Metabolic modulation of tumors with engineered bacteria for immunotherapy

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Metabolic modulation of tumors with engineered bacteria for immunotherapy

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3 **The availability of L-arginine in tumors is a key determinant of an efficient anti-tumor**
4 **T cell response¹⁻³. Consequently, elevation of typically low L-arginine levels within the**
5 **tumor may greatly potentiate the anti-tumor responses of immune checkpoint**
6 **inhibitors, such as PD-L1 blocking antibodies. However, currently no means are**
7 **available to locally increase intra-tumoral L-arginine levels. Here, we used a synthetic**
8 **biology approach to develop an engineered probiotic *Escherichia coli* Nissle 1917 strain**
9 **that colonizes tumors and continuously converts ammonia, a metabolic waste product**
10 **that accumulates in tumors⁴, into L-arginine. Colonization of tumors with these bacteria**
11 **elevated intra-tumoral L-arginine concentrations, increased the amount of tumor-**
12 **infiltrating T cells, and had striking synergistic effects with PD-L1 blocking antibodies in**
13 **the clearance of tumors. The anti-tumor effect of the living therapeutic was mediated by**
14 **L-arginine and was dependent on T cells. These results show that engineered microbial**
15 **therapies enable metabolic modulation of the tumor microenvironment leading to**
16 **enhanced efficacy of immunotherapies.**

17
18 Tumors create a suppressive metabolic microenvironment that contributes to ineffective anti-
19 tumor immunity⁵⁻⁹. For example, glucose and L-arginine are often scarce in the tumor
20 microenvironment (TME), which hampers the functionality of T cells^{7,10-12}. Thus, a potential
21 strategy to improve anti-tumor T cell responses is to design interventions that alter the
22 metabolic milieu in tumors in favor of T cell function^{13,14}.

23
24 The amino acid L-arginine plays an important role in the regulation of the immune system². A
25 meta-analysis of 11 clinical trials revealed that dietary supplementation of L-arginine increases
26 the CD4⁺ T-cell proliferative response and reduces the incidence of infectious complications¹⁵.
27 In tumors, L-arginine levels are typically low because tumor-associated macrophages, myeloid-

28 derived suppressor cells and tumor cells can express high levels of arginase-1, which breaks
29 down L-arginine and thus dampens the anti-tumor activity of T cells^{2,16,17}. We have previously
30 shown that increasing intracellular L-arginine levels enhances mitochondrial function of T cells
31 and endows them with a higher survival capacity and anti-tumor functionality³. In agreement
32 with this, oral administration of L-arginine to tumor-bearing mice enhances anti-tumor T cell
33 responses and slows down the progression of melanoma, osteosarcoma, colon and breast
34 cancer^{3,18-20}.

36 To investigate whether L-arginine administration synergizes with PD-L1 blockade, we first
37 compared the therapeutic efficacy of oral L-arginine administration in combination with a PD-
38 L1 blocking antibody against established MC38 tumors (Fig. 1a-c). Mice bearing small, visible
39 MC38 tumors were randomly assigned to four groups. Two groups received oral gavage of
40 water combined with intraperitoneal (i.p.) injections of either PBS or anti-PD-L1 antibodies.
41 The other two groups received daily an L-arginine solution (2 mg/g of body weight) by oral
42 gavage either combined with PBS-injections or with anti-PD-L1 antibodies (i.p.). In agreement
43 with previous findings^{3,21}, mice that received daily oral L-arginine administration or anti-PD-
44 L1 antibodies exhibited reduced MC38 tumor growth as compared to the control group (Fig. 1a
45 and b). Remarkably, combinatorial administration of L-arginine and anti-PD-L1 antibodies
46 reduced tumor growth further and significantly increased the percentage of mice that
47 completely rejected tumors and survived long-term (Fig. 1a-c). These data suggest that L-
48 arginine and PD-L1 blockade cooperate to restrict tumor growth and provide an effective
49 combinatorial anti-tumor treatment in mice.

51 To achieve the desired anti-tumor effect, relatively high doses of L-arginine have to be
52 administered to mice. In comparison, a patient weighing 75 kg would need to ingest 150 g of

53 L-arginine daily, which is nearly impracticable. Therefore, we tested whether injecting a
54 saturated L-arginine solution directly into tumors has a similar therapeutic effect. However, we
55 did not observe a reduction in tumor growth when administered alone or combined with anti-
56 PD-L1 treatment (Extended Fig. 1a-c), likely because L-arginine rapidly diffused out of tumors.
57 This highlights the need to develop alternative means to locally and continuously increase
58 L-arginine concentrations in tumors.

59

60 Because bacteria can survive and thrive in tumors²², we aimed to use a non-pathogenic strain
61 of *E. coli* (*E. coli* Nissle 1917, EcN) as an intra-tumor, synthetic biology-based cellular therapy²³
62 to produce high local concentrations of arginine in the TME (Fig. 2a). Tumors commonly
63 accumulate high levels of ammonia as a metabolic byproduct⁴, a phenomenon that has been
64 primarily linked to increased glutaminolysis in proliferating cancer cells^{24,25}. Capitalizing on
65 the availability of ammonia within the TME, we engineered EcN to efficiently channel ammonia
66 into the synthesis of high levels of arginine under anaerobic conditions found in tumors. Since
67 the ArgR protein negatively regulates the expression of all genes in the arginine biosynthesis
68 pathway, the *argR* gene was deleted^{26,27}. Additionally, the key arginine biosynthesis pathway
69 enzyme N-acetylglutamate synthase (ArgA) is inhibited by high levels of internal L-arginine,
70 thus preventing excess arginine production in *E. coli*²⁸. To circumvent this, a feedback resistant,
71 dominant mutant of ArgA, designated as ArgA^{fbr}, which is not inhibited by high levels of
72 L-arginine, was integrated in the bacterial genome²⁸. This L-arginine-producing strain is
73 hereafter referred to as L-Arg bacteria.

