, percentages of F480+ CD38hi cells are displayed for the different treatments. (e) M1/M2 ratios were calculated and indicated for the different conditions. (f) Percentages of live cells were showed for the different treatments. For (b–d) statistical significance for experiments was evaluated using one-tailed paired Student’s t test (n = 3/group) and indicated with *P ≤ 0.01.

Figure 6. Treatment with P4D2 mAb hinders tumor growth and improves survival in MM animal models. BALB/c mice with subcutaneous CRH5 MM tumors were treated with P4D2 or P1D9 mAbs. Control mice were left untreated. (a) Tumor size is shown for the different treatments. Differences between control and P4D2 mAb groups were compared with two-way ANOVA followed by the Bonferroni multiple comparison test (n = 5/group) and indicated with *P ≤ 0.01. (b) Mice treated with P4D2 mAb (n = 5/group) show increased survival compared to controls *P ≤ 0.01. (c) Survival curves for mice carrying intraperitoneal CRH5 MM tumors, treated or not-treated with P4D2 mAb. Differences in P4D2 mAb treated vs. controls were evaluated using Kaplan-Meier curves with log-rank test and indicated with *P ≤ 0.01 and n = 5.
tumors started growing and spreading within the peritoneal cavity, mice were treated with P4D2 or left untreated. Survival analyses showed a significant increase in median survival of P4D2-treated mice compared to controls (Figure 6c). In all animals treated with P4D2 no adverse events were observed such as acute effects, distress, or weight loss, and gross tissue examination failed to indicate any toxicity in the organs (kidney, brain, spleen, liver, and lungs).

**Treatment with P4D2 induces mesothelioma apoptosis in vivo and alters intratumor macrophages M1/M2 ratio with increased production of iNOS**

We next characterized tumors from mice treated with P4D2 and untreated. MM cells were identified using mesothelin mAbs and the frequency of apoptosis revealed using annexin V staining methods. These flow cytometry assays confirmed that P4D2 induce MM apoptosis also in animal models (Supplementary Figure 11). When we analyzed TAMs using F480 antibodies in immunofluorescence we observed a reduced number of these immune cells in tumors from mice treated with P4D2 compared with controls (Figure 7a). Flow cytometry results confirmed these data showing lower percentages of F480+ macrophages in tumors from P4D2-treated mice compared with controls (Figure 7b). Analysis of TAMs for markers for M1 (CD38hi) and M2 (Egr2) macrophages showed that mice injected with P4D2 had reduced percentages of F480+ CD38hi M1 cells and F480+Egr2+ M2 macrophages compared with controls (Figure 7c). Analysis of iNOS and arginase-1 mRNA produced by M1 and M2 macrophages, respectively, showed higher production of iNOS mRNA for P4D2-treated mice compared to controls, but no differences in arginase-1 mRNA levels. To evaluate if P4D2 skews macrophage differentiation toward the M1 phenotype in vivo, we calculated ratios between M1 and M2 macrophages using data from flow cytometry and real-time PCR. In both cases, M1:M2 ratios were significantly higher for P4D2-treated mice indicating a prevalence of M1 iNOS-secreting TAMs in these animals (Figure 7d,e).

Importantly, T-cell frequency, proliferation, and granzyme B secretion were analyzed in tumors from either P4D2-treated or untreated mice. No differences were recorded for any of these parameters. In addition, markers for T regulatory cells (CD25 and FoxP3) were analyzed and no differences were detected between the two groups (Supplementary Figure 12). Similarly, in our analysis of other immune cell types that included dendritic cells, neutrophils, or monocytic myeloid-derived suppressor cells (MDSC), no differences were observed between P4D2-treated and untreated mice (Supplementary Figure 13).

**Discussion**

MM is a devastating cancer related to asbestos exposure. Clinical symptoms arise late in the course of the disease, 30–40 years after
asbestos exposure, when the efficacy of therapeutic interventions is limited. In this study, we found that human MM tumors express high levels of galectin-9, a lectin that recently emerged as a promising target for cancer immunotherapy due to its dual role in cancer cell apoptosis\textsuperscript{5-11} and TAMs differentiation.\textsuperscript{14,38}

We generated and tested a novel P4D2 mAb that binds galectin-9 C-terminal CRD and shows unique agonistic properties with recombinant galectin-9 in inducing cancer cell apoptosis and in modulating macrophage polarization. In contrast, the P1D9 mAb that binds galectin-9 N-terminal CRD exhibited negligible biological activity.

