Title: Granzyme B skews $CD4^+$ T cell differentiation resulting in increased intestinal pathogenicity

Authors: Kristen L. Hoek,[a] Michael J. Greer,[b*] Kathleen G. McClanahan,[a*] Ali Nazmi,[a] M. Blanca Piazuelo,[c,e] Kshipra Singh,[c,e] Keith T. Wilson,[a,c,e,f,g] and Danyvid Olivares-Villagómez.[a,e,g,h]

*These two authors contributed equally to this report

Affiliations: [a]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, A5301 Medical Center North, Nashville, Tennessee 37232, USA; [b]Department of Biomedical Informatics, Vanderbilt University, Nashville, Tennessee, 37232, USA; [c]Division of Gastroenterology, Hepatology and Nutrition, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee 37232, USA; [e]Center for Mucosal Inflammation and Cancer, Vanderbilt University Medical Center, Nashville, Tennessee 37232; [f]Veterans Affairs Tennessee Valley Healthcare System, Nashville, Tennessee 37232; [g]Vanderbilt Institute for Infection, Immunology and Inflammation, Vanderbilt University Medical Center, A5301 Medical Center North, Nashville, Tennessee 37232, USA. [h]ORCID, 0000-0002-1158-8976

Corresponding author: Danyvid Olivares-Villagómez. Address: Vanderbilt University Medical center, A5211 Medical Center North, Nashville Tennessee 37232-0001. Fax number: 615-343-7392. Telephone number: 615-936-0134. email: danyvid.olivares-villagomez@vumc.org.

Abstract: $CD4^+$ T cell activation and differentiation are important events that set the stage for proper immune responses. Many factors are involved in the activation and differentiation of T cells, and these events are tightly controlled to prevent unwanted and/or exacerbated immune responses that may harm the host. It has been well documented that granzyme B, a potent serine protease involved in cell-mediated cytotoxicity, is readily expressed by certain $CD4^+$ T cells, such as regulatory T cells and $CD4^+CD8\alpha\alpha^+$ intestinal intraepithelial lymphocytes, both of which display cytotoxicity associated with granzyme B. However, because not all $CD4^+$ T cells expressing granzyme B are cytotoxic, additional roles for this protease in $CD4^+$ T cell biology remain unknown. Here, using a combination of *in vivo* and *in vitro* approaches, we report that granzyme B-deficient $CD4^+$ T cells display increased IL-17 production. In the adoptive transfer model of intestinal inflammation, granzyme B-deficient $CD4^+$ T cells triggered a more rapid disease onset than their WT counterparts, and presented a differential transcription profile. Similar results were also observed in granzyme B-deficient mice infected with *Citrobacter rodentium*. Our results suggest that granzyme B modulates $CD4^+$ T cell differentiation, providing a new perspective into the biology of this enzyme.

Introduction

45 Granzymes are a family of serine proteases expressed by many immune cell populations.
46 There are five different granzymes in humans (A, B, H, K and M) and 11 in mice (A, B, C, D, E,
47 F, G, K, L, M and N), which differ in function and substrate specificity.[1] Most research has focused
48 on granzymes A and B due to their hallmark role in $CD8^+$ T and NK cell-mediated cytotoxicity
49 against tumor cells or virus-infected cells.[2] However, mounting evidence indicates that granzymes
50 are also involved in other processes, including inflammatory responses,[3-5] remodeling of the
51 extracellular matrix,[6] and cardiovascular disorders.[7]

52 Activated $CD4^+$ T cells express granzyme B, and some of these cells exhibit lytic activity
53 similar to $CD8^+$ T cells.[8, 9] Antigen-specific $CD4^+$ T cell lytic activity mediated by granzyme B
54 can be triggered by viral and bacterial infections.[10-12] Similarly, some tumor-infiltrating $CD4^+$ T
55 cells also present anti-tumor cytotoxicity mediated by granzyme B.[13] In addition, one of the
56 mechanisms by which regulatory $CD4^+$ T cells in humans and mice control immune responses is
57 through granzyme B-mediated cell death.[14-18]

58 After activation, $CD4^+$ T cells can differentiate into a diverse set of T helper (Th) cells
59 depending on environmental cues and transcription factors, which ultimately leads Th cells to
60 produce a specific cytokine profile.[19] In certain populations of differentiated $CD4^+$ T cells,
61 granzyme B controls $CD4^+$ T cell immune responses by initiating activation-induced cell death.[20]
62 Downregulation of granzyme B, for example when Th2 cells are cultured in the presence of
63 vasoactive intestinal peptide, reduces Th2 cell death.[20] Moreover, granzyme B expression in Th2
64 cells is enhanced by IL-10R signaling, which results in cell death.[21] These studies clearly indicate
65 that granzyme B is involved in controlling some immune $CD4^+$ T cells responses. However, it is
66 unclear whether this enzyme is actively involved during the process of Th differentiation.

67 In this report, we present data indicating that granzyme B is expressed during $CD4^+$ T cell
68 differentiation. Cells lacking granzyme B show a different cytokine profile than granzyme B-
69 competent cells, primarily presenting increased IL-17 production. Moreover, using the adoptive
70 transfer model of intestinal inflammation, we show that granzyme B-deficient $CD4^+$ T cells
71 activated *in vivo* have a different gene expression profile and display increased pathogenicity
72 relative to WT cells. Similarly, we present evidence indicating that granzyme B deficiency
73 predisposes mice to higher susceptibility to *Citrobacter rodentium* infection. These results indicate
74 that granzyme B is required for proper $CD4^+$ T cell differentiation, and in its absence, $CD4^+$ T cells
75 may acquire an aberrant phenotype characterized by increased IL-17 production and pathogenicity.
76

77 **Results**

78 **Th0 and Th1, but not Th17 $CD4^+$ T cells express granzyme B during differentiation**

79 To determine the role of granzyme B in T cell activation and differentiation, we cultured
80 naïve $CD4^+$ T cells in Th0, Th1 and Th17 differentiation conditions. One and three days after
81 culture, RNA was extracted to determine granzyme B expression relative to naïve $CD4^+$ T cells.
82 Under Th0 conditions, expression of granzyme B at day 1 did not increase above the levels
83 observed for naïve $CD4^+$ T cells (Figure 1a, top), but presented an almost two-fold increase at day
84 3 (Figure 1a, bottom). Th1 cells expressed a 6-fold increase in granzyme B mRNA over naïve T
85 cells at day 1, decreasing to 2-fold at day 3. On the other hand, $CD4^+$ T cells under Th17
86 differentiation conditions did not induce granzyme B mRNA expression at either time point
87 (Figure 1a).

88 Around 15% of Th0 and Th1 cells expressed granzyme B intracellularly at day 5 (Figure
89 1b), whereas this enzyme was not detected in Th17 cells, confirming the results for granzyme B

90 mRNA expression. Granzyme B in Th1 cells was not detected at 1 day after differentiation but it
91 was conspicuously expressed at day 3 and day 5 (Figure 1c). On the other hand, Th17 cells did not
92 express granzyme B at any of the time points analyzed (Figure 1c). Because Th1 and Th17 cultures
93 contain different cytokine/antibody cocktails, and granzyme B is expressed in the former but not
94 in the latter helper cells, we investigated what factors present in these culture conditions induce or
95 repress granzyme B expression. For this purpose, we activated $CD4^+$ T cells under Th0 conditions
96 (anti-CD3/CD28, IL-2, and irradiated spleen cells), in the presence of individual factors present in
97 Th1 and Th17 differentiation cocktails (Figure 1d). IL-2 alone induced ~7.5% of $CD4^+$ T cells to
98 express granzyme B, which was not significantly different from cells cultured without IL-2 or in
99 the presence of IL-12, IL-1β, and IL-23, these last two cytokines involved in Th17 differentiation.
100 However, Th17-inducing cytokines IL-6 and TGFβ, as well as the blocking anti-IFNγ antibody
101 significantly ablated granzyme B expression. These results indicate that $CD4^+$ T cell activation
102 induces granzyme B activation, whereas some cytokines and anti-IFNγ inhibit its expression.
103 Because most of our differentiation cultures included spleen cells as a source of APC, we
104 determined whether these cells influence granzyme B expression during $CD4^+$ T cell activation.
105 As shown in Figure 1e, $CD4^+$ T cell activation in the absence of APC (but including IL-2), resulted
106 in less than 2% of $CD4^+$ T cells expressing granzyme B, whereas irradiated spleen APC
107 significantly increased granzyme B expression.
108 Overall, these results indicate that granzyme B is expressed during Th0 and Th1
109 differentiation, with irradiated APC being an important expression-enhancing factor. On the other
110 hand, Th17 differentiation conditions ablate granzyme B expression. This effect can be attributed
111 to IL-6, TGFβ, and anti-IFNγ.

112 As shown in Figure 1b, during Th0 and Th1 differentiation, two populations of $CD4^+$ T
113 cells can be distinguished: WT-gzmB^{pos} and WT-gzmB^{neg}. To determine whether these
114 populations present different cytokine profiles, we determined the frequencies of IFNγ and IL-17-
115 producing cells in each of these subpopulations. Around half of Th0 WT-gzmB^{pos} expressed IFNγ,
116 whereas only around 5% of the WT-gzmB^{neg} $CD4^+$ T cells were positive for IFNγ (Figure 1f). Th0
117 cells derived from granzyme B-deficient (*Gzmb*$^{-/-}$) mice presented decreased levels of IFNγ-
118 producing cells in comparison to WT-gzmB^{pos} cells, but significantly more IFNγ$^+$ cells than WT-
119 gzmB^{neg} cells (Figure 1f). On the other hand, IL-17 production was not significantly different
120 among Th0 cells from any of the $CD4^+$ T cell populations.
121 Th1 differentiation induced high levels of IFNγ$^+$ cells in all $CD4^+$ T cell populations
122 analyzed; however, WT-gzmB^{pos} cells presented the highest levels of IFNγ$^+$ cells in comparison
123 to WT-gzmB^{neg} or cells derived from *Gzmb*$^{-/-}$ mice (Figure 1g). Interestingly, under Th1
124 conditions, cells from *Gzmb*$^{-/-}$ mice presented significantly greater levels of IL-17$^+$ cells when
125 compared to WT-gzmB^{pos} and WT-gzmB^{neg} cells (Figure 1g). These results suggest that granzyme
126 B may be involved in preventing IL-17 production when $CD4^+$ T cells are cultured under Th1
127 conditions.
128
129 **Mesenteric lymph node APC enhance granzyme B and IL-17 expression**
130 As shown in the previous section, addition of irradiated spleen APC to the cultures
131 enhanced granzyme B expression by activated $CD4^+$ T cells under Th0 conditions (Figure 1e).
132 Because APC activity varies between lymphoid organs, we investigated whether APC from spleen
133 and mesenteric lymph nodes (MLN) induced differential granzyme B expression, and if so,
134 whether IFNγ and IL-17 expression is affected. Spleen APC induced greater granzyme B

135 expression than their MLN counterparts in WT $CD4^+$ T cells (Supp. Figure 1a). IFNγ production
136 between WT-$gzmB^{pos}$ $CD4^+$ T cells was similar regardless of the source of irradiated APC, which
137 was also observed between WT-$gzmB^{neg}$ $CD4^+$ T cells (Supp. Figure 1b). However, irradiated
138 APC from MLN induced higher IL-17 production than spleen APC in the WT-$gzmB^{pos}$ $CD4^+$ T
139 cell population (Supp. Figure 1c). Granzyme B-deficient $CD4^+IFN\gamma^+$ T cells were similar between
140 cultures containing spleen or MLN APC (Supplemental Fig. 1d). However, increased IL-17
141 production was also observed in granzyme B-deficient $CD4^+$ T cells differentiated in the presence
142 of MLN APC (Supp. Figure 1e).