74

75 *In vitro*, when cultured in a medium containing ammonia chloride as the only nitrogen source,
76 the engineered L-Arg bacteria produced higher levels of L-arginine as compared to non-
77 engineered EcN and continued to accumulate L-arginine after three hours (Fig. 2b). In

78 agreement with its *in vitro* activity, a proteomic analysis using high-resolution mass
79 spectrometry²⁹ confirmed that engineered L-Arg bacteria were devoid of the arginine
80 repressor (ArgR) and expressed high levels of ArgA^{fbr} (Fig. 2c, d). In addition, engineering these
81 two critical components of the arginine biosynthesis pathway resulted in significant
82 upregulation of additional enzymes within the pathway (such as ArgB-E and ArgG-I) and
83 several subunits of the arginine transporter (ArtJ, ArtP, ArtQ) as compared to non-engineered
84 EcN that were cultured under the same conditions (Fig. 2c, d). Only a few enzymes were
85 downregulated including ArcA, which can convert arginine back into citruline. In general, the
86 genetic manipulations had specific and targeted effects on the proteome and did not globally
87 alter the features of the EcN bacteria (Fig. 2c).

88
89 Following intratumoral injection, L-Arg bacteria colonized MC38 tumors to similar levels as
90 non-engineered EcN and persisted long-term (Fig. 2e). Importantly, homogenates of MC38
91 tumors colonized with L-Arg bacteria contained significantly higher levels of arginine as
92 compared to tumors treated with non-engineered EcN (Fig. 2f). Collectively, these data
93 demonstrate that EcN can be engineered to produce high levels of L-arginine and that treatment
94 of tumors with L-Arg bacteria represents a unique therapeutic approach to increase local levels
95 of this immunomodulatory metabolite within the tumor.

96
97 An analysis of tissue sections showed that MC38 tumors colonized with L-Arg bacteria for
98 72 h contained more tumor-infiltrating T cells than EcN-colonized or untreated tumors (Fig.
99 3a), which was confirmed by a quantification of tumor-infiltrating CD4⁺ and CD8⁺ T cells from
100 dissociated tumors using flow cytometry (Fig. 3b, Extended Fig. 1d). Another effect caused by
101 L-Arg bacteria was a decrease in the percentage of Foxp3⁺ regulatory T cells in tumors from

102 about 50% to 20% (Fig 3c.), consistent with previous findings in mice with increased L-arginine
103 levels³⁰.

104

105 We next investigated whether colonization of MC38 tumors with L-Arg bacteria synergizes
106 therapeutically with blockade of the PD-1 pathway. Mice bearing established MC38 tumors
107 received four intratumoral injections of EcN or L-Arg bacteria either alone or combined with
108 i.p. administration of anti-PD-L1 antibodies. Strikingly, the combined administration of L-Arg
109 bacteria and PD-L1 blocking antibodies synergistically reduced tumor growth, and 74% of the
110 mice completely eradicated tumors (Fig. 3d, e). In contrast, complete tumor rejection was
111 observed for only 44% and 39% of mice that received anti-PD-L1 antibodies alone or combined
112 with non-engineered EcN, respectively (Fig. 3d, e). Thus, the ~30% increase in mice that
113 rejected tumors could be exclusively attributed to the L-arginine producing capacity of the
114 engineered bacteria. Administration of EcN alone did not influence tumor growth when
115 compared to a PBS control (Extended Fig. 2d). Together, these results demonstrate that L-Arg
116 bacteria significantly improve checkpoint inhibitor-based immunotherapy.

117

118 To test whether the therapeutic effect of the combinatorial treatment with L-Arg bacteria and
119 anti-PD-L1 antibodies is caused by an improved anti-tumor T cell response, or by inhibition of
120 cancer cell growth⁴, we repeated the experiments in *Cd3e*^{-/-} mice that lack all T cells but exhibit
121 organized lymphoid organ structures and normal B cell development³¹. Following injection of
122 EcN and L-Arg bacteria, MC38 tumors in *Cd3e*^{-/-} mice were colonized to a similar extent as
123 tumors of wild type mice (Extended Fig. 3a). Yet, in *Cd3e*^{-/-} mice there was no difference in
124 MC38 tumor growth between mice that received L-Arg bacteria combined with PD-L1
125 antibodies and all other groups (Extended Fig. 3b, c), indicating that the anti-tumor effect of
126 L-Arg bacteria was T cell-dependent. We then asked whether L-Arg bacteria influence effector

127 functions of T cells. Ten days after initiating different treatments of MC38 tumor-bearing wild
128 type mice, T cells were isolated from tumors and their phenotype was analyzed by flow
129 cytometry. The combinatorial treatment of L-Arg bacteria with anti-PD-L1 antibodies increased
130 the frequency of tumor-infiltrating T cells that produce the pro-inflammatory cytokine
131 TNF- α (Fig. 3f) and decreased the percentage of T cells co-expressing the inhibitory receptors
132 PD-1 and LAG-3 (Fig. 3g). Thus, the therapeutic effect of L-Arg bacteria combined with anti-PD-
133 L1 antibodies was mediated through T cells, most likely by sustaining their effector functions.

