Title: A regulatory B cell subpopulation expressing CD301b lectin promotes breast cancer growth

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Abstract

Tumor-associated carbohydrate antigens (TACAs) can promote tumor growth by regulating the anti-tumor immune response. The accumulation of immune suppressor cells within the tumor in response to TACAs suggests a critical pathway to suppress immune targeting of the tumor. Employing murine breast cancer models, we isolated a regulatory B cell subpopulation in the breast tumor microenvironment that displays an immune suppressive phenotype through its Tn TACA-binding lectin, CD301b. We then demonstrated that depleting CD301b^{+} cells facilitated tumor control and mouse survival, whereas increasing Tn antigen expression decreased mouse survival. As tumor cells use Tn expression to overcome immune targeting, interfering with or blocking the Tn-CD301 axis may unleash the immune system, specifically within the aberrantly glycosylated tumor microenvironment, offering new immunotherapy for breast and other cancers.

MAIN TEXT

Introduction

A major setback observed in many cancers arises from the modulation of the immune system to prevent effective anti-cancer responses. Vaccines, CAR T cells, and checkpoint blockade prime the immune system against cancer-associated antigens, promoting cancer cell destruction via immune cytotoxicity (*1*). Breast cancer is a prime example of a "cold tumor" that doesn't trigger a robust immune response (*2*). Immune therapies for breast cancer remain largely ineffective due to the ability of the tumor to grow unimpeded and eventually metastasize. Immune regulation can occur through cytokine secretion (such as IL-10) (*3, 4*), signaling mechanisms, or checkpoints (such as through PD-1) (*5*). During the malignant transformation of a mammalian cell, a dramatic and aberrant modification of cellular glycosylation is observed. Tumor-associated carbohydrate antigens, or TACAs, can induce immune suppression, allowing cancer cells to evade immune cells (*6-8*). Lectins, such as galectins, are carbohydrate-binding proteins that function as immune cell receptors and can activate immune regulatory pathways through their interactions with TACAs (*9, 10*). Similarly, modulating immune profiles in the tumor microenvironment (TME) by engaging sialic-acid-binding immunoglobulin-like lectins (Siglecs) is a known TACA mechanism (*11-13*). Thus, elucidating the protein-carbohydrate interactions in regulating the immune system within the TME is becoming increasingly important.

Tn antigen is a TACA ranked as a high-priority cancer-associated antigen based on its antigenicity and oncogenicity (*14-18*). Tn is a truncated form of the normal, extended cell surface O-glycan. Tn is the terminal O-linked N-acetylgalactosamine (GalNAc) on serine or threonine residues. The aberrant glycosylation associated with Tn may occur when the enzyme responsible for O-glycan elongation—T-synthase—or its associated chaperone—cosmc (C1GALT1C1)—becomes functionally inhibited (*19, 20*). Tn-expressing mucin 1 (MUC1) has been associated with breast

49 cancer cells (*21, 22*), especially in triple-negative breast cancer (*23, 24*) , and modulating MUC1
50 expression or Tn glycosylation can inhibit tumor growth (*25-27*). However, developing immune
51 responses against Tn-MUC1 has been problematic (*27, 28*).
52
53 CD301, also known as macrophage galactose-type lectin, or MGL, is a C-type lectin receptor (CLR)
54 that binds Tn antigen on the surface proteins (*29*). In mice, the Tn-recognizing homolog to the
55 human CD301 is CD301b, or MGL2 (*30*). CD301b has been considered to be expressed exclusively
56 by myeloid cells such as dendritic cells (DCs) and macrophages (*17*). Previously, MGL2-expressing
57 myeloid cell populations have been linked with immunosuppressive responses (*16, 17, 31-33*). DCs
58 and macrophages were shown to suppress the proliferation of $CD4^+$ effector T lymphocytes through
59 the interaction of MGL with terminal GalNAc residues on CD45 expressed by T cells (*17*). This
60 cell-specific glycosylation of CD45 provided an immunoregulatory pathway of MGL-mediated
61 control of T_{eff} cell function. In another study, $CD301b^+$ dendritic cells were shown to suppress T
62 follicular helper cells and antibody responses to protein antigens (*31*). Recently, an
63 immunosuppressive DC subset expressing CD301b was shown to accumulate at secondary sites
64 and promote metastasis in pancreatic cancer (*33*) and lung cancer (*34*).
65
66 Here, we demonstrate the accumulation of $CD301b^+$ immune cells in the tumor microenvironment
67 as a response to Tn-expression by breast cancer cells. We characterize these cells into populations
68 of cells expressing markers associated with regulatory innate-like B cells and produce suppressive
69 cytokines. As a subset of B lymphocytes, regulatory B cells (Bregs) exhibit immunosuppressive
70 functions in homeostasis (*35*) and numerous disease states, including colitis (*36*), arthritis (*37*), HIV
71 (*38, 39*), and multiple cancers (*40-43*). Thus, these cells are broadly relevant in multiple tissues,
72 systems, and microenvironments throughout the body (*35*). Breg cells are typically characterized
73 by their production of IL-10 (*37, 44*), a cytokine most frequently implicated in immune suppression.

74 Inhibiting IL-10-producing Bregs has been shown to disrupt normal anti-inflammatory processes
75 and can result in chronic inflammation (*45*). Bregs are often identified by key cell surface markers,
76 including CD1d(*46*), CD24 (*47*), and/or CD5 (*46*). A heterogeneous subpopulation of B cells
77 expressing innate cell markers such as CD11b and demonstrating immunoregulatory functions has
78 been identified (*37, 45, 48-54*). Of note, a subpopulation of innate-like B cells may produce high
79 levels of IgM as one mechanism for decreasing inflammatory responses (*55*).
80
81 This work offers a method of immune suppression similar to checkpoint regulation, which has
82 represented critical targets in the rapidly advancing field of cancer immunotherapy. Immune
83 interactions of TACAs have been severely understudied. Elucidating immune suppression
84 mechanisms through protein-carbohydrate interactions has important implications for discovering
85 new immune therapies to boost immune anti-cancer function through overcoming regulation
86 mechanisms.
87
88 **Results**
89
90 ***CD301b$^+$ cells accumulate in a murine breast cancer model***
91 We first sought to establish the relationship between CD301b$^+$ immune cells and Tn glycoantigen-
92 expressing breast cancer cells. We used a CRISPR-Cas9 gene editing model to knockout cosmc
93 chaperone expression in AT3 murine breast cancer cells. Cosmc is a chaperone essential in
94 elongating the core O-glycan beyond the truncated Tn form of α-GalNAc (*19, 20, 56*). When cosmc
95 function/expression is disrupted, elongation of the O-glycan is not observed; instead, the Tn antigen
96 is observed at significantly higher levels. After disrupting cosmc expression in these cell lines, we
97 confirmed increased Tn cell surface expression through flow cytometry using both a reBaGs6 IgM
98 antibody (Supplementary Fig. 1A) (*57*) as well as a complementary biotinylated VVL lectin
99 coupled with fluorescent streptavidin (Supplementary Fig. 1B). Both staining methods indicated

100 significantly higher Tn expression on the cosmc KO cell line. We also isolated RNA from each cell
101 line and confirmed decreased cosmc expression in the Tn^{hi} cell line through qPCR using cosmc
102 primers (Supplementary Fig. 1C). We then tested whether knocking out cosmc expression changes
103 the proliferation rate of the AT3 breast cancer cells *in vitro*. After culturing both cell lines at the
104 same concentrations for three days, we observed no significant differences in cell proliferation
105 between the two lines (Supplementary Fig. 1D), indicating that Tn expression alone does not
106 promote cancer cell growth.
107 To determine the *in vivo* tumor cell growth, we injected AT3 (Tn^{lo}) and AT3 cosmc KO (Tn^{hi})
108 murine breast tumor cells into the mammary pads of C57BL/6 mice (n=25) and monitored tumor
109 growth for up to 23 days. We observed that Tn^{hi} tumors grew significantly faster than Tn^{lo} tumors
110 (Fig. 1A), indicating that Tn expression impacts tumor growth rate. Tumor-bearing and tumor-free
111 control mice were then euthanized to assess CD301b on multiple immune cell subsets in the spleen,
112 draining lymph nodes (or analogous lymph nodes in tumor-free mice), bone marrow, or breast
113 tumors by flow cytometry (for gating strategy, please see Supplementary Fig. 2). A significantly
114 higher accumulation of $CD45^{+}CD301b^{+}$ immune cells was observed in the Tn^{hi} tumor
115 microenvironments compared to the Tn^{lo} tumor tissue (Fig. 1B-C). Low levels of cell surface
116 CD301b were observed on immune cell subsets in the spleens or lymph nodes of tumor-free mice
117 (Fig. 1C). However, a significant population of $CD45^{+}CD301b^{+}$ cells was observed in the tumor
118 tissues and the bone marrow of the tumor-bearing mice. $CD301b^{+}$ immune cell accumulation in the
119 TME correlates with tumor Tn expression.
120

121 ***Tumor-infiltrating CD301b^{+} cells display a regulatory B-cell phenotype***

122 We next observed that essentially all tumor-infiltrating $CD45^{+}CD301b^{+}$ immune cells express the
123 B-cell markers B220 and CD19, and they do not express markers associated with other major
124 immune cell types CD3, NK1.1 and CD11c (Fig. 2A-D). CD11b expression on the tumor-

125 infiltrating $CD45^{+}CD301b^{+}$ varied (Fig. 2B, 2D). Since CD301b function has primarily been
126 attributed to myeloid cells, and particularly to DCs, we assessed and observed that the tumor-
127 infiltrating CD45+CD301b+B220+CD19+ immune cells do not express CD11c (Fig. 2E). The
128 absolute number of B220+CD19+ cells per mg of tumor tissue is highly increased in Tn^{hi} tumor
129 tissues compared to Tn^{lo} tumors, with approximately a third of them expressing CD301b+ in the
130 Tn^{hi} tumors (Fig. 2F).
131
132 The tumor-infiltrating $B220^{+}CD19^{+}CD301b^{+}$ cells express cell surface IgM, a B cell-specific
133 surface receptor (Fig. 3A). Next, we observed the $CD301b^{+}$ B cells express surface proteins
134 associated with regulatory B cells, such as CD5, CD24, Gr1 with varying levels, while other surface
135 markers traditionally associated with Bregs (CD1d, CD25, CD21, Ly6C and Ly6G) were minimally
136 expressed on these $B220^{+}CD19^{+}CD301b^{+}$ B cells (Fig. 3B-F). The tumor-infiltrating $CD301b^{+}$ cells
137 may represent a previously uncharacterized subset of B cells with a phenotype indicating an innate-
138 like regulatory B cell population (*48-55*).
139
140 ***Tn-Expressing Cancer Cells Induces Expression of CD301b on B cells***
141 CD301b mean fluorescence intensity (MFI) was significantly higher in Tn^{hi} tumor-infiltrating cells
142 than Tn^{lo} (Fig. 3G), indicating that CD301b expression is tumor Tn-associated. To further elucidate
143 if B cells can express CD301b in response to Tn expression, we obtained splenocytes from healthy
144 WT C57Bl/6 mice and used magnetic cell sorting protocols to positively select for $CD19^{+}$ cells or
145 isolate pan B cell populations through negative selection, compared to unselected total splenic
146 mononuclear cells (Supplementary Fig. 3). These splenocyte populations were then co-cultured
147 with either AT3 Tn^{lo} or AT3 Tn^{hi} cancer cells, compared to cultured alone (unstimulated). After
148 three days of co-culture, cells were analyzed using flow cytometry: viable singlet
149 $CD45^{+}B220^{+}CD19^{+}$ cells were gated out, and CD301b MFI was assessed in each group. We

