MAIT Cells Modulate Innate Immune Cells and Inhibit Colon Cancer Growth

Olivia J. Cheng[1,2], Eric J. Lebish[3], Owen Jensen[1,2], Damian Jacenik[3,4], Shubhanshi Trivedi[2], Jackson Cacioppo[6], Jeffrey Aubé[6], Ellen J. Beswick*[3,5], Daniel T. Leung*[1,2]

[1]Division of Microbiology & Immunology, Department of Pathology, University of Utah, Salt Lake City, Utah, United States

[2]Division of Infectious Disease, Department of Internal Medicine, University of Utah, Salt Lake City, Utah, United States

[3]Division of Gastroenterology, Department of Internal Medicine, University of Utah, Salt Lake City, Utah, United States

[4]Department of Cytobiochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

[5]Division of Digestive Diseases and Nutrition, Department of Internal Medicine, University of Kentucky, Lexington, Kentucky, United States

[6]Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States

Corresponding Authors: Daniel T. Leung, MD

26 N Medical Drive Room 517, Salt Lake City, Utah 84132

+1 801-581-8812, Daniel.leung@utah.edu

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*Co-senior authors

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Study Approval

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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 and have potentials for use as adoptive cell therapy. However, studies into their anti-cancer activity, including their role in colon cancer, are limited. Using an animal model of colon cancer, we show that peritumoral injection of *in vivo*-expanded MAIT cells into RAG1$^{-/-}$ mice with MC38-derived tumors inhibits tumor growth compared to control. 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. In a human peripheral leukocyte co-culture model, we show that leukocytes stimulated with MAIT ligand show an increase in eotaxin-1 production and activation of eosinophils, associated with increased cancer cell killing. In conclusion, we show that MAIT cells have a protective role in a murine colon cancer model, associated with modulation of the immune response to cancer, potentially involving eosinophil-associated mechanisms. Our results highlight the potential of MAIT cells for non-donor restricted 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.

52 **Introduction**

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

65

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

74

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

89

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

96 innate immunity by affecting eosinophils in a mixed immune cell environment and provide
97 protection in a murine model of colon cancer, highlighting the potential of MAIT cells to be a
98 candidate for CRC immunotherapy.
99
100 **Results**
101 **Peritumoral injection of MAIT cells leads to inhibition of tumor growth in the MC38 model of**
102 **CRC**
103 To test the effect of MAIT cells on colon cancer growth, MAIT cells were expanded in C57BL/6J
104 wildtype (WT) mice for 7 days, isolated (Supplemental Fig. 1), and were peritumorally injected
105 into RAG1$^{-/-}$ mice with MC38 cells-derived tumor 1 day after cancer cell injection (Fig. 1A). We
106 observed that the growth of MC38 cells-derived tumors was significantly lower ($p<0.05$) when
107 mice were treated with MAIT cells, as shown in the final tumor volume and weight at harvest
108 (Fig. 1B-E). The same effect was also observed when MAIT cells were injected with ligand 5-A-
109 RU and methylglyoxal (MGO) into both MC38-bearing RAG1$^{-/-}$ and C57BL/6J WT mice
110 (Supplemental Fig. 2). These data suggest that MAIT cells provide a protective role in this
111 murine subcutaneous model of colon cancer.
112
113 **MAIT cell peritumoral injection induces cell death and cytokine production in the tumor**
114 **microenvironment.**
115 Consistent with the observed inhibition of tumor growth (Fig. 1), caspase 3/7 protein activity
116 was detected to be significantly higher in tumors in the MAIT cells-treated group compared to

117 the control group ($p<0.01$; Fig. 2A), suggesting that the treatment of MC38-bearing mice with
118 MAIT cells leads to increased cell death.
119
120 To assess any functional changes in the tumor microenvironment, we analyzed released
121 cytokines and chemokines from the extracted tumor. Tumor pieces (8 mg) were incubated in
122 complete media for 18 hours and the resulting supernatants were analyzed using Luminex
123 multiplex cytokine and chemokine analysis. Out of the 31 cytokines and chemokines analyzed
124 (Supplemental Table 1), we observed a significant increase in the production of various
125 cytokines, including the proinflammatory cytokines IFN-γ, and IL-17, and GM-CSF ($p<0.05$; Fig.
126 2B, C, and D), which provides further evidence that the introduction of MAIT cells into MC38
127 cells-derived tumors leads to the reprogramming of the tumor microenvironment. Additionally,
128 a higher level of eotaxin-1 ($p = 0.0524$) was detected in the tumors extracted from mice in the
129 MAIT treatment group (Fig. 2E). To determine the plausibility that these cytokines could be
130 produced by activated MAIT cells, we cultured sorted mouse MAIT cells alone *in vitro* with a T
131 cell activation cocktail. We confirmed significantly higher levels of each of the above cytokines
132 compared to unstimulated conditions (Supplemental Fig. 3). Together, we observed functional
133 changes of immune cells and reprogramming of the tumor microenvironment associated with
134 *in vivo* tumor inhibition.
135
136 **Activation of human MAIT cells with a MAIT ligand enhances the killing of colon cancer cells**
137 To model the effect of human MAIT cells on colon cancer cells in the presence of other immune
138 cells, we utilized human whole leukocytes co-cultured with the human colon cancer cell line