134

135 We next asked whether the bacterial combination treatment enhances the formation of long-
136 term protective T cell memory. Mice in which tumors regressed completely were re-challenged
137 after 90 days with a subcutaneous injection of MC38. Notably, MC38 tumors did not grow in
138 these mice, indicating that long-term immunological antitumor T cell memory had been formed
139 (Fig. 4a). The antitumor T cell memory was specific to MC38 as a subsequent challenge with a
140 different tumor cell line (B16) resulted in similar growth kinetics as in naïve control mice (Fig.
141 4b).

142

143 Next, we isolated CD8⁺ and CD4⁺ memory T cells from mice 100 days after they rejected MC38
144 tumors in response to a treatment with either L-Arg bacteria + anti PD-L1 or EcN + anti- PD-L1.
145 Then, we transferred 5 x 10⁵ total memory T cells into new mice with established MC38 tumors
146 (Fig 4c). Memory T cells from mice that rejected tumors in response to L-Arg bacteria and anti-
147 PD-L1 had a superior anti-tumor activity in recipient mice, suggesting that colonization of
148 tumors with L-Arg bacteria enhances the formation of tumor-specific T cell memory (Fig. 4c).
149 Collectively, these results demonstrate that the combination of L-Arg bacterial treatment with
150 PD-1-based immunotherapy results in substantial tumor control resulting in a 75% complete
151 response rate and formation of tumor-antigen-specific immunological memory. This supports

152 the notion of using this synthetic biology approach to increase durable responses in cancer
153 patients undergoing immunotherapy.

154

155 While intratumoral injection of bacteria is suitable for tumors that are accessible
156 percutaneously or via specific procedures like colonoscopy³², systemic administration would
157 enable the colonization of tumors that are not readily accessible. To evaluate intravenous (i.v.)
158 administration of engineered bacteria as a potential therapeutic approach, we injected
159 increasing amounts of bacteria into mice bearing MC38 tumors of different size. While small
160 tumors were not consistently colonized (data not shown), tumors with a volume of $\geq 100 \text{ mm}^3$
161 were efficiently colonized long-term upon i.v. administration of 5×10^7 CFU of EcN or L-Arg
162 bacteria (Fig. 4d). Notably, other organs contained no detectable bacteria, indicating that EcN
163 and L-Arg bacteria specifically colonized tumors (Fig. 4d). While intratumoral injections of
164 bacteria were very well tolerated, i.v. injections caused a reduction in body weight, which was
165 reverted after four days (Extended Fig. 5a, b).

166

167 Since small tumors were not consistently colonized, we tested the therapeutic efficacy of i.v.
168 administered bacteria in mice bearing relatively large tumors ($\geq 100 \text{ mm}^3$). Mice were treated
169 with anti-PD-L1 blocking antibodies alone or combined with a single i.v. injection of EcN or
170 L-Arg bacteria. Under these conditions, anti-PD-L1 antibodies reduced tumor growth but did
171 not cause complete rejections of tumors (Fig. 4e), likely due to initial tumor size being
172 approximately twice as large as compared to previous experiments. Treatment with
173 anti-PD-L1 antibodies and L-Arg bacteria (i.v.) reduced tumor growth and 40% of mice rejected
174 tumors completely. These results demonstrate that i.v. administered L-Arg bacteria specifically
175 colonize tumors and have a synergistic therapeutic anti-tumor effect in combination with
176 PD-L1 blockade.

177

178 To extend our analysis to a different mouse tumor model, we subcutaneously injected B16-OVA
179 melanoma cells, which form aggressive and poorly immunogenic tumors. B16-bearing mice
180 were treated bi-weekly with EcN or L-Arg bacteria either alone or combined with anti-PD-L1
181 antibodies. No differences in tumor growth were observed between the groups, likely because
182 there was no effective anti-tumor T cell response that could be enhanced by L-arginine and
183 anti-PD-L1 antibodies (Extended Fig. 4). Therefore, we repeated these experiments and
184 adoptively transferred OT-I T cells into mice with established B16-OVA tumors to initiate an
185 anti-tumor T cell response. Under these conditions, the combined treatment of L-Arg bacteria
186 with anti-PD-L1 antibodies had a therapeutic effect and significantly decreased tumor growth
187 as compared to a treatment with anti-PD-L1 antibodies alone or combined with EcN (Figs. 4d-
188 f), confirming the efficacy of L-Arg bacteria in a different tumor type.

189

190 In summary, synthetic biological approaches and the utilization of engineered microbial
191 therapies represent a unique means of local metabolic modulation of the tumor
192 microenvironment. In this study we demonstrate the synergy between L-Arg bacterial therapy
193 and PD-L1 blockade, and their ability to generate robust T-cell dependent, long-term anti-
194 tumor immunity. As compared to daily oral administration of large amounts of L-arginine, a
195 single i.v. injection of L-Arg bacteria was sufficient to colonize tumors long-term and achieve a
196 therapeutic response. The manufacture of these synthetic biotic medicines is easily scalable, as
197 evidenced by the initiation and completion of several recent clinical trials^{33,34}. These findings
198 support the utilization of synthetic biotic medicines as immunotherapies in cancer and warrant
199 further development towards evaluation in the clinic.

