MAIT Cells Modulate Innate Immune Cells and Inhibit Colon Cancer Growth

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Abstract

Mucosal-associated invariant T (MAIT) cells are innate-like T cells that can be activated by microbial antigens and cytokines and are abundant in mucosal tissues including the colon. MAIT cells have cytotoxic and pro-inflammatory functions. Despite their potential for adoptive cell therapy, studies into their anti-cancer activity, including their role in colon cancer, are limited. We expanded MAIT cells *in vivo* and injected them into RAG1$^{-/-}$ mice with MC38-derived tumors and tumor growth was assessed. Peritumoral injection of MAIT cells inhibits tumor growth compared to control. Genomic and multiplex cytokine analyses show that tumors from the MAIT cell-treated group have higher expression of markers for eosinophil-activating cytokines, suggesting an association between eosinophil recruitment and tumor inhibition. Multiplex cytokine and flow cytometry analyses of human peripheral leukocytes stimulated with MAIT ligand show an increase in eotaxin-1 production and activation of eosinophils, associated with increased cancer cell killing. We documented that MAIT cells have a protective role in a murine colon cancer model, potentially involving novel eosinophil-associated mechanisms. This study provides evidence of the immune modulating potential of MAIT cells within the tumor microenvironment, leading to compositional and functional changes. Our results highlight the potential of MAIT cells for colon cancer immunotherapy.

Brief summary

In models of colon cancer, MAIT cells have anti-tumor activity, associated with increased production of proinflammatory and eosinophil-modulating cytokines.

47 **Introduction**

48 Mucosal-associated invariant T (MAIT) cells are innate-like T cells that recognize microbial-
49 derived products in a TCR-dependent manner via MR-1, a highly conserved MHC class I-related
50 molecule (1, 2). MAIT cells can be activated with the ligand 5-OP-RU, a microbial riboflavin
51 metabolite, for specific stimulation (3, 4). MAIT cells can also be activated in a TCR-independent
52 manner by cytokines, such as interleukins (IL)-7, IL-12, IL-15, and IL-18 (5-7), broadening the
53 modes of activation and the target cell types. MAIT cells are known for their rapid effector
54 function and cytotoxicity upon activation and have been shown to kill infected target cells (2, 8,
55 9). Additionally, MAIT cells have the capacity to interact with and modulate other immune cell
56 types, including B cells, neutrophils, and monocytes (10-13). In humans, MAIT cells are
57 abundant in the peripheral blood (14), as well as in mucosal tissues such as the liver (15), lung
58 (16), and gut (17, 18). In mice, MAIT cell frequencies vary by strain and tissue type, but overall
59 have lower frequencies than in humans (19, 20).

60

61 Due to the abundance of MAIT cells in various mucosal tissues, interest in MAIT cells has
62 expanded beyond its anti-microbial functions, and recent studies have examined their potential
63 role in mucosal tumors. Reports have suggested both pro- and anti-tumor activity of MAIT cells,
64 with some studies demonstrating a modulating effect on Natural Killer (NK) cell anti-tumor
65 activity (21-23). In addition, due to their lack of alloreactive potential and cytotoxic capacity,
66 MAIT cells have been considered for use in immunotherapy (24-26). Evidently, the role of MAIT
67 cells in cancer is complex and context-dependent, warranting further studies in various types of
68 cancer, particularly their potential to interact with other immune cell types.

69

70 As of 2020, human colorectal cancer (CRC) represents about 10% of new cancer cases and
71 death worldwide (27). In addition to surgical resection and adjuvant chemotherapy, the use of
72 immunotherapies is available but limited in efficacy; thus, further understanding of the immune
73 cells involved could help advance therapy (28-30). In human CRC, studies have demonstrated
74 both positive and negative associations between tumor-infiltrating MAIT cells and overall
75 survival (31, 32). MAIT cells accumulate in CRC tumors (17, 31, 33) and tumor-infiltrating MAIT
76 cells display bacterial antigen recognition signatures (34), but the impact of these cells in the
77 tumor microenvironment is not well understood. Tumor-infiltrating MAIT cells retain their
78 cytotoxicity and production of cytokines (17, 35), but have impaired interferon- γ (IFN-γ)
79 production (17, 36) and high levels of exhaustion markers (33, 36). A multi-omics analysis of
80 CRC demonstrated an abundance of intratumor microbes and their immune modulation
81 potential on tumor-infiltrating immune cells, including MAIT cells (32). The prevalence of
82 intratumoral bacteria in CRC (32, 34) and the specificity of MAIT cells for microbial antigens
83 warrant further investigation into the role of MAIT cells in CRC.

84

85 Here, we show that peritumoral injection of purified MAIT cells inhibits MC38 cells-derived
86 tumor growth in a mouse model of colon cancer. We show that this tumor inhibition is
87 associated with changes in the immune cell profile and functional changes, including higher
88 levels of myeloid cell-modulating cytokines and chemokines. Culture of human peripheral
89 leukocytes with colon cancer cell line resulted in the activation of eosinophils upon MAIT cell
90 stimulation. Together, our data suggest a novel mechanism by which MAIT cells can modulate