The agonistic properties of P4D2 are supported by data showing that MM cells treated with either P4D2 mAb or with recombinant galectin-9 undergo apoptosis and show reduced viability. It has been already demonstrated in different types of tumors that recombinant galectin-9, through its interaction with the endogenous galectin-9, induces cancer cell apoptosis. Here, we show for the first time that a unique antigalactin-9 mAb can be used as a substitute to recombinant galectin-9 or even used in combination to further decrease viability of cancer cells.

Similarities between P4D2 and recombinant galectin-9 were not limited to the induction of apoptosis as both treatments also modulated monocyte-macrophage differentiation in a similar way by depleting human macrophages expressing CCR5 a receptor crucial for the migration of M2 TAMs in the tumor microenvironment.\textsuperscript{32,35} In contrast, when we evaluated CD71 expression in differentiated macrophages, we observed a significant increase only following P4D2 treatment. Significant differences between P4D2 and recombinant galectin-9 were also observed for other well-established macrophage markers including CD11b, CD11c, CD14, and CD206. These data, together with those obtained in mouse macrophages differentiated with higher concentrations of M-CSF, indicate that P4D2 and recombinant galectin-9 have very similar but not identical mechanisms of action.

The capacity of P4D2 to modulate macrophage differentiation was also evaluated by culturing hBDMs with supernatants from MM cells treated or untreated with the antigalactin-9 mAb. In these experiments, we hypothesized that P4D2 would interfere with the cytokines secreted by the tumor cells and indirectly reduce CCR5+ macrophage formation. The results supported this hypothesis, with the supernatant from P4D2-treated MM cells showing a lower number of CCR5+ macrophages compared with the supernatant from untreated MM cells. To definitively demonstrate that P4D2 does not have a direct effect on monocytes differentiation, these experiments should be repeated using MM cell supernatants in which the antigalactin-9 antibodies have been removed. However, even if it is unclear that P4D2 has an indirect effect on monocyte differentiation, this antibody clearly inhibits the formation of protumor macrophages as demonstrated by the reduction in MARCO mRNA levels. MARCO expression defines a subtype of TAMs with an M2-like immunosuppressive gene signature. Targeting these TAMs with MARCO mAbs have been shown to induce antitumor activity in carcinoma and melanoma tumor models,\textsuperscript{33} suggesting that treatment with P4D2 may achieve similar results. In our experiments, macrophages differentiated with P4D2 also showed increased motility and IL-8 secretion when compared with hBDMs differentiated with only AB serum. This is another clue that P4D2 decreases the differentiation of hBDMs to protumor macrophages, which are known to have reduced motility and lower production of IL-8.\textsuperscript{39,40}

We further confirmed in animal models of MM our findings that P4D2 induces two different beneficial effects: tumor cell apoptosis and polarization of tumor macrophages toward M1-like myeloid cells. In these animal experiments, P4D2 treatment induced MM cell apoptosis, reduced tumor growth, and improved survival, with tumors from P4D2-treated mice exhibiting reduced infiltration of TAMs. Further characterization of tumors from P4D2-treated mice showed complete depletion of M2-like (F480\textsuperscript{+}Egr2\textsuperscript{+}) cells, as observed in experiments of monocyte-macrophage differentiation in vitro. Polarization toward an M1 phenotype was also confirmed by an increased production of the antitumor enzyme, iNOS.

Our data collectively suggest that targeting galectin-9 using antibodies against its C-terminal CRD could be an attractive strategy for cancer immunotherapy. However, there are still important limitations to consider in targeting galectin-9 in MM as animals do eventually progress based on our survival experiments. Indeed, the complex network of immunosuppressive pathways present in tumors is unlikely to be overcome by intervention with a single immune-modulatory agent.\textsuperscript{41} Since our data suggest that P4D2 acts on macrophage polarization but does not interfere with T-cell activity, a synergistic increase in the antitumor efficacy of this treatment is likely to be achieved by T-cell checkpoint blockade using PD-1 or PDL-1 mAbs. Recent findings revealed that macrophages are essential when targeting the PD1-PDL1 axis. In one study, it was shown that macrophages can remove anti-PD1 antibodies from T cells, blunting their response. A second study demonstrated that macrophages also express PD1 on their surface, which impairs their phagocytic activity.\textsuperscript{42,43} Moreover, another recent report suggested that durable regression of established tumors requires concurrent immunotherapy with four distinct agents, which target complementary aspects of innate and adaptive immunity.\textsuperscript{14,41}

Thus, the development of novel therapies that include anti-galectin 9 P4D2 mAb to modulate TAMs will provide a new path toward development of more effective immunotherapeutic options for the treatment of human cancers.

