EBV-Associated Tumors Increase Regulatory T cell Recruitment via CCR4 Ligand Expression and are a Promising Indication for Treatment with Small Molecule CCR4 Inhibitors

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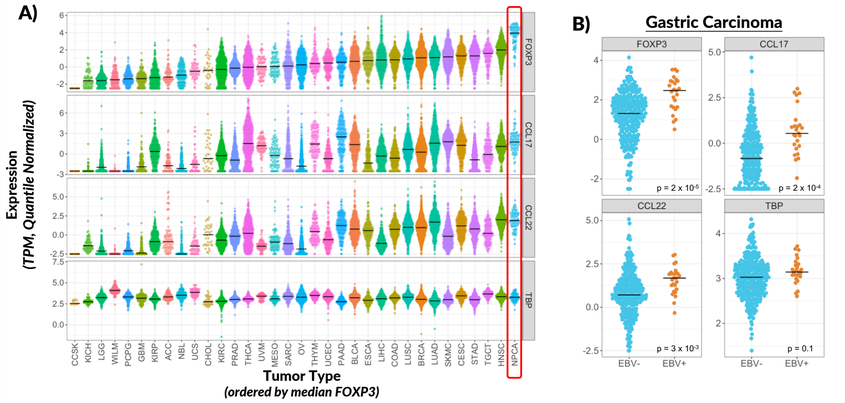
Introduction

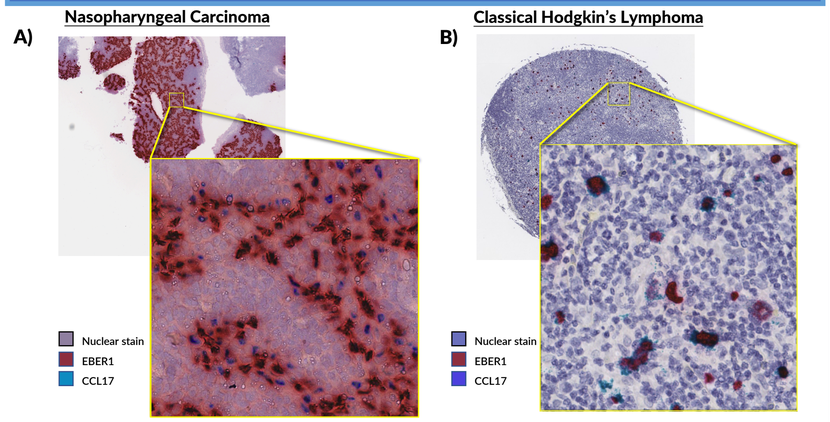
- Regulatory T cells (T_{reg}) are a major immune-suppressive cell found in the tumor microenvironment (TME)
- FLX Bio has developed potent and selective small molecule antagonists of CCR4, a key T_{reg} chemotactic receptor that helps them migrate into tumors
- Epstein-Barr Virus (EBV) positivity is seen in approximately 95% of Nasopharyngeal Carcinoma, 40% of classical Hodgkin's Lymphoma, and 10% of Gastric Carcinomas
- The EBV gene LMP1 has been shown to drive high expression of the CCR4 ligands, CCL17 and CCL22
- **EBV-positive tumors may be particularly sensitive to treatment with the FLX Bio CCR4 antagonist in the clinic**

CCR4 Ligands and FOXP3 Expression are Elevated in EBV$^+$ Tumors



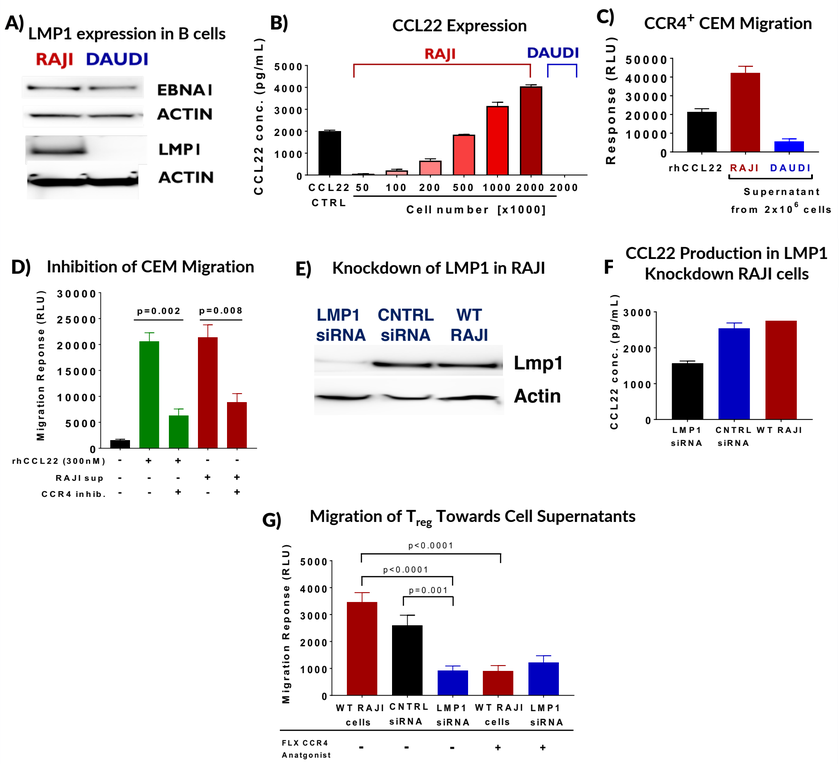
A) RNA-Seq data from 79 Nasopharyngeal Carcinomas (NPCA) from two published studies normalized with TCGA/TARGET tumor data. NPCA had the 1st, 3rd, and 2nd highest median expression of FOXP3, CCL17, and CCL22, respectively. **B)** Gastric carcinoma was divided into EBV$^-$ and EBV$^+$ subsets based on TCGA annotation. FOXP3, CCL17, and CCL22 expression was significantly increased in EBV$^+$ tumors. Housekeeping genes such as TBP and GAPDH (not shown) were not significantly different.