150 observed significantly higher CD301b expression in all splenocyte groups co-cultured with Tn^{hi}
151 cancer cells compared with unstimulated or Tn^{lo} cancer cell co-cultures (Fig. 3H). These results
152 indicate an important role for Tn in the expression of CD301b on B cell populations.
153

154 ***B Cell Deficient Mice did not Exhibit CD301b+ Cell Accumulation in Response to Tn^{hi} Tumor***

155 Towards elucidating the role of CD301b+ B cells in the context of Tn-expressing tumor growth,
156 we used RAG1 KO (B6.129S7-Rag1tm1Mom/J) mice that lack functional T or B cells. We injected
157 Tn^{hi} tumor cells into the mammary pads of RAG1 KO mice alongside a WT control group. We
158 observed significantly decreased tumor growth (Fig. 4A) and no observable CD301b+ immune cells
159 infiltrating the tumors (Fig. 4B-C) compared to WT mice. No observable B220+CD19+ cells were
160 present within the RAG1 KO mouse tumors (Fig. 4D). These results further demonstrate that the
161 tumor-infiltrating CD301b+B220+CD19+IgM+ immune cells constitute a B cell population as they
162 are absent when mice lack the lymphocyte-specific *rag1* gene. Our results also suggest that
163 CD301b+ B cells play a regulatory function in promoting tumor growth. However, along with the
164 CD301b+ B cells, all lymphocytes, including T cells and CD301b- B cells, are absent in RAG1 KO
165 mice. Therefore, attributing such an immunoregulatory role to tumor-infiltrating CD301b+ B cells
166 requires elucidating their functional properties specifically, as laid out in the next two sections.
167

168 ***Tumor-infiltrating $CD301b^{+}$ B cells exhibit immunosuppressive characteristics***

169 To better understand the functional suppressive characteristics of tumor-infiltrating CD301b+ B
170 cells, we used intracellular staining of Tn^{hi} breast tumor-bearing mouse tumor tissue to stain for
171 regulatory cytokines. We gated and compared the following populations: $CD45^{+}B220^{+}CD19^{+}$ B
172 cells in tumor-free spleens and $CD45^{+}CD301b^{+}B220^{+}CD19^{+}$ from Tn^{hi} tumors. Of note, we
173 observed that tumor-associated $CD19^{+}B220^{+}CD301b^{+}$ produce significantly higher levels of IL10,
174 TGFβ, and subunits of IL35 than splenic-derived B cells from tumor-free mice as controls (Fig.

175 5A-B, Supplementary Fig. 4), suggesting a role for immune suppression in this tumor-infiltrating
176 B cell subset.
177
178 ***CD301b/Tn Axis Impacts Tumor Growth and Mouse Survival***
179 To illuminate how CD301b immune cells contribute to tumor growth, we employed a mouse model
180 in which CD301b immune cells can be depleted: B6(FVB)-*Mgl2*$^{tm1.1(HBEGF/EGFP)Aiwsk}$/J mice
181 (MGL2-DTR)(*31, 32*). We injected AT3 Tn^{lo} or Tn^{hi} tumor cells (Fig. 6A) into MGL2-DTR mice
182 that were/ were not CD301b-depleted with diphtheria toxin treatment. We measured tumor growth
183 and observed a significant decrease in tumor size when CD301b^{+} immune cells were depleted in
184 the Tn^{hi} group. We also injected AT3 Tn^{hi} cells into mammary pads of CD301b null MGL2-DTR
185 homozygous mice and observed significantly lower breast tumor growth than WT mice (Fig. 6B).

186 In a complementary experiment, we used a mouse model expressing human MUC1: C57BL/6-
187 Tg(MUC1)79.24Gend/J (*58*). These mice display aberrant glycosylation on MUC1, such as Tn
188 antigen, unlike wild-type mouse MUC1 glycosylation (*59-62*). We used methylcholanthrene
189 (MCA) as a carcinogen to induce spontaneous tumors in the mammary pads of both WT and
190 Muc1.Tg mice to assess how Tn expression affects tumor growth. We observed significantly
191 increased tumor growth rates to endpoint tumor sizes over 21 weeks in the Muc1.Tg mice (Fig.
192 6C), indicating that higher Tn expression on spontaneous tumors—not just cancer cell lines—can
193 impact breast cancer prognosis. To our point, tumor-infiltrating B cells from MUC1.Tg
194 spontaneous tumors showed significantly higher percentages of CD301b^{+} cells than the
195 corresponding B cell populations from WT MCA-induced tumors (Fig. 6D). These data together
196 indicate an important role for the CD301b-Tn axis in breast tumor development and the high
197 therapeutic potential for modulating it to improve the anti-tumor immune response in breast cancer.
198 However, other factors, such as differences in tumorigenicity, cell-intrinsic tumor growth, or
199 metastatic activity, can potentially contribute to the difference in survival in conjunction with or

200 independent from the interaction of MUC1-Tn with CD301b+ cells. Modulating CD301b and Tn
201 expression in this mouse model would enable further evaluation of the CD301b-Tn axis in tumor
202 immunoregulation.

203 **Discussion**

204 Numerous recent research studies have established the importance of Tn as a key antigen in relation
205 to human cancer development and progression (*20, 21, 63-65*). Additionally, CD301 plays an
206 immune regulatory role in human infection (*31, 32*) and the progression of human cancer (*33*). In a
207 recent study, CD301b^{+} DCs were shown to accumulate in response to higher levels of Tn in lung
208 tumor cells, and the Tn-CD301b interactions influence the development of pro-angiogenic
209 programs (*34*). The link between Tn and CD301-mediated immune responses thus needs further
210 elucidation, particularly in cancer models known to express aberrant glycosylation, such as breast
211 cancer. Overcoming immune regulation in breast cancer can potentially turn these "cold" tumors
212 into immunotherapeutic-responsive "hot" tumors, potentially improving clinical responses to
213 checkpoint blockade and tumor antigen vaccines. This study shows an accumulation of CD301b^{+}
214 immune cells in response to high Tn antigen expression by developing breast tumors in mice. These
215 CD301b^{+} immune cells phenotypically and functionally display an innate-like B cell phenotype
216 with regulatory functions, including producing immunoregulatory cytokines. Innate-like B cells
217 may express CD11b and CD5 but low levels of CD11c (*53, 66*). Innate-like B cells are also
218 associated with high levels of IgM and low levels of IgD (*55*) These cells have been shown to
219 produce natural IgMs in response to tumor-associated gangliosides (*67*). Of note, extensive research
220 studies have shown that B cells with regulatory function cannot currently be defined based on any
221 specific combination of extracellular markers or transcription factors; rather, the heterogeneity of
222 regulatory B cells suggests that these cells arise in response to environmental stimuli (*35*). We
223 observe suppressive CD301b^{+} cells accumulating in the tumor microenvironment specifically, and
224 particularly in response to higher Tn expression by the developing breast tumor. However, as some

accumulation is observed in bone marrow in tumor-bearing mice, studies investigating the origin of these cell populations would be warranted. MGL2/CD301b function has primarily and predominantly been attributed to myeloid cells in the past decades. Our results indicating that a previously uncharacterized regulatory CD301b+ lymphoid cell population plays an important role in the tumor immune escape warrants further exploration of the function and regulation of *mgl2* gene expression. Particularly, the results obtained in the RAG1 KO mouse model would suggest that CD301b+ B cells promote tumor growth. However, the direct role of B cell-specific CD301b and/or CD301b+ B cells could be further assessed by modulating CD301b expressing cells and B cells independently, such as developing a muMt-/Mgl2-DTR chimeric mouse model.

The MUC1.Tg mouse model is limited because it may not account for CD301b-Tn interaction alone and likely exhibits distinct tumor microenvironment development. Understanding the innate-like characteristics of these cells could have implications for their mechanisms of suppressive functions in the context of Tn^{hi} tumors. Analysis (e.g., via transcriptomics) of these tumor-infiltrating cell populations in relation to the tumor immune microenvironment could thus describe the novel population of suppressive cells and their mechanisms of action. Eliminating these cells can improve tumor response in mice, which has important implications for targeting the CD301b-Tn axis to improve immunotherapy. Further characterization of how these cells modulate immune responses in the TME will help determine optimal therapies to target and overcome their accumulation and immunosuppressive mechanisms. Furthermore, therapies targeting CD301 and/or Tn could improve breast cancer response to breakthrough therapies, such as PD-1 or CTLA-4 checkpoint blockade. Future investigation of these therapeutic strategies will thus be a priority in this research. Understanding how these $B220^{+}CD19^{+}IgM^{+}CD301b^{+}$ immune cells modulate the immune pathways in TME and impact disease progression could have important implications for cancer immunotherapy.

Materials and Methods

251

252 ***Mice***

253 Eight-week-old female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME)
254 and housed at Emory University Whitehead Biomedical Research Building. MGL2-DTR mutant
255 mice (*Mgl2*$^{tm1.1(HBEGF/EGFP)Aiwsk}$) were a generous gift from Akiko Iwasaki at Yale University.
256 Muc1.Tg mice (C57BL/6-Tg(MUC1)79.24Gend/J) were a generous gift from Sandra Gendler at
257 Mayo Clinic. Mice were kept in microisolator cages and handled under biosafety level 2 (BSL2)
258 hoods. For tissue processing and subsequent flow cytometry, mice were euthanized by carbon
259 dioxide inhalation in accordance with IACUC guidelines. For applicable experiments, serum
260 samples, spleens, lymph nodes, bone marrow, and/or tumors were harvested. Where applicable, cell
261 suspensions were generated through mechanical tissue disruption and collagenase D digestion. Red
262 blood cells were lysed, and samples were filtered through 60 μm nylon filters to obtain single-cell
263 suspensions. For depletion of CD301b^{+} cells, heterozygous MGL2-DTR mice were treated with
264 diphtheria toxin (0.5μg/mouse) in sterile PBS intraperitoneally every two to three days, starting at
265 day -1 before tumor injection. Effective MGL2 cell depletion was confirmed using flow cytometry
266 in bone marrow and tumor samples (Supplementary Fig. 5A-B). For RAG1 KO experiments, 6-8
267 weeks old RAG1 KO mice (B6.129S7-Rag1tm1Mom/J) were obtained from Jackson Laboratory.
268 The lack of B cell populations was confirmed using flow cytometry in bone marrow and lymph
269 node samples (Supplementary Fig. 5C).

270

271 All mouse experiments were in compliance with the Emory University Institutional Animal Care
272 and Use Committee under an approved animal use protocol. Our animal use protocol adheres to the
273 principles outlined in *U.S. Government Principles for the Utilization and Care of Vertebrate*
274 *Animals Used in Testing*, *Research and Training*, the Animal Welfare Act, the *Guide for the Care*
275 *and Use of Laboratory Animals*, and the *AVMA Guidelines for the Euthanasia of Animals*.