91 innate immunity affecting eosinophils n a mixed immune cell environment and provide
92 protection in a murine model of colon cancer, highlighting the potential of MAIT cells to be a
93 candidate for CRC immunotherapy.
94
95 **Results**
96 **Peritumoral injection of MAIT cells leads to inhibition of tumor growth in the MC38 model of**
97 **CRC**
98 To test the effect of MAIT cells on colon cancer growth, MAIT cells were expanded in C57BL/6J
99 wildtype (WT) mice for 7 days, isolated (Supplemental Fig. 1), and were peritumorally injected
100 into RAG1$^{-/-}$ mice with MC38 cells-derived tumor 1 day after cancer cell injection (Fig. 1A). We
101 observed that the growth of MC38 cells-derived tumors was significantly lower ($p<0.05$) when
102 mice were treated with MAIT cells, as shown in the final tumor volume and weight at harvest
103 (Fig. 1B-E). The same effect was also observed when MAIT cells were injected with ligand 5-A-
104 RU and methylglyoxal (MGO) into both MC38-bearing RAG1$^{-/-}$ and C57BL/6J WT mice
105 (Supplemental Fig. 2). These data suggest that MAIT cells provide a protective role in this
106 murine subcutaneous model of colon cancer.
107
108 **MAIT cell peritumoral injection leads to changes in the tumor microenvironment.**
109 To examine potential functional and compositional changes induced by MAIT cell peritumoral
110 injection, RNA was isolated from extracted tumors and quantitative real-time reverse-
111 transcription PCR (qRT-PCR) was performed to evaluate the expression of apoptosis- and
112 oxidative stress-related genes relative to saline treated control group. We observed higher gene

113 expression of *Caspase3* ($p < 0.01$) *and Fas* ($p < 0.05$) in tumors treated with MAIT cells
114 (Supplemental Fig. 3A). Consistently, caspase 3/7 protein activity was detected to be
115 significantly higher in tumors in the MAIT cells-treated group compared to the control group
116 ($p<0.01$; Fig. 2A), supporting the tumor inhibition effect observed *in vivo*. Using gene markers
117 for various innate immune cells, we observed a non-significant increase in *Siglecf* and *Ccr3*
118 expression in tumors in mice from the MAIT treatment group compared to control, suggesting
119 an impact on eosinophils, while there was no evidence of changes in NK (*Klrb1c*) or macrophage
120 *(Adgre1*) cell markers (Supplemental Fig. 3B). Consistent with the increase in eosinophil
121 markers mentioned above, an increased expression of eotaxin-2 (also known as *Ccl24)* and
122 cytokine *Il5* ($p = 0.052$) was observed in the MAIT cells treatment group (Supplemental Fig 3C).
123 Overall, our results suggest that the treatment of MC38-bearing mice with MAIT cells leads to
124 increased cell death and eosinophil-activating cytokine.
125
126 To confirm functional changes at the protein level, we analyzed released molecules from the
127 extracted tumor. Tumor pieces (8 mg) were incubated in complete media for 18 hours and the
128 resulting supernatants were analyzed using Luminex multiplex cytokine and chemokine
129 analysis. We observed a significant increase in the production of various cytokines, including
130 the proinflammatory cytokines IFN-γ, and IL-17, and GM-CSF ($p<0.05$; Fig. 2B, C, and D), which
131 provides further evidence that the introduction of MAIT cells into MC38 cells-derived tumors
132 leads to the reprogramming of the tumor microenvironment. Consistent with the increase in
133 eosinophil gene markers mentioned above, a higher level of eotaxin-1 was detected in the
134 tumors extracted from mice in the MAIT treatment group (Fig. 2E). To determine the

135 plausibility that these cytokines could be produced by activated MAIT cells, we cultured sorted
136 mouse MAIT cells alone *in vitro* with a T cell activation cocktail. We confirmed significantly
137 higher levels of each of the above cytokines compared to unstimulated conditions
138 (Supplemental Fig. 4). Together, we observed functional changes of immune cells and
139 reprogramming of the tumor microenvironment associated with *in vivo* tumor inhibition.
140

141 **Activation of human MAIT cells with a MAIT ligand enhances the killing of colon cancer cells**

142 To model the effect of human MAIT cells on colon cancer cells in the presence of other immune
143 cells, we utilized human whole leukocytes co-cultured with the human colon cancer cell line
144 COLO 205 (Fig. 3A). Calcein release assay was used to examine cytotoxicity and culturing with
145 human whole leukocytes for 4 hours leads to specific lysis of COLO 205 cells (Fig. 3B). Activating
146 MAIT cells with the MAIT ligand, 5-A-RU and MGO, significantly increased the specific lysis
147 capacity of the leukocytes ($p<0.05$; Fig. 3B). Flow cytometry analysis also demonstrates
148 enhanced killing of COLO 205 by whole leukocytes with the addition of the MAIT ligand in a 16-
149 hour co-culture, with a significant reduction in the frequency of CFSE-labeled cells ($p<0.05$; Fig.
150 3C). Together, these data show that MAIT cell-specific activation with 5-A-RU enhances the
151 killing of colon cancer cells.
152

153 **Stimulation of MAIT cells in human peripheral leukocytes induces eotaxin-1 production in**
154 **MAIT cells and activation of eosinophils in an MR-1 dependent manner**

155 To determine the immune modulating potential of human MAIT cell in a mixed cell population,
156 we used human whole leukocyte cultures to examine the effect of MAIT cell activation in a cell