CCR4 Ligands are Coexpressed with EBV Genes in CHL and NPC



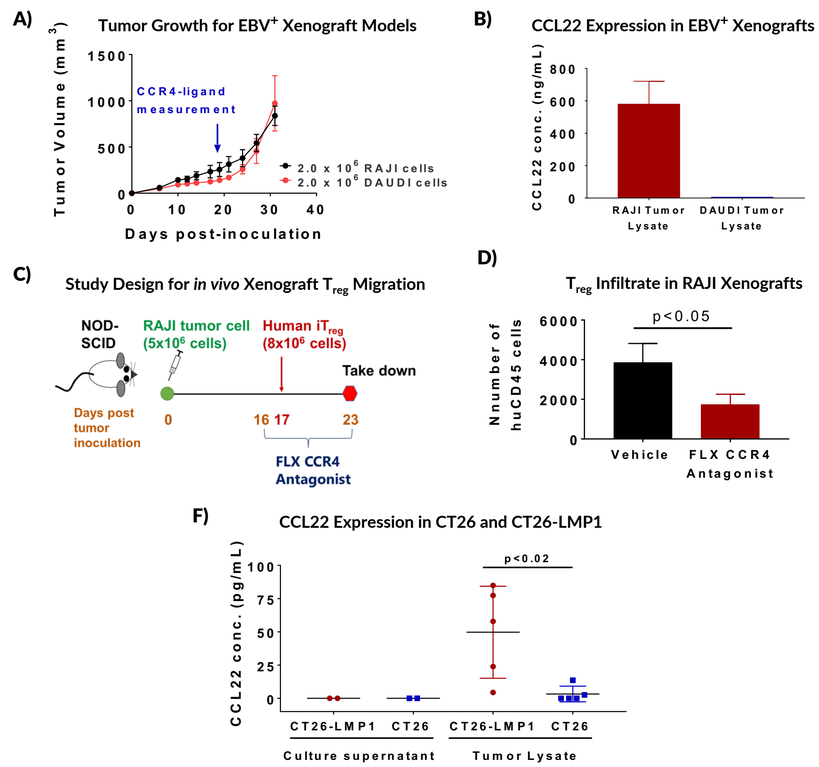
Sections of Nasopharyngeal carcinomas (**A**) or cores from classical Hodgkin's Lymphoma (**B**) were used for RNA *in situ* hybridization by ACD RNAScope technology. CCL17 coexpression with EBER1, a constitutive EBV transcript, can clearly be seen in both tumor types. CCL22 coexpression (not shown) could similarly be seen in both tumor types.

Expression of LMP1 in Human EBV$^+$ B Cells Induces CCL22



A) LMP1 expression was detected by Western Blot in EBV$^+$ RAJI cells but not in EBV$^+$ DAUDI cells. **B)** LMP1$^+$ RAJI cells produced high levels of CCL22 and **C)** CCR4$^+$ CEM cells migrated toward the RAJI supernatant. **D)** Cell migration was significantly blocked by a CCR4 antagonist. **E)** LMP1 siRNA significantly reduced LMP1 expression in transfected RAJI cells **F)** which produced reduced levels of CCL22. **G)** Induced human T_{reg} migrated towards cell supernatant from RAJI cells but not from LMP1-siRNA treated RAJI cells.

LMP1$^+$ Tumors Produce High Levels of CCL22 in Mouse Models



A) 2 x 10^6 RAJI or DAUDI cells were inoculated subcutaneously into NOD-SCID mice and had comparable tumor growth. **B)** RAJI tumors produced high levels of hCCL22 compared to DAUDI. **C)** Study design for RAJI xenograft model to test human T_{reg} migration *in vivo* **D)** Human T_{reg} cells transferred into RAJI tumor-bearing mice migrated into the tumor which could be blocked with FLX CCR4 antagonist. **F)** Murine colon CT26 cells were engineered to express LMP1 (CT26-LMP1). CT26-LMP1 cells in culture did not produce mouse CCL22 but high levels of CCL22 were detected from CT26-LMP1 tumor lysates.

Summary and Conclusion

- EBV$^+$ tumors in humans, including Nasopharyngeal, Classical Hodgkin's Lymphoma, and Gastric Carcinoma show expression of the CCR4 ligands, CCL22 and CCL17, and increased T_{reg} infiltration, as measured by FOXP3 expression.
- Supporting observations that the Epstein-Barr Virus (EBV) LMP1 gene can drive expression of CCR4 ligands, we find high production of CCL22 by EBV$^+$ LMP1$^+$ RAJI cells *in vitro* and *in vivo*, but not by EBV$^+$ LMP1$^-$ DAUDI cells or EBV$^+$ LMP1-siRNA RAJI cells. RAJI supernatant drove migration by CCR4$^+$ CEM cells that could be blocked by a FLX Bio CCR4 small-molecule antagonist.
- An engineered LMP1$^+$ CT26 mouse cell line produced significant levels of CCL22 *in vivo* but not *in vitro*, suggesting that, directly or indirectly, LMP1 also has effects on non-B cell tumors. Further studies will be performed to demonstrate antitumor effects of the FLX Bio CCR4 small-molecule antagonist in this system.
- **Thus, EBV$^+$ tumors may modulate the tumor microenvironment by recruiting suppressive T_{reg} via CCL17 and CCL22. EBV$^+$ tumor patients may represent a population preferentially sensitive to CCR4-antagonist treatment.**