143 Overall, these results indicate that APC present in distinct immunological compartments
144 stimulate differential granzyme B expression. Moreover, APC that induce $CD4^+$ T cells to express
145 less granzyme B also seem to promote increased IL-17 production (particularly in the WT-$gzmB^{pos}$
146 and granzyme B-deficient $CD4^+$ T cell populations), suggesting an inverse relationship between
147 granzyme B expression and IL-17 production.

148

149 ***In vivo* activation of granzyme B-deficient $CD4^+$ T cells results in increased pathogenicity**

150 To further understand the role of granzyme B in $CD4^+$ T cell differentiation, we adoptively
151 transferred naïve $CD4^+CD45RB^{hi}$ T cells from WT or *Gzmb*$^{-/-}$ mice into *Rag2*$^{-/-}$ recipient mice.
152 Because it is well-established that transfer of naïve $CD4^+$ T cells into immunocompromised
153 recipients results in chronic intestinal inflammation, [22, 23] this set up allows us to: 1) determine
154 whether granzyme B-deficiency alters $CD4^+$ T cell pathogenicity, and 2) whether absence of
155 granzyme B influences the cytokine profile of $CD4^+$ T cells activated *in vivo* (discussed in the next
156 section).

157 In our animal colony, transfer of naïve $CD4^+$ T cells from WT mice typically results in
158 disease symptoms by 6 weeks after transfer. However, when naïve $CD4^+$ T cells from *Gzmb*$^{-/-}$ mice
159 were transferred into *Rag2*$^{-/-}$ mice, weight loss was evident starting at 14 days after transfer, and
160 by day 21 these mice had lost around 20% of their original weight (Figure 2a). At this point, the
161 experiments were terminated to comply with our institution's animal welfare regulations. In
162 contrast, at day 21, *Rag2*$^{-/-}$ mice that received WT cells were either gaining or maintaining their
163 weight (Figure 2a). These results suggest that absence of granzyme B resulted in more pathogenic
164 T cells. To determine whether this was a $CD4^+$ T cell intrinsic effect, we adoptively transferred
165 naïve $CD4^+$ T cells from WT or *Gzmb*$^{-/-}$ donor mice into *Rag2*$^{-/-}$ *Gzmb*$^{-/-}$ mice. Donor $CD4^+$ T cells
166 from WT mice did not cause early weight loss in the double knock-out recipient mice, while $CD4^+$
167 T cells deficient in granzyme B caused similar weight loss as in *Rag2*$^{-/-}$ recipient mice (Figure 2a).
168 These results indicate a granzyme B-intrinsic effect in $CD4^+$ T cell pathogenesis. Weight loss in
169 mice receiving granzyme B-deficient cells was accompanied by other signs of disease, such as
170 diarrhea, pilo-erection, and hunching (Figure 2b). As expected, colon pathology based on immune
171 cell infiltration, loss of goblet cells, epithelial damage and hyperplasia was higher in mice
172 receiving $CD4^+$ T cells from *Gzmb*$^{-/-}$ donors (Figure 2c). Finally, granzyme B-deficient $CD4^+$ T
173 cells reconstituted the recipient mouse colons better than WT cells, regardless of the recipient mice
174 (Figure 2d).
175 In summary, our data indicate that absence of granzyme B in $CD4^+$ T cells activated *in vivo*
176 results in accelerated pathogenicity and increased cell reconstitution.
177
178 **Granzyme B-deficient $CD4^+$ T cells present increased IL-17 production *in vivo*.**

179 To determine whether granzyme B deficiency results in differential $CD4^+$ T cell cytokine
180 profiles *in vivo*, we adoptively transferred naïve $CD4^+CD45RB^{hi}$ T cells from WT or *Gzmb*$^{-/-}$ mice
181 into *Rag2*$^{-/-}$ recipient mice. Three weeks after transfer, donor-derived cells were recovered from
182 the MLN and lamina propria and analyzed for IFNγ and IL-17 expression. As shown in Figure 3,
183 IFNγ was the predominant cytokine produced by donor $CD4^+$ T cells derived from WT and *Gzmb*$^{-}$
184 $^{/-}$ mice. In cells recovered from the MLN, there were similar levels of IFNγ production between
185 WT and *Gzmb*$^{-/-}$ $CD4^+$ T cells (Figure 3a). However, $CD4^+$ T cells from *Gzmb*$^{-/-}$ donor mice
186 presented significantly increased IL-17 production in comparison to WT $CD4^+$ T cells (Figure 3a).
187 A similar pattern was observed in donor-derived cells recovered from the lamina propria (Figure
188 3b).
189 In summary, *in vivo* activated $CD4^+$ T cells deficient in granzyme B presented increased
190 IL-17 production. These results suggest that absence of granzyme B results in skewed *in vivo*
191 differentiation.
192
193 **Granzyme B deficient $CD4^+$ T cells present a distinct gene expression profile**
194 Because granzyme B-deficient $CD4^+$ T cells had increased IL-17 production when
195 activated *in vivo*, we investigated whether these cells presented a distinct gene expression profile
196 in comparison to *in vivo* activated $CD4^+$ T cells from WT mice. For this purpose, $CD4^+CD45RB^{hi}$
197 T cells from WT and *Gzmb*$^{-/-}$ donor mice were adoptively transferred into *Rag2*$^{-/-}$ mice. Three
198 weeks after transfer, cells were recovered from the MLN, their RNA extracted and gene expression
199 profile determined by RNAseq. As shown in Figure 4a, WT and granzyme B-deficient $CD4^+$ T
200 cells presented significantly different gene expression profiles. We also performed gene set
201 enrichment analysis to determine whether WT and granzyme B-deficient $CD4^+$ T cells recovered

202 from recipient mice segregated in specific groups. As indicated in Figure 4b, GSEA indicated that
203 these two $CD4^+$ T cell populations presented distinct differential gene expression within several
204 GSEA groups (Figure 4b). These results indicate that granzyme B-deficient $CD4^+$ T cells, in the
205 adoptive transfer system, follow a distinct differentiation pathway compared to WT $CD4^+$ T cells.

206 We validated the results generated from the RNAseq analysis by comparing the expression
207 of selected genes in naïve $CD4^+$ T cells (Figure 4c, top), and donor-derived cells recovered 21d
208 post-adoptive transfer (Figure 4c, bottom). Hallmark genes involved in Th17 responses, such as
209 *Rorc* and *Il17a* were similarly expressed in naïve $CD4^+$ T cells, regardless of the granzyme B
210 status, but their expression had significantly increased in recovered granzyme B-deficient $CD4^+$ T
211 cells. *Ddit4*, a gene important for optimal T cell proliferation,[24] is highly expressed in T cells from
212 multiple sclerosis patients, and is important in Th17 differentiation.[25] *Ddit4* expression was 3-fold
213 higher in activated $CD4^+$ T cells derived from *Gzmb*$^{-/-}$ mice in comparison to WT $CD4^+$ T cells
214 (Figure 4c). Granzyme B-deficient $CD4^+$ T cells presented a 2-fold increase in *Il7r* expression
215 (Figure 4c), which mediates IL-7 signaling, a well-known lymphopoietic cytokine also expressed
216 in T cells undergoing homeostatic expansion.[26] Plexin D1, encoded by the *Plxnd1* gene, binds
217 semaphorins[27] and is critical for directing thymocyte migration.[28] Although the role of *Plxnd1* is
218 not well known in mature T cell biology, granzyme B-deficient $CD4^+$ T cells presented
219 approximately a 4-fold increase in expression. Interestingly, donor $CD4^+$ T cells derived from
220 *Gzmb*$^{-/-}$ mice presented decreased *Il10ra* expression in comparison to WT counterparts (Figure
221 4c).

222 Overall, these results show that in the adoptive transfer model of $CD4^+$ T cell activation,
223 granzyme B deficiency results in a distinct gene expression profile, characterized by genes relevant
224 to Th17 differentiation and T cell proliferation.

225

226 **Granzyme B-deficient CD4$^+$ T cells possess better reconstitution capabilities**

227 As indicated in Figure 2d, granzyme B-deficient CD4$^+$ T cells showed greater
228 reconstitution in the colon than WT CD4$^+$ T cells when transferred into Rag-2$^{-/-}$ recipient mice.
229 However, this observation could be due to inflammation-driven proliferation, which was more
230 prevalent in *Rag2*$^{-/-}$ mice receiving CD4$^+$ T cells from *Gzmb*$^{-/-}$ mice. To further determine the
231 reconstitution potential of CD4$^+$ T cells derived from WT and *Gzmb*$^{-/-}$ mice, we performed
232 competitive adoptive T cell transfer experiments. For this purpose, naïve CD4$^+$ T cells from WT
233 CD45.1 and *Gzmb*$^{-/-}$ CD45.2 mice (1x10^5 each) were adoptively co-transferred into the same
234 recipient mice. Of the total donor-derived cells obtained from the MLN, granzyme B-deficient
235 CD4$^+$ T cells presented ~60% reconstitution, whereas WT-derived cells showed ~40%
236 reconstitution (Figure 5a). Although the IFNγ profile of co-transferred cells was similar to cells
237 transferred alone, co-transferred WT cells presented increased IL-17 production in comparison to
238 WT CD4$^+$ T cells transferred alone (Figure 5b). These results raise the possibility that granzyme
239 B-deficient CD4$^+$ T cells influenced the behavior of granzyme-competent CD4$^+$ T cells. *Rag2*$^{-/-}$
240 mice co-transferred with WT and GzB$^{-/-}$ CD4$^+$ T cells displayed similar weight loss (Figure 5c)
241 and colon pathology (Figure 5d) to mice transferred only with CD4$^+$ T cells from *Gzmb* $^{-/-}$ mice,
242 suggesting that the increased pathogenicity of the latter cells was maintained even in the presence
243 of granzyme-competent CD4$^+$ T cells.