157 culture model of CRC. MAIT cells were selectively stimulated with 5-A-RU overnight and culture
158 supernatant was analyzed using multiplex cytokine and chemokine assay. In 5-A-RU stimulated
159 cultures, we found a higher level of Macrophage inflammatory protein (MIP)-1α, MIP-1β, and
160 Monocyte chemoattractant protein (MCP)-1 ($p< 0.05$ and $p<0.01$; Fig. 4A), suggesting a capacity
161 of MAIT cells to modulate macrophages and monocytes and granulocytes (37-40). Consistent
162 with the recruitment of eosinophils with MAIT treatment in our murine model (Fig 2A), we
163 found that concentrations of IL-5, GM-CSF, and eotaxin-1 levels are significantly higher in
164 cultures stimulated with the MAIT ligand 5-A-RU/MGO compared to unstimulated leukocytes
165 ($p<0.05$ and $p<0.01$; Fig. 4A). In addition, we observed that cytokines known to activate and
166 attract eosinophils, including IL-4 ($p<0.01$) and IL-8 ($p=0.0547$) (41, 42), are also significantly
167 higher in 5-A-RU/MGO stimulated cultures compared to control (Fig. 4A). Together, these data
168 demonstrate the immune modulating capacity of human MAIT cell activation in a mixed cell
169 population, specifically the potential to modulate eosinophils.
170
171 Previous studies have demonstrated the ability of eosinophils to kill COLO205 cells in a
172 granzyme A-dependent manner (43). To determine if MAIT cell activation can induce eosinophil
173 activation and cytotoxic function in a setting with mixed cell population of leukocytes, we
174 cultured human peripheral leukocytes with or without the MAIT ligand 5-A-RU/MGO overnight
175 and analyzed cytokine expression and levels of activation of MAIT cell and eosinophil using flow
176 cytometry (Supplemental Fig. 1). Ligand stimulated MAIT cells had significantly higher
177 expression of the activation marker CD69 ($p<0.01$) and of eotaxin-1 ($p<0.001$) (Fig. 4B).
178 Compared to unstimulated cultures, whole leukocyte cultures stimulated with the MAIT ligand

179 also shows a significant increase in eosinophil activation, marked by CD69 and granzyme A
180 expression ($p<0.0001$; Figure 4C). These data show that in peripheral leukocyte populations, the
181 addition of the MAIT ligand induces the expression of eosinophil-activating cytokines in MAIT
182 cells and correspondingly, eosinophil activation.
183
184 To confirm if eosinophil activation upon 5-A-RU/MGO stimulation is mediated via MR1 or a
185 broad consequence of antigen processing, anti-MR1 neutralizing antibody was added to the
186 human leukocyte cultures 1 hour before stimulation with 5-A-RU/MGO. In the presence of anti-
187 MR1, MAIT cell activation and the expression of eotaxin-1 are significant reduced ($p < 0.001$ and
188 $p < 0.0001$; Fig. 4B). Similarly, the activation and the induction of effector molecule granzyme A
189 in eosinophils induced by MAIT cell stimulation are significantly reduced in the presence of anti-
190 MR1 antibody ($p < 0.001$; Fig. 4C), suggesting that the effect of 5-A-RU/MGO on eosinophils is
191 dependent on MR1.
192
193 Together, these data show that the addition of 5-A-RU/MGO, a MAIT-specific ligand, leads to an
194 increased production of eosinophil-recruiting chemokines and cytokines by MAIT cells and
195 eosinophil activation, highlighting the ability of MAIT cells to modulate eosinophils in a MR1-
196 dependent manner.
197
198 **Discussion**
199 While MAIT cells are primarily known for their ability to kill microbially infected cells, other
200 potential roles have emerged. Specifically, due to their abundance at mucosal tissues, there has

201 been an increasing interest in the role of MAIT cells in mucosal tissue-associated cancers. MAIT
202 cells are a promising potential candidate for cancer immunotherapies: MAIT cells lack
203 alloreactive potential, thus having an advantage over the use of conventional T cells with HLA-
204 restrictions for the application of universal, non-donor-restrictive adoptive cell therapy (24).
205 MAIT cells also express higher levels of the multi-drug efflux protein MDR1, conferring higher
206 persistence against certain chemotherapy drugs compared to conventional $CD8^+$ T cells and
207 thus an ideal target for use in immunotherapy coupled with chemotherapy (25, 26, 44).
208 Additionally, the checkpoint inhibitor, anti-PD1, has been shown to enhance the cytotoxicity of
209 MAIT cells in patients with prostate cancer (45), basal and squamous cell carcinoma, and
210 hepatocellular carcinoma (46-48). Recent studies have demonstrated the ability to generate
211 chimeric antigen receptor (CAR)-MAIT cells, which retain their anti-tumor capacity even in the
212 presence of immunosuppressive tumor-associated macrophages (49, 50). Furthermore,
213 protocols for MAIT cell expansion have been established, including demonstration that, when
214 expanded with a human serum replacement, MAIT cells display enhanced functionality and low
215 levels of exhaustion markers, making them optimized for adoptive therapy (51, 52).
216
217 In this study, we provided evidence that MAIT cells have an anti-tumoral role both in an MC38
218 cell-derived allograft tumor model in $RAG1^{-/-}$ mice and against human colon cancer cells. We
219 used RAG deficient mice which are characterized by the lack of mature B and T cells to exclude
220 the potential impact of the presence of endogenous T cells. In the murine model of colon
221 cancer, we observed tumor inhibition, associated with a significant increase in the production
222 of anti-tumor cytokines, and evidence suggesting the recruitment of eosinophils with increases

223 in both gene and protein expression of eotaxin, IL-5, and SiglecF, among others. In human
224 leukocyte and human colon cancer cell co-culture, we observed enhanced levels of COLO 205
225 lysis after stimulation with MAIT ligand 5-A-RU/MGO, demonstrating their cytotoxic capacity
226 against colon cancer cells. In addition, MAIT cell activation in human whole leukocytes leads to
227 the production of a wide range of cytokines and chemokines, including eotaxin-1, GM-CSF, and
228 Mip-1, highlighting the potential for anti-tumor effects through their immune-modulating
229 properties (10). Particularly, we observed that MAIT cell activation resulted in an increase in the
230 production of IL-5 and eotaxin-1, and GM-CSF, and an increase in activation and effector
231 function of eosinophils, the effect of which was MR1-dependent.
232
233 We observed the capacity of MAIT cell to modulate innate cell populations in our models of
234 colon cancer. We found significantly higher levels of eotaxin-1, known to recruit and activate
235 eosinophils, in both mouse *in vivo* and human *in vitro* models. *In vitro* studies have
236 demonstrated that eosinophils have the ability to induce direct killing on colon cancer cells (43,
237 53), supported by an *in vivo* study of two colon cancer models showing that eosinophil
238 contributes to tumor suppression (53). Another study using a subcutaneous MC38 cells-derived
239 tumor model in WT mice has shown that eosinophil inversely correlates with tumor growth and
240 the observed tumor suppression was dependent on GM-CSF and eosinophil recruitment (54).
241 This is consistent with our experiment where we observed an association between MAIT cell-
242 mediated inhibition of MC38-derived tumors and an increase in tumor production of GM-CSF
243 and eotaxin-1 production in the tumors. With eosinophils' known ability to release effector
244 molecules like major basic protein (MBP) into the tumor microenvironment to induce cancer