139 COLO 205 (Fig. 3A). Calcein release assay was used to examine cytotoxicity and culturing with
140 human whole leukocytes for 4 hours leads to specific lysis of COLO 205 cells (Fig. 3B). Activating
141 MAIT cells with the MAIT ligand, 5-A-RU and MGO, significantly increased the specific lysis
142 capacity of the leukocytes ($p<0.05$; Fig. 3B). Flow cytometry analysis also demonstrates
143 enhanced killing of COLO 205 by whole leukocytes with the addition of the MAIT ligand in a 16-
144 hour co-culture, with a significant reduction in the frequency of CFSE-labeled cells ($p<0.05$; Fig.
145 3C). Together, these data show that MAIT cell-specific activation with 5-A-RU enhances the
146 killing of colon cancer cells.
147
148 **Stimulation of MAIT cells in human peripheral leukocytes induces eotaxin-1 production in**
149 **MAIT cells and activation of eosinophils in an MR-1 dependent manner**
150 To determine the immune modulating potential of human MAIT cell in a mixed cell population,
151 we used human whole leukocyte cultures to examine the effect of MAIT cell activation in a cell
152 culture model of CRC. MAIT cells were selectively stimulated with 5-A-RU overnight and culture
153 supernatant was analyzed using multiplex cytokine and chemokine assay. In 5-A-RU stimulated
154 cultures, we found a higher level of Macrophage inflammatory protein (MIP)-1α, MIP-1β, and
155 Monocyte chemoattractant protein (MCP)-1 ($p< 0.05$ and $p<0.01$; Fig. 4A), suggesting a capacity
156 of MAIT cells to modulate macrophages and monocytes and granulocytes (37-40). Consistent
157 with increase in eotaxin-1 with MAIT treatment in our murine model (Fig 2A), we found that
158 concentrations of IL-5, GM-CSF, and eotaxin-1 levels are significantly higher in cultures
159 stimulated with the MAIT ligand 5-A-RU/MGO compared to unstimulated leukocytes ($p<0.05$
160 and $p<0.01$; Fig. 4A), highlighting the capacity of MAIT cell activation on modulating

161 eosinophils. In addition, we observed that other cytokines known to activate and attract
162 eosinophils, including IL-4 ($p<0.01$) and IL-8 ($p=0.0547$) (41, 42), are also significantly higher in
163 5-A-RU/MGO stimulated cultures compared to control (Fig. 4A). Together, these data
164 demonstrate the immune modulating capacity of human MAIT cell activation in a mixed cell
165 population, specifically the potential to modulate eosinophils.
166
167 Previous studies have demonstrated the ability of eosinophils to kill COLO205 cells in a
168 granzyme A-dependent manner (43). To determine if MAIT cell activation can induce eosinophil
169 activation and cytotoxic function in a setting with mixed cell population of leukocytes, we
170 cultured human peripheral leukocytes with or without the MAIT ligand 5-A-RU/MGO overnight
171 and analyzed cytokine expression and levels of activation of MAIT cell and eosinophil using flow
172 cytometry (Supplemental Fig. 1). Ligand stimulated MAIT cells had significantly higher
173 expression of the activation marker CD69 ($p<0.01$) and of eotaxin-1 ($p<0.001$) (Fig. 4B).
174 Compared to unstimulated cultures, whole leukocyte cultures stimulated with the MAIT ligand
175 also shows a significant increase in eosinophil activation, marked by CD69 and granzyme A
176 expression ($p<0.0001$; Figure 4C). These data show that in peripheral leukocyte populations, the
177 addition of the MAIT ligand induces the expression of eosinophil-activating cytokines in MAIT
178 cells and correspondingly, eosinophil activation.
179
180 To confirm if eosinophil activation upon 5-A-RU/MGO stimulation is mediated via MR1 or a
181 broad consequence of antigen processing, anti-MR1 neutralizing antibody was added to the
182 human leukocyte cultures 1 hour before stimulation with 5-A-RU/MGO. In the presence of anti-