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Author Contributions

R.G. and J.M.L. conceived the project. F.P.C., C.B., G.A., M.P., S.G., J.N, and W.J. performed experiments with tumor models. N.L. and M.J.J. generated bacterial strains. J.P.T analyzed tissue sections. R.G., F.S., A.S., D.D.L., K.A.W. and J.M.L. supervised the project. D.S.L., J.M.L and R.G wrote the manuscript.

Data availability

Proteome raw data will be available in the PRIDE database.

Figure Legends

Fig. 1: L-arginine synergizes with PD-L1 blockade to promote MC38 tumor rejection.
5 x 10⁵ MC38 colon adenocarcinoma cells were injected s.c. in C57BL/6 WT mice and tumors were allowed to grow for 9-11 days until they reached a size of about 50 mm³ before treatments were initiated. Control mice (n=13) received daily oral gavage of H₂O (100µl) and bi-weeekly 200µl of PBS i.p. The L-arginine group received daily an oral gavage of L-arginine (2g/kg body weight in 100µl) (n=19). The PD-L1 group received 200µg of αPD-L1 mAb i.p. bi-weekly (four injections in total) (n=16). A fourth group received L-arginine (oral) and anti-PD-L1 (i.p.) (n=17). **(a)** *In vivo* MC38 tumor growth (number of mice are indicated in the graph). Arrows on

top indicate time points of treatments. Values represent mean tumor volume $\pm$ SEM. *P* values were determined by two-way ANOVA. **(b)** Tumor growth curves of individual mice. **(c)** Survival analysis of different groups of mice. *P* values were determined by log-rank comparisons between curves. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

Fig. 2: Metabolically engineered bacteria produce L-arginine and colonize tumors

A metabolically engineered strain of *E. coli* Nissle was generated to produce high levels of L-arginine in the tumor microenvironment. **(a)** Schematic of L-Arg-bacterial strain where the arginine biosynthesis negative regulator protein ArgR was deleted and the feedback resistant ArgA^{fbr} was introduced. **(b)** An *in vitro* assay was performed with 1×10^9 cells/mL L-Arg bacteria or non-engineered control bacteria (EcN) that were pre-induced in M9 medium + 0.5% glucose + 5 mM NH₄Cl. Reaction mixture was incubated at 37°C with shaking for 3 h. Samples were taken at 0, 1.5 and 3 hours respectively and arginine production was measured by LC-MS/MS. Non-engineered EcN was used as a negative control. n=2 **(c)** Pre-induced EcN and L-Arg bacteria were grown for 3h in M9 medium + 0.5% glucose + 5 mM NH₄Cl. Then, their total proteome was analyzed by LC-MS/MS. The volcano plot shows the results of differential abundance analysis (two-tailed Welch's *t*-test) between EcN and L-Arg bacteria. Red and blue dots represent proteins whose abundances were significantly higher or lower in L-Arg bacteria as compared to EcN (*P* < 0.01, Log₂ |FC| > 4). n=4. **(d)** Illustration of the L-arginine biosynthesis pathway. The color code displays the Log₂ foldchange as determined by LC-MS/MS shown in Fig. 2c. The arrows between enzymes indicate the direction of the reaction according to the KEGG database. **(e)** MC38 tumors were established and treated via intratumoral injection with 5×10^6 CFUs of either L-Arg bacteria or EcN. Tumors were harvested and homogenized after 24, 72 and 120 h and bacterial abundance was measured by colony forming unit (CFU) assay. **(f)** MC38 tumors were established and treated via intratumoral injection with 5×10^6 CFUs of

254 either L-Arg bacteria or EcN. Tumors were harvested and homogenized after 24 h and arginine
255 levels were measured by LC-MS/MS. *P* values were determined using a two-tailed t test. **P*<
256 0.05

257

258 **Fig. 3: L-Arg bacteria synergize with PD-L1 blockade to promote MC38 tumor rejection.**

259 **(a)** H&E staining and CD3 immunohistochemistry of control MC38 tumors and tumors
260 colonized with L-Arg bacteria and EcN (72 h). MC38 tumors were established in C57BL/6 WT
261 mice and treated via intratumoral injection with 5×10^6 CFUs of either L-Arg bacteria or EcN.
262 After 72 h, tumors were harvested, fixed and stained. Control tumors have low numbers of CD3-
263 positive T-cells. EcN colonized tumors show similar numbers of T-cells, while tumors colonized
264 with L-Arg bacteria are rich in T cells (bar represents 20 micrometer, arrow = CD3-positive T-
265 cells). **(b)** MC38 tumors were established in C57BL/6 WT mice and treated via intratumoral
266 injection with 5×10^6 CFUs of either L-Arg bacteria or EcN. After 24 h, tumors were harvested,
267 dissociated and immune infiltrates were purified and stained with monoclonal antibodies to
268 CD4 and CD8. The number of T cells per gram tumor tissue was quantified by flow cytometry.
269 The gating strategy is shown in Extended Fig. 1d. *P* values were determined using a two-tailed
270 t test. *n*=8 for Ctrl bacteria, *n*=11 for L-arg bacteria. Data are from three independent
271 experiments. **(c)** Same as in b but the frequency of FOXP3⁺ Tregs was quantified by flow
272 cytometry. *P* value was determined using a two-tailed t test. *n*= 6 for no bacteria and EcN
273 controls, *n*=4 for L-Arg bacteria. **(d)** MC38 tumors were established in C57BL/6 WT mice.
274 Tumors of the control group (*n*=15) were treated with 5×10^6 CFU EcN (i.t.) twice a week. A
275 second group (*n*=11) was treated with 5×10^6 CFU L-Arg bacteria (i.t.) twice a week. A third
276 group (*n*=9) received anti-PD-L1 antibodies. A fourth group (*n*=17) received anti-PD-L1
277 antibodies and 5×10^6 CFU Ctrl bacteria i.t. (*E. coli* Nissle + α PD-L1). A fifth group (*n*=16)
278 received anti-PD-L1 antibodies(i.p.) and 5×10^6 L-Arg bacteria i.t (L-Arg-bacteria + α PD-L1).