**Materials and methods**

**Immunohistochemistry (IHC)**

IHC was performed on paraffin-embedded tissue sections from human MM tumor biopsies and normal human peritoneal mesothelium (a kind gift from Dr. Harvey Pass, New York University, NY). Assessment of tumor content was based on hematoxylin-eosin staining, combined with immunohistochemical features (WilmTumor-1, Calretinin, Cytokeratin5/6 stains). Expert pathologists in pleural pathology, independently evaluated the biopsies (Dr. Pass and M.C.). Immunohistochemistry for galectin-9 (LS-B6275 mAb, LS Bio) was performed as previously described.\textsuperscript{44} Presence of galectin-9 positive cells was evaluated on 10 fields/slide at 200× magnification with a BX43 microscope (Olympus, PA, ISA).
**Mice**

Female 6–8 weeks old BALB/c mice, were obtained from the Jackson Laboratory. Galectin-9 KO BALB/c mice were provided by GalPharma, Co. (Takamatsu, Kagawa, Japan). Animal experiments were performed in accordance with institutional guidelines and approved by the University of Hawaii IACUC (#16-2355).

**Recombinant proteins**

Recombinant human galectin-8, stable galectin-9 (hG9NC), and mouse stable galectin-9 (mG9NC) were obtained from GalPharma. The recombinant hG9NC consists of an N- and C-terminal CRDs linked by His-Met residues, where N-CRD and C-CRD correspond to the 1st–148th and 178th–323rd amino acids respectively of the human galectin-9 sequence (GenPept-BAB83624.1). mG9NC is composed of N-CRD and C-CRD linked by Gly-Ser residues where N-CRD and C-CRD correspond to 1st–147th and 177th–322nd amino acids, respectively of the mouse galectin-9 sequence (GenPept-AAH03754.1). hG8G9 and hG9G8 are artificial structures generated by replacing the N-CRD and C-CRD of galectin-8 and -9. Briefly, the open reading frames of hG9NC and hG8NC cloned in pET11a vector were digested by NdeI to cut off DNA fragments coding for N-CRDs of human galectin-8 and human galectin-9, respectively, then ligated with the rest of the DNA fragments in alternate combinations. All of these molecules were purified from endotoxin using Cellulose ETclean (Chisso). Wild-type galectin-9 was purchased from R&D Systems (Minneapolis, MN, USA).

**Production, purification, and titration of antigalectin-9 mAbs**

Galectin-9 knock out (KO) female BALB/c mice were immunized with hG9NC or mG9NC. ELISA was performed on sera to determine hG9NC and mG9NC reactivity with preimmunization sera used as controls. The mouse responding best to galectin-9 was boosted for 5 d, then sacrificed and its splenocytes fused with P3X63Ag8.653 cells at a ratio of 5:1 as previously described. Hybridoma supernatants were assessed via ELISA. Mouse and human MM cell viability was assessed by MTT assay. Briefly, 2000 cells were plated in each well of 96 well plates in Ham’s F12 culture medium. After 24 h, cells were treated in reduced FBS (2%) with 20 µg/ml of either P4D2 or P1D9 mAb. In experiments with mouse cells, mG9NC was also used at 1 µg/ml. In experiments with human cells, hG9NC was used at 2 µg/ml. Controls were left untreated. In these assays, the lowest mAb that induced significant effects in our titration experiments were used (Supplementary Figure 14). Viability was evaluated in triplicate for each condition every 24 h. Fold increases of viability were calculated by dividing the value of each day with the viability measured at day 1, before mAb treatment. Apoptosis vs. necrosis was evaluated in MM cells using flow cytometric analysis of 106 MM cells cultured as above described. These cells were collected after 48 h using Cell Stripper (Corning) and stained with V500 conjugated MG2a-53 antibodies conjugated with FITC or PE using the lighting link labeling kit (Innova Biosciences). Percentages of early (PI+/Annexin V−) and late (PI+/Annexin V+) apoptotic cells were assessed using LSRFortessa (BD Biosciences) Flow Cytometer and FlowJo software.