183 MR1, MAIT cell activation and the expression of eotaxin-1 are significant reduced ($p < 0.001$ and
184 $p < 0.0001$; Fig. 4B). Similarly, the activation and the induction of effector molecule granzyme A
185 in eosinophils induced by MAIT cell stimulation are significantly reduced in the presence of anti-
186 MR1 antibody ($p < 0.001$; Fig. 4C), suggesting that the effect of 5-A-RU/MGO on eosinophils is
187 dependent on MR1.
188
189 Together, these data show that the addition of 5-A-RU/MGO, a MAIT-specific ligand, leads to an
190 increased production of eosinophil-recruiting chemokines and cytokines by MAIT cells and
191 eosinophil activation, highlighting the ability of MAIT cells to modulate eosinophils in a MR1-
192 dependent manner.
193
194 **Discussion**
195 While MAIT cells are primarily known for their ability to kill microbially infected cells, other
196 potential roles have emerged. Specifically, due to their abundance at mucosal tissues, there has
197 been an increasing interest in the role of MAIT cells in mucosal tissue-associated cancers. MAIT
198 cells are a promising potential candidate for universal cancer immunotherapies: MAIT cells lack
199 alloreactive potential, thus having an advantage over the use of conventional T cells with HLA-
200 restrictions for the application of non-donor-restrictive adoptive cell therapy (24). MAIT cells
201 also express higher levels of the multi-drug efflux protein MDR1, conferring higher persistence
202 against certain chemotherapy drugs compared to conventional $CD8^+$ T cells and thus an ideal
203 target for use in immunotherapy coupled with chemotherapy (25, 26, 44). Additionally, the
204 checkpoint inhibitor, anti-PD1, has been shown to enhance the cytotoxicity of MAIT cells in

205 patients with prostate cancer (45), basal and squamous cell carcinoma, and hepatocellular
206 carcinoma (46-48). Recent studies have demonstrated the ability to generate chimeric antigen
207 receptor (CAR)-MAIT cells, which retain their anti-tumor capacity even in the presence of
208 immunosuppressive tumor-associated macrophages (49, 50). Furthermore, protocols for MAIT
209 cell expansion have been established, including demonstration that, when expanded with a
210 human serum replacement, MAIT cells display enhanced functionality and low levels of
211 exhaustion markers, making them optimized for adoptive therapy (51, 52).
212
213 In this study, we provided evidence that MAIT cells have an anti-tumoral role both in an MC38
214 cell-derived allograft tumor model in RAG1$^{-/-}$ mice and against human colon cancer cells. We
215 used RAG deficient mice which are characterized by the lack of mature B and T cells to exclude
216 the potential impact of the presence of endogenous T cells. In the murine model of colon
217 cancer, we observed tumor inhibition, associated with a significant increase in the production
218 of anti-tumor cytokines, and evidence suggesting the recruitment of eosinophils with increases
219 in both gene and protein expression of eotaxin, IL-5, and SiglecF, among others. In human
220 leukocyte and human colon cancer cell co-culture, we observed enhanced levels of COLO 205
221 lysis after stimulation with MAIT ligand 5-A-RU/MGO, demonstrating their cytotoxic capacity
222 against colon cancer cells. In addition, MAIT cell activation in human whole leukocytes leads to
223 the production of a wide range of cytokines and chemokines, including eotaxin-1, GM-CSF, and
224 Mip-1, highlighting the potential for anti-tumor effects through their immune-modulating
225 properties (10). Particularly, we observed that MAIT cell activation resulted in an increase in the

226 production of IL-5 and eotaxin-1, and GM-CSF, and an increase in activation and effector
227 function of eosinophils, the effect of which was MR1-dependent.
228
229 We observed the capacity of MAIT cell to modulate innate cell populations in our models of
230 colon cancer. We found significantly higher levels of eotaxin-1, known to recruit and activate
231 eosinophils, in both mouse *in vivo* and human *in vitro* models. *In vitro* studies have
232 demonstrated that eosinophils have the ability to induce direct killing on colon cancer cells (43,
233 53), supported by an *in vivo* study of two colon cancer models showing that eosinophil
234 contributes to tumor suppression (53). Another study using a subcutaneous MC38 cells-derived
235 tumor model in WT mice has shown that eosinophil inversely correlates with tumor growth and
236 the observed tumor suppression was dependent on GM-CSF and eosinophil recruitment (54).
237 This is consistent with our experiment where we observed an association between MAIT cell-
238 mediated inhibition of MC38-derived tumors and an increase in tumor production of GM-CSF
239 and eotaxin-1. With eosinophils' known ability to release effector molecules like major basic
240 protein (MBP) into the tumor microenvironment to induce cancer cell lysis (55), these data
241 support the involvement of eosinophils in MAIT-mediated tumor suppression.
242
243 In addition to the production of eosinophil modulating effectors, we report MAIT cell
244 production of other pro-inflammatory and immune-modulating cytokines, GM-CSF, IFN-γ, and
245 IL-17. GM-CSF regulates the proliferation, differentiation, recruitment, and effector functions of
246 myeloid cell populations (11, 12, 56), and its addition of GM-CSF to human primary monocytes
247 inhibits COLO 205 proliferation (57). In murine colon cancer, neutrophils play a role in limiting