279 **Left panel:** *In vivo* MC38 tumor growth (number of mice are indicated in the graph). Values
280 represent mean tumor volume $\pm$ SEM. *P* values were determined by two-way ANOVA.
281 **p* < 0.05. **Right panel:** Survival curves of mice. A log-rank test comparison between curves was
282 applied. **(e)** Tumor growth curves of individual mice. **(f)** MC38 tumor-bearing mice were
283 treated as indicated in the graph. Ten days after the treatment T cells were isolated from tumors
284 and stimulated with PMA and ionomycin for 5 hours in the presence of brefeldin A. The
285 percentage of TNF- α producing CD8⁺ T cells was analyzed by flow cytometry. **(g)** Same
286 experiment as in (f) but PD-1 and LAG-3 expression was analyzed in non-stimulated CD8⁺
287 T cells.

288
289 **Fig. 4: L-Arg bacteria enhance anti-tumor immunity and can be administered**
290 **systemically. (a)** Mice in which tumors completely regressed were re-challenged with 5×10^5
291 MC38 colon adenocarcinoma cells 90 days after the first injection of MC38 cells. As a control
292 5×10^5 MC38 cells were injected into naïve C57BL/6 WT mice. **(b)** Mice in which MC38 tumors
293 completely regressed were re-challenged with 5×10^5 B16 melanoma. **(c)** Left side: Schematic
294 of the adoptive T cell transfer experiment. CD4⁺ and CD8⁺ memory T cells were isolated from
295 spleens of mice 100 days after they completely rejected MC38 tumors in response to different
296 bacterial combination treatments. 5×10^5 total memory T cells were adoptively transferred into
297 mice with established MC38 tumors. Right side: Graph shows MC38 tumor growth curves.
298 Number of mice are indicated on the graph. ***P* < 0.01 as determined by two-way ANOVA. **(d)** 5
299 $\times 10^7$ EcN or L-Arg bacteria were intravenously injected into mice with MC38 tumors with a size
300 of ≥ 100 mm³. Eleven days later, tumors, kidneys and spleens were collected, and bacterial
301 abundance was analyzed by CFU assay. **(e)** Mice with established MC38 tumors (≥ 100 mm³)
302 were treated with anti-PD-L1 antibodies (i.p) and i.v. injections of EcN or L-Arg bacteria as
303 indicated in the graph. Left panel: tumor growth curves. Right panel: Survival curves. **(f)** Same

304 experiment as in (e) but tumor growth curves of individual mice are shown. **(g)** B16-OVA
305 tumors were injected s.c. into C57BL/6 WT mice. Seven days later, 2.5×10^5 OT-I T cells were
306 adoptively transferred into tumor-bearing mice. Mice were treated four times with i.t.
307 injections of 5×10^6 CFU of EcN or L-Arg bacteria and i.p. injections of anti-PD-L1 antibodies.
308 Tumor growth curves are shown. Bars represent the SEM, P values were determined by two-
309 way ANOVA. **(h)** Same experiment as in (g) but tumor growth curves of individual mice are
310 shown.

311

312 **Extended Data Fig. 1: Intratumoral injections of an L-arginine solution does not affect the**
313 **growth of MC38 tumors. (a)** MC38 tumors were established in C57BL/6 WT mice. Ten days
314 later, 50 μ l of a saturated L-arginine solution was injected into tumors. This corresponds to 0.25
315 mg L-arginine/g body weight and is the maximal volume that tumors can take up. Where
316 indicated, mice were also treated with 200 μ g anti-PD-L1 antibodies or isotype control
317 antibodies. Mice received a total of four injections (bi-weekly). Graph shows MC38 tumor
318 growth curves. Bars represent SEM. Numbers of mice per group are indicated in the graph. **(b)**
319 Same experiment as in (a) but survival curves are shown. **(c)** Same experiment as in (a) but
320 growth curves of individual mice are shown. **(d)** Example of the gating strategy to quantify CD4
321 and CD8 T cells.

322

Extended Data Fig. 2: EcN colonization does not affect the growth of MC38 tumors. (a)
MC38 tumors were established in C57BL/6 WT mice. Mice were treated with either 5×10^6 CFU
EcN (i.t.) or with i.t. injections of PBS, bi-weekly (four treatments in total).

Extended Data Fig. 3: The anti-tumor effect of L-Arg bacteria is T cell-dependent. (a)
MC38 tumors were established in C57BL/6 WT mice and in *CD3e*^{-/-} mice and treated via

intratumoral injection with 5×10^6 CFUs of either L-Arg bacteria or EcN. Tumors were harvested and homogenized after 24 hours and bacterial abundance was measured by CFU assay. n=2 **(b)** MC38 tumors were established in *CD3e*^{-/-} mice. When tumors started to be visible and palpable, a homogenous group of tumor-bearing mice was chosen for the different treatments. Tumors of the control group were treated with 5×10^6 CFU EcN (i.t.) twice a week. A second group was treated with 5×10^6 CFU L-Arg bacteria (i.t.) twice a week. A third group received anti-PD-L1 antibodies i.p. and 5×10^6 CFU EcN i.t. (EcN + α PD-L1) and a fourth group received anti-PD-L1 antibodies i.p. and 5×10^6 L-Arg bacteria i.t (L-Arg-bac. + α PD-L1). Tumor growth over time. n=5 for each group. **(c)** Survival curves of mice from (b).