245 cell lysis (55), these data support the involvement of eosinophils in MAIT-mediated tumor
246 suppression.
247
248 In addition to the production of eosinophil modulating effectors, we report MAIT cell
249 production of other pro-inflammatory and immune-modulating cytokines, GM-CSF, IFN-γ, and
250 IL-17. GM-CSF regulates the proliferation, differentiation, recruitment, and effector functions of
251 myeloid cell populations (11, 12, 56), and its addition of GM-CSF to human primary monocytes
252 inhibits COLO 205 proliferation (57). In murine colon cancer, neutrophils play a role in limiting
253 tumor progression, invasion and proliferation (58). We found higher GM-CSF in tumors from
254 the MAIT cell-injection group, suggesting a potential role for myeloid cell populations in
255 mediating the observed anti-tumor effect. We also found increased expression of IFN-γ in
256 MAIT-treated tumors. IFN-γ is a cytotoxic cytokine that can act directly on tumor cells to induce
257 tumor elimination (59-62). Specifically, IFN-γ inhibits MC38-derived tumor growth in mice (63)
258 and COLO 205 in vitro (64). In addition to the direct effects on tumor cells, IFN-γ also has
259 immune modulating effects on innate immune cells, including the enhancement of the anti-
260 tumor activity of eosinophils against colon cancer cell lines, including MC38 (53). Finally, our
261 demonstration of increased IL-13 and IL-17 in MAIT-treated tumors is consistent with studies
262 showing their production by MAIT cells (26, 65, 66). In line with our murine model of colon
263 cancer, a prior study showed that treatment with recombinant IL-17 in MC38-bearing mice
264 results in tumor inhibition (63).
265

266 Taken together, our study employing both murine model and human cell cultures demonstrates
267 a protective role of MAIT cells in colon cancer. While further analysis of immune cell activity in
268 the tumor microenvironment is needed to fully understand the interaction between MAIT cells
269 and other immune cells, our findings suggest a novel role of MAIT cells in recruitment and
270 activation of eosinophils. The mechanism by which MAIT cell mediates MC38-derived tumor
271 inhibition, particularly the role of eosinophils, needs further study, including how MAIT cells
272 could be harnessed in future therapeutic applications against colon cancer.
273

274 **Methods and Materials**

275 Mouse and cell lines

276 CL57BL/6 wild type and B6.129S7-*Rag1*^{tm1Mom}/*J* (*Rag1*$^{-/-}$) mice were purchased from the
277 Jackson Laboratory (Bar Harbor, MA, USA). Mice were housed at the University of Utah CMC
278 Animal Facility on a 12h – 12h light-dark cycle and were provided with water and normal rodent
279 chow ad libitum. Mouse and human cell lines, MC38 and Colo205, were purchased from the
280 American Type Culture Collection (ATCC, Manassas, Virginia, United States) and maintained in
281 RPMI complete media supplemented with 10% fetal bovine serum (FBS, Thermo Fisher
282 Scientific, Waltham, MA, USA), 1% penicillin/streptomycin cocktail (Corning, Tewksbury, MA,
283 USA) and 25mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA).
284

285 *In vivo* MAIT cell expansion and isolation

286 On day 0, C57BL/6J mice were anesthetized with isoflurane and intranasally inoculated with a
287 50 mL solution (25 mL per nare) of PBS containing 200 nmol 5-A-RU (67), 50 mM methylglyoxal

288 (Sigma-Aldrich, St. Louis, MO, USA), and 16.67 mg Pam2CSK4 (InvivoGen, San Diego, CA, USA).
289 The reaction of methylglyoxal with 5-A-RU results in the formation of the MAIT-activating ligand
290 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (68). On days 1, 2 and 4, mice
291 were again inoculated with 200 nmol 5-A-RU and 50 mM methylglyoxal. On day 7, mice were
292 euthanized, lungs were perfused with 5 mL of PBS, and then single-cell lung suspensions were
293 prepared using the gentleMACS Mouse Lung Dissociation Kit (Miltenyi Biotech, Bergisch
294 Gladbach, North Rhine-Westphalia, Germany) according to the manufacturer's protocol. Lung
295 suspensions were then pooled into male and female groups and stained with fluorochrome-
296 conjugated antibodies for MAIT isolation using fluorescent-associated cell sorting (FACS). Lung
297 suspensions were first incubated with Fixable Viability Dye eFluor 780 (BD Biosciences, Franklin
298 Lakes, NJ, USA) and then blocked with anti-mouse CD16/CD32 Fc block (Biolegend, San Diego,
299 CA, USA) and unlabeled MR1-6-formylpterin-tetramer (6-FP) (NIH Tetramer Core Facility at
300 Emory University, Atlanta, GA, USA) to minimize non-specific binding. Suspensions were then
301 stained for 30 minutes at room temperature with the MAIT cell TCR binding MR1-5-OP-RU-
302 tetramer (NIH Tetramer Core Facility at Emory University, Atlanta, GA, USA) and the following
303 antibodies: anti-CD3-PE-Dazzle594, anti-TCRb-BV421, anti-CD4-FITC, anti-CD8-APC, anti-TCRgd-
304 PE-Cy7, anti-CD44-BV650, and anti-CD45R-PE-Cy5 (Supp. Table 1). MAIT cells were gated as
305 single cell lymphocyte, live, B220-, TCR γδ-, $CD44^{hi}$, CD3+, TCRβ+ and MR1-5OPRU+. Following
306 FACS, sorted MAIT cells were washed in sterile PBS and resuspended at $1x10^6$ cells per ml for
307 use for peritumoral injection in the MC38 cancer model.
308
309 MAIT cell peritumoral injection in MC38 mouse model.

