**HFB9-2, a novel Galectin-9 neutralizing antibody to reverse immune suppression in the tumor microenvironment**

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**Introduction**

Although monoclonal antibodies targeting immune checkpoints have demonstrated clinical success in a range of tumor types, sustained responses are only observed in a fraction of patients due to primary or secondary resistance to treatment. Recent evidence implicates the pleiotropic immunosuppressive mediator Galectin-9 (Gal-9) as a key factor present in the tumor microenvironment that renders tumors resistant to current immunotherapies. High Gal-9 expression has been reported in different types of cancers including hematological malignancies such as Acute Myeloid Leukemia (AML) and Acute Lymphocytic Leukemia (ALL), and multiple solid tumors. We hypothesize that targeting Gal-9 may represent a valuable strategy to reduce immunosuppression and improve clinical response in selected cancer patients.

Gal-9 has been reported to play a dual role in AML as both a self-renewal factor for leukemic stem cells and a suppressor of anti-cancer immune responses. Analysis of AML patient serum samples demonstrated that Gal-9 expression was significantly higher in healthy controls and that Gal-9 levels dropped at complete remission. Higher levels of Gal-9 were found in French-American-British (FAB) M4 and M5 AML samples, and the lowest levels were observed in 36 patients samples.

We present a humanized monoclonal antibody, HFB9-2, that specifically binds to human Gal-9 with sub-micromolar affinity, recognizes recombinant Gal-9 and produced by human tumor cells, and is cross-reactive with mouse and monkey Gal-9 orthologs. HFB-3 blocks the interaction of Gal-9 with its receptors TREM2 and CD44 in a dose-dependent manner. These two receptors have been described to mediate Gal-9-immunosuppressive signals in effector and regulatory T cells. Treatment of human PBMCs with HFB-2 prevents Gal-9-induced Th cell apoptosis and suppresses the expansion of regulatory T cells induced by Gal-9. Moreover, HFB-2 has a favorable developability profile, demonstrating stability for 32 days at 4°C, as well as for several freeze-thaw cycles. High plasma exposure following a single dose administration to mice was observed. HFB-2 exhibits significant anti-tumor efficacy in the WEHI-164 syngeneic mouse model as a single agent or in combination with anti-CD3e antibody. Further analysis of the novel HFB-2 treatment in the AML patients is currently ongoing to guide the selection of patients most likely to benefit from HFB-2 treatment in the clinic.

After the data presented here provide evidence that neutralization of Gal-9 with HFB-2 blocks key immunosuppressive mechanisms known to favor cancer progression and to limit the efficacy of current immunotherapies, and position HFB-2 as a drug candidate for clinical evaluation in AML and other indications.

**Results**

### 1. Gal-9 Is Broadly Expressed in Tumor Infiltrating Immune Cells

Gal-9 has been recently shown to impair the immunological activities of cytotoxic T cells and natural killer (NK) cells, thus facilitating AML cells to escape immune attack. Moreover, a TMI-3/Gal-9 autoimmune stimulus loop has been developed to regulate self-renewal of human myeloid leukemia stem cells (LSCs) and to promote leukemic progression. We have analyzed AML patient serum samples and demonstrated that Gal-9 levels are significantly higher than in healthy controls and drop at complete remission.

**Figure 1.** Single-well ELISA analysis of Gal-9 expression in multiple solid tumors, including non-small cell lung cancer (NSCLC), breast cancer (BC), and melanoma. Single-well ELISA analysis demonstrated that Gal-9 is expressed broadly across tumor infiltrating immune cells such as macrophages, B cells, and CD8+ T cells.

### 2. High Levels of Gal-9 Are Circulating in AML Patients

Gal-9 has been recently shown to impair the immunological activities of cytotoxic T cells and natural killer (NK) cells, thus facilitating AML cells to escape immune attack. Moreover, a TIM-3/Gal-9 autoimmune stimulus loop has been developed to regulate self-renewal of human myeloid leukemia stem cells (LSCs) and to promote leukemic progression. We have analyzed AML patient serum samples and demonstrated that Gal-9 levels are significantly higher than in healthy controls and drop at complete remission.

**Figure 2.** High levels of Gal-9 are circulating in AML patients.

### 3. HFB-9-2 Binding Affinity and Cross-reactivity

**Figure 3.** Binding and cross-reactivity of HFB9-2, a humanized neutralizing antibody derived from chimeric antibody WEHI-2a. (A) HFB9-2 binds to recombinant Gal-9 with an EC50 of 0.15 nM. (B) HFB9-2 binds to recombinant human CRD1 with an EC50 of 1.1 nM. (C) HFB9-2 binds to recombinant B cells with an EC50 of 0.15 nM determined by ELISA. IC50 values for HFB9-2 and HFB9-3 were determined by ELISA. (A) HFB9-2 IC50 values for CD8, CD44, and CD23 determined by ELISA (nM). (B) MFI was measured using flow cytometry. (C) HFB9-2 IC50 values for 10×10^6 cells/mL, 10×10^7 cells/mL, and 10×10^8 cells/mL, respectively.

### 4. Effects on Gal-9-Induced Tumor Escape Mechanisms

**Figure 4.** Effects of HFB9-2 on Gal-9-induced T cell escape mechanisms. (A) HFB9-2 and 15 nM IL-2 increases the ability of tumor infiltrating T cells to kill tumor cells. (B) HFB9-2 and 15 nM IL-2 increases the ability of T cells to kill tumor cells. (C) HFB9-2 and 15 nM IL-2 increases the ability of T cells to kill tumor cells. (D) HFB9-2 and 15 nM IL-2 increases the ability of T cells to kill tumor cells.

### 5. Developmental Assessment of HFB9-2

**Figure 5.** Maturation of HFB9-2 enables mouse model studies including multiple tumor models, including colon and liver cancer, (A) Using the BA1166 and BA1168), and HFB9-2 (B). HFB9-2 demonstrated good stability under all tested conditions. Stability under different storage conditions and chemical stability have been determined.

### 6. Antitumor Activity in s.c. Syngeneic Tumor Model

**Figure 6.** In vivo evaluation of HFB9-2. (A) HFB9-2 administration in the B16F10 melanoma mouse model decreases tumor growth (B). HFB9-2 adminstered for 14 days to mice bearing the B16F10 melanoma xenograft. HFB9-2 treatment was initiated at day 1 and was continued for 14 days. (C) B16F10 melanoma xenograft in mice treated with vehicle (100 µL of PBS) or HFB9-2 (5 mg/kg, 3 times per week). (D) HFB9-2 administration reduces tumor growth in a dose-dependent manner. (E) B16F10 melanoma xenograft in mice treated with vehicle (100 µL of PBS) or HFB9-2 (5 mg/kg, 3 times per week).

**Conclusion**

Here we describe a humanized Gal-9 neutralizing antibody, HFB9-2, with the following properties:

- Sub-micromolar affinity for human Gal-9
- Stable in plasma and sera of multiple species orthologs
- Favorable developability and pharmacokinetic profiles
- Inhibitory activity in two key immunomodulatory functions of Gal-9, Th1 apoptosis and Th expansion
- Anti-tumor efficacy in s.c. WEHI-164 syngeneic model

We also demonstrate, in collaboration with the Gustave Roussy Institute, that Gal-9 levels are significantly higher in AML patients compared to healthy controls. Together, we propose Gal-9 neutralizing antibody therapy as an approach to treat AML and other cancer types where Gal-9 expression associates with immunosuppression and resistance to immune-therapies.

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