248 tumor progression, invasion and proliferation (58). We found higher GM-CSF in tumors from
249 the MAIT cell-injection group, suggesting a potential role for myeloid cell populations in
250 mediating the observed anti-tumor effect. We also found increased expression of IFN-γ in
251 MAIT-treated tumors. IFN-γ is a cytotoxic cytokine that can act directly on tumor cells to induce
252 tumor elimination (59-62). Specifically, IFN-γ inhibits MC38-derived tumor growth in mice (63)
253 and COLO 205 in vitro (64). In addition to the direct effects on tumor cells, IFN-γ also has
254 immune modulating effects on innate immune cells, including the enhancement of the anti-
255 tumor activity of eosinophils against colon cancer cell lines, including MC38 (53). Finally, our
256 demonstration of increased IL-13 and IL-17 in MAIT-treated tumors is consistent with studies
257 showing their production by MAIT cells (26, 65, 66). In line with our murine model of colon
258 cancer, a prior study showed that treatment with recombinant IL-17 in MC38-bearing mice
259 results in tumor inhibition (63).
260
261 Taken together, our study employing both murine model and human cell cultures demonstrates
262 a protective role of MAIT cells in colon cancer. While further analysis of immune cell activity in
263 the tumor microenvironment is needed to fully understand the interaction between MAIT cells
264 and other immune cells, our findings suggest the potential for MAIT cells in recruitment and
265 activation of eosinophils. The mechanism by which MAIT cell mediates MC38-derived tumor
266 inhibition, particularly the role of eosinophils, needs further study, including how MAIT cells
267 could be harnessed in future therapeutic applications against colon cancer.
268
269 **Methods and Materials**

270 Mouse and cell lines

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

279

280 *In vivo* MAIT cell expansion and isolation

281 On day 0, C57BL/6J mice were anesthetized with isoflurane and intranasally inoculated with a
282 50 uL solution (25 uL per nare) of PBS containing 200 nmol 5-A-RU (67), 50 mM methylglyoxal
283 (Sigma-Aldrich, St. Louis, MO, USA), and 16.67 mg Pam2CSK4 (InvivoGen, San Diego, CA, USA).
284 The reaction of methylglyoxal with 5-A-RU results in the formation of the MAIT-activating ligand
285 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (68). On days 1, 2 and 4, mice
286 were again inoculated with 200 nmol 5-A-RU and 50 mM methylglyoxal. On day 7, mice were
287 euthanized, lungs were perfused with 5 mL of PBS, and then single-cell lung suspensions were
288 prepared using the gentleMACS Mouse Lung Dissociation Kit (Miltenyi Biotech, Bergisch
289 Gladbach, North Rhine-Westphalia, Germany) according to the manufacturer's protocol. Lung
290 suspensions were then pooled into male and female groups and stained with fluorochrome-
291 conjugated antibodies for MAIT isolation using fluorescent-associated cell sorting (FACS). Lung

292 suspensions were first incubated with Fixable Viability Dye eFluor 780 (BD Biosciences, Franklin
293 Lakes, NJ, USA) and then blocked with anti-mouse CD16/CD32 Fc block (Biolegend, San Diego,
294 CA, USA) and unlabeled MR1-6-formylpterin-tetramer (6-FP) (NIH Tetramer Core Facility at
295 Emory University, Atlanta, GA, USA) to minimize non-specific binding. Suspensions were then
296 stained for 30 minutes at room temperature with the MAIT cell TCR binding MR1-5-OP-RU-
297 tetramer (NIH Tetramer Core Facility at Emory University, Atlanta, GA, USA) and the following
298 antibodies: anti-CD3-PE-Dazzle594, anti-TCRb-BV421, anti-CD4-FITC, anti-CD8-APC, anti-TCRgd-
299 PE-Cy7, anti-CD44-BV650, and anti-CD45R-PE-Cy5 (Supp. Table 1). MAIT cells were gated as
300 single cell lymphocyte, live, B220-, TCR γδ-, $CD44^{hi}$, CD3+, TCRβ+ and MR1-5OPRU+. Following
301 FACS, sorted MAIT cells were washed in sterile PBS and resuspended at $1x10^6$ cells per ml for
302 use for peritumoral injection in the MC38 cancer model.
303
304 MAIT cell peritumoral injection in MC38 mouse model.
305 MC38 cell inoculation and MAIT cell peritumoral injection were performed as previously
306 described (69). Briefly, in Rag1-/- mice, $1x10^6$ MC38 cells were injected subcutaneously on Day
307 0. $1x10^5$ FACS-sorted, sex-matched MAIT cells were then injected peritumorally (same site as
308 MC38 injection) on Day 1. For control group, mice were injected with an equivalent volume of
309 PBS. Tumor growth was monitored by measuring tumor size with caliper until Day 15-21 and
310 the mice were sacrificed to obtain the tumors. Extracted tumors were weighed at the end of
311 the experiment.
312
313 Mouse MAIT cell in vitro stimulation