310 In Rag1-/- mice, $1x10^6$ MC38 cells were injected subcutaneously on Day 0. $1x10^5$ FACS-sorted,
311 sex-matched MAIT cells were then injected peritumorally on Day 1. For control group, mice
312 were injected with an equivalent volume of PBS. Tumor growth was monitored until Day 15-21
313 and the mice were sacrificed to obtain the tumors. Extracted tumors were weighed at the end
314 of the experiment.
315
316 Mouse MAIT cell in vitro stimulation
317 Expanded lung MAIT cells from C57BL/6J donor mice were cultured in vitro at 50,000 cells/well.
318 Cells were either left unstimulated or stimulated with 1X eBioscience Cell Stimulation Cocktail
319 (500X, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in 100uL complete RPMI
320 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, supplemented with 10% FBS, 1%
321 penicillin/streptomycin, 1mM HEPES). After overnight culture, supernatants were analyzed
322 using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel - Premixed 25 Plex -
323 Immunology Multiplex Assay (EMD Millipore Corporation, Billerica, MA, USA). and custom FAS
324 panel on the Luminex MAGPIX.
325
326 Detection of tumor-released cytokines
327 8 mg (± 0.5mg) of each mouse tumor was incubated in 500 µL of complete RMPI medium for 18
328 hours and 25 µL of the resulting supernatant was analyzed using Luminex bead assay for
329 various soluble markers using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead
330 Panel - Premixed 25 Plex - Immunology Multiplex Assay (EMD Millipore Corporation, Billerica,

331 MA, USA). and custom FAS panel on the Luminex MAGPIX. Measurements below detectable
332 limits were considered as 0.
333
334 Caspase activity measurement
335 Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega Corporation, Madison, WI, USA) was
336 used to determine the caspase activity in the extracted tumors. 8 mg tumor pieces were
337 homogenized in Apo-ONE® Caspase-3/7 Reagent using a bead beater. After 1 freeze-thaw to
338 ensure lysis, samples were incubated for 30 minutes on a shaker and analyzed according to
339 manufacturer's protocol. Caspase 3/7 activity is measured as arbitrary fluorescent unit (AFU)
340 using a SpectraMax Mini plate reader (Molecular Devices, San Jose, CA, USA).
341
342 RNA isolation and qRT-PCR
343 RNA was extracted from tumor pieces stored in RNAlater or flash-frozen using Trizol (Thermo
344 Fisher Scientific, Waltham, MA, USA). Extracted RNA was converted to cDNA using High-
345 Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA)
346 according to the manufacturer's protocol. qRT-PCR was performed using TaqMan Real Time
347 PCR Assays (Thermo Fisher Scientific, Waltham, MA, USA) with comparative Ct (ΔΔCt) analysis
348 on QuantStudio5.
349
350 Human whole leukocyte preparation and culture

351 Human whole leukocytes from anonymous healthy donors were isolated from apheresis cones
352 from ARUP using RBC sedimentation with HetaSep (STEMCELL Technologies, Vancouver, British
353 Columbia). Freshly isolated whole leukocytes were used fresh for *in vitro* culture.
354
355 Whole leukocytes were cultured with or without 5-A-RU (50 nM) and MGO (50 µM) in 200 µL
356 RPMI complete media and incubated at 37 °C overnight. CD69, and eotaxin, granzyme A, and in
357 MAIT cells and Eosinophils were analyzed using flow cytometry. To detect released cytokines in
358 the medium, 25 µL of supernatant was analyzed using Cytokine 35-Plex Human Panel for the
359 Luminex™ platform (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) on the Luminex
360 MAGPIX. To block interactions activation of MAIT cell via MR1, anti-MR1 (Clone 26.5, Biolegend,
361 San Diego, CA, USA) or an isotype control was used at 10 µg/ml.
362
363 COLO 205 Killing Assays
364 For Calcein Release Assay, 50,000 Calcein-labeled (2 µM, Biolegend, San Diego, CA, USA) COLO
365 205 cells were co-cultured with 500,000 human whole leukocytes for 4 hours with or without 5-
366 A-RU (50 nM) and MGO (50 µM) in 200 µL RPMI complete media at 37 °C. Maximum release
367 was set up with 2% Triton X-100 (Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA).
368 All samples and controls were set up in triplicates. Cultures were gently mixed and centrifuged,
369 and 100 µL of culture supernatant was transferred to a new plate and analyzed for fluorescence
370 intensity using BioTek Synergy H1 Multimode Reader (BioTek Instruments, Inc, Winooski, VT,
371 USA). Percent Specific Lysis was expressed as a percent relative to maximum release control,
372 calculated as [(Fluorescence $_{Sample}$ – Fluorescence $_{Untreated}$)/ (Fluorescence $_{max}$ – Fluorescence

373 $_{Untreated}$)] x 100. For flow cytometry-based killing assay, 100,000 CFSE-stained (Immunochemistry

374 Technologies, Davis, California, CA, USA) COLO 205 was co-cultured with 10^6 human whole

375 leukocytes for 16 hours and the frequency of CFSE+ cells was analyzed.