**Cells**

Murine AB12 cells derived from asbestos-induced tumors in a BALB/c mouse were kindly provided by Dr. B. Robinson (University of Western Australia, Nedlands, Australia). Human REN cells were kindly provided by Dr. A. Albelda (University of Pennsylvania, PA, USA). Human Mill and ROB cells were characterized and provided by Dr. H. Pass. Human Hmeso cells were from ATCC. Murine CRH5, EOH6, and EOH9 cells were isolated from peritoneal asites developed in asbestos- or erionite-injected mice in carcinogenesis experiments previously described. All cells were cultured in Ham’s F12 medium (Corning) containing 10% FBS and antibiotics. All the MM cells used in this study were provided to our laboratories or purchased in years between 2004 and 2007.

**Flow cytometry analysis of antigalectin-9 mAb binding to MM cells or monocytes**

Mouse cells or monocytes were washed and stained for 1 h at 4°C with either P4D2 mAb or IgG2 isotype control clone MG2a-53 antibodies conjugated with FITC or PE using the lighting link labeling kit (Innova Biosciences). Cells were then analyzed using LSRFortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (BD).

**Evaluation of MM cell viability and apoptosis**

Mouse and human MM cell viability was assessed by MTT assay. Briefly, 2000 cells were plated in each well of 96 well plates in Ham’s F12 culture medium. After 24 h, cells were treated in reduced FBS (2%) with 20 µg/ml of either P4D2 or P1D9 mAb. In experiments with mouse cells, mG9NC was also used at 1 µg/ml. In experiments with human cells, hG9NC was used at 2 µg/ml. Controls were left untreated. In these assays, the lowest mAb that induced significant effects in our titration experiments were used (Supplementary Figure 14). Viability was evaluated in triplicate for each condition every 24 h. Fold increases of viability were calculated by dividing the value of each day with the viability measured at day 1, before mAb treatment. Apoptosis vs. necrosis was evaluated in MM cells using flow cytometric analysis of 106 MM cells cultured as above described. These cells were collected after 48 h using Cell Stripper (Corning) and stained with V500 conjugated Annexin V (BD Biosciences) and Propidium Iodide cell viability dye (Biolegend). Percentages of early (PI+/Annexin V−) and late (PI+/Annexin V+) apoptotic cells were assessed using LSRFortessa (BD Biosciences) Flow Cytometer and FlowJo software.

**Monocyte-to-macrophage differentiation**

The effects of P4D2 and P1D9 mAbs as well as those of recombinant galectin-9 on monocyte-to-macrophage differentiation were evaluated on both mouse bone marrow-derived monocytes (mBMM) and human blood-derived monocytes (hBDM). For mBMM, marrow was flushed from femurs and tibiae with HBSS, using a syringe with a 25-gauge needle, and cell suspensions were then passed through a 40 µm pore cell strainer to remove tissue debris. mBMMs were plated in DMEM (Thermo Fisher), containing 10% FBS and antibiotics. Differentiation of mBMMs was induced with either 20 g/ml or 40 ng/ml M-CSF (BioLegend). In these experiments, cells were also incubated for 7 d with either 20 µg/ml P4D2, 20 µg/ml P1D9, or 1 µg/ml mG9NC. Control cells were untreated and media replaced at day 4. In other experiments, differentiation of mBMMs were induced using 50% of media from CRH5 cells
treated with 20 µg/ml P4D2 mAb for 48 h. Media from untreated CRH5 cells was used as control. After 7 d, differentiated mBMMs were incubated with Fc-block (BD Biosciences) for 15 min on ice, followed by incubation with anti-F480-FITC clone BM8 and anti-CD38-PE clone 90 (BioLegend) for M1 macrophage staining. A Fixation/Permeabilization kit was used in combination with anti-Egr-2-APC clone erongr2 (Thermo Fisher) to stain M2 macrophages. Live cells were distinguished from debris using ZOMBIE® violet cell viability dye (Biolegend). Cells were analyzed on an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (BD). Blood for hBDM was obtained from healthy volunteers under an approved protocol (CHS#19442). Monocytes were isolated using Histopaque-1077 (Sigma-Aldrich) and cultured in X-Vivo-10 media (BioWhittaker) containing 5% AB serum (Sigma-Aldrich) with either P4D2 or P1D9 mAbs (20 µg/ml), hG9NC (2 µg/ml), or no antibody for untreated control cells. Cells were incubated with fresh media on day 3 of culture, and fully differentiated human macrophages were analyzed by flow cytometry on day 6 of culture. In other experiments, differentiation of hBDMs were induced using 50% of media from ROB cells treated with 20 µg/ml P4D2 mAb for 48 h. Media from untreated ROB cells was used as control. Anti-CD71 clone CY1G4, anti-CCR5 clone J418F1, anti-CCR2 clone K036C2, anti-CD68 clone FA-11, anti-CD11b clone ICRF44, anti-CD 11c clone 3.9, anti-CD206 clone 15-2, and anti-CD14 clone M5E2 antibodies (all from Biolegend) were used to characterize mature human macrophages. Surface/intracellular staining and flow cytometer analysis were performed as above described. Migration of hBDMs differentiated with AB serum with or without P4D2 was measured using 8 µm Transwell inserts (Greiner). In these assays, 3,000 cells were seeded in serum-free X-Vivo-10 media in the upper chamber, while media with 5% AB serum was used in the lower chamber. Following 24 h incubation at 37°C, the inserts were removed and the non-migrating cells wiped away with a cotton swab, while cells attached to the under-surface were methanol-fixed and stained. Migrated cells were counted from four randomly selected fields using a ×20 objective. Cytokine secretion of hBDMs differentiated with AB serum with or without P4D2 was assessed by cytometric bead array using the human inflammatory cytokine kit (BD).