276

277 ***Generation of Tn^{hi} breast cancer cells***

278 To express Tn glycans at high levels in tumor cells, we used a CRISPR/Cas9 methodology to stably
279 silence cosmc expression in AT3 cells using established protocols and reagents. Mouse cosmc guide
280 RNA and CRISPR/Cas9 plasmid were obtained from Santa Cruz Technology (sc-425587). AT3
281 murine breast cancer cells were a generous gift from the Kebin Liu lab at Augusta University. AT3
282 cells were transfected with cosmc CRISPR/Cas9 KO plasmid according to the manufacturer's
283 protocol. Puromycin was used to select transfected cells. We then used flow cytometry to confirm
284 higher expression of Tn on the AT3 cell surfaces using the ReBaG6 antibody (generously provided
285 by Richard Cummings at Harvard University) (Supplementary Fig. 1A) (*57*) and VVL lectin
286 (Vector Laboratories) (Supplementary Fig. 1B). The proliferation of each transfected cell line (AT3
287 and AT3 Tn^{hi}) was tested in an MTT proliferation assay for three days, and colorimetric analysis
288 was performed with a CytoTek plate reader according to protocol; no significant differences in
289 proliferation compared to parent cells were observed (Supplementary Fig. 1D). Cell lines were
290 maintained in RPMI media supplemented with 10% FBS, sodium pyruvate, HEPES buffer, NEAA,
291 β-mercaptoethanol, and penicillin/streptomycin at 37°C, 5% CO2.

292

293 ***AT3 Tn^{lo} and/or AT3 Tn^{hi} Tumor Challenge***

294 Breast tumor cells were harvested and washed in sterile PBS. Cells were suspended in a final
295 concentration of 2.5E6/ml sterile PBS. Cells were subcutaneously injected into the mammary pads
296 of mice at 2.5E5/100µl/mouse. Mice were observed and euthanized at the tumor endpoint
297 (maximum tumor dimension between 0.9 cm and 1.2 cm, typically after Day 17). For instance, Tn^{lo}
298 tumor-bearing mice analyzed for Fig. 2 were sacrificed on Day 23, and Tn^{hi} tumor-bearing mice
299 were sacrificed on Day 17. RAG1 KO mice in Fig. 4 were sacrificed on Day 23. In cases where
300 tumors were observed to ulcerate or reach maximum growth sooner than the earliest endpoint

301 (before Day 17), the mice were euthanized immediately, and samples were excluded. Tumor
302 volumes were calculated as ((length x width x width)/2) in mm^3.
303
304 ***Flow Cytometry***
305 Cells were stained in PBS with TruStain fcX (BioLegend, Cat. No. 101320) to reduce non-specific
306 antibody binding. Cell samples were stained with the following antibodies and stains (in multiple
307 sets to prevent fluorophore overlap): B220-FITC (BioLegend, clone RA3-6B2), CD11c-PacBlue
308 (BioLegend, clone N418), CD11b-FITC (BioLegend, clone M1/70), CD3e-APC (BioLegend, 145-
309 2C11), CD45-APCCy7 (BioLegend, clone 30-F11), CD19-PE (BioLegend, clone 6D5), NK1.1-
310 PerCP (BioLegend, PK136), Gr1-PECy5 (BioLegend), CD19-APC (BioLegend, clone 1D3),
311 Ly6C-BV605 (BioLegend, clone HK1.4), CD11b-PacBlue (BioLegend M1/70), LycG-BV605
312 (BioLegend clone RTK2758), CD1d-PacBlue (BioLegend, clone 1B1), CD5-PECy5 (BioLegend,
313 clone 53-7.3), CD24-PECy7 (BioLegend, clone 30-F1), CD301b-APC (BioLegend, clone URA-1),
314 CD301b-PE (BioLegend, clone URA-1), CD25-BV605 (BioLegend, clone PC61), CD21-BV510
315 (BioLegend), IL10 PECy7 (BioLegend, clone JES5-16E3), TGFb-BV421 (BioLegend, clone TW7-
316 16B4), IgM-PECy5 (BioLegend, clone RMM-1), IgD-PECy7 (BioLegend, clone 11-26c.2a)
317 IL35:EBI3 subunit – PerCP (R&D, clone 355022), IL35:p35 subunit – AF594 (R&D, clone 27537),
318 and Ghost Red 710 (Tonbo Biosciences). For intracellular staining, cell samples were treated with
319 GolgiStop for four hours prior to the staining protocol. Intracellular staining was performed
320 according to the protocol (BD Biosciences). All isotype controls were obtained from BioLegend
321 except isotypes for IL35 subunits, which were obtained from R&D. Samples were washed and
322 analyzed with flow cytometry (Cytek Aurora or Agilent Novocyte Penteon). Fluorescence minus
323 one (FMO) plus specific isotype control antibody-stained samples were used as negative staining
324 controls, and single stains were used for compensation. Flow cytometry data were analyzed using

325 FlowJo Single Cell Analysis Software (Treestar, Inc., Ashland, Oregon) with gating strategies
326 shown in Supplementary Fig. 2 (example showing tumor tissue from Tn^{hi}-tumor bearing mice).
327

328 ***Splenocyte B Cell Selection and Co-Culture***

329 Splenocytes were obtained from healthy WT C57Bl/6 mice. BioLegend MojoSort antibody kits
330 were used to positively select CD19 cells or negatively isolate Pan B cells, according to
331 manufacturer protocols. Flow cytometry was used to determine the B cell purity of each sample
332 (Supplementary Fig. 3). Viable cells were counted and cultured in 96-well plates at 1E5 cells/100 µl
333 complete RPMI (supplemented with Ca+) /well. Tumor cells were harvested, and viable cells were
334 counted. Indicated cell populations were then plated in each splenocyte sample at 1E5 cells/100 µl
335 complete RPMI (for the "Unstimulated" sample, 100 µl complete RPMI was used without tumor
336 cells), for a final volume of 200 µl /well. Plates were cultured for 3 days at 37°C, 5% CO2. Samples
337 were washed and prepared for flow cytometry analysis.
338

339 ***Statistical Analysis***

340 GraphPad Prism v8 was used for statistical analyses. Two-way ANOVA with Tukey's multiple
341 comparisons test was used to determine statistical significance between experimental groups in
342 each of the applicable experimental models (Fig. 1C, Fig. 2F, Fig. 4D, Fig. 6A). Ordinary one-way
343 ANOVA with Tukey's multiple comparisons test was used to analyze Fig. 5. Mouse survival in
344 Fig. 6C was compared using Log-rank (Mantel-Cox) test. An unpaired parametric two-tailed t-test
345 was used for Figs. 1A, 3G, 4A, 4C, 6B, 6D. Significance is indicated on each graph based on p-
346 value: >0.05 = ns; <.05 = *; <0.01 = **; <0.001 = ***; <0.0001 = ****.
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504
505

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511

512 **Figure legends:**

513 **Fig. 1. Tumor growth and CD301b expression in Tn^{lo} and Tn^{hi} breast cancer models.** AT3
514 (Tn^{lo}) and AT3 cosmc KO cells (Tn^{hi}) (2.5E5/mouse) were injected into the mammary pads of
515 C57BL/6 mice (n = 25). Tumor sizes were monitored. **A.** Tumor sizes were calculated based on
516 (length times $width^2$)/2 for volumes in mm^3. **B-C.** Tumor-free and tumor-bearing mice were
517 euthanized, and tissues were obtained from spleens, lymph nodes, bone marrow, and tumors. Flow
518 cytometry was performed to assess immune profiles. CD45+ cells were gated from total
519 mononuclear cells (FSC/SSC) and viable cells according to the gating strategy in Supplementary
520 Fig. 2. CD301b+ cells were then gated from CD45+ cells and assessed in tumor-free and tumor-
521 bearing tissues.

522

523 **Fig. 2. Characterization of CD45+CD301b+ immune cells.** Tn^{hi} AT3 tumor-bearing mice were
524 euthanized, and tumor tissues were harvested. Single-cell suspensions were stained for indicated
525 immune cell markers. CD45+CD301b+ immune cells were gated according to fluorescence minus
526 one (FMO) plus isotype staining controls (Supplementary Fig. 2) in: **A-C.** CD45+CD301b+ cells
527 were gated in Tn^{hi} tumor tissues, and immune cell populations (B220, CD19, CD11c, CD11b, CD3,
528 NK1.1) were assessed compared to indicated controls. **D.** Quantification of A-C**. E.** Gated
529 $CD45^+CD301b^+B220^+CD19^+$ cells were assessed for CD11c and CD11b. F. Comparison between
530 the total numbers of $CD45^+B220^+CD19^+$ cells, $CD45^+B220^+CD19^+CD301b^+$ cells, and all other
531 $CD45^+$ cells in Tn^{hi} and Tn^{lo} tumors per mg of tumor.

532

533 **Fig. 3. Phenotypes of Tn^{hi} tumor-infiltrating $CD45^+CD301b^+$ B cells.** Tn^{hi} AT3 tumor-bearing
534 mice were euthanized, and tumor tissues were harvested. Single-cell suspensions were stained for
535 indicated immune cell markers. **A-E.** $CD45^+CD301b^+$ B cells ($B220^+CD19^+$) were gated according
536 to FMO) plus isotype staining controls (Supplementary Fig. 2) and assessed for the presence of the
537 surface proteins associated with regulatory B cells **F.** Quantification of **A-E**. **G.** Mean fluorescent
538 intensity (MFI) of CD301b was assessed in gated $CD45^+B220^+CD19^+$ cells infiltrating Tn^{lo} and
539 Tn^{hi} tumors **H**. Splenocytes were obtained from tumor-free C57BL/6 mice. Cells were unselected,
540 positively selected for $CD19^+$ cells, or negatively isolated based on pan B cell markers (please see
541 Supplementary Fig. 3 for cell purity). Each splenocyte sample was co-cultured with or without Tn^{hi}
542 or Tn^{lo} AT3 tumor cells for three days. Flow cytometry was used to analyze $CD301b^+$ expression
543 in $CD45^+B220^+CD19^+$ cells. Indicated values represent the mean fluorescent intensity of $CD301b^+$.