376

377 Flow Cytometry and FACS

378 Isolated mouse lung cells were stained and MAIT cells were sorted using FACS Aria III Cell Sorter

379 (BD Bioscience). Stained human *in vitro* samples were analyzed using Cytek Aurora (Cytek

380 Biosciences, Fremont, CA, USA). FCS files were analyzed using FlowJo™ v10.8 Software (BD Life

381 Sciences, Franklin Lakes, NJ, USA). MAIT cells were defined from the lymphocyte as live singlet,

382 $CD45^+$, $CD3^+$, MR1-5-OP-RU-Tetramer$^+$, Vα7.2$^+$ cells. Eosinophils were gated from the

383 granulocyte population as live singlet, $CD45^+$, $CD3^-$, $CD44^+$, Siglec8$^+$ cells. Gating strategies are

384 included in Supplemental Figure 1.

385

386 Antibodies used for FACS and Flow Cytometry

387 List of antibodies used in FACS of mouse MAIT cells.

Antibody/Viability dye	Clone	Vendor
eBioscience Fixable Viability Dye eFluor™ 780	NA	Invitrogen, Thermo Fisher Scientific
Anti-CD45R/B220	RA3-6B2	Biolegend
Anti-gd	GL3	Biolegend

Anti-CD3	17A2	Biolegend
Anti-CD44	IM7	Biolegend
MR1-5-OP-RU	NA	NIH Tetramer Core
Anti-TCRb	H57-597	Biolegend

388

389 List of antibodies used in flow cytometry analysis of human leukocyte cultures.

Antibody/Tetramer	Clone	Vendor
Zombie UV	NA	Biolegend
Anti-CD45	HI30	Biolegend
Anti-CD45RO	UCHL1	BD Biosciences
Anti-CD3	SK7	BD Biosciences
Anti-CD4	SK3	BD Biosciences
Anti-CD8	RPA-T8	Biolegend
MR1-5OPRU	NA	NIH Tetramer Core
Anti-Vα7.2	3C10	Biolegend
Anti-CD161	HP-3G10	Biolegend
Anti-CD25	BC96	Biolegend
Anti-CD69	FN50	BD Biosciences
Anti-CD44	IM7	Biolegend
Anti-Siglec8	7C9	Biolegend
Anti-Granzyme A	CB9	Biolegend

Anti-Eotaxin-1	IC3201U	R&D Systems

390

391 Statistical Analysis

392 Statistical analysis was performed using GraphPad Prism version 8 for Mac (GraphPad Software,
393 Boston, Massachusetts USA). Two-tailed Mann-Whitney U test was used to compare tumor
394 weights between groups, and Wilcoxon ranked t test was used to compare variables between
395 treatment groups in experiment involving paired human samples.

396

397 Study Approval

398 Mouse studies were approved by the University of Utah IACUC, protocol numbers 21-09-16.
399 The protocol to use human whole leukocytes from anonymous blood donors was reviewed by
400 the Institutional Review Board of the University of Utah and determined to be exempt
401 (IRB_00097389)

402

403 Author Contributions

404 OJC, EJB, and DTL designed the experiments. OJC, EJL, OJ, DJ, and ST performed the
405 experiments. OJC analyzed the data. EJB and DTL supervised the work. OJC, OJ, EJB and DTL
406 wrote the paper. EJB and DTL obtained funding. All authors reviewed and approved the final
407 manuscript.

408

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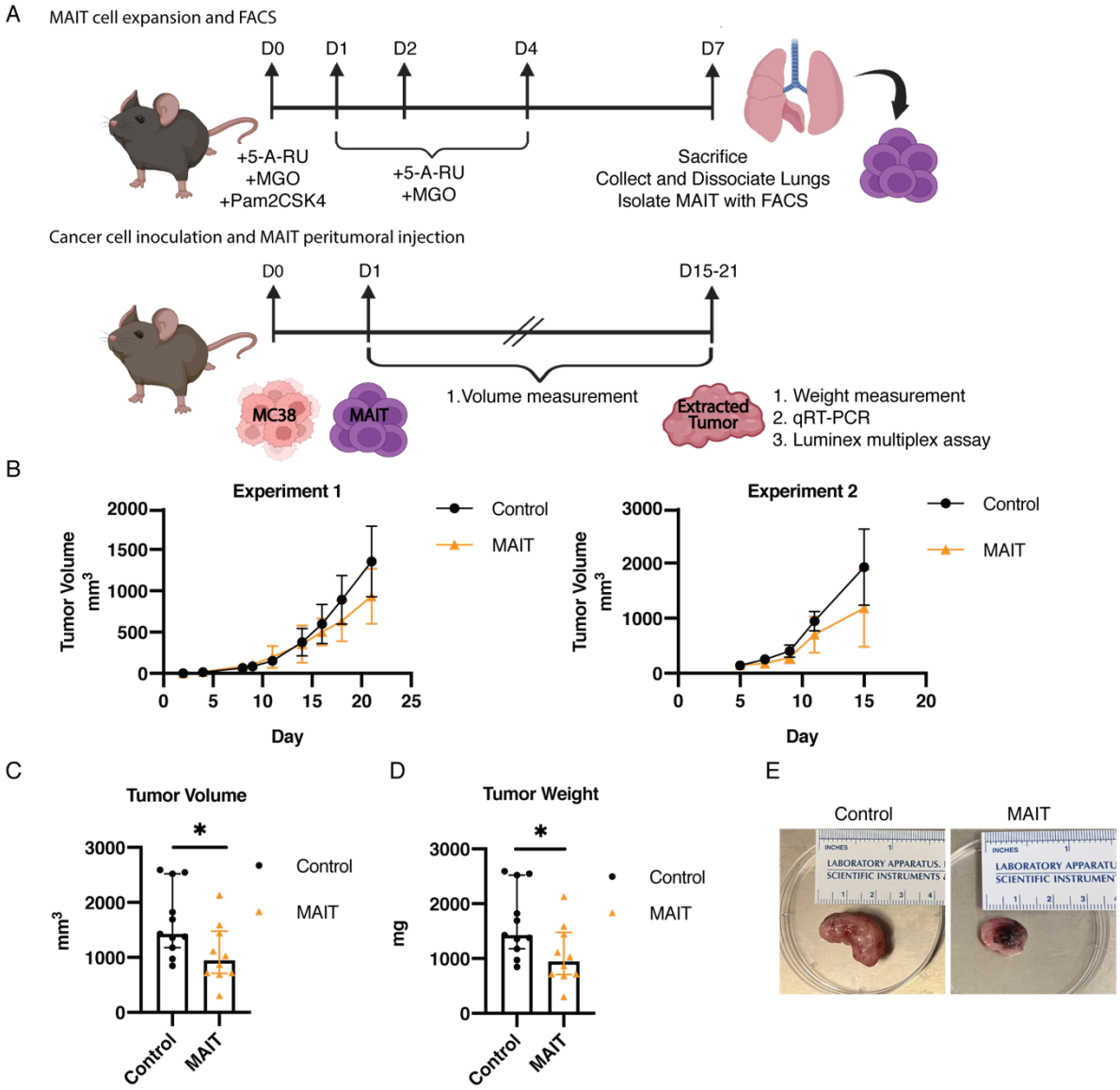