244

245 **Granzyme B-deficient mice present normal cellularity and IFNγ /IL-17 production**

246 Since *in vivo* activated granzyme B-deficient CD4$^+$ T cells possess differential gene
247 expression and cytokine profiles, it is possible that these differences are also observed in naïve

248 cells. To investigate this possibility, we enumerated the $CD4^+$ T cell cellularity of spleen, MLN,
249 and IEL compartments from WT and *Gzmb*$^{-/-}$ mice. As indicated in Figure 6a, frequencies and
250 numbers of $TCR\beta^+CD4^+$ T cells were similar among the two groups of mice. *Ex vivo* IFNγ and IL-
251 17 production by non-stimulated (ns) or stimulated (s) naïve $CD4^+$ T cells derived from the MLN
252 and IEL showed no distinguishable difference between cells from WT and *Gzmb*$^{-/-}$ mice (Figure
253 6b).

254 In summary, naïve WT and *Gzmb*$^{-/-}$ animals appear to have similar $CD4^+$ T cell cellularity
255 and basal IFNγ and IL-17 production in peripheral lymphoid organs and the intestines.

256

257 **Granzyme B deficiency results in increased disease severity during *Citrobacter rodentium***
258 **infection**

259 As demonstrated in the previous section, naïve *Gzmb*$^{-/-}$ mice have a normal $CD4^+$ T cell
260 compartment compared to WT mice. However, because we observed that activated T cells from
261 *Gzmb*$^{-/-}$ mice became more pathogenic and had a distinct gene expression profile in the adoptive
262 transfer model of colitis, we investigated whether *Gzmb*$^{-/-}$ mice respond properly to antigenic
263 stimulus. For this purpose, we infected WT and *Gzmb* $^{-/-}$ mice with the extracellular bacterium
264 *Citrobacter rodentium*, which preferentially colonizes the colon of mice and induces an IL-17/IL-
265 22 immune response. After infection, WT mice maintained similar weight throughout the course
266 of the experiment; however, starting at 9 days post-infection, *Gzmb*$^{-/-}$ mice lost weight, reaching
267 approximately ~20% loss of the starting weight by day 12 (Figure 7a). *Gzmb*$^{-/-}$ mice also presented
268 other signs of disease, such as diarrhea, hunched posture, and pilo-erection, which were mostly
269 absent in WT mice (Figure 7b). Despite similar colon bacterial burden (Figure 7c) *Gzmb*$^{-/-}$ mice
270 developed higher pathology characterized by increased infiltration and epithelial injury than WT

271 mice (Figure 7d). Analysis of cytokine production showed that in the spleen, MLN and LP of
272 infected *Gzmb*$^{-/-}$ mice, there was a significant increase in CD4^{+} T cells expressing IL-17, while
273 IFNγ production was increased only in the MLN (Figure 7e). Because innate immune cells are
274 important for the response against *C. rodentium*, we investigated whether the absence of granzyme
275 B in innate immune cells was responsible for the observed increased pathology in infected *Gzmb*$^{-}$
276 $^{/-}$ mice. For this purpose, *Rag-2*$^{-/-}$ and *Rag-2*$^{-/-}$*Gzmb*$^{-/-}$ mice were infected with *C. rodentium* and
277 monitored for 14 days. Both groups of mice maintained similar weights throughout the course of
278 the experiment (Supplemental Figure 2a), indicating that the effect caused by granzyme B-
279 deficiency is most likely associated with adaptive immune cells. To further confirm these results,
280 we treated *Rag-2*$^{-/-}$ and *Rag-2*$^{-/-}$*Gzmb*$^{-/-}$ mice with anti-CD40 antibodies, which induce acute
281 intestinal inflammation in the absence of T or B cells.[29] As shown in Supplemental Figure 2b, *Rag-*
282 *2*$^{-/-}$ and *Rag-2*$^{-/-}$*Gzmb*$^{-/-}$ mice presented similar weight loss throughout the course of the experiment.
283 Overall, these results recapitulate what was observed in the adoptive transfer model of
284 colitis, and indicate that the absence of granzyme B increased pathogenicity, which is associated
285 with adaptive immune responses.
286
287 **Discussion**
288 Granzyme B expression in CD4^{+} T cells is well-documented, and has been primarily
289 studied in the context of granzyme B/perforin-dependent regulatory T cell suppressor activity.[30]
290 However, as our data indicate, granzyme B expression appears to be a common feature among a
291 significant fraction of activated CD4^{+} T cells. Interestingly, not all differentiation conditions
292 induced granzyme B expression. For example, Th0 and Th1 cells rapidly express granzyme B
293 starting at 1-day post activation, whereas Th17 cells do not express this enzyme at any time after

294 activation. In the former conditions, expression of granzyme B is primarily driven by the presence
295 of irradiated splenocytes, indicating the possibility that cell-to-cell contact is important for
296 granzyme B expression.

297 Although none of the cytokines involved in Th1 differentiation induced granzyme B
298 expression above the levels observed with the addition of irradiated splenocytes, anti-IFNγ and
299 some cytokines present in Th17 cocktails, such as IL-6 and TGFβ prevented the expression of the
300 enzyme, which suggests that restriction of granzyme B expression is necessary for Th17
301 polarization. Studies in cytotoxic $CD8^+$ T cells have shown that activation of these cells with anti-
302 CD3 in the presence of IL-6 induces granzyme B expression, but not in the presence of TGF-β.[31]
303 Here we show that in $CD4^+$ T cell differentiation, IL-6 does not enhance the expression of
304 granzyme B, indicating that $CD8^+$ and $CD4^+$ T cells possess different mechanisms for modulating
305 granzyme B production.

306 Granzyme B expression may not only be limited during the initial $CD4^+$ T cell priming and
307 differentiation. For example, TGFβ is known for its role in regulatory T cell differentiation, and
308 some of these cells require granzyme B for their suppressor functions. However, as our results
309 indicate, TGFβ ablates expression of this enzyme, suggesting that regulatory T cells must acquire
310 granzyme B expression during post-priming events. Similarly, a fraction of effector $CD4^+$ T cells
311 that migrate into the intestinal intraepithelial lymphocyte compartment acquire granzyme B
312 expression after transcriptional reprogramming.[32]

313 One of the most intriguing questions is: Why do $CD4^+$ T cells express granzyme B during
314 Th0/Th1 activation and differentiation? During *in vitro* Th0/Th1 differentiation WT-gzmB^{pos} and
315 WT-gzmB^{neg} cells have a distinct IFNγ profile, suggesting that these cells may represent different
316 lineages, and the activity of granzyme B influences their outcome. This is better exemplified when

317 analyzing granzyme B-deficient $CD4^+$ T cells, where *in vivo* and *in vitro* activation skew them
318 towards an IL-17-producing phenotype, with a distinct gene expression signature. We postulate
319 that granzyme B-deficient $IL\text{-}17^+$ cells represent a lineage of cells that underwent aberrant
320 differentiation. Therefore, expression of granzyme B during Th0/Th1 activation serves as a
321 checkpoint for proper $CD4^+$ T cell differentiation, preventing IL-17 production and increased
322 pathology. These observations have important significance because it has been reported that allelic
323 variants of granzyme B correlate with autoimmune disorders,[33-35] raising the possibility that in
324 certain $CD4^+$ T cell-mediated disorders, lack of proper granzyme B function increases the
325 probability of improper differentiation and increased pathogenesis potential. If this idea is correct,
326 then WT-$gzmB^{pos}$ $CD4^+$ T cells may represent cells in which the differentiation signals have the
327 potential to skew cells into an unwanted phenotype, and granzyme B expression in these cells
328 ensures the correct differentiation process.

329 Granzyme B activity in lymphocyte development and/or differentiation has been
330 previously reported.[36] These authors showed that granzyme A and B are important for the
331 development of a subset of TCR^{neg} intestinal intraepithelial lymphocytes known for the expression
332 of intracellular CD3γ. Development of these cells requires granzyme B to cleave and inactivate
333 the intracellular domain of Notch1. This raises the interesting possibility that during $CD4^+$ T cell
334 differentiation, granzyme B prevents unwanted phenotypes by disrupting signals coming from the
335 environment, such those provided by Notch and its ligands, which are known to influence $CD4^+$ T
336 cell differentiation.[37] Our observation that granzyme B expression was increased in the presence
337 of irradiated splenocytes, supports this hypothesis and we are currently investigating what signals
338 derived from APC enhance granzyme B expression.

339 It has been reported that granzyme B-deficient $CD4^+CD25^-$ T cells used in murine models
340 of graft versus host disease (GVHD), induced faster disease onset with increased lethality, in
341 comparison to their WT counterparts.[38] Although this group did not provide a mechanistic
342 explanation for the increased pathogenicity observed, they suggested that increased proliferation
343 and decreased cell death may be involved. According to our data, we believe that in the GVHD
344 model, differential proliferation and survival of granzyme B-deficient $CD4^+$ T cells are the result
345 of aberrant $CD4^+$ T cell differentiation, which allows escape of highly pathogenic clones. Further
346 investigations in the GVDH model are needed to test this hypothesis.

347 In summary, granzyme B has been known for its role in cell mediated-cytotoxicity, and
348 while many groups have reported potential extracellular roles for this enzyme,[39] here we present
349 *in vitro* and *in vivo* evidence supporting a novel intrinsic function for granzyme B during $CD4^+$ T
350 cell differentiation. Although many questions remain to be answered regarding how granzyme B
351 activity is involved in this process, our results provide a strong foundation for a better
352 understanding of the function of this enzyme in $CD4^+$ T cell biology.

353

354 **Methods**

355 *Mice*. C57BL/6J and C57BL/6J.CD45.1 mice were originally purchased from The Jackson
356 Laboratory (000664, and 002014 respectively) and have been maintained and acclimated in our
357 colony for several years. Granzyme B (*Gzmb*)$^{-/-}$ mice were kindly provided by Dr. Xuefang Cao.
358 CD57BL/6J *Rag2*$^{-/-}$ mice were kindly provided by Dr. Luc Van Kaer. *Rag2*$^{-/-}$*Gzmb*$^{-/-}$ were
359 generated in our colony. All mice were between 6 to 10 weeks of age at the time of
360 experimentation. Male and female mice were used for all experiments, except for *Citrobacter*

361 *rodentium* infection where 7-week-old females were used. Mice were maintained in accordance
362 with the Institutional Animal Care and Use Committee at Vanderbilt University.
363
364 *Reagents and flow cytometry.* Fluorochrome-coupled anti-mouse CD4 (GK1.5), CD8α (53-6.7),
365 CD45RB (C363.16A), granzyme B (NGZB), IFNγ (XMG1.2), IL-17a (TC11-18H10), TCRβ
366 (H57-597) and ghost viability dyes were purchased from ThermoFisher, BD Bioscences or Tonbo.
367 Biotinylated anti-CD8α (53-1.7) and CD19 (1D3) antibodies were purchased from Tonbo. Anti-
368 CD40 antibody was purchased from BioXcell. Surface cell staining was performed following
369 conventional techniques. For intracellular cytokine staining, cells were stimulated with PMA and
370 ionomycin in the presence of Golgi Stop (BD Biosciences) for 4hr prior to staining. Extracellular
371 markers were stained, cells were fixed briefly with 2% paraformaldehyde, followed by
372 permeabilization and intracellular staining using the BD Cytofix/Cytoperm kit, according to
373 manufacturer's instructions. All stained samples were acquired using BD FACS Canto II, 3- or 4-
374 Laser Fortessa, or 5-Laser LSR II flow cytometers (BD Bioscences). Data were analyzed using
375 FlowJo software (Tree Star).
376
377 *Lymphocyte isolation*. Spleen and MLN lymphocytes were isolated by conventional means. IEL
378 and LP cells were isolated by mechanical disruption as previously reported.[40] Briefly, after
379 flushing the intestinal contents with cold HBSS and removing excess mucus, the intestines were
380 cut into small pieces (~1 cm long) and shaken for 45 minutes at 37°C in HBSS supplemented with
381 5% fetal bovine serum and 2 mM EDTA. Supernatants were recovered and cells isolated using a
382 discontinuous 40/70% Percoll (General Electric) gradient. To obtain lamina propria lymphocytes,