544

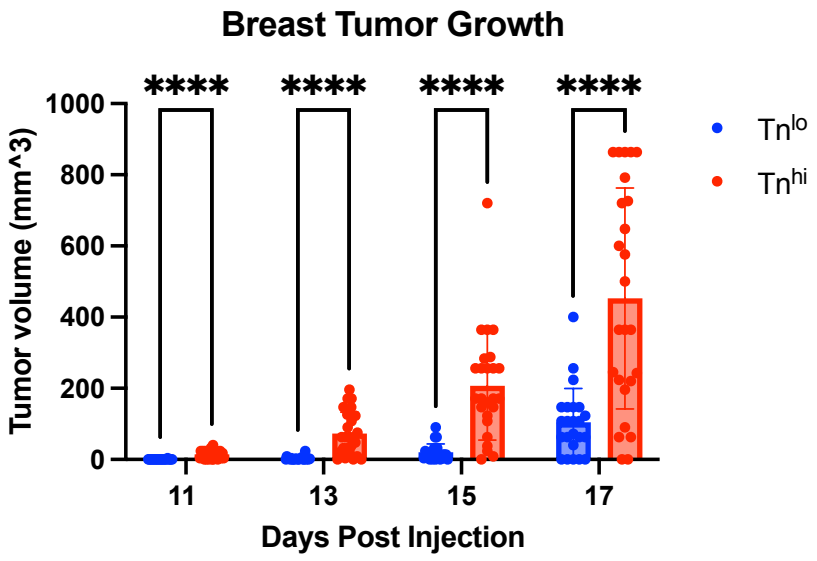
545 **Fig. 4**. **Tumor growth is attenuated in a B cell-deficient mouse model.** Tn^{hi} tumors were injected
546 into the mammary pads of WT or RAG KO mice. Tumor growth was **A.** monitored. When tumor
547 sizes reached a maximum dimension of 1.2 cm, tumor tissues were collected, and **B-C** analyzed for
548 CD301b+ immune cell infiltration. **D.** Total numbers of CD45+B220+CD19+ cells in Tn^{hi} tumors
549 in WT C57BL/6 and RAG KO mice per mg of the tumor.

550

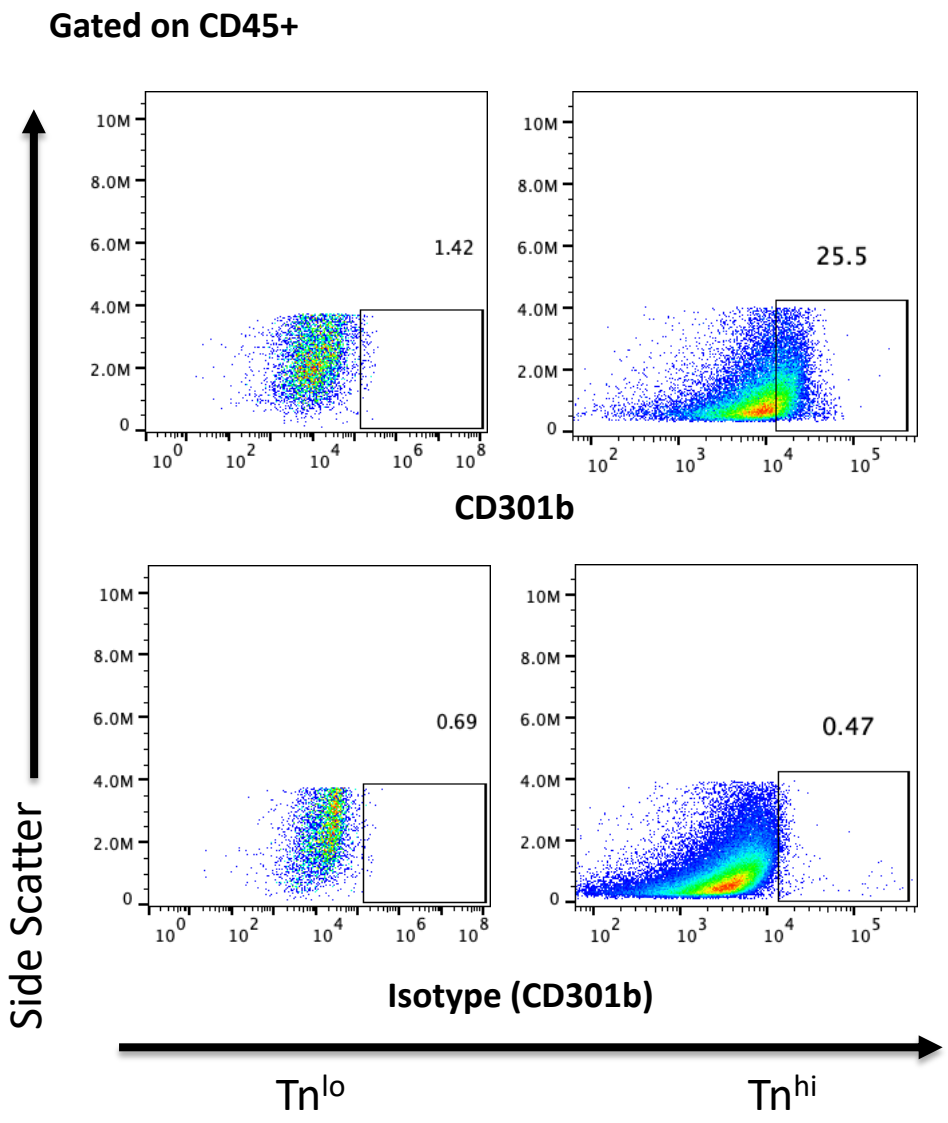
551 **Fig. 5. Tn-associated Regulation by $CD45^+CD301b^+$ tumor-infiltrating immune cells. A-B**
552 AT3 Tn^{hi} (cosmc KO) cells (2.5E5/mouse) were injected into the mammary pad in 4-6 mice per
553 group. Mice were euthanized, and tumors were processed for single-cell suspensions. Cells were
554 stained for extracellular markers, and intracellular staining was used to assess **A.** Mean Fluorescent
555 Intensity (MFI) and **B.** % positive of indicated parent populations of cytokines IL10, IL35 (EBI3

556 subunit), IL35 (p35 subunit), and TGFβ in gated cell populations (please see our gating strategy in
557 Supplementary Fig.4).
558
559 **Fig. 6. Tumor growth in CD301b-depleted and Tn^{hi} breast cancer models. A.** Tn^{lo} (AT3) and
560 Tn^{hi} (AT3 cosmc KO) tumor cells ($2.5x10^5$/mouse) were injected into heterozygous MGL2-DTR
561 mice with or without $CD301b^+$ cells depleted (n= 4 or 5) mice per group into the mammary pad.
562 Tumor sizes were monitored. **B.** Tn^{hi} tumor cells ($2.5x10^5$/mouse) were injected into the mammary
563 pads of homozygous MGL2-DTR mice (n=5). Tumor sizes were monitored. **C**. Methylcholanthrene
564 (MCA) was injected into the mammary pads of WT C57BL/6 and MUC1.Tg (Tn^{hi}) mice to generate
565 spontaneous breast tumors. Survival of mice (tumor dimensions > 1.2cm) were monitored. **D.**
566 MCA-induced tumor tissues were obtained from mice, and flow cytometry was used to assess
567 $CD301b^+$ expression on tumor-infiltrating B cells.

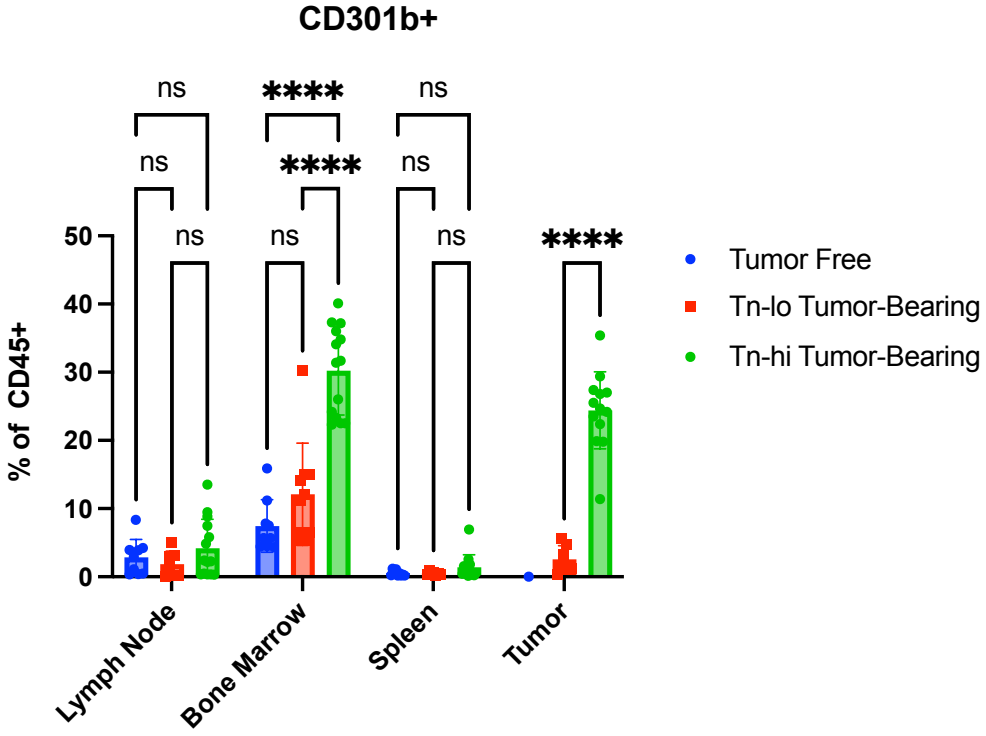
A


Breast Tumor Growth
****
****
****
****
Tumor volume (mm^3)
1000
800
600
400
200
0
11
13
15
17
Days Post Injection
Tn^lo
Tn^hi

B

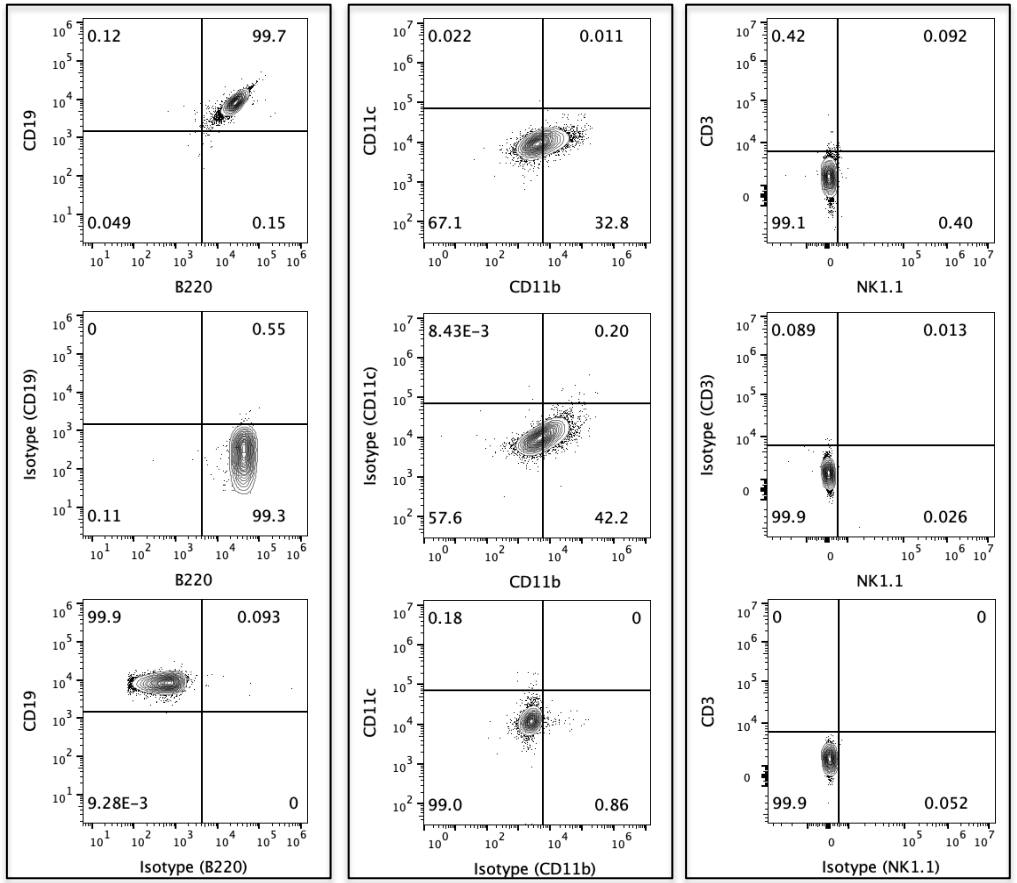

Gated on CD45+
1.42
25.5
CD301b
0.69
0.47
Isotype (CD301b)
Side Scatter
Tn^lo
Tn^hi