**Murine therapeutic experiments**

To evaluate tumor dimensions and survival, s.c. mouse models of MM were employed. In these experiments, 105 CRH5 or EOH6 cells were injected in the hind flank in cohorts of five BALB/c mice. When tumors became palpable on day 7 (3–4 mm in maximal diameter), mice received an i.p. injection of 400 µg of P4D2. A second dose of mAb was given 7 d later and tumors excised after another 7 d. Tissues were washed with PBS, minced and incubated for 1 h at 37°C in digestion buffer consisting of 1 mg/ml collagenase IV, 100 µg/ml hyalurodinase, and 15 mg/ml DNase I (all from Roche Applied Sciences) in PBS. After digestion, tumors were forced through a 40 µm cell strainer. A total of 106 cells were stained for flow cytometer analysis with macrophages were characterized as described above.

Isolation and analysis of mesothelioma and tumor-infiltrating immune cells from mice

CRH5 cells (10<sup>5</sup>) were injected s.c. in cohorts of 5 BALB/c mice. When tumors reached 50 mm in maximal diameter, mice received an i.p. injection of 400 µg of P4D2. A second dose of mAb was given 7 d later and tumors excised after another 7 d. Tissues were washed with PBS, minced and incubated for 1 h at 37°C in digestion buffer consisting of 1 mg/ml collagenase IV, 100 µg/ml hyalurodinase, and 15 mg/ml DNase I (all from Roche Applied Sciences) in PBS. After digestion, tumors were forced through a 40 µm cell strainer. A total of 106 cells were stained for flow cytometer analysis with macrophages were characterized as described above.

Analysis of RNA expression

RNA was extracted using the RNeasy Mini kit and treated with RNase-free DNasel (all from Qiagen). Synthesis of cDNA was performed using Superscript III (Invitrogen) and oligo dT primers. For real-time PCR, 1 µL of cDNA was used in 10 µL reactions using Platinum SYBR Green qPCR SuperMix (Invitrogen) carried out in a LightCycler 480 II thermal cycler (Roche). Oligonucleotides used for PCR included primers specific for the housekeeping gene β-actin, murine iNOS, and arginase-1. For the analysis of hBDM maturation, specific primers were used for the housekeeping gene ubiquitin c (UBC), human CD68 and MARCO. Cycling conditions were used as suggested in the SYBR Green kit instructions and results analyzed using Relative Quantification Software (Roche).