383 intestinal tissue was recovered and digested with collagenase (187.5 U/ml, Sigma) and DNase I
384 (0.6 U /ml, Sigma). Cells were isolated using a discontinuous 40/70% Percoll gradient.
385
386 In vitro *CD4 T cell activation/differentiation.* Naïve CD4 T cells were isolated from the spleens of
387 the indicated mice by magnetic sorting using the Miltenyi or StemCell naïve $CD4^+$ T cell isolation
388 kits according to the manufacturer's instructions. Splenocytes or MLN cells used as APC were
389 incubated for 1hr at 37°C, non-adherent cells were removed, adherent cells were washed and
390 resuspended in complete RPMI, and irradiated with 7 Gy (700 rads). $5x10^5$ $CD4^+$ T cells with or
391 without $5x10^5$ irradiated APC were incubated in the presence of plate bound anti-CD3 (5μg/ml)
392 and soluble anti-CD28 (2.5μg/ml), for the indicated times, under the following conditions: Th0
393 (10ng/mL 1L-2), Th1 (10ng/mL 1L-2, 20ng/mL IL-12, and 10μg/mL anti-IL-4) , and Th17
394 (5ng/mL TGFβ, 20ng/mL IL-6, 10ng/mL IL-1β, 10ng/mL IL-23, 10μg/mL anti-IL-4, and
395 10μg/mL anti-IFNγ).
396
397 *Adoptive transfer of naïve CD4 T cells*. Splenocytes from WT or *Gzmb*$^{-/-}$ mice were depleted of
398 $CD19^+$ and $CD8\alpha^+$ cells using magnetic bead sorting (Miltenyi). Enriched cells were stained with
399 antibodies directed against TCRβ, CD4 and CD45RB, and naïve $CD4^+$ T cells were sorted as
400 $CD4^+CD45RB^{hi}$ using a FACSAria III at the Flow Cytometry Shared Resource at VUMC. $1x10^5$
401 cells were adoptively transferred into the indicated recipient mice. Starting weight was determined
402 prior to injection. Mice were monitored and weighed weekly. At the end time point, mice were
403 sacrificed and donor-derived cells were isolated from the indicated organs or tissues. Colon
404 histopathology was performed in a blind fashion by a GI pathologist (MBP), following established
405 parameters.[41] For competitive transfer experiments, $1x10^5$ $CD4^+CD45RB^{hi}$ cells from both WT-

406 CD45.1 and *Gzmb*$^{-/-}$ CD45.2 mice were adoptively co-transferred into *Rag2*$^{-/-}$ mice and analyzed
407 as indicated above.
408
409 *Transcription profile analysis.* For gene expression analysis, TCRβ^{+}CD4^{+} T cells were purified by
410 flow cytometry from a pool of MLN cells comprised of 1 male and 1 female animal with similar
411 disease progression 21 days after adoptive transfer. RNA was isolated using the RNeasy micro kit
412 (Qiagen). cDNA library preparation and RNAseq was performed by the VANTAGE Core at
413 VUMC on an Illumina NovaSeq 6000 (2 x 150 base pair, paired-end reads). The tool Salmon[42]
414 was used for quantifying the expression of RNA transcripts. The R project software along with the
415 edgeR method[43] was used for differential expression analysis. For gene set enrichment analysis
416 (GSEA), RNAseq data was ranked according to the t-test statistic. The gene sets curated (C2), GO
417 (C5), immunological signature collection (C7) and hallmarks of cancer (H) of the Molecular
418 Signatures Database (MSigDB) were used for enrichment analysis. GSEA enrichment plots were
419 generated using the GSEA software[44] from the Broad Institute with 1000 permutations.
420
421 *Real time PCR*. For *in vitro* analysis, RNA was extracted from MACS-enriched, differentiated
422 CD4^{+} T cells at day 1 and 3 of culture. For *in vivo* analysis, RNA was isolated from flow cytometry
423 purified donor-derived TCRβ^{+}CD4^{+} T cells as described above. cDNA was synthesized using the
424 RT2 First Strand kit (Qiagen). Real time PCR was performed using RT2 SYBR Green Mastermix
425 (Qiagen) on an Applied BioSystems Proflex PCR cycler. All primers were purchased from Qiagen.
426
427 Citrobacter rodentium *infection*. Seven-week old female WT and *Gzmb*$^{-/-}$ animals were infected
428 with *C. rodentium* as previously described[45]. Briefly, mice were infected with $5x10^{8}$ CFU

429 exponentially grown bacteria by oral gavage. Starting weight was determined prior to gavage.
430 Mice were monitored and weighed daily for 14d. At the end time point, cells were isolated from
431 the indicated organs or tissues. Cellularity was determined, intracellular cytokine staining was
432 performed, and colon histopathology was performed in a blind fashion by a GI pathologist (MBP)
433 as previously described.[46]
434
435 *Anti-CD40 disease induction*. *Rag-2*$^{-/-}$ and *Rag-2*$^{-/-}$*Gzmb*$^{-/-}$ mice were injected i.p. with
436 150μg/mouse of anti-CD40 (BioXcell). Mice were monitored and weighed for daily for 7 days.
437
438 **Acknowledgments**
439 This work was supported by NIH grant R01DK111671 (D.O-V.); National Library of Medicine
440 T15 LM00745 (M.J.G.); Vanderbilt Training in Cellular, Biochemical and Molecular Sciences
441 Training Program, T32 GM008554-25 (K.G.M.); Vanderbilt Training in Gastroenterology T32
442 grant NIH T32 DK007673 (A.N.); and the Digestive Disease Research Center at Vanderbilt
443 University Medical Center, NIH grant P30DK058404 (M.B.P., K.T.W., and D.O-V.). We thank
444 the Flow Cytometry Shared Resource and the Vanderbilt Technologies for Advanced Genomics
445 (VANTAGE) for their invaluable support.
446
447 **Author contributions**: K.L.H., and D.O-V. designed research; K.L.H., M.J.G., K.M., A.N., and
448 K.S. performed research; K.L.H., M.J.G., M.B.P. and D.O-V. analyzed data; K.S. and K.T.W.
449 provided expertise with *Citrobacter rodentium* infections; K.L.H. and D.O-V. wrote the
450 manuscript.
451

Competing interests: The authors declare no competing interests.

References

1. Anthony DA, Andrews DM, Watt SV, Trapani JA, Smyth MJ. Functional dissection of the granzyme family: cell death and inflammation. *Immunol Rev* 2010; **235**(1)**:** 73-92.

2. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol* 2008; **26:** 389-420.

3. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. *Immunol Rev* 2010; **235**(1)**:** 105-116.

4. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate proinflammatory cytokine responses. *J Immunol* 2015; **194**(2)**:** 491-497.

5. Kumar AA, Delgado AG, Piazuelo MB, Van Kaer L, Olivares-Villagomez D. Innate CD8alphaalpha(+) lymphocytes enhance anti-CD40 antibody-mediated colitis in mice. *Immun Inflamm Dis* 2017; **5**(2)**:** 109-123.

6. Parkinson LG, Toro A, Zhao H, Brown K, Tebbutt SJ, Granville DJ. Granzyme B mediates both direct and indirect cleavage of extracellular matrix in skin after chronic low-dose ultraviolet light irradiation. *Aging Cell* 2015; **14**(1)**:** 67-77.

7. Chamberlain CM, Ang LS, Boivin WA, Cooper DM, Williams SJ, Zhao H *et al.* Perforin-independent extracellular granzyme B activity contributes to abdominal aortic aneurysm. *Am J Pathol* 2010; **176**(2)**:** 1038-1049.

8. Lancki DW, Hsieh CS, Fitch FW. Mechanisms of lysis by cytotoxic T lymphocyte clones. Lytic activity and gene expression in cloned antigen-specific CD4+ and CD8+ T lymphocytes. *J Immunol* 1991; **146**(9)**:** 3242-3249.

9. Ebnet K, Chluba-de Tapia J, Hurtenbach U, Kramer MD, Simon MM. In vivo primed mouse T cells selectively express T cell-specific serine proteinase-1 and the proteinase-like molecules granzyme B and C. *Int Immunol* 1991; **3**(1)**:** 9-19.

10. Sun Q, Burton RL, Pollok KE, Emanuel DJ, Lucas KG. CD4(+) Epstein-Barr virus-specific cytotoxic T-lymphocytes from human umbilical cord blood. *Cell Immunol* 1999; **195**(2)**:** 81-88.

11. Canaday DH, Wilkinson RJ, Li Q, Harding CV, Silver RF, Boom WH. CD4(+) and CD8(+) T cells kill intracellular Mycobacterium tuberculosis by a perforin and Fas/Fas ligand-independent mechanism. *J Immunol* 2001; **167**(5)**:** 2734-2742.

12. Yasukawa M, Ohminami H, Yakushijin Y, Arai J, Hasegawa A, Ishida Y *et al.* Fas-independent cytotoxicity mediated by human CD4+ CTL directed against herpes simplex virus-infected cells. *J Immunol* 1999; **162**(10)**:** 6100-6106.

13. Dorothee G, Vergnon I, Menez J, Echchakir H, Grunenwald D, Kubin M *et al.* Tumor-infiltrating CD4+ T lymphocytes express APO2 ligand (APO2L)/TRAIL upon specific stimulation with autologous lung carcinoma cells: role of IFN-alpha on APO2L/TRAIL expression and -mediated cytotoxicity. *J Immunol* 2002; **169**(2)**:** 809-817.

14. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* 2004; **104**(9)**:** 2840-2848.

15. Koonpaew S, Shen S, Flowers L, Zhang W. LAT-mediated signaling in CD4+CD25+ regulatory T cell development. *J Exp Med* 2006; **203**(1)**:** 119-129.

16. Kawamura K, Kadowaki N, Kitawaki T, Uchiyama T. Virus-stimulated plasmacytoid dendritic cells induce CD4+ cytotoxic regulatory T cells. *Blood* 2006; **107**(3)**:** 1031-1038.

17. Price JD, Schaumburg J, Sandin C, Atkinson JP, Lindahl G, Kemper C. Induction of a regulatory phenotype in human CD4+ T cells by streptococcal M protein. *J Immunol* 2005; **175**(2)**:** 677-684.

18. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 2005; **174**(4)**:** 1783-1786.

19. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 2010; **28:** 445-489.

20. Sharma V, Delgado M, Ganea D. Granzyme B, a new player in activation-induced cell death, is down-regulated by vasoactive intestinal peptide in Th2 but not Th1 effectors. *J Immunol* 2006; **176**(1)**:** 97-110.

21. Coomes SM, Kannan Y, Pelly VS, Entwistle LJ, Guidi R, Perez-Lloret J *et al.* CD4(+) Th2 cells are directly regulated by IL-10 during allergic airway inflammation. *Mucosal immunology* 2017; **10**(1)**:** 150-161.

22. Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 1994; **179**(2)**:** 589-600. PMC2191378.

23. Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1994; **1**(7)**:** 553-562.

24. Reuschel EL, Wang J, Shivers DK, Muthumani K, Weiner DB, Ma Z *et al.* REDD1 Is Essential for Optimal T Cell Proliferation and Survival. *PLoS One* 2015; **10**(8)**:** e0136323.

25. Zhang F, Liu G, Li D, Wei C, Hao J. DDIT4 and Associated lncDDIT4 Modulate Th17 Differentiation through the DDIT4/TSC/mTOR Pathway. *J Immunol* 2018; **200**(5)**:** 1618-1626.

26. Fry TJ, Mackall CL. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol* 2005; **174**(11)**:** 6571-6576.

27. Carvalheiro T, Rafael-Vidal C, Malvar-Fernandez B, Lopes AP, Pego-Reigosa JM, Radstake T *et al.* Semaphorin4A-Plexin D1 Axis Induces Th2 and Th17 While Represses Th1 Skewing in an Autocrine Manner. *Int J Mol Sci* 2020; **21**(18).

28. Choi YI, Duke-Cohan JS, Ahmed WB, Handley MA, Mann F, Epstein JA *et al.* PlexinD1 glycoprotein controls migration of positively selected thymocytes into the medulla. *Immunity* 2008; **29**(6)**:** 888-898.

29. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R *et al.* Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 2006; **25**(2)**:** 309-318.

30. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR *et al.* Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 2007; **27**(4)**:** 635-646.

31. Huber M, Heink S, Grothe H, Guralnik A, Reinhard K, Elflein K *et al.* A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity. *Eur J Immunol* 2009; **39**(7)**:** 1716-1725.

32. Mucida D, Husain MM, Muroi S, van Wijk F, Shinnakasu R, Naoe Y *et al.* Transcriptional reprogramming of mature CD4(+) helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. *Nat Immunol* 2013; **14**(3)**:** 281-289.

33. Jin Y, Birlea SA, Fain PR, Gowan K, Riccardi SL, Holland PJ *et al.* Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. *The New England journal of medicine* 2010; **362**(18)**:** 1686-1697.

34. Li S, Yao W, Pan Q, Tang X, Zhao S, Wang W *et al.* Association analysis revealed one susceptibility locus for vitiligo with immune-related diseases in the Chinese Han population. *Immunogenetics* 2015; **67**(7)**:** 347-354.

35. Shen C, Gao J, Sheng Y, Dou J, Zhou F, Zheng X *et al.* Genetic Susceptibility to Vitiligo: GWAS Approaches for Identifying Vitiligo Susceptibility Genes and Loci. *Front Genet* 2016; **7:** 3.

36. Ettersperger J, Montcuquet N, Malamut G, Guegan N, Lopez-Lastra S, Gayraud S *et al.* Interleukin-15-Dependent T-Cell-like Innate Intraepithelial Lymphocytes Develop in the Intestine and Transform into Lymphomas in Celiac Disease. *Immunity* 2016; **45**(3)**:** 610-625.

37. Amsen D, Helbig C, Backer RA. Notch in T Cell Differentiation: All Things Considered. *Trends Immunol* 2015; **36**(12)**:** 802-814.

38. Du W, Leigh ND, Bian G, O'Neill RE, Mei L, Qiu J *et al.* Granzyme B-Mediated Activation-Induced Death of CD4+ T Cells Inhibits Murine Acute Graft-versus-Host Disease. *J Immunol* 2015; **195**(9)**:** 4514-4523.

39. Boivin WA, Cooper DM, Hiebert PR, Granville DJ. Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. *Laboratory investigation; a journal of technical methods and pathology* 2009; **89**(11)**:** 1195-1220.

40. Nazmi A, Greer MJ, Hoek KL, Piazuelo MB, Weitkamp J, Olivares-Villagomez D. Osteopontin and iCD8alpha cells promote intestinal intraepithelial lymphocyte homeostasis. *J Immunol* 2020; **In press**.

41. Aranda R, Sydora BC, McAllister PL, Binder SW, Yang HY, Targan SR *et al.* Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. *J Immunol* 1997; **158**(7)**:** 3464-3473.

42. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods* 2017; **14**(4)**:** 417-419.

43. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010; **26**(1)**:** 139-140.

44. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005; **102**(43)**:** 15545-15550.

632 45. Olivares-Villagomez D, Algood HM, Singh K, Parekh VV, Ryan KE, Piazuelo MB
633 *et al.* Intestinal epithelial cells modulate CD4 T cell responses via the thymus
634 leukemia antigen. *J Immunol* 2011; **187**(8)**:** 4051-4060.
635
636 46. Singh K, Gobert AP, Coburn LA, Barry DP, Allaman M, Asim M *et al.* Dietary
637 Arginine Regulates Severity of Experimental Colitis and Affects the Colonic
638 Microbiome. *Front Cell Infect Microbiol* 2019; **9:** 66.
639
640

641

642 **Figure legends**

643

644 **Figure 1**. Granzyme B expression during T cell differentiation. (a) Naïve $CD4^+$ T cells from WT
645 mice were cultured in the presence of plate-bound anti-CD3 and soluble CD28 under the
646 indicated differentiation conditions, and in the absence of irradiated splenocytes. At day 1 (top)
647 and 3 (bottom), cells were recovered, RNA extracted and granzyme B mRNA expression
648 determined. (b) Intracellular granzyme B in cells cultured as in (a), but in the presence of $5x10^5$
649 irradiated splenocytes. Left, representative dot plot; live, $TCR\beta^+CD4^+$ cells are displayed. Right,
650 data summary. Each dot represents an individual sample. (c) Granzyme B expression of cells
651 cultured in Th1 and Th17 conditions in the presence of irradiated splenocytes at different time
652 points. (d) Cells cultured in Th0 conditions in the presence of the indicated individual cytokines,
653 harvested 5 days after culture. (e) Cells cultured as indicated in (d) in the presence or absence of
654 irradiated APC from spleen. Intracellular IFNγ and IL-17 staining of representative samples
655 cultured in (f) Th0 or (g) Th1 conditions in the presence of irradiated splenocytes. Top,
656 intracellular IFNγ and IL-17 staining representative samples; bottom, data summary. Each dot
657 represents an individual sample. Data are representative of at least 3 independent experiments,
658 performed in triplicate. n=6. For (e), ***P<0.001, Student's t test; for (g) and (h), *P<0.05;
659 **P<0.01; ***P<0.001; ****P<0.001, One-way ANOVA.

660

661 **Supplemental Figure 1**. Granzyme B and cytokine expression in the presence of $5x10^5$
662 irradiated spleen or MLN APC. Naïve $CD4^+$ T cells from WT mice were cultured in the presence
663 of plate-bound anti-CD3 and soluble anti-CD28 under Th1 differentiation conditions. (a)
664 Granzyme B expression 5 days after culture. (b) IFNγ and (c) IL-17 production from the same
665 cultures. (d) IFNγ and (e) IL-17 expression from granzyme B-deficient $CD4^+$ T cells under
666 similar cultures as in (a). Each dot represents an individual sample. Data are representative of at
667 least 2 independent experiments, performed in triplicate. n=3-6. *P<0.05; **P<0.01;
668 ***P<0.001. For (a) and (e), Student's t-test; for (b) and (c), One-way ANOVA.

669

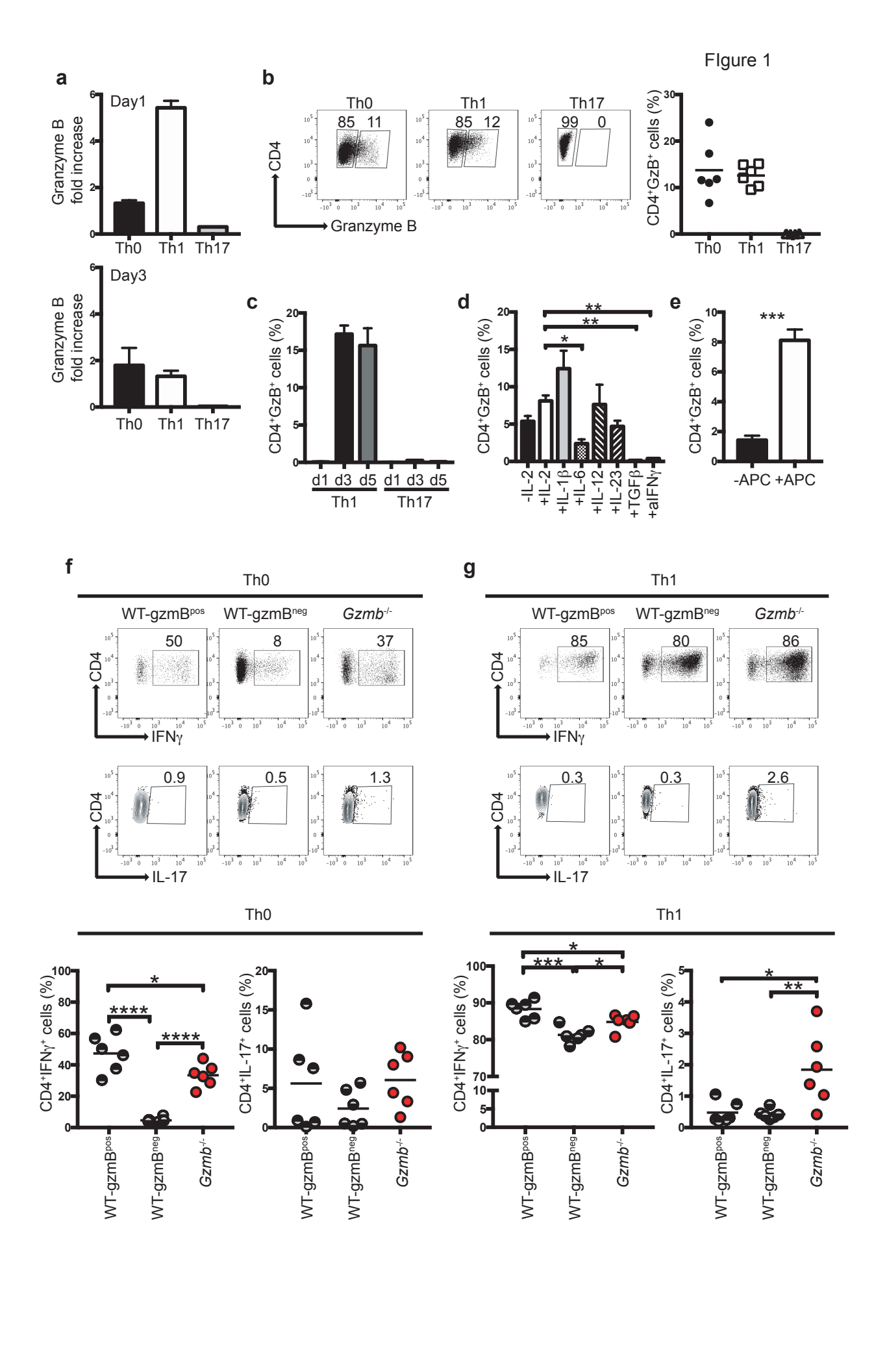
670 **Figure 2**. *In vivo* activated granzyme B-deficient $CD4^+$ T cells present increased pathogenicity.
671 $1x10^5$ $CD4^+CD45RB^{hi}$ T cells from WT or *Gzmb*$^{-/-}$ donor mice were adoptively transferred into
672 *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Gzmb*$^{-/-}$ recipient mice. (a) Mice were weighed weekly, and (b) monitored for