C

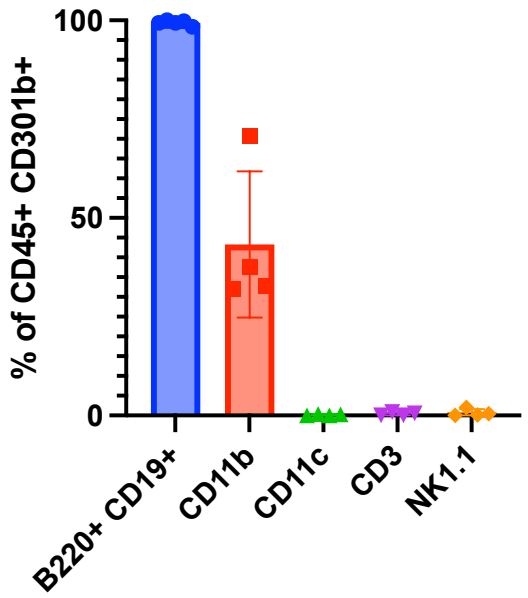

CD301b+
ns
****
ns
ns
****
ns
ns
ns
ns
****
% of CD45+
50
40
30
20
10
0
Lymph Node
Bone Marrow
Spleen
Tumor
Tumor Free
Tn-lo Tumor-Bearing
Tn-hi Tumor-Bearing

Tn^{hi} Tumor-infiltrating Gated on CD45+ CD301b+

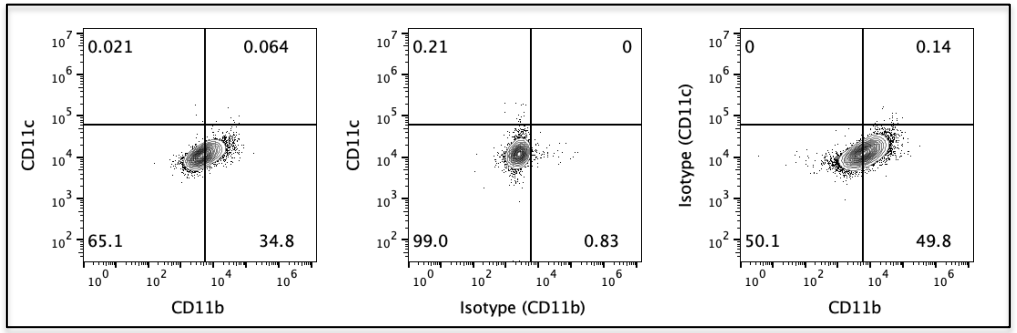
A CD19+ B220+ B CD11c+ CD11b+ C CD3+ NK1.1+



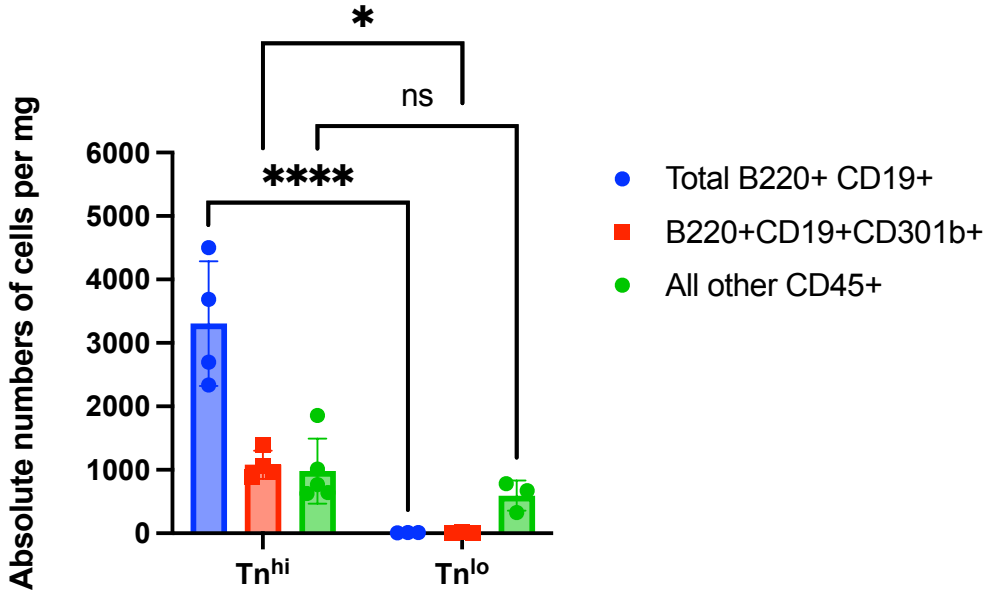
D

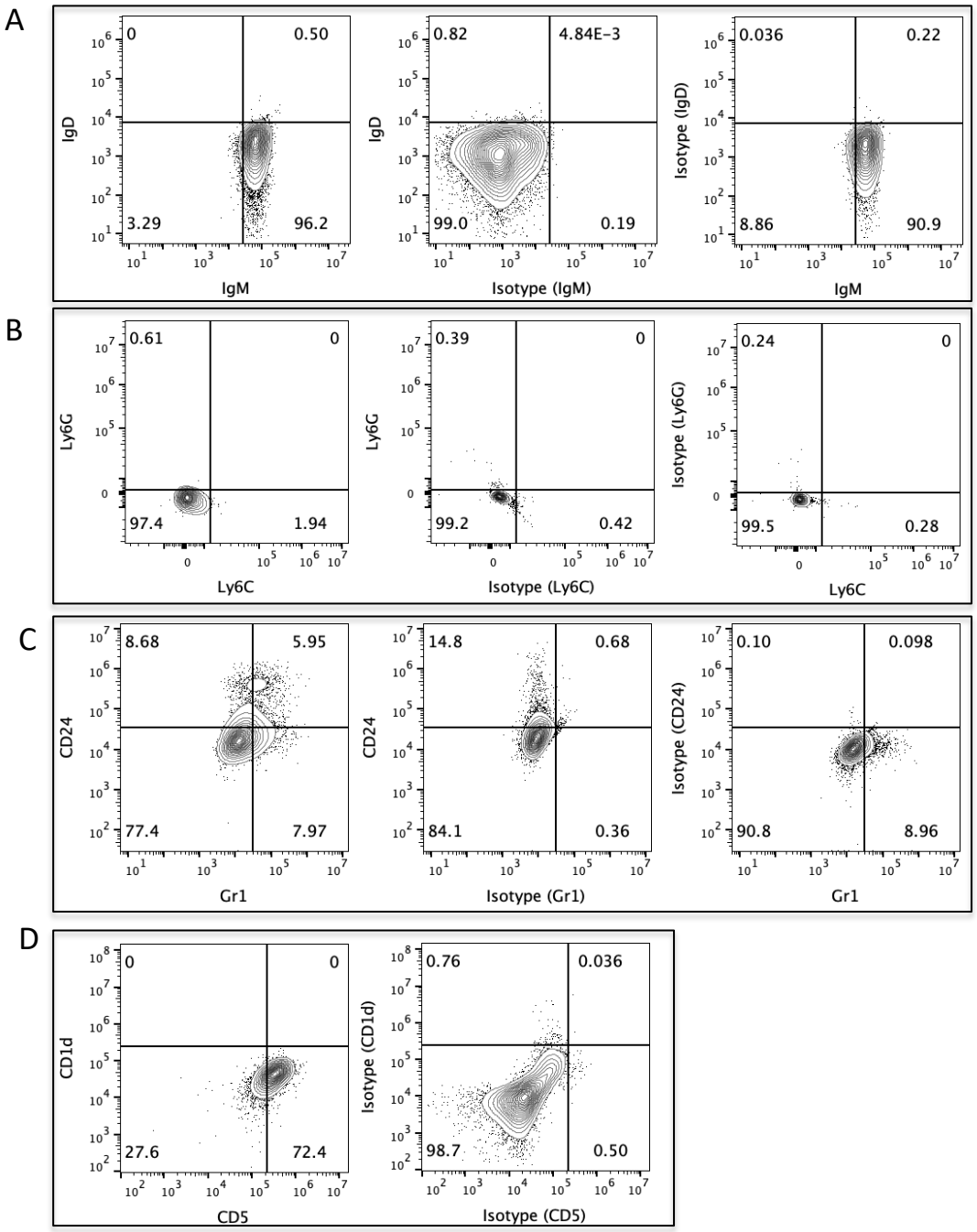


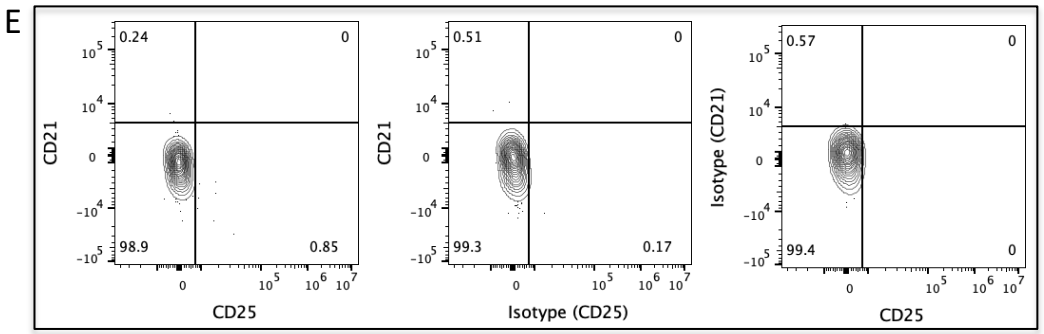
E Tn^{hi} Tumor-infiltrating Gated on CD45+ CD301b+ B220+ CD19+