314 Expanded lung MAIT cells from C57BL/6J donor mice were cultured in vitro at 50,000 cells/well.
315 Cells were either left unstimulated or stimulated with 1X eBioscience Cell Stimulation Cocktail
316 (500X, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in 100uL complete RPMI
317 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, supplemented with 10% FBS, 1%
318 penicillin/streptomycin, 1mM HEPES). After overnight culture, supernatants were analyzed
319 using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel - Premixed 25 Plex -
320 Immunology Multiplex Assay (EMD Millipore Corporation, Billerica, MA, USA). and custom FAS
321 panel on the Luminex MAGPIX. A complete list of cytokines and chemokines analyzed is listed in
322 Supplemental Table 1.
323
324 Detection of tumor-released cytokines
325 8 mg (± 0.5mg) of each mouse tumor was incubated in 500 µL of complete RMPI medium for 18
326 hours and 25 µL of the resulting supernatant was analyzed using Luminex bead assay for
327 various soluble markers using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead
328 Panel - Premixed 25 Plex - Immunology Multiplex Assay (EMD Millipore Corporation, Billerica,
329 MA, USA). and custom FAS panel on the Luminex MAGPIX. A complete list of cytokines and
330 chemokines analyzed is listed in Supplemental Table 1. Measurements below detectable limits
331 were considered as 0.
332
333 Caspase activity measurement
334 Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega Corporation, Madison, WI, USA) was
335 used to determine the caspase activity in the extracted tumors. 8 mg tumor pieces were

336 homogenized in Apo-ONE® Caspase-3/7 Reagent using a bead beater. After 1 freeze-thaw to
337 ensure lysis, samples were incubated for 30 minutes on a shaker and analyzed according to
338 manufacturer's protocol. Caspase 3/7 activity is measured as arbitrary fluorescent unit (AFU)
339 using a SpectraMax Mini plate reader (Molecular Devices, San Jose, CA, USA).
340
341 Human whole leukocyte preparation and culture
342 Human whole leukocytes from anonymous healthy donors were isolated from apheresis cones
343 from ARUP using RBC sedimentation with HetaSep (STEMCELL Technologies, Vancouver, British
344 Columbia). Freshly isolated whole leukocytes were used fresh for *in vitro* culture.
345
346 Whole leukocytes were cultured with or without 5-A-RU (50 nM) and MGO (50 µM) in 200 µL
347 RPMI complete media and incubated at 37 °C overnight. CD69, and eotaxin, granzyme A, and in
348 MAIT cells and Eosinophils were analyzed using flow cytometry. To detect released cytokines in
349 the medium, 25 µL of supernatant was analyzed using Cytokine 35-Plex Human Panel for the
350 Luminex™ platform (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) on the Luminex
351 MAGPIX. A complete list of cytokines and chemokines analyzed is listed in Supplemental Table
352 2. To block interactions activation of MAIT cell via MR1, anti-MR1 (Clone 26.5, Biolegend, San
353 Diego, CA, USA) or an isotype control was used at 10 µg/ml.
354
355 COLO 205 Killing Assays
356 For Calcein Release Assay, 50,000 Calcein-labeled (2 µM, Biolegend, San Diego, CA, USA) COLO
357 205 cells were co-cultured with 500,000 human whole leukocytes for 4 hours with or without 5-