Figure 1. MAIT cell peritumoral injection leads to inhibition of MC38 cells-derived tumor growth

A) Experimental setup of MAIT cell expansion in C57BL/6J mice and MAIT cell peritumoral injection in MC38-bearing RAG1$^{-/-}$ mice. MAIT cells were expanded in C57BL/6J mice for 7 days and sorted MAIT cells were injected peritumorally in RAG1$^{-/-}$ mice 1 day after MC38 cell injection. Control: n = 11 males. MAIT: n = 10 (9 males and 1 female). Tumor volume was monitored during the course of the experiment. Tumors were extracted at the end of the experiment for weight measurement, qRT-PCR analysis and Luminex multiplex analysis. B) MC38 cells-derived tumor volume in mm^3 over time from 2 independent experiments. C-D) Tumor measurements. Each dot represents a tumor sample and data is shown as median with interquartile range. C) Volume of extracted tumors in mm^3 and D) Weight of extracted tumors in mg. *$p<0.05$ by two-tailed Mann-Whitney U test. E) Representative photos of extracted tumors from each experimental group. Figure created using BioRender.com

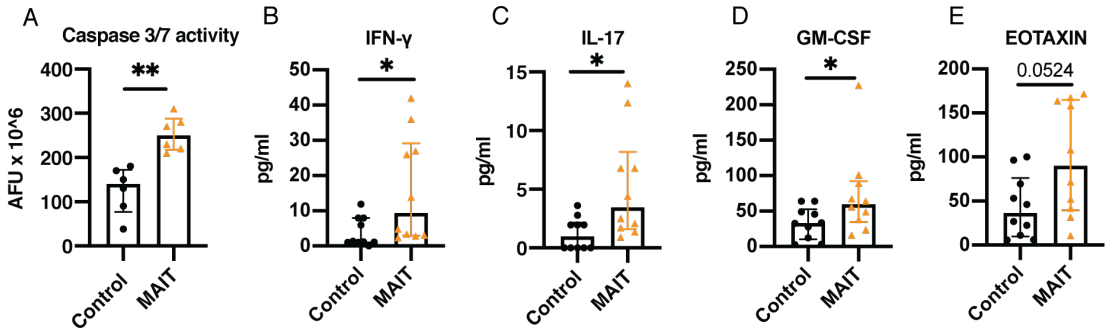

Figure 2. MAIT cell peritumoral injection induces changes in cytokines produced within tumors.

Comparison of (A) caspase activity and (B – E) concentration of various cytokines and chemokines released from extracted tumors between control group and MAIT group. A) Caspase activity is measured in arbitrary fluorescent unit (AFU). B) GM-CSF, C) eotaxin, D) IFN-γ, and E) IL-17. 25 µL of supernatants from 18-hour culture of 8 mg tumor pieces were analyzed using Luminex multiplex assay. Each dot represents an individual sample of supernatant and data is shown as median with interquartile range. *$p<0.05$ **$p<0.01$ by two-tailed Mann-Whitney U test.

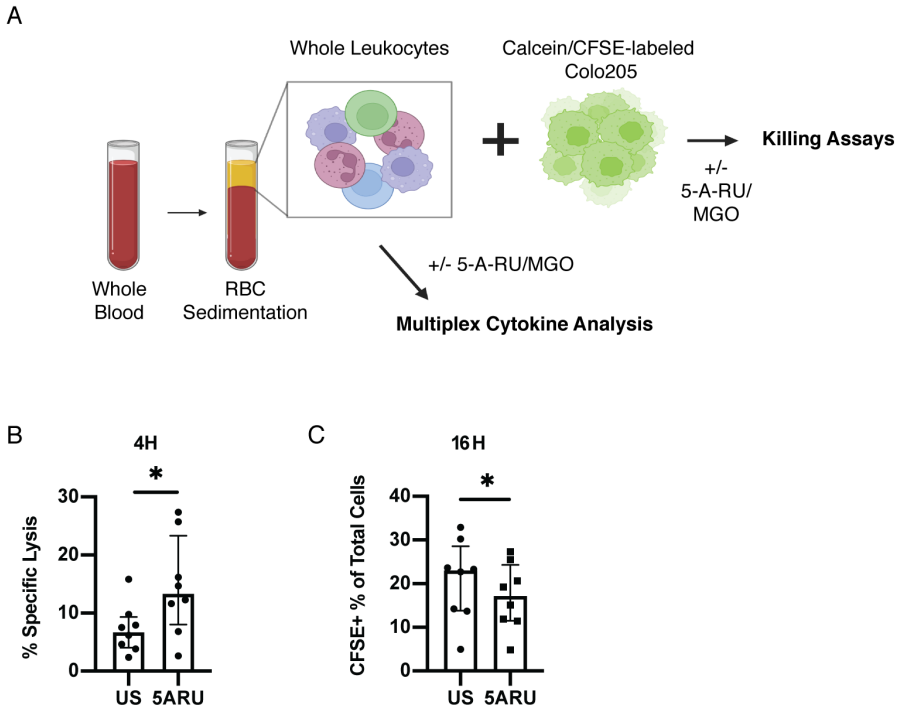

Figure 3. Activation of MAIT cells enhance killing against colon cancer cells.