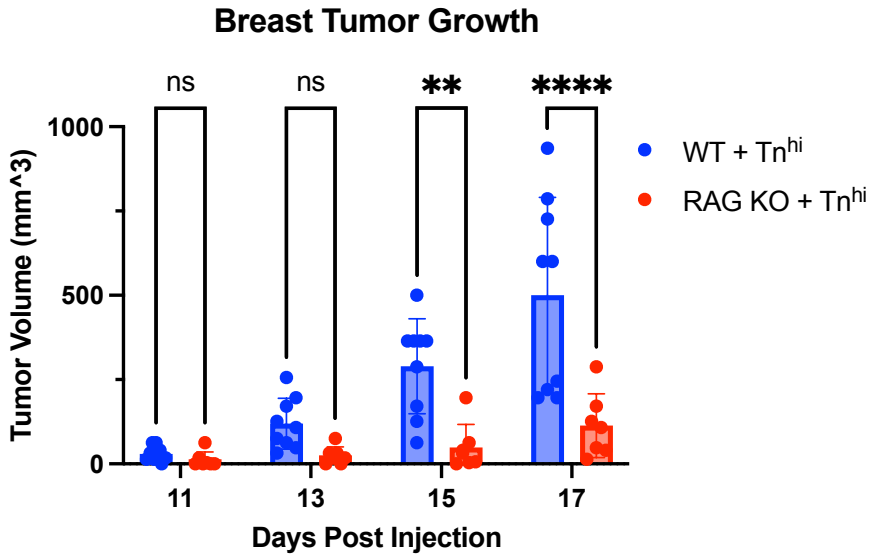
F



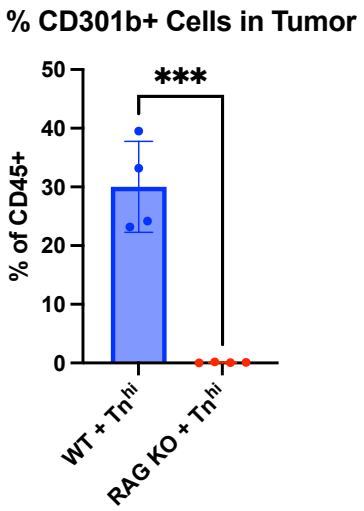

A
IgD
IgM
0
0.50
3.29
96.2
IgD
Isotype (IgM)
0.82
4.84E-3
99.0
0.19
Isotype (IgD)
IgM
0.036
0.22
8.86
90.9
B
Ly6G
Ly6C
0.61
0
97.4
1.94
Ly6G
Isotype (Ly6C)
0.39
0
99.2
0.42
Isotype (Ly6G)
Ly6C
0.24
0
99.5
0.28
C
CD24
Gr1
8.68
5.95
77.4
7.97
CD24
Isotype (Gr1)
14.8
0.68
84.1
0.36
Isotype (CD24)
Gr1
0.10
0.098
90.8
8.96
D
CD1d
CD5
0
0
27.6
72.4
Isotype (CD1d)
Isotype (CD5)
0.76
0.036
98.7
0.50


E
CD21
CD25
0.24
0
98.9
0.85
CD21
Isotype (CD25)
0.51
0
99.3
0.17
Isotype (CD21)
CD25
0.57
0
99.4
0
F
Tumor-Infiltrating CD301b+ B Cell Phenotype
% of CD45+ CD301b+ B220+ CD19+
100
50
0
CD1d
CD5
CD25
CD24
CD21
Gr1
Ly6C
Ly6G
IgM
IgD
G
MFI (E5)
10
8
6
4
2
0
****
Tn^lo
Tn^hi
H
MFI of CD301b-APC on B220+CD19+ Gated Cells from CD45+
MFI
20000
15000
10000
5000
0
Unselected
CD19+ Selected
Pan B Cell Isolated
Unstimulated
AT3 Tn Low
AT3 Tn High

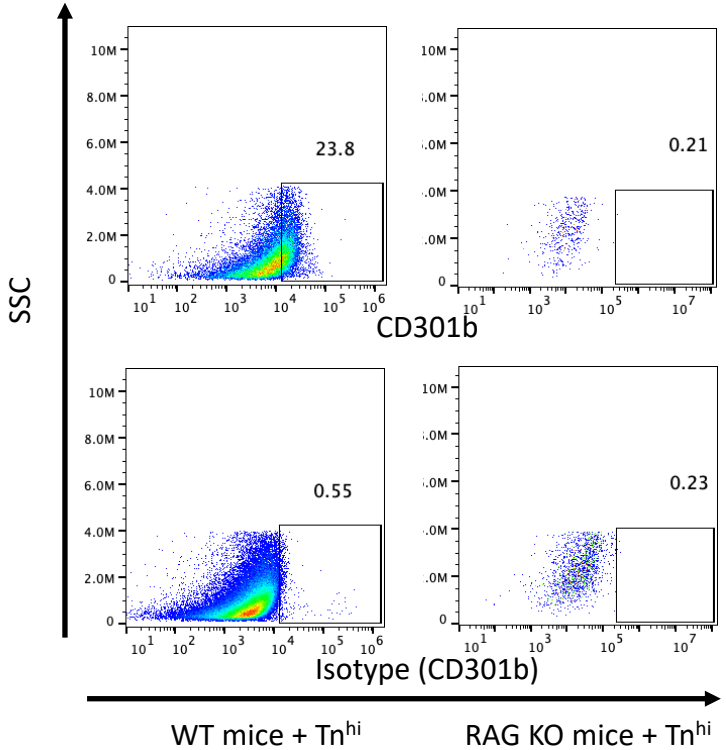
A


Breast Tumor Growth
ns
ns
**
****
1000
500
0
Tumor Volume (mm^3)
11
13
15
17
Days Post Injection
WT + Tn^hi
RAG KO + Tn^hi

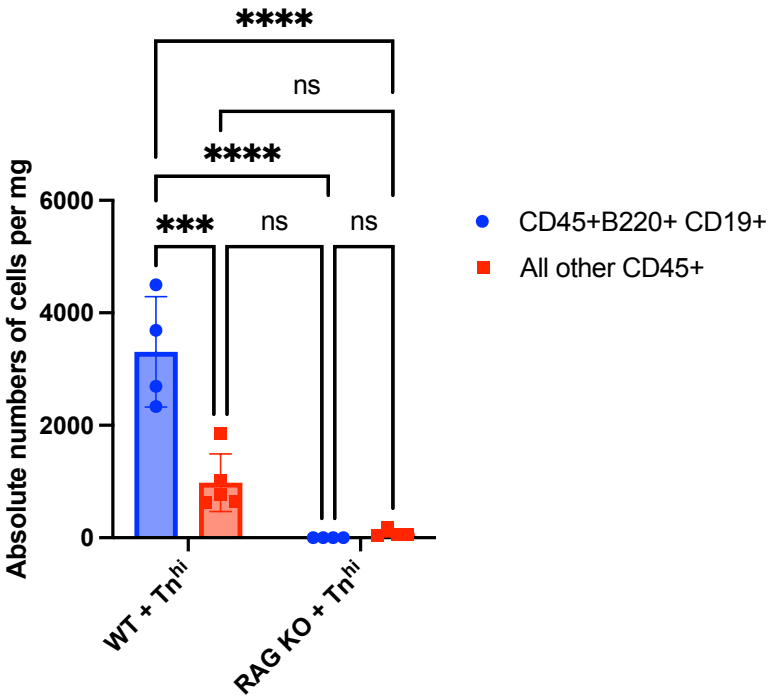
C

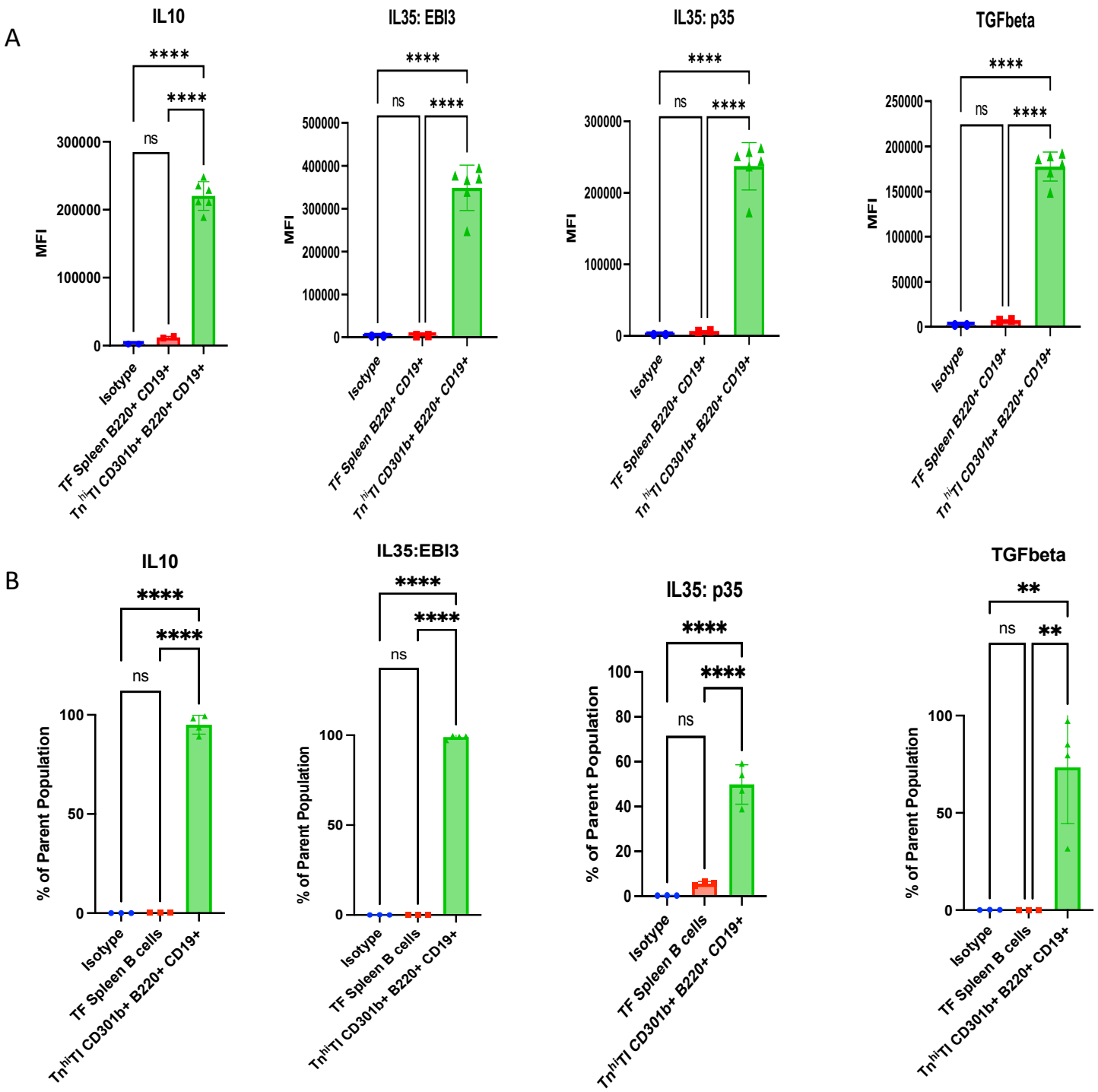

% CD301b+ Cells in Tumor
***
50
40
30
20
10
0
% of CD45+
WT + Tn^hi
RAG KO + Tn^hi