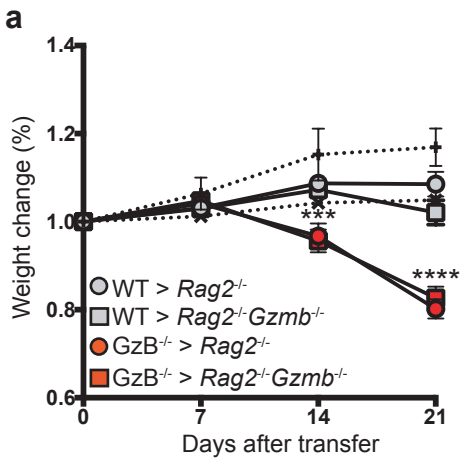
673 signs of disease. Twenty-one days after transfer, (c) colon pathology was scored (left,
674 representative micrographs, 100X magnification; right, data summary), and (d) donor-cell
675 reconstitution of the IEL compartment in the colon was determined. For (a), dotted lines
676 represent untreated *Rag2*$^{-/-}$ and *Rag-2*$^{-/-}$*Gzmb*$^{-/-}$ mice. Each dot represents an individual mouse.
677 Data are representative of at least 3 independent experiments. n=8-10. For (a), ***P<0.001;
678 ****P<0.0001; Student's t-test for each time point. For (b), # compares WT > *Rag2*$^{-/-}$ to *Gzmb*$^{-/-}$
679 > *Rag2*$^{-/-}$ and WT > *Gzmb*$^{-/-}$*Rag2*$^{-/-}$ at 14 days, and ## compares WT > *Rag2*$^{-/-}$ to *Gzmb*$^{-/-}$ > *Rag2*$^{-/-}$
680 and WT > *Gzmb*$^{-/-}$*Rag2*$^{-/-}$ at 21 days; Two-way ANOVA. For (c) and (d), *P<0.05;
681 ***P<0.001; One-way ANOVA.
682
683 **Figure 3**. Granzyme B-deficient CD4^{+} T cells present skewed IL-17 differentiation *in vivo.*
684 1x10^{5} CD4^{+}CD45RB^{hi} T cells from WT or *Gzmb*$^{-/-}$ donor mice were adoptively transferred into
685 *Rag2*$^{-/-}$ recipient mice. Three weeks after transfer, donor-derived cells were recovered from (a)
686 MLN, and (b) lamina propria, and their IFNγ/IL-17 profile was determined by intracellular
687 staining. Dot plots indicate a representative sample. Live, TCRβ^{+}CD4^{+} cells are displayed.
688 Graphs represent the summary. Each dot indicates an individual mouse. For MLN, n=11-13; for
689 lamina propria, n=7-11. Data are representative of at least 3 independent experiments. **P<0.01;
690 ****P<0.001. Student's t-Test.
691
692 **Figure 4**. Granzyme B-deficient CD4^{+} T cells activated *in vivo* present a distinct gene expression
693 profile. 1x10^{5} CD4^{+}CD45RB^{hi} T cells from WT or *Gzmb*$^{-/-}$ donor mice were adoptively
694 transferred into *Rag2*$^{-/-}$ recipient mice. Three weeks after transfer, donor-derived cells were
695 recovered from the MLN, sorted for live TCRβ^{+}CD4^{+}, their RNA isolated and sequenced. (a)
696 Representative heat map. (b) Representative gene set enrichment analysis. (c) Real-time PCR
697 validation of selected genes. n=6-7. *P<0.05; **P<0.01. Student's t-Test.
698
699 **Figure 5**. Granzyme B-deficient CD4^{+} T cells present greater *in vivo* reconstitution.
700 1x10^{5} CD4^{+}CD45RB^{hi} T cells from WT (CD45.1) and *Gzmb*$^{-/-}$ (CD45.2) donor mice were co-
701 transferred into the same *Rag2*$^{-/-}$ recipient mice. As controls, cells from the same donors were
702 independently transferred into *Rag2*$^{-/-}$ recipient mice. Three weeks after transfer, (a) MLN
703 reconstitution (right, representative dot plot displaying live, TCRβ^{+}CD4^{+} cells; left, summary),

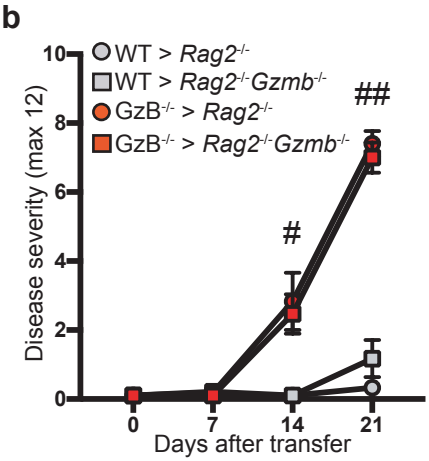
704 and (b) IFNγ/IL-17 production was determined. (c) *Rag2*$^{-/-}$ mice that received CD4^{+} T cells from
705 both WT/*Gzmb*$^{-/-}$ or only *Gzmb*$^{-/-}$ mice were weighed weekly. (d) Colon pathology was
706 determined 3 weeks post transfer. Each dot represents an individual mouse. For (a) to (c), n=8-
707 10; for (e) to (g), n=5-11. Data are representative of at least 2 independent experiments.
708 **P<0.01; Student's t-Test.
709
710 **Figure 6**. Naïve granzyme B-deficient mice present a normal CD4^{+} T cell compartment. (a)
711 CD4^{+} T cell frequencies and cellularity from spleen, MLN, and IEL compartment were
712 determined by flow cytometry. (b) *Ex vivo* IFNγ and IL-17 production from CD4^{+} T cells derived
713 from naïve WT and *Gzmb*$^{-/-}$ mice. Cells were non-stimulated (ns) or stimulated (s) for 4 h with
714 PMA/ionomycin. Each dot represents an individual mouse. For (a), spleen and MLN, n=6-10; for
715 IEL, n=12-13. For (b), n=6.
716
717 **Figure 7.** Granzyme B-deficient mice present severe disease after *C. rodentium* infection.
718 Indicated mice were orogastrically infected with *C. rodentium* (5x10^{8} CFU/mouse) and
719 monitored for (a) weight change, and (b) signs of disease. At 14 days post infection, colons were
720 dissected, (c) colonization/g of colon was determined, and (d) pathology scored as indicated in
721 the Methods section; left, representative micrographs (200X magnification); right, data
722 summary. (e) CD4^{+} T cell IFNγ and IL-17 expression in the indicated organs was determined.
723 Cells were non-stimulated (ns) or stimulated (s) for 4 h with PMA/ionomycin. Each dot
724 represents an individual mouse. For (a-d), n=7-9; for (e), 3-5. Data are representative of at least 3
725 independent experiments. For (a): **P<0.01; ***P<0.01; ****P<0.001; Student's t-Test for each
726 time point. For (b): ****P<0.001; Student's t-Test for each time point. For (d) and (e): *P<0.05;
727 **P<0.01; One-way ANOVA.
728
729 **Supplemental Figure 2.** (a) Absence of adaptive immune cells rescues granzyme B-deficient
730 mice from severe disease. The indicated mice were orogastrically infected with *C. rodentium*
731 (5x10^{8} CFU/mouse) and monitored for weight change for 14 days. Data are representative of at
732 least 2 independent experiments. n=3-4 for sham groups. n=5-7 for infected mice. (b) Granzyme
733 B deficiency does not alter disease progression in the anti-CD40 model of colitis. The indicated

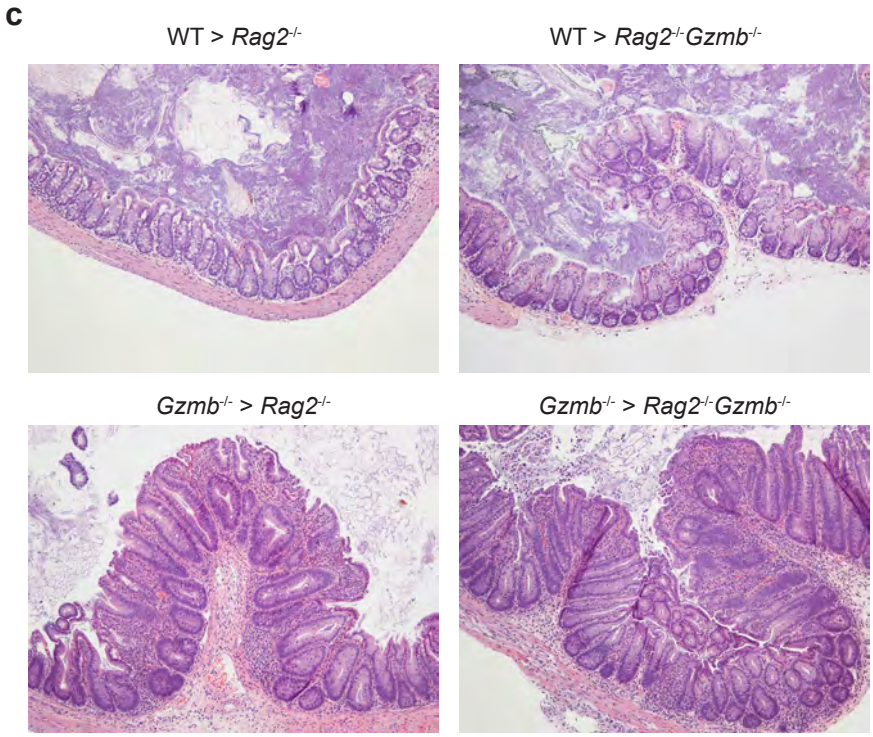
734 mice were treated i.p. with 150 μg/mouse and weighed daily. Data are representative of at least 3
735 independent experiments. n=2 for sham groups. n=14 for treated group.
736
737
738
739
740
741
742
743
744
745