Immunofluorescence

Frozen sections of CRH5 tumors were fixed with 4% paraformaldehyde, blocked for endogenous biotin activity and then incubated overnight at 4°C with 1:300 anti-F480-FITC clone BM8 (Biolegend). Slides were counterstained and mounted using DAPI mounting medium (VectorLab). Expression of F480 was evaluated with an Axiostarp2+ fluorescent microscope (Zeiss). Percentage of F480<sup>+</sup> cells was evaluated on 10 fields with at least 100 cells in the same slide using ImageJ.
**Statistical analyses**

All statistical tests were performed using GraphPad Prism (GraphPad5.0). Means of two groups were compared using one-tailed paired Student’s t test. When more than two groups were compared, two-way ANOVA followed by the Bonferroni multiple comparison test was conducted. For survival, differences were evaluated using Kaplan-Meier curves with log-rank test. Data are represented as mean ± s.e.m. with statistical significance values indicated in the figure legends together with the n values used to calculate the statistics. All in vitro experiments with MM cells have been repeated at least three times using samples from the same source as technical replicates. In vivo studies as well as experiments with primary cells were also repeated at least three times using different sources as biological replicates.

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**Disclosure of Interest**

The authors have declared that no conflict of interest exists.

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**References**

1. Tureci O, Schmitt H, Fadde N, Pfreundschuh M, Sahin U. Molecular definition of a novel human galectin which is immunogenic in patients with Hodgkin’s disease. J Biol Chem. 1997;272:6416–6422.

2. Wada J, Kanwar YS. Identification and characterization of galectin-9, a novel beta-galactoside-binding mammalian lectin. J Biol Chem. 1997;272:6078–6086.

3. Bi S, Earl LA, Jacobs L, Baum LG. Structural features of galectin-9 and galectin-1 that determine distinct T cell death pathways. J Biol Chem. 2008;283:12248–12258. doi:10.1074/jbc.M800523200.

4. Li Y, Feng J, Geng S, Geng S, Wei H, Chen G, Li X, Wang L, Wang R, Peng H, et al. The N- and C-terminal carbohydrate recognition domains of galectin-9 contribute differently to its multiple functions in innate immunity and adaptive immunity. Mol Immunol. 2011;48:670–677. doi:10.1016/j.molimm.2010.11.011.

5. Kuroda J, Yamamoto M, Nagoshi H, Kobayashi T, Sasaki N, Shimura Y, Horike S, Kimura S, Yamauchi A, Hirashima M, et al. Targeting activating transcription factor 3 by galectin-9 induces apoptosis and overcomes various types of treatment resistance in chronic myelogenous leukemia. Mol Cancer Res. 2010;8:994–1001. doi:10.1158/1541-7786.MCR-10-0049.

6. Kobayashi T, Kuroda J, Ashihara E, Oomizu S, Terui Y, Taniyama A, Adachi S, Takagi T, Yamamoto M, Sasaki N, et al. Galectin-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. Leukemia. 2010;24:835–850. doi:10.1038/leu.2010.25.

7. Wiersma VR, de Bruyn M, van Ginkel RJ, Sigar E, Hirashima M, Niki T, Nishi N, Samplonius DP, Helfrich W, Bremer E. The galectin-binding protein galectin-9 has direct apoptotic activity toward melanoma cells. J Invest Dermatol. 2012;132:2302–2305. doi:10.1038/jid.2012.133.

8. Fujita K, Iwama H, Sakamoto T, Okura K, Kobayashi K, Takano J, Katsura A, Tatsuta M, Maeda E, Mimura S, et al. Galectin-9 suppresses the growth of hepatocellular carcinoma via apoposis in vitro and in vivo. Int J Oncol. 2015;46:2419–2430. doi:10.3892/ijo.2015.2941.

9. Kobayashi K, Morishita A, Iwama H, Fujita K, Okura R, Fujihara S, Yamashita T, Fujimori T, Kato K, Kamada H, et al. Galectin-9 suppresses cholangiocarcinoma cell proliferation by inducing apoptosis but not cell cycle arrest. Oncol Rep. 2015;34:1761–1770. doi:10.3892/or.2015.4197.

10. Tadokoro T, Morishita A, Fujihara S, Iwama H, Niki T, Fujita K, Akashi E, Mimura S, Oura K, Sakamoto T, et al. Galectin-9: an anticancer molecule for gallbladder carcinoma. Int J Oncol. 2016;48:1165–1174. doi:10.3892/ijo.2016.3347.

11. Takano J, Morishita A, Fujihara S, Iwama H, Kokado F, Fujikawa K, Fujita K, Chiyot T, Tadokoro T, Sakamoto T, et al. Galectin-9 suppresses the proliferation of gastric cancer cells in vitro. Oncol Rep. 2016;35:851–860. doi:10.3892/orno.2015.4452.