B

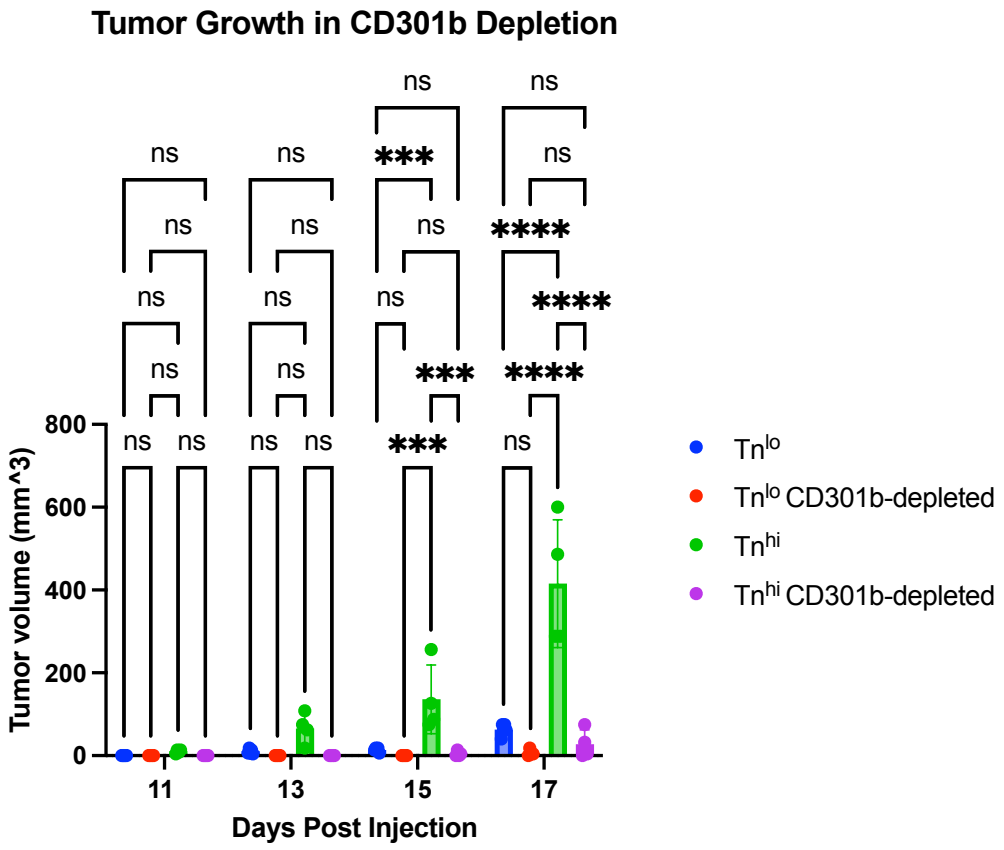

SSC
23.8
0.21
CD301b
0.55
0.23
Isotype (CD301b)
WT mice + Tn^hi
RAG KO mice + Tn^hi

D

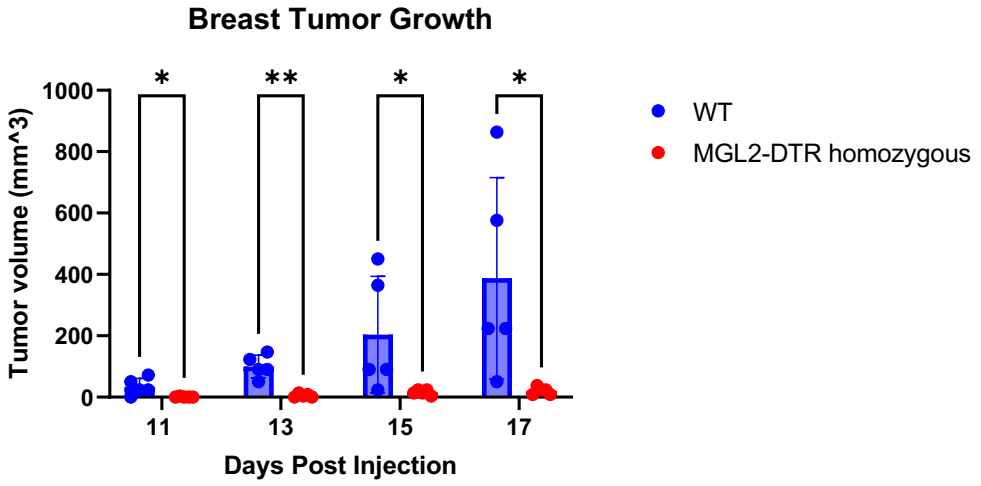

****
ns
****
***
ns
ns
6000
4000
2000
0
Absolute numbers of cells per mg
WT + Tn^hi
RAG KO + Tn^hi
CD45+B220+ CD19+
All other CD45+


A
IL10
IL35: EBI3
IL35: p35
TGFbeta
MFI
****
ns
Isotype
TF Spleen B220+ CD19+
Tn hiTI CD301b+ B220+ CD19+
B
IL10
IL35:EBI3
IL35: p35
TGFbeta
% of Parent Population
**
TF Spleen B cells

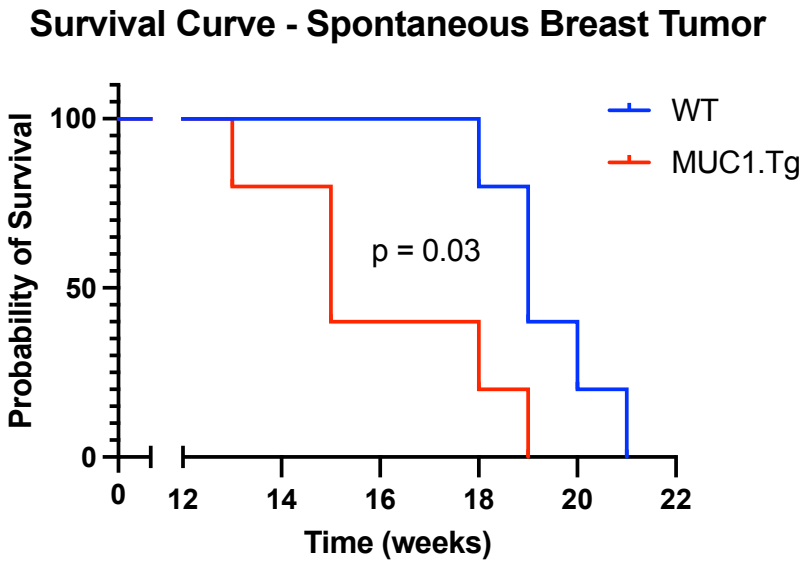
A


Tumor Growth in CD301b Depletion
Tumor volume (mm^3)
Days Post Injection
Tn^lo
Tn^lo CD301b-depleted
Tn^hi
Tn^hi CD301b-depleted

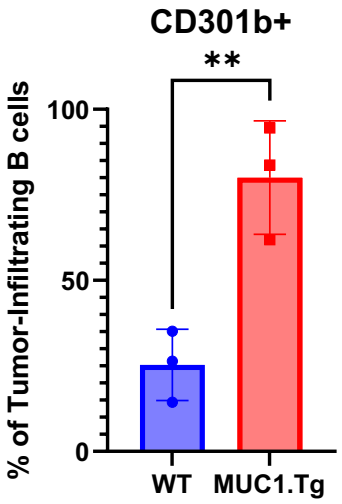
B


Breast Tumor Growth
Tumor volume (mm^3)
Days Post Injection
WT
MGL2-DTR homozygous

C


Survival Curve - Spontaneous Breast Tumor
Probability of Survival
Time (weeks)
p = 0.03
WT
MUC1.Tg

D


CD301b+
% of Tumor-Infiltrating B cells
WT
MUC1.Tg

Supplementary Materials for

A regulatory B cell subpopulation expressing CD301b lectin promotes breast cancer growth

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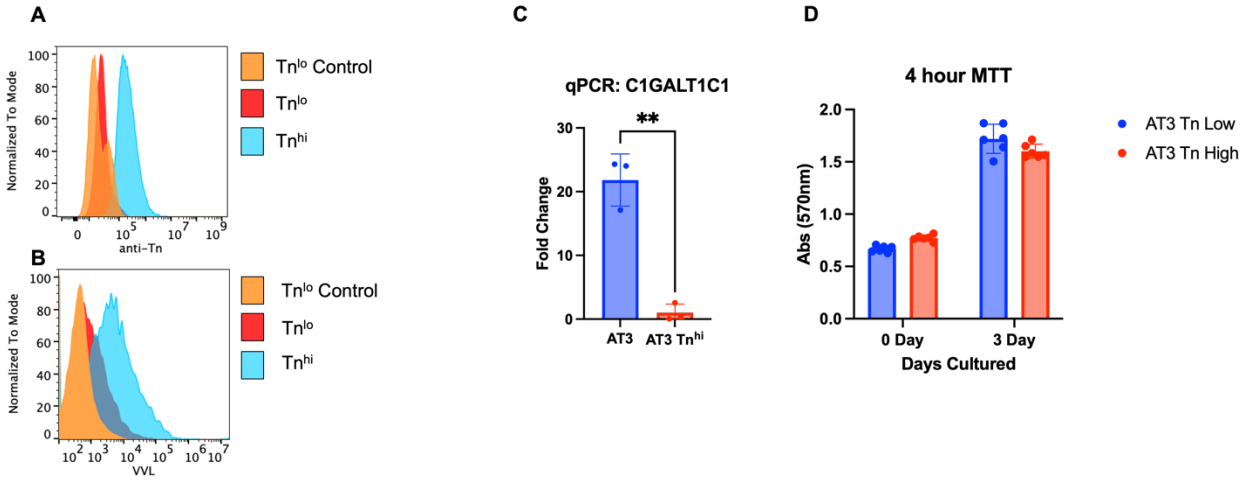
This PDF file includes:

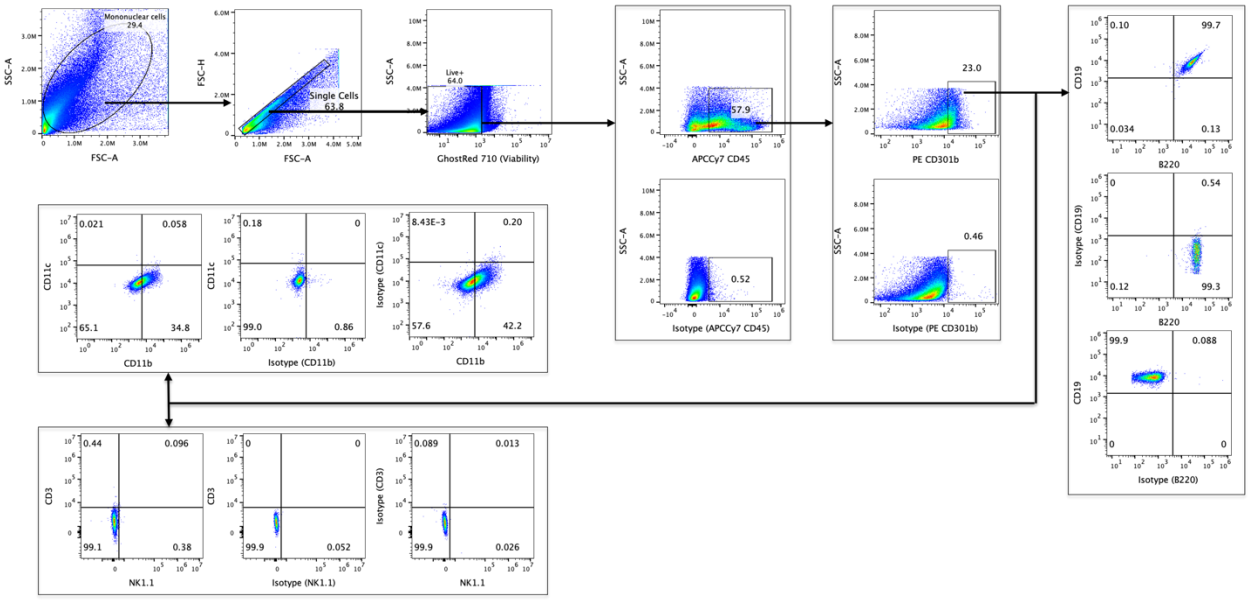
Figs. S1 to S5

Fig. S1.