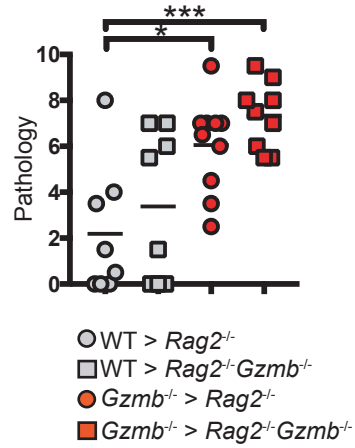
FIgure 1


a
Day1
Granzyme B fold increase
Th0
Th1
Th17
Day3
b
Th0
Th1
Th17
85 11
85 12
99 0
CD4
Granzyme B
CD4+GzB+ cells (%)
c
d1 d3 d5
Th1
Th17
d
-IL-2
+IL-2
+IL-1β
+IL-6
+IL-12
+IL-23
+TGFβ
+aIFNγ
e
-APC
+APC
f
Th0
WT-gzmBpos
WT-gzmBneg
Gzmb-/-
50
8
37
IFNγ
0.9
0.5
1.3
IL-17
CD4+IFNγ+ cells (%)
CD4+IL-17+ cells (%)
g
Th1
85
80
86
0.3
0.3
2.6


a
Weight change (%)
1.4
1.2
1.0
0.8
0.6
0
7
14
21
Days after transfer
***
****
WT > Rag2-/-
WT > Rag2-/-Gzmb-/-
GzB-/- > Rag2-/-
GzB-/- > Rag2-/-Gzmb-/-


b
Disease severity (max 12)
10
8
6
4
2
0
0
7
14
21
Days after transfer
#
##
WT > Rag2-/-
WT > Rag2-/-Gzmb-/-
GzB-/- > Rag2-/-
GzB-/- > Rag2-/-Gzmb-/-


c
WT > Rag2-/-
WT > Rag2-/-Gzmb-/-
Gzmb-/- > Rag2-/-
Gzmb-/- > Rag2-/-Gzmb-/-


Pathology
10
8
6
4
2
0
***
*
WT > Rag2-/-
WT > Rag2-/-Gzmb-/-
Gzmb-/- > Rag2-/-
Gzmb-/- > Rag2-/-Gzmb-/-

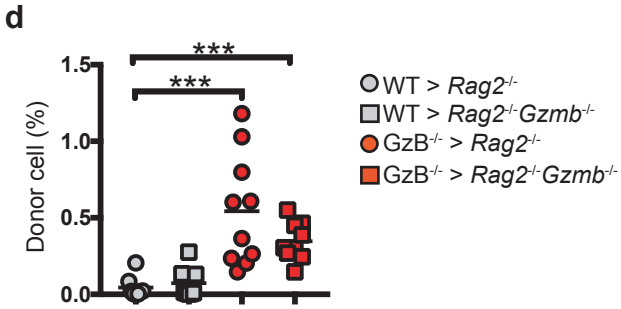

d
Donor cell (%)
1.5
1.0
0.5
0.0
***
***
WT > Rag2-/-
WT > Rag2-/-Gzmb-/-
GzB-/- > Rag2-/-
GzB-/- > Rag2-/-Gzmb-/-

Figure 2

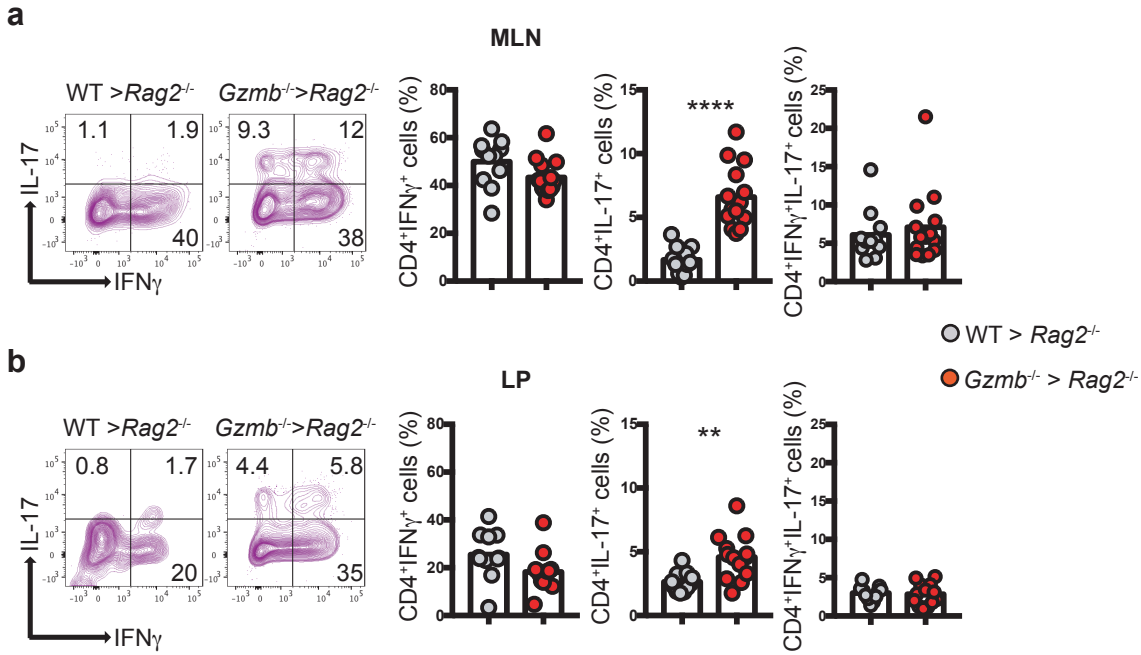

a
MLN
WT >Rag2-/-
Gzmb-/->Rag2-/-
1.1
1.9
40
9.3
12
38
IL-17
IFNγ
CD4+IFNγ+ cells (%)
CD4+IL-17+ cells (%)
****
CD4+IFNγ+IL-17+ cells (%)
WT > Rag2-/-
Gzmb-/- > Rag2-/-
b
LP
WT >Rag2-/-
Gzmb-/->Rag2-/-
0.8
1.7
20
4.4
5.8
35
IL-17
IFNγ
CD4+IFNγ+ cells (%)
CD4+IL-17+ cells (%)
**
CD4+IFNγ+IL-17+ cells (%)

Figure 3

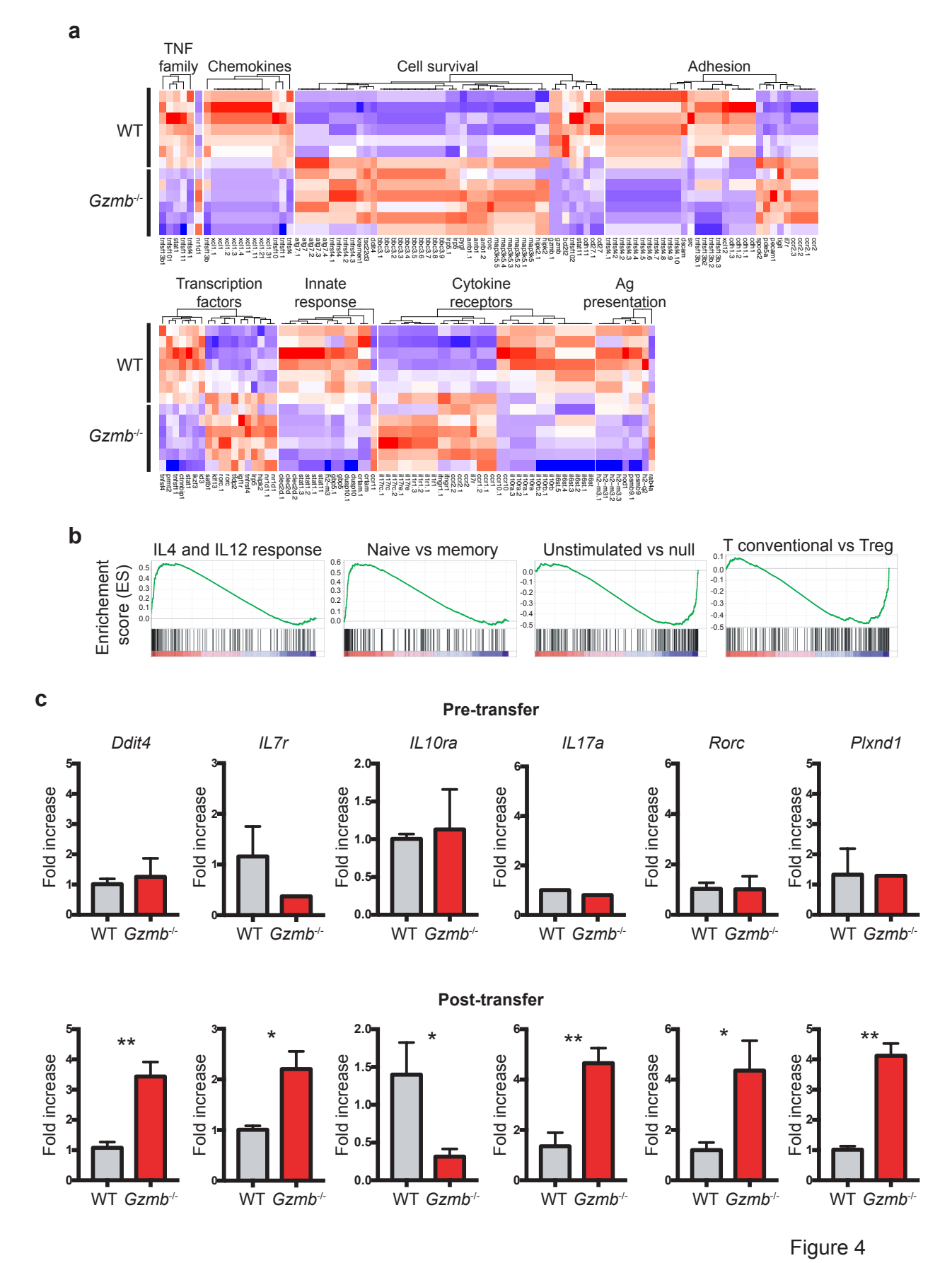

a
TNF family
Chemokines
Cell survival
Adhesion
WT
Gzmb-/-
Transcription factors
Innate response
Cytokine receptors
Ag presentation
b
IL4 and IL12 response
Naive vs memory
Unstimulated vs null
T conventional vs Treg
Enrichement score (ES)
c
Pre-transfer
Ddit4
IL7r
IL10ra
IL17a
Rorc
Plxnd1
Fold increase
WT
Gzmb-/-
Post-transfer
**
*