358 A-RU (50 nM) and MGO (50 µM) in 200 µL RPMI complete media at 37 °C. Maximum release
359 was set up with 2% Triton X-100 (Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA).
360 All samples and controls were set up in triplicates. Cultures were gently mixed and centrifuged,
361 and 100 µL of culture supernatant was transferred to a new plate and analyzed for fluorescence
362 intensity using BioTek Synergy H1 Multimode Reader (BioTek Instruments, Inc, Winooski, VT,
363 USA). Percent Specific Lysis was expressed as a percent relative to maximum release control,
364 calculated as [(Fluorescence $_{Sample}$ – Fluorescence $_{Untreated}$)/ (Fluorescence $_{max}$ – Fluorescence
365 $_{Untreated}$)] x 100. For flow cytometry-based killing assay, 100,000 CFSE-stained (Immunochemistry
366 Technologies, Davis, California, CA, USA) COLO 205 was co-cultured with 10^6 human whole
367 leukocytes for 16 hours and the frequency of CFSE+ cells was analyzed.
368
369 Flow Cytometry and FACS
370 Isolated mouse lung cells were stained and MAIT cells were sorted using FACS Aria III Cell Sorter
371 (BD Bioscience). Stained human *in vitro* samples were analyzed using Cytek Aurora (Cytek
372 Biosciences, Fremont, CA, USA). FCS files were analyzed using FlowJo™ v10.8 Software (BD Life
373 Sciences, Franklin Lakes, NJ, USA). MAIT cells were defined from the lymphocyte as live singlet,
374 $CD45^+$, $CD3^+$, MR1-5-OP-RU-Tetramer$^+$, Vα7.2$^+$ cells. Eosinophils were gated from the
375 granulocyte population as live singlet, $CD45^+$, $CD3^-$, $CD44^+$, Siglec8$^+$ cells. Gating strategies are
376 included in Supplemental Figure 1.
377
378 Antibodies used for FACS and Flow Cytometry
379 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

380

381 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

382

383 Statistical Analysis

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

388

389 Author Contributions

390 OJC, EJB, and DTL designed the experiments. OJC, EJL, OJ, DJ, and ST performed the
391 experiments. OJC analyzed the data. EJB and DTL supervised the work. OJC, OJ, EJB and DTL
392 wrote the paper. EJB and DTL obtained funding. All authors reviewed and approved the final
393 manuscript.

394

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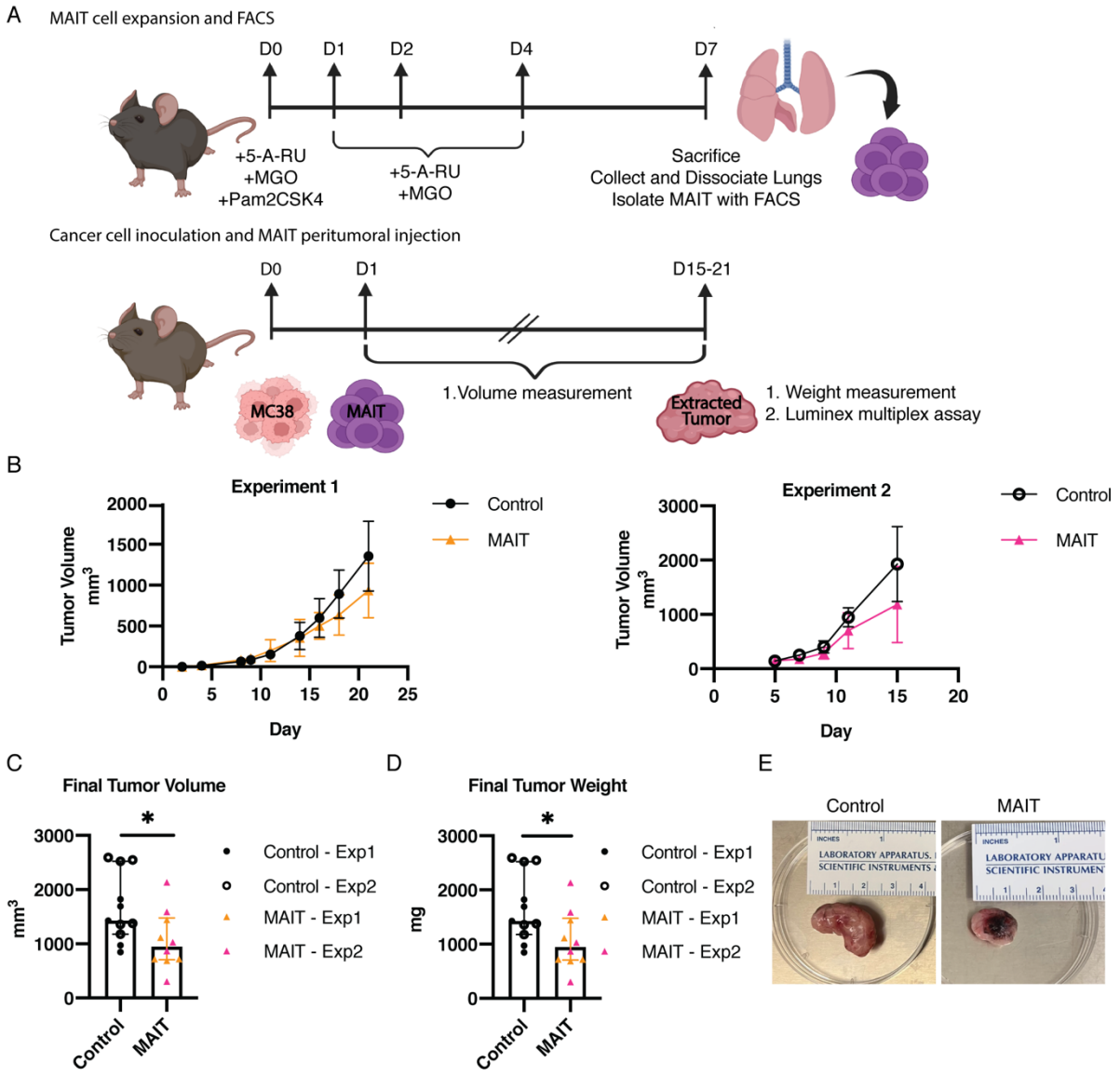
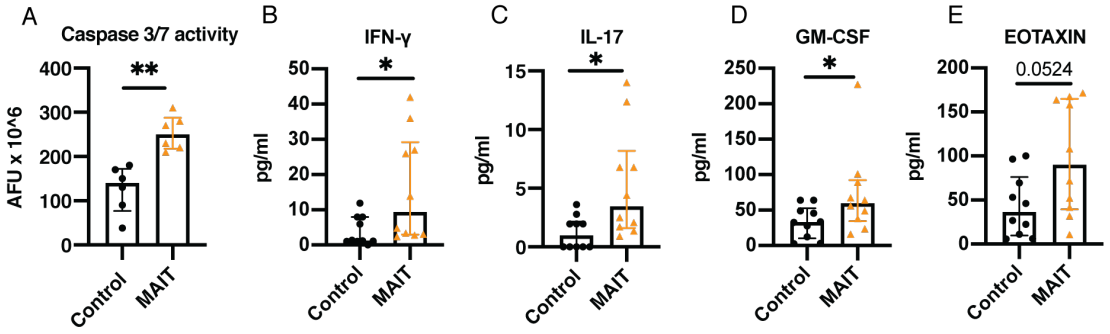