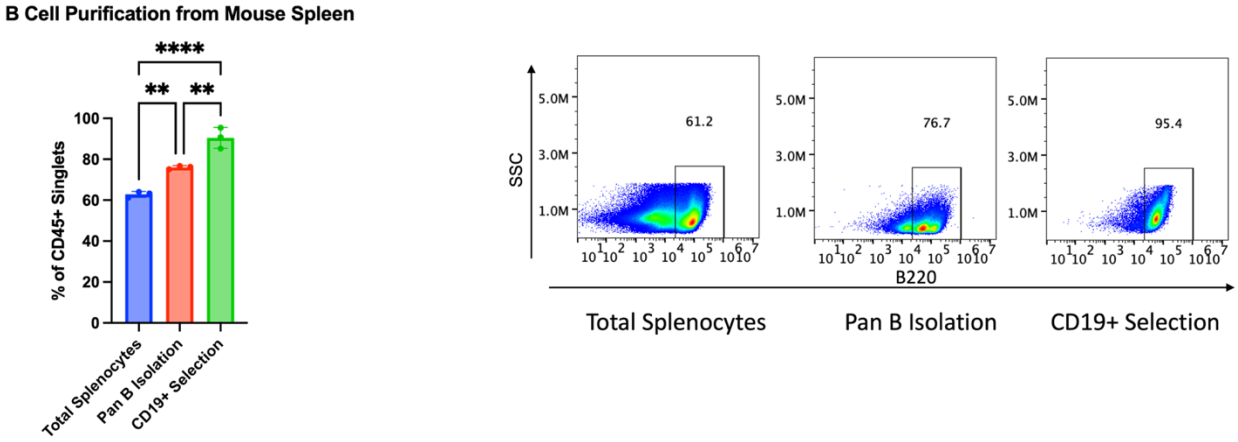
Generation of Tn^{hi} murine breast cancer cells. A-B. CRISPR-Cas9 KO of cosmc chaperone increases Tn cell surface expression in AT3 murine breast cancer cells. AT3 (Tn^{lo}) and AT3 cosmc KO (Tn^{hi}) cells were stained with A. mouse anti-Tn (reBaGs6 IgM) primary and anti-IgM secondary antibodies, with anti-IgM fluorescent antibody only as a control, and B. biotinylated VVL followed by fluorescently-conjugated streptavidin, with fluorescent streptavidin only as a control. **C**. RNA was isolated from AT3 and AT3 Tn^{hi} (cosmc KO) cell lines. qPCR was performed in triplicate wells using validated C1GALT1 (cosmc) qPCR primers and using β-actin as a control for each sample. Fold changes were normalized against the average of Tn^{hi} group. **D.** AT3 (Tn^{hi})and AT3 cosmc KO cells (Tn^{hi}) were cultured in 96 well plates (n=5) at 1E4 cells/200mL for 3 days at 37 degrees. MTT proliferation assay was performed to determine cell growth.

Fig. S2.



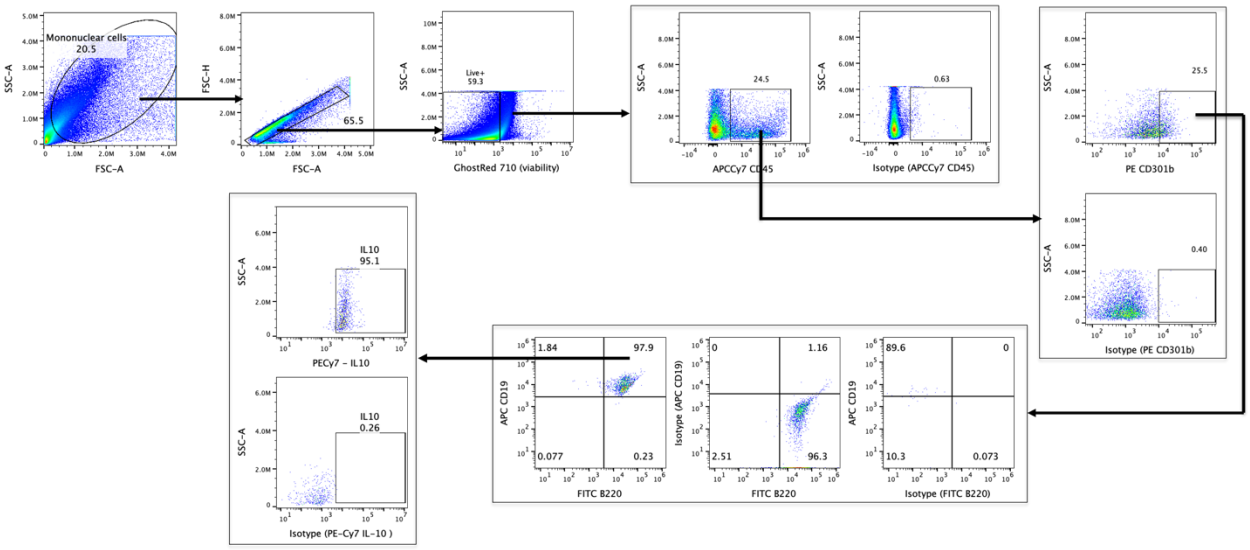
Gating strategy and FMO isotype control representation for flow cytometry analysis of mouse tissues. Bone marrow, lymph nodes, spleens, and tumors were harvested from mice. Single-cell suspensions were generated and stained for flow cytometry using an Fc blocker to minimize background staining. Shown is a representative tumor sample. Immune cells were gated based on the forward and side scatter areas. Singlets were further gated based on forward scatter height. Viable cells were further gated based on GhostRed710 negative cells. CD45+ cells were then gated, and immune populations were gated on CD45+ cells as described.

Fig. S3.



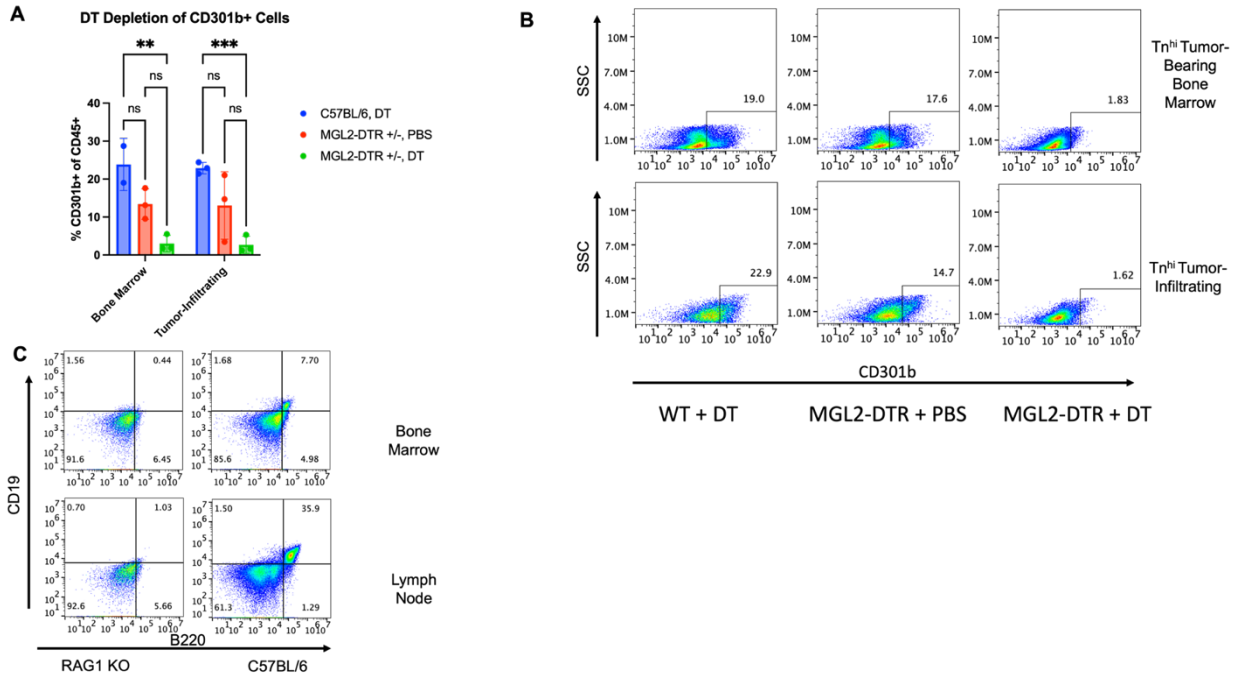
B Cell purification from Mouse Spleen. Spleens were obtained from healthy, tumor-free C57BL/6 mice (n=3). Spleens were processed into single-cell suspensions under sterile conditions. Spleen samples were then aliquoted for unpurified, pan B isolation (according to the BioLegend Pan B Isolation kit for mice) or CD19+ selection (according to the BioLegend CD19+ Selection kit for mice). After magnetic sorting, cell samples were stained with anti-CD45 and anti-B220 fluorescently conjugated antibodies. B cell populations were assessed using flow cytometry to analyze B220+ populations out of CD45+ gated live immune singlets.

Fig. S4.



Intracellular staining of regulatory cytokines. Cell samples were obtained from tissues of Tn^{hi} tumor-bearing mice. Single-cell suspensions were obtained and stained for extracellular markers. Cell samples were then fixed, permeabilized, and stained for indicated intracellular cytokines. Flow cytometry was performed. CD45+ CD301b+ B220+ CD19+ from tumor tissues of tumor-bearing mice were compared to CD45+ B220+ CD19+ gated cells from spleens of tumor-free C57BL/6 mice. Shown is the representative gating strategy along with the appropriate controls.

Fig. S5.



A-B. Depletion of CD301b+ cells following DT treatment in MGL2-DTR mice. Tn^{hi} **(**AT3 cosmc KO cells) tumor cells ($2.5x10^5$/mouse) were injected into the mammary pads of mice. DT was injected intraperitoneally (I.P.) at day -1 before tumor injection and administered every 2-3 days during tumor growth. Mice were euthanized and indicated tissues were processed for single-cell suspensions. Flow cytometry assessed CD301b expression from CD45+ immune cells in bone marrow and tumor. **C.** Validation of B cell absence in RAG1 KO mice. RAG1 KO mice (B6.129S7-Rag1tm1Mom/J) were obtained from Jackson Laboratory. Spleens were obtained, and flow cytometry was used to confirm the absence of CD45+ B220+ CD19+ B cells. <insert page break here>