Figure 4

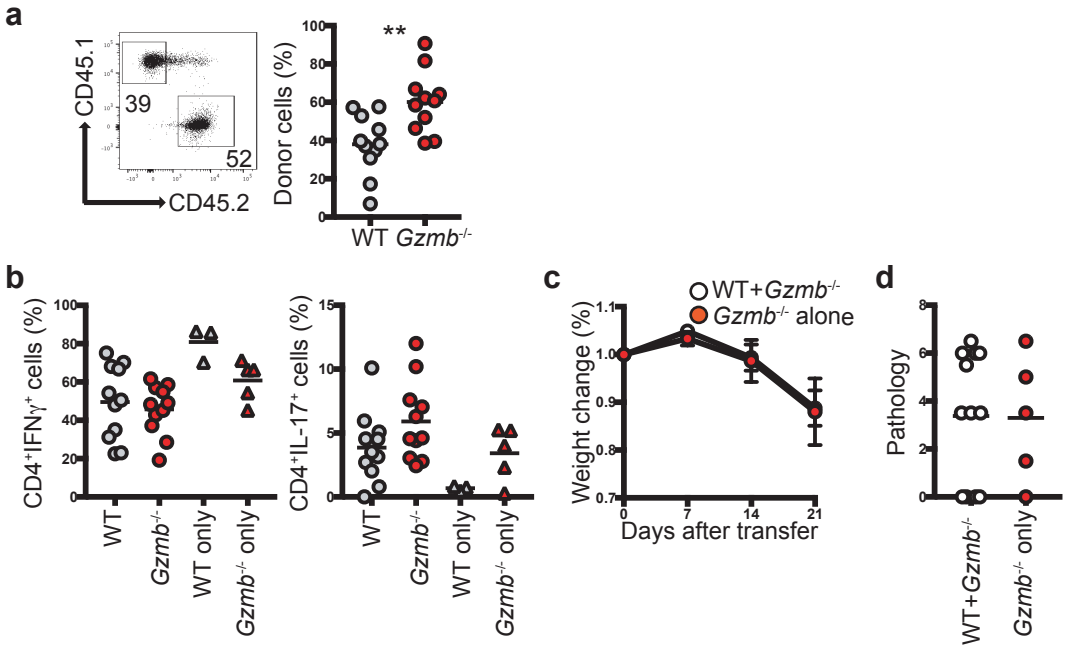

a
CD45.1
CD45.2
39
52
Donor cells (%)
**
WT
Gzmb-/-
b
CD4+IFNγ+ cells (%)
CD4+IL-17+ cells (%)
WT
Gzmb-/-
WT only
Gzmb-/- only
c
WT+Gzmb-/-
Gzmb-/- alone
Weight change (%)
Days after transfer
d
Pathology
WT+Gzmb-/-
Gzmb-/- only

Figure 5

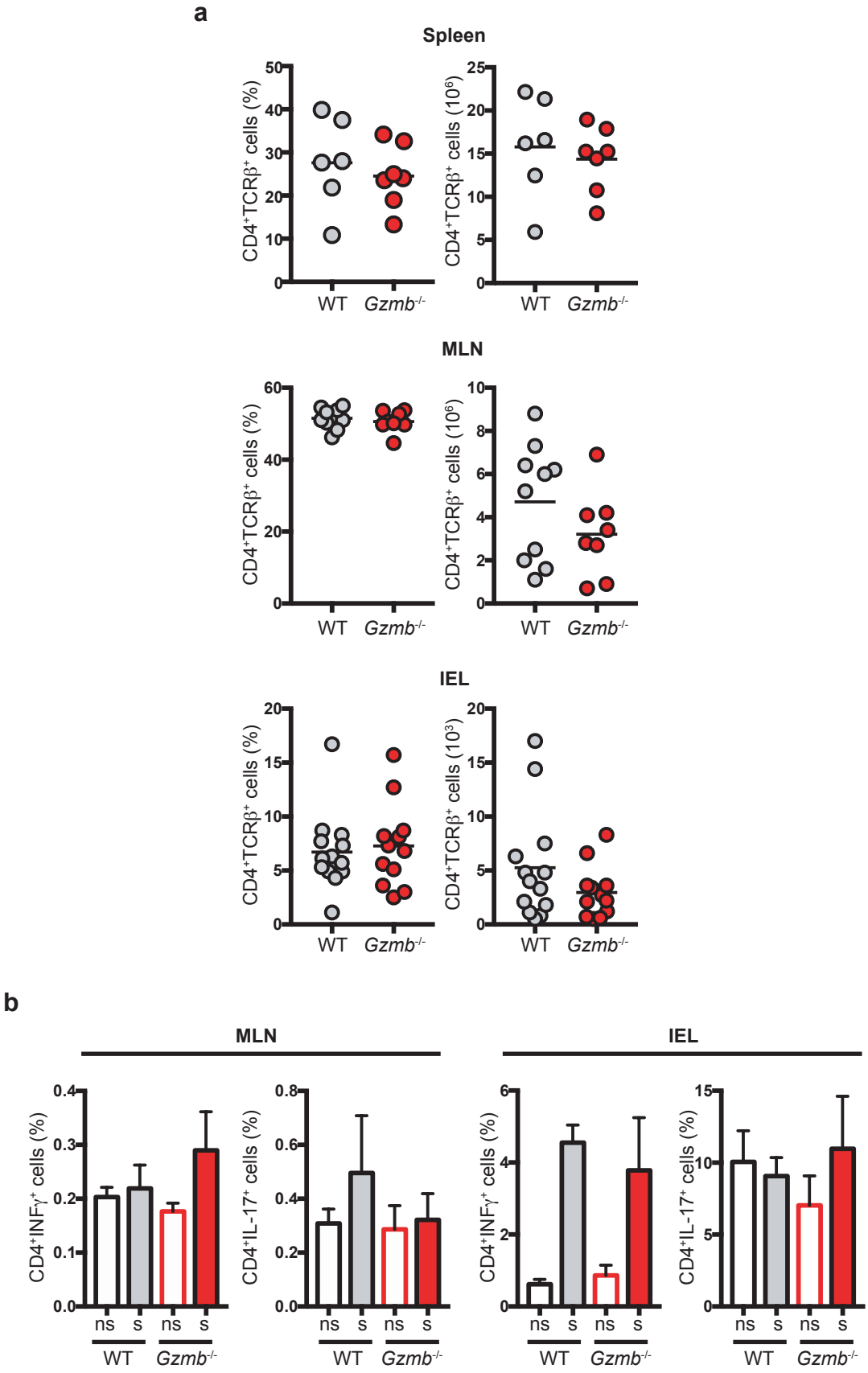

a
Spleen
CD4+TCRβ+ cells (%)
CD4+TCRβ+ cells (10^6)
WT
Gzmb-/-
MLN
IEL
CD4+TCRβ+ cells (10^3)
b
MLN
IEL
CD4+INFγ+ cells (%)
CD4+IL-17+ cells (%)
ns
s

Figure 6

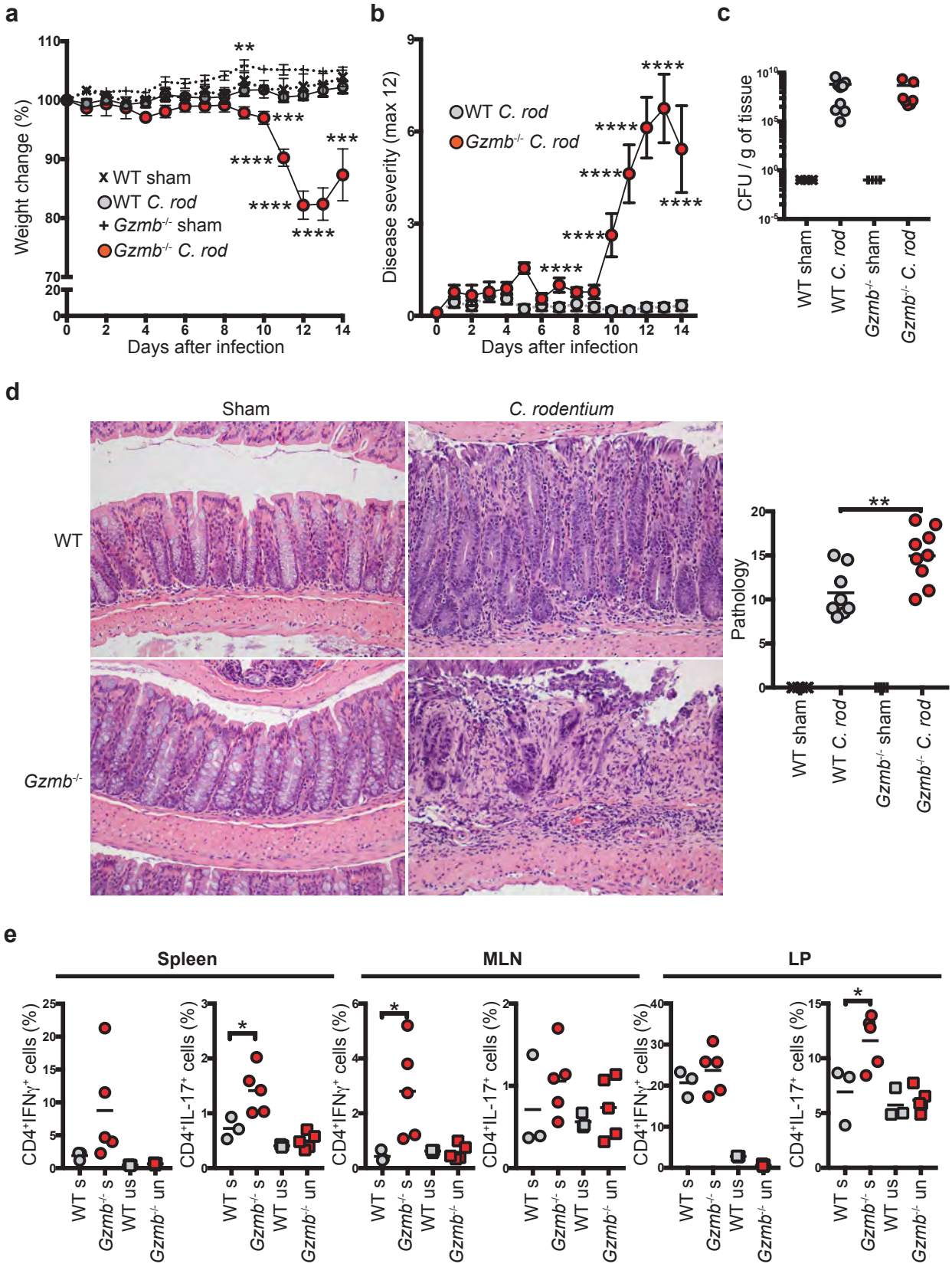

a
Weight change (%)
Days after infection
x WT sham
WT C. rod
+ Gzmb-/- sham
Gzmb-/- C. rod
b
Disease severity (max 12)
Days after infection
WT C. rod
Gzmb-/- C. rod
c
CFU / g of tissue
WT sham
WT C. rod
Gzmb-/- sham
Gzmb-/- C. rod
d
Sham
C. rodentium
WT
Gzmb-/-
Pathology
e
Spleen
MLN
LP
CD4+IFNγ+ cells (%)
CD4+IL-17+ cells (%)
WT s
Gzmb-/- s
WT us
Gzmb-/- un

Figure 7