Extended Data Fig. 4: Bacterial treatments and PD-L1 blockade have no effect on B16 tumor growth. C57BL/6 WT mice with established B16-OVA tumors were treated four times with i.t. injections of 5×10^6 EcN or L-Arg bacteria and i.p. injections of anti-PD-L1 antibodies. Tumor growth curves. Bars represent the SEM, n=5.

Extended Data Fig. 5: Effect of bacterial treatments on mouse health.

(a) C57BL/6 WT mice with established MC38 tumors were treated with four i.t. injections of 5×10^6 EcN or L-Arg bacteria, or with PBS. The weight of mice was followed over time. Bars represent the SEM, throughout. **(b)** C57BL/6 WT mice with established MC38 tumors were treated with on i.v. injection of 5×10^7 EcN or L-Arg bacteria, or with PBS. The weight of mice was followed over time.

Material and methods

Mice

C57BL/6 mice were obtained from Charles River (Italy). *CD3e*^{-/-} mice have been described previously³⁵. Mice were maintained under specific pathogen-free conditions in the animal facility of the Institute for Research in Biomedicine (IRB). Five mice per cage were housed in ventilated cages under standardized conditions (20±2°C, 55±8% relative humidity, 12 h light/dark cycle). Food and water were available ad libitum, and mice were examined daily. Female mice were used between 6 and 10 weeks of age. Mice were treated in accordance with guidelines of the Swiss Federal Veterinary Office and experiments were approved by the Dipartimento della Sanita` e Socialita`.

L-Arginine administration and α PD-L1 therapy experiments

MC38 murine colon adenocarcinoma cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, GlutaMax and 25 mmol/L Hepes. Before injection into mice, cells were trypsinized and washed twice with PBS. Then, 5×10^5 cells were subcutaneously injected in the dorsal region of C57BL/6 WT mice. The size of tumors was measured in a blinded fashion using calipers. Nine to eleven days post injection, when tumors started to be visible and palpable, mice were selected based on tumor size ranging between 30 and 80 mm³. Every second day, mice were injected i.p. with 200µg of mAb binding PD-L1 (α PD-L1 mAb from Bio X Cell, clone B7-H1), treated daily by oral gavage with an L-arginine solution (2 g/Kg body weight) in H₂O or received a combination of the two therapies (α PD-L1 mAb + L-Arg) until the end of the experiment. Age-matched control mice were treated with PBS i.p. and gavaged with H₂O.

Flow cytometry of TILs

323 Tumors were disaggregated mechanically and enzymatically with 2 mg/mL collagenase IV
324 (Roche) and 50 U/mL DNase I (Roche). Single-cell suspensions were stained with the following
325 antibodies: CD8 Super Bright 702, CD4 Super Bright 780, Foxp3 eFluor450, CD45 PerCP-

326 Cy5.5/APC-eFluor 780, CD44 Super Bright 600, PD-1 PE, LAG3 FITC, TNF APC, TCRb PE-Cy7
327 (ThermoFisher). Dead cells were excluded using Zombie Red viability dye (Biolegend). For
328 intracellular stainings, T cells were fixed and permeabilized with True-Nuclear™ Transcription
329 Factor Buffer Set (Biolegend) according to the manufacturer's instructions. For *ex*
330 *vivo* intracellular cytokine stainings, cells were stimulated with 50 ng/mL PMA (SIGMA), 1
331 µg/mL Ionomycin (SIGMA), Brefeldin A and Monensin (Life Technologies) for 5 hours at 37°C.
332 Samples were acquired on a BD Symphony flow cytometer (BD Biosciences) and data was
333 analyzed using FlowJo software.

Tumor homogenization and CFUs count.

Excised tumors from C57BL/6 and *CD3ε*^{-/-} mice were weighed and then chopped into small pieces using sterile scissors. Tumor pieces were placed into 2 mL round-bottom tubes (Eppendorf) with 500 µL of PBS and one 5 mm Steel Bead per tumor (Qiagen). Samples were homogenized in a Tissue Lyzer II device (Qiagen) at 300 rpm for 15 min. Serial dilutions of tumor homogenates were plated in LB medium (MP Biomedicals) on Bacto® Agar (BD) plates to which 30 µg/mL chloramphenicol was added for L-Arg bacteria selection or 300 µg/mL streptomycin (Sigma) for control *E. coli* Nissle. Plates were incubated 12 h at 37° C and then bacterial colonies were counted.

CD3-Immunohistochemistry

Paraffin-embedded tissue samples of syngeneic mouse tumors were analyzed for the abundance of intratumoral T-cell infiltration using a polyclonal anti-CD3 antibody (Dako A0452). After dewaxing and rehydration of tissue sections, antigen retrieval was performed using a water bath at 98°C and an EDTA-based buffer at pH 9 for 20 min. Thereafter, antigen blocking was performed for 10 min at RT using serum free protein block and the primary

antibody incubated at a dilution of 1:800 for an hour. After washing with PBS and Tween 20, slides were incubated for 30 min at RT using a biotinylated anti-rabbit antibody (Vector BA-9100) before visualization of biotin with Vectastain ABC kit (A 1:150 + B 1:150 in PBS).