12. Zhu C, Anderson AC, Schubart A, Xiong H, Imotila J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK. The tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol. 2005;6:1245–1252. doi:10.1038/ni1271.

13. Fujita K, Iwama H, Oura K, Tadokoro T, Yamakawa K, Sakamoto T, Nomura T, Tani J, Yoneyama H, Morishita A, Hiroto T. Cancer therapy due to apoptosis: galectin-9. Int J Mol Sci. 2017;18:74. doi:10.3390/ijms18010074.

14. Daley D, Mani VR, Mohan N, Akkad N, Ochi A, Heindel DW, Lee KB, Zambrinis CP, Pandian GSB, Savadkar S, et al. Dectin 1 activation on macrophages by galectin 9 promotes pancreatic carcinoma and peripheral immune tolerance. Nat Med. 2017;23:556–567. doi:10.1038/nm.4314.

15. Wada J, Ota K, Kumar A, Wallner EL, Kanwar YS. Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin. J Clin Invest. 1997;99:2452–2461. doi:10.1172/JCI119429.

16. Kashio Y, Nakamura K, Abedin MJ, Seki M, Nishi N, Yoshida N, Nakamura T, Hirashima M. Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. J Immunol. 2003;170:3631–3636.

17. Yap TA, Aerts JG, Popat S, Fennell DA. Novel insights into mesothelioma biology and implications for therapy. Nat Rev Cancer. 2017;17:475–488. doi:10.1038/nrc.2017.42.

18. Carbone M, Ly BH, Dodson RF, Pagano I, Morris PT, Dogan UA, Gazdar AF, Pass HI, Yang H. Malignant mesothelioma: facts, myths, and hypotheses. J Cell Physiol. 2012;227:44–58. doi:10.1002/jcp.22724.

19. Lievensa LA, Cornelissen R, Rezemer K, Kaijen-Lambers ME, Hegmans JP, Aerts JG. Pleural effusion of patients with malignant mesothelioma biology and implications for therapy. Nat Rev Oncol. 2012;16:1755–1764. doi:10.1038/nrct.2016.021.

20. Chen E, d’Almeida S, Bloody T, Tabiasco J, Deshayes S, Fonteneau JF, Cellerin L, Delneste Y, Grégoire M, Blanquet C. Pleural effusions from patients with mesothelioma induce recruitment of monocytes and their differentiation into M2 macrophages. J Thorac Oncol. 2016;11:1765–1773. doi:10.1016/j.jto.2016.06.022.

21. Chang CI, Liao JC, Kuo L. Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. Cancer Res. 2001;61:1100–1106.

22. Rathi M, Muller I, Kropp P, Closs E, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. Front Immunol. 2014;5:332. doi:10.3389/fimmu.2014.00532.
23. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev. 2008;222:180–191. doi:10.1111/j.1600-065X.2008.00608.x.

24. Cornelissen R, Lieverse LA, Maat AP, Hendriks RW, Hoogsteden HC, Bogers AJ, Hegmans JP, Aerts JG, Gangopadhyay N. Ratio of intratumoral macrophage phenotypes is a prognostic factor in epithelioid malignant pleural mesothelioma. PLoS One. 2014;9:e106742. doi:10.1371/journal.pone.0106742.

25. Ye J, Ma N, Madden TL, Ostell JM. IgBLAST: an immunoglobulin variable domain sequence analysis tool. Nucleic Acids Res. 2013;41:W34–40. doi:10.1093/nar/gkt382.

26. Essono S, Frobert Y, Grassi J, Créminon C, Boquet D. A general method allowing the design of oligonucleotide primers to amplify the variable regions from immunoglobulin cDNA. J Immunol Methods. 2003;279:251–266.

27. Dunbar J, Krawczyk K, Leem J, Marks C, Nowak J, Regep C, Georges G, Kelm S, Popovic B, Deane CM. SAbPred: a structure-based antibody prediction server. Nucleic Acids Res. 2011;39:W474–W483. doi:10.1093/nar/gkw361.

28. Sacchettini JC, Baum LG, Brewer CF. Multivalent protein-carbohydrate interactions. A new paradigm for supermolecular assembly and signal transduction. Biochemistry. 2001;40:3009–3015.

29. Collins BE, Paulson JC. Cell surface biology mediated by low affinity multivalent protein-glycan interactions. Curr Opin Chem Biol. 2004;8:617–625. doi:10.1016/j.cbpa.2004.10.004.