Figure 1. MAIT cell peritumoral injection leads to inhibition of MC38 cells-derived tumor growth

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 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



633

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

635 **tumors.**

636 Comparison of (A) caspase activity and (B – E) concentration of various cytokines and

637 chemokines released from extracted tumors from Figure 1 between control group and MAIT

638 group. A) Caspase activity is measured in arbitrary fluorescent unit (AFU). B) GM-CSF, C)

639 eotaxin, D) IFN-γ, and E) IL-17. 25 µL of supernatants from 18-hour culture of 8 mg tumor pieces

640 were analyzed using Luminex multiplex assay. Each dot represents an individual sample of

641 supernatant. Data was collected from 2 independent experiments and is shown as median with

642 interquartile range. *$p<0.05$ **$p<0.01$ by two-tailed Mann-Whitney U test.

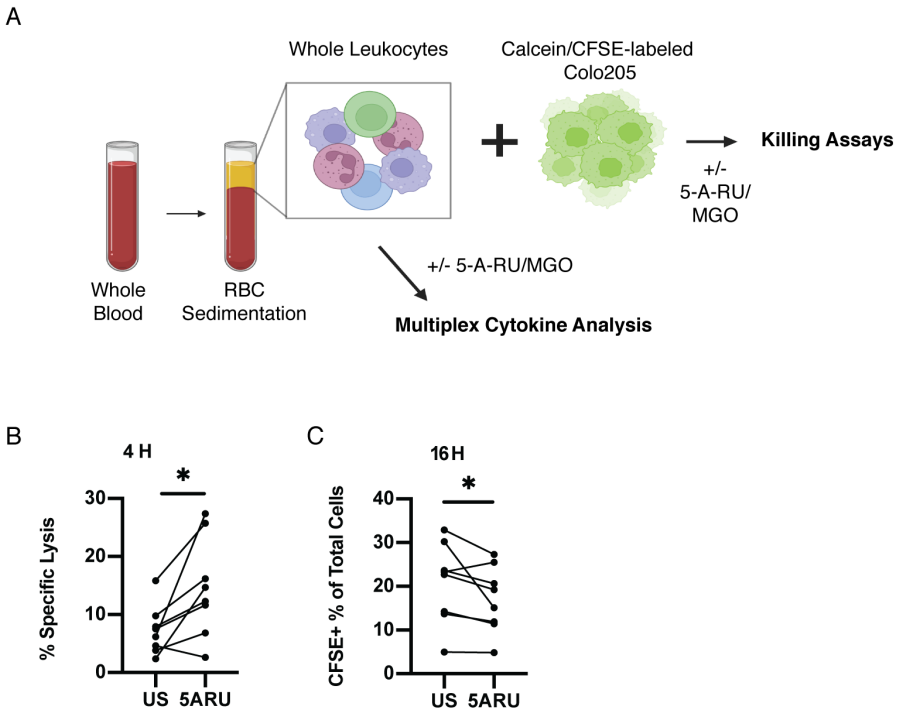


Figure 3. Activation of MAIT cells enhance killing against colon cancer cells.