334 *B16 model and adoptive transfer of OT-I T-cells:*

335 B16.OVA cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco), 1X
336 GlutaMax (Gibco), 25 mmol/L HEPES (Gibco) and Penicilin/Streptomycin. For *in vivo*
337 experiments, 5×10^5 cells were injected s.c. in 100 uL of PBS in the back of C57BL/6 mice.

338 For adoptive transfer of OT-I T-cells into B16.OVA tumor-bearing mice, spleens were obtained
339 from CD45.1+ OT-I Rag-/- mice (B6.129S7-Rag1tm1Mom/J, C57BL/6-Tg(TcraTcrb)1100Mjb/J
340 were crossed with B6.SJL-Ptprca Pepcb/BoyJ; all strains from The Jackson Laboratory). Mice
341 were euthanized and spleens were harvested and mechanically disaggregated. The resulting
342 cell suspension was filtered using 40 µm cell strainers. Then, CD8+ OT-I T-cells were isolated
343 at a purity >95% by negative selection using the EasySep™ mouse CD8+ T Cell Isolation Kit
344 (Stem Cell Technologies) following manufacturer's instructions. OT-I T-cells were activated *in*
345 *vitro* for 48 h prior to adoptive transfer by plating 150,000 cells per well in flat bottom 96-well
346 plates pre-coated with 1 µg/mL anti-CD3e (Life Technologies) and 0,5 µg/mL anti-CD28 (Life
347 Technologies) in the presence of 10 ng/mL of rhIL-2 (Sino Biological). After activation, cells
348 were counted and resuspended in PBS for adoptive transfer. At day 7 p.t.i. mice received 100
349 uL of PBS containing 250,000 activated OT-I T-cells by i.v. injection in the tail vein. Two days
350 later bacteria, anti-PD-L1 treatments were started and continued twice a week for a total of
351 four treatments.

Engineering of E. coli Nissle to generate L-arginine producing bacteria

Escherichia coli Nissle 1917 (Nissle) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, *E. coli* DSM 6601). Two modifications of the native arginine biosynthesis pathway, deletion of *argR* and insertion of a feedback resistant version of *argA* (*argA^{fbr}*), were engineered into the chromosome of Nissle in order to convert ammonia to excess arginine. Control of *argA^{fbr}* was placed under the oxygen responsive promoter P_{fnrS} to allow for repression of the gene during biomass production and induction in the hypoxic conditions of the tumor microenvironment. One copy of the *fnr-argA^{fbr}* with chloramphenicol resistant marker was introduced between the *malE* and *malK* genes. Both deletion of *argR* and chromosomal insertion of *argA^{fbr}* were done by standard *E. coli* manipulation method such as lambda red recombineering.

Bacterial cell preparation for In vitro and In vivo assays

For smaller scale production, bacterial cultures were grown in LB media supplemented with selective antibiotics (either streptomycin or chloramphenicol) in flasks overnight at 37°C with shaking at 250 rpm. The overnight cultures were then diluted 100-fold into 100 mL rich media with antibiotics in 1 L baffled flasks. The diluted cultures were expanded by shaking at 250 rpm, 37 °C, until reaching an OD₆₀₀ ~ 6. Cells were harvested by centrifugation and washed once with cold PBS. The cells were then resuspended in 15% glycerol + PBS, aliquoted into cryovials and stored at -80°C. For larger scale production, bioreactor was used to generate more cells with higher density. Bacterium was inoculated into 50 ml of FM1 medium in a 500-ml Ultra-Yield flask (Thomson) which was then grown at 37 °C with shaking at 350 rpm overnight. The overnight culture was used to inoculate FM1 in an Eppendorf BioFlow 115 bioreactor (starting OD₆₀₀ of ~0.15). The fermenter was controlled at 37 °C, 60% dissolved oxygen with agitation, air, and oxygen supplementation, and controlled to pH 7 using ammonium hydroxide for 4 – 5

hours. At the end of fermentation, cells were harvested by centrifugation at 4,500 g for 30 min at 4 °C, resuspended in formulation buffer, and stored at -80 °C.

In Vitro Arginine Production Assay

Bacterial stocks stored at -80°C were thawed on ice and diluted 20-fold into 20 mL 2YT media with no antibiotics in a baffled 125 mL flask. The flask was incubated at 37°C while shaking at 250 rpm for 1.5 hours to recover from frozen state and then moved to anaerobic chamber to incubate at 37°C statically for 2 h for pathway induction. At the end of these two incubations, cells were pelleted by centrifugation and washed once with PBS. Cell number was measured by cellometer, and bacteria were resuspended into a final concentration of 1×10^9 cells/mL using M9 + 0.5% glucose + 5 mM NH₄Cl media into 96 deepwell plates. Representative samples of 0 hours were taken immediately. The deep well plate was covered by breathable membrane and immediately moved to 37°C with shaking at 250 rpm. Representative samples were then taken at 1.5 hours and 3 hours after culture. All samples were spun down to pellet cells and supernatants were collected in a new 96 well plate to be analyzed by LC-MS/MS for arginine quantification.