30. Garner OB, Baum LG. Galectin-glycan lattices regulate cell-surface glycoprotein organization and signalling. Biochem Soc Trans. 2008;36:1472–1477. doi:10.1042/BST0361472.

31. Holness CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood. 1993;81:1607–1613.

32. Aldinucci D, Colombatti A. The inflammatory chemokine CCL5 and cancer progression. Mediators Inflamm. 2014;2014:1–12. doi:10.1155/2014/292376.

33. Georgoudaki A-M, Prokopec KE, Boura VF, Hellevist E, Sohn S, Östling J, Dahan R, Harris RA, Rantalainen M, Klevebring D, et al. Reprogramming tumor-associated macrophages by antibody targeting inhibits cancer progression and metastasis. Cell Rep. 2016;15:2000. doi:10.1016/j.celrep.2016.04.084.

34. Wang J, Pantopoulos K. Regulation of cellular iron metabolism. Biochem J. 2011;434:365–381. doi:10.1042/BJ20110182.

35. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity. 2014;41:49–61. doi:10.1016/j.immuni.2014.06.010.

36. Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado Jde D, Popovich PG, Partida-Sanchez S, Gueriu-de-Arellano M, Olszewski MA. Novel markers to delineate murine M1 and M2 macrophages. PLoS One. 2015;10:e0145342. doi:10.1371/journal.pone.0145342.

37. Chockalingam S, Ghosh SS. Macrophage colony-stimulating factor and cancer: a review. Tumour Biol. 2014;35:10635–10644. doi:10.1007/s13277-014-2627-0.

38. Enninga EAL, Chatzopoulos K, Butterfield JT, Sutor SL, Leontovich AA, Nevala WK, Flotte TJ, Markovic SN. CD206-positive myeloid cells bind galectin-9 and promote a tumor-supportive microenvironment. J Pathol. 2018;245. doi:10.1002/path.2018.245.issue-4.

39. Go A, Ryu Y-K, Lee J-W, Moon E-Y. Cell motility is decreased in macrophages activated by cancer cell-conditioned medium. Biomol Ther (Seoul). 2013;21:481–486. doi:10.4062/biomolther.2013.076.

40. Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML, Lee Y-C, Yang P-C. Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. Clin Cancer Res. 2003;9:729–737.

41. Moynihan KD, Opel CF, Szeto GL, Tzeng A, Zhu EF, Engreitz JM, Williams RT, Rakhra K, Zhang MH, Rothschilds AM, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. Nat Med. 2016;22:1402–1410. doi:10.1038/nm.4200.

42. Arlauckas SP, Garris CS, Kohler RH, Kitaoka M, Cuccarese MF, Yang KS, Miller MA, Carlson JC, Freeman GI, Anthony RM et al. In vivo imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy. Sci Transl Med. 2017;9 pii: eaal3604. doi:10.1126/scitranslmed.aal3604.

43. Mayer AT, Natajaran A, Gordon SR, Maute RL, McCracken MN, Ring AM, Weissman IL, Gambhir SS. Practical immuno-PET radiotracer design considerations for human immune checkpoint imaging. J Nucl Med. 2017;58:538–546. doi:10.2967/jnumed.117.177659.

44. Bertino P, Panigada M, Soprana E, Bianchi V, Bertilaccio S, Sanvito F, Rose AH, Yang H, Gaudino G, Hoffmann PR, et al. Fowlpox-based survivin vaccination for malignant mesothelioma therapy. Int J Cancer. 2013;133:612–623. doi:10.1002/ijc.20846.

45. Nishi N, Itoh A, Fujiyama A, Yoshida N, Araya S, Hirashima M, Shoji H, Nakamura T. Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins. FEBS Lett. 2005;579:2058–2064. doi:10.1016/j.febslet.2005.02.054.

46. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. Biotechnology.

47. Bertino P, Panigada M, Soprana E, Bianchi V, Bertilaccio S, Sanvito F, Rose AH, Yang H, Gaudino G, Hoffmann PR, et al. Fowlpox-based survivin vaccination for malignant mesothelioma therapy. Int J Cancer. 2013;133:612–623. doi:10.1002/ijc.20846.

48. Davis MR, Manning LS, Whitaker D, Garlepp MJ, Robinson BW. Imaging antibody of predefined specificity. 1975. Biotechnology.