A) Experimental setup of human whole leukocyte isolation and killing assay co-culture with COLO 205. B) Percent specific lysis of COLO 205 co-cultured with human whole leukocytes with or without 5-A-RU/MGO stimulation using a 4-hour Calcein Release Assay. C) Frequency of CFSE+ cells after overnight co-culture of human whole leukocytes with COLO 205 with or without 5-A-RU/MGO stimulation for 16 hours by flow cytometry. Figure created using BioRender.com. *$p<0.05$ by Wilcoxon ranked test.

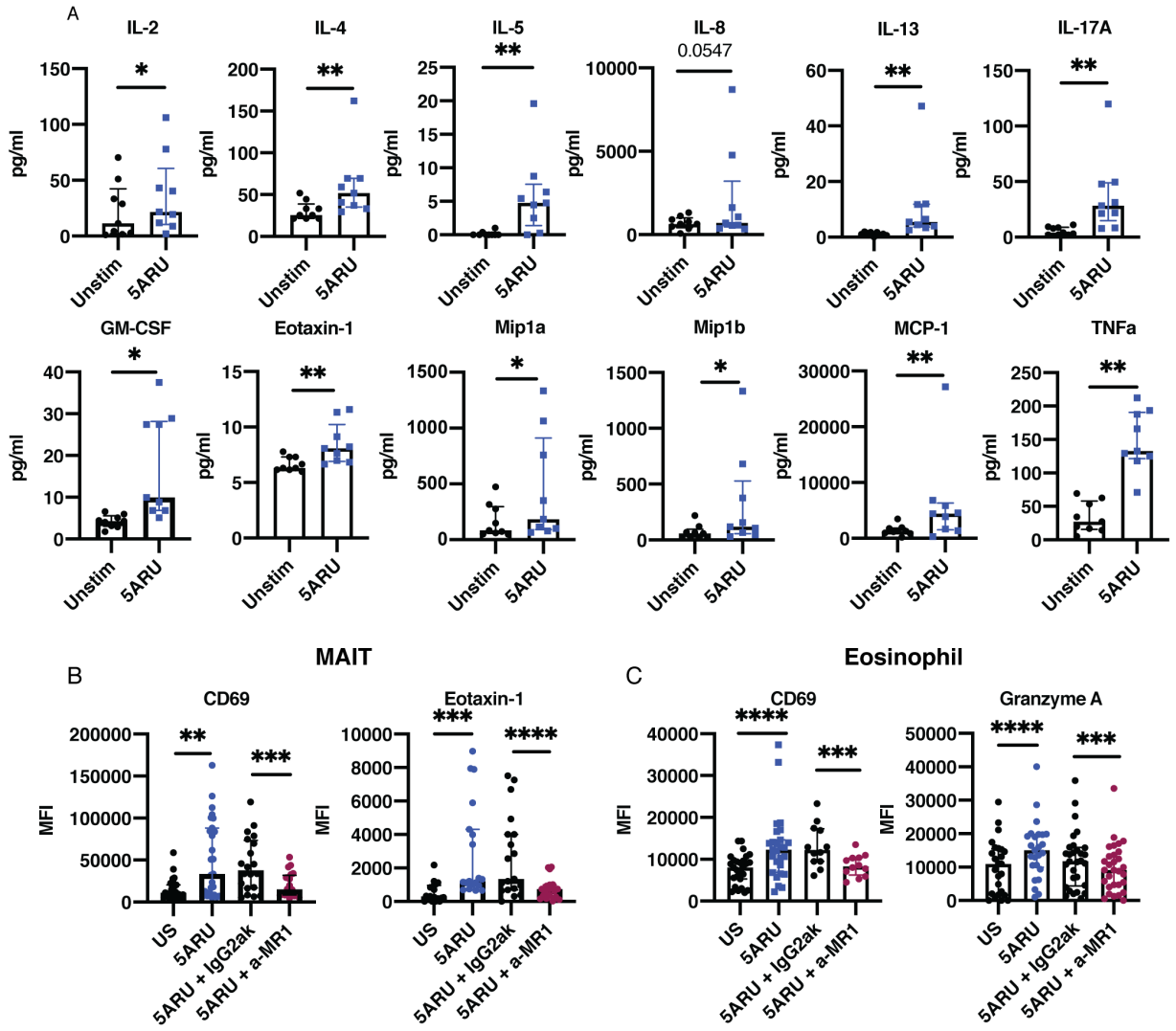

Figure 4. Stimulation of human peripheral leukocytes with MAIT ligand induces eotaxin-1 production in MAIT cells and activation of eosinophils in an MR-1 dependent manner.

A) Supernatant concentration of various cytokines and chemokines detected in overnight cultures of human whole leukocytes with or without 5-A-RU/MGO stimulation analyzed by Luminex multiplex assay. Each dot represents an individual sample of supernatant from a healthy donor and data is shown as median with interquartile range. B) Flow cytometry analysis of CD69 and eotaxin-1 expression in MAIT cells. C) Flow cytometry analysis of CD69 and granzyme A expression in eosinophils. (B and C) Expression of each marker is shown as MFI (median fluorescence intensity). Each dot represents a sample from an individual healthy donor.
**p<0.01 *** p<0.001 **** p<0.0001 by Wilcoxon ranked test.