LC-MS/MS analysis of L-arginine in bacterial cultures and tumor homogenates

Bacterial supernatant or tumor homogenate was extracted with 4 parts 70 % acetonitrile containing 1 µg/mL 15N₄,13C₆-arginine (Cambridge Isotope Laboratories), vortexed, and centrifuged at 2300 g for 5 minutes at 4°C. Supernatants were diluted 10 fold with 0.1% formic acid prior to analysis. Arginine was separated and detected on a Vanquish UHPLC/TSQ Altis LC-MS/MS system (Thermo Fisher Scientific). Samples were injected (2 µL) and separated on an

Accucore aQ 2.6 μ m C18, 2.1 x 100 mm column (Thermo Fisher Scientific). Analytes were eluted using a linear gradient of 95% solution A shifting over to 95% solution B over 2 minutes (Solution A: 0.1% formic acid (FA), 0.02% perfluoropentanoic acid (PFPeA); Solution B: acetonitrile, 0.1% FA, 0.02% PFPeA) at 0.4 mL/min at 40°C. Arginine was detected using selected reaction monitoring (SRM) of compound specific mass transitions in positive electrospray ionization mode (arginine: 175>70; 15N₄,13C₆-arginine: 185>75). Peaks were integrated and arginine/15N₄,13C₆-arginine peak area ratios were used to calculate concentrations of the unknowns using a 1/X weighted linear 7-point standard curve from 0.032 to 250 ng/mL using Xcalibur Quan Browser (Thermo Fisher Scientific).

LC-MS analysis of the E. coli Nissle proteome

L-Arg bacteria and non-engineered E. coli Nissle were pre-induced according to the method described above. At the end of induction, the bacterial cells were washed by cold PBS and centrifuged at 8000 rpm, 4 °C for 5 min. The PBS was discarded, and the cell pellets were then stored at -80 °C. Bacterial pellets were re-suspended in a ammonium bicarbonate buffer pH= 8 (ABC) containing 4% SDS. Then, samples were boiled at 95°C for 10 min followed by sonication in Bioruptor (15 cycles, 30s on, 30s off, high mode). Non-soluble cell debris were spun down at 16,000g for 10 min and supernatants were precipitated with 5 volumes cold acetone at -80°C over night. Precipitated proteins were spun down at 16,000 g for 20 min at 4°C and pellets was washed twice with ice cold 80% acetone. The pellet was dried in a SpeedVac centrifuge and then re-suspended in ABC buffer containing 8 M urea followed by sonication. Protein amount was determined using a Nanodrop. Proteins were pre-digested with LysC (1:50, w/w) followed by reduction of disulfide bonds with 10mM DTT and subsequent alkylation with 50 mM iodacetamide. Then samples were diluted 1:5 with ABC and trypsin (1:50, w/w) was added for overnight digestion at RT. The resulting peptide mixtures were acidified and loaded on C18

StageTips³⁶. Peptides were eluted with 80% acetonitrile (ACN), dried using a SpeedVac centrifuge, and resuspended in 2% ACN, 0.1% trifluoroacetic acid (TFA), and 0.5% acetic acid.

Peptides were separated on an EASY-nLC 1200 HPLC system (Thermo Fisher Scientific, Odense) coupled online to a Q Exactive HF mass spectrometer via a nanoelectrospray source (Thermo Fisher Scientific)³⁷. Peptides were loaded in buffer A (0.5% formic acid) on in house packed columns (75 μ m inner diameter, 50 cm length, and 1.9 μ m C18 particles from Dr. Maisch GmbH). Peptides were eluted with a non-linear 270 min gradient of 5%–60% buffer B (80% ACN, 0.5% formic acid) at a flow rate of 250 nl/min and a column temperature of 50°C. The Q Exactive HF was operated in a data dependent mode with a survey scan range of 300-1750 m/z and a resolution of 60'000 at m/z 200.

MaxQuant software (version 1.5.3.54) was used to analyze MS raw files³⁸. MS/MS spectra were searched against the E. coli Nissle Uniprot FASTA database (UP000011176, the sequence for argA^{fbr} was manually added) and a common contaminants database (247 entries) by the Andromeda search engine³⁹. A false discovery rate (FDR) of 1% was required for peptides and proteins. Peptide identification was performed with an allowed initial precursor mass deviation of up to 7 ppm and an allowed fragment mass deviation of 20 ppm. Nonlinear retention time alignment of all measured samples was performed in MaxQuant. Peptide identifications were matched across different replicates within a time window of 1 min of the aligned retention times. Protein identification required at least 1 razor peptide. A minimum ratio count of 1 was required for valid quantification events via MaxQuant's Label Free Quantitation algorithm (MaxLFQ)³⁸. Data were filtered for common contaminants and peptides only identified by side modification were excluded from further analysis.

Data analysis was performed using the R statistical computing environment. Missing values were imputed with a normal distribution of 30% in comparison to the SD of measured values and a 1.8 SD down-shift of the mean to simulate the distribution of low signal values.

In vivo experiments with L-Arg bacteria

MC38 tumor cells were cultured and transferred s.c. into C57BL/6 WT mice as described above. Tumors were allowed to grow for 9-11 days prior to the treatments (tumor volume between 50-80 mm³). 5 x 10⁶ control or L-Arg bacteria were injected in 50 uL of PBS intratumorally (i.t.) with a 26-gauge needle twice a week for a total of four treatments. L-Arg bacteria and control bacteria were injected alone or in combination with 200µg of αPD-L1 mAb i.p in 100 uL of PBS.

Statistics

Data were analysed with Prism 5 (GraphPad Software) using Student's t test, 2way ANOVA and log-rank test comparison between curves statistical analysis. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Graphs show the mean + standard error of the mean (s.e.m.).

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Figure 1

Figure 2

Figure 3

Figure 4

Extended Figure 1

Extended Figure 2

Extended Figure 3

Extended Figure 4

Extended Figure 5