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. Each dot represents a sample from an individual healthy donor and data was collected from 2 independent experiments. Figure created using BioRender.com. *$p<0.05$ by Wilcoxon ranked test.

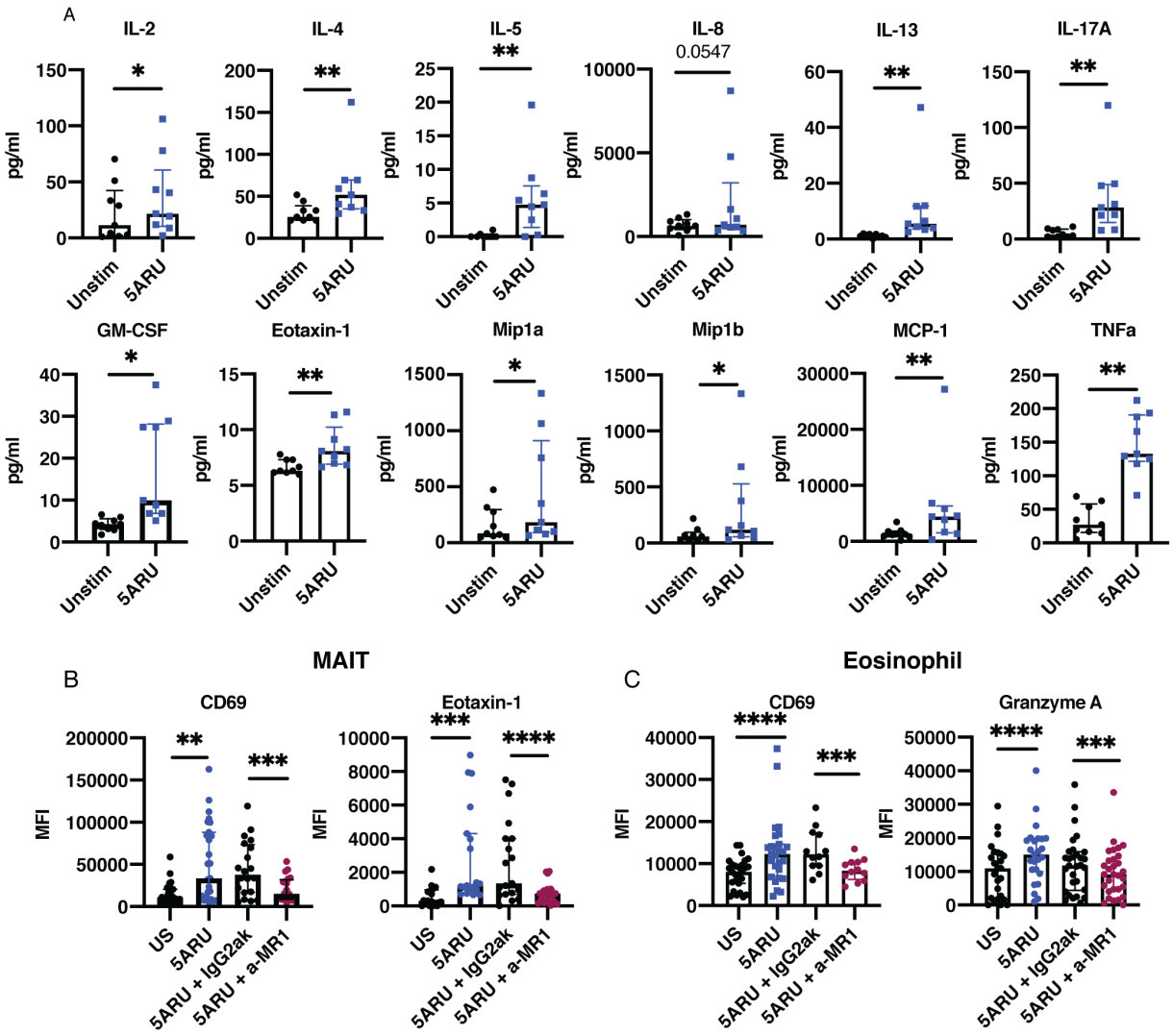


Figure 4. Stimulation of human peripheral leukocytes with MAIT ligand induces eotaxin-1 production in MAIT cells and activation of eosinophils in an MR-1 dependent manner.

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. Data was collected from 2 independent experiment and 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. Data was collected from 4 independent experiments.
**p<0.01 *** p<0.001 **** p<0.0001 by Wilcoxon ranked test.