Vγ1$^+$ γδ T cell-derived IL-4 initiates CD8 T cell immunity

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Abstract

Dendritic cells (DC) are pivotal for initiating adaptive immunity, a process triggered by the activation of DC via pathogen products or damage. Here, we describe an additional layer to this process, essential when pathogen-derived signals alone cannot directly achieve full DC activation. Immunisation with sporozoites from *Plasmodium* leads to CD8 T cell priming in a complex response that is initiated by a collaboration between conventional type 1 DC (cDC1) and γδ T cells. We unveil a pivotal initiating role for Vγ1^{+} γδ T cells, as they directly supply IL-4 to DC and CD8 T cells. IL-4 synergises with a CD4 T cell-derived CD40L signal to induce IL-12 production by cDC1. Both IL-12 and IL-4 then directly signal CD8 T cells, with synergy between these cytokines driving enhanced IL-12 receptor expression and expansion of responding CD8 T cells. This study reveals a key role for Vγl^{+} γδ T cells in initiating CD8 T cell immunity to *Plasmodium*. More broadly, it shows that responses to some pathogens require help from innate-like T cells to pass an initiation threshold and further amplify the response in a process underscored by IL-4 production.

Introduction

Priming of CD8 T cells is a complex process that relies on antigen presentation by DC that are sufficiently activated to provide all the necessary costimulatory signals. Classically, DC activation involves pattern recognition receptor (PRR) based detection of the pathogen itself, and inflammatory cues associated with the encounter. These signals increase costimulatory molecule expression on the DC and enhance MHC-II expression for antigen presentation to CD4 T cells[1]. The CD4 T cells then provide CD40L, thereby licensing the DC[2-4] and stimulating production of cytokines that contribute to T cell differentiation and expansion[5]. DC that are mature but not completely activated (or 'immunogenic') fail to produce pro-inflammatory cytokines[1], resulting in impaired T cell-mediated immunity[6]. Immune responses to pathogens that induce strong pattern recognition receptor (PRR) signalling in DC are less dependent upon CD40L-mediated help from CD4 T cells[7]. Conversely, the responses to some pathogens[7] and other poor immunogens, are more dependent upon CD4 T cell help for full DC activation and licensing[8]. CD8 T cell responses to radiation attenuated *Plasmodium* sporozoites (RAS), are completely dependent upon help from CD4 T cells[9,10], placing *Plasmodium* sporozoites in the category of poor immunogens.

The cytokines produced in response to DC activation and classically associated with differentiation and expansion of CD8 T cells include type I interferons, IL-2, IL-12 and IFNγ[11], but not commonly IL-4. Regarded as a prototypical Th2 cytokine, IL-4 has well-defined roles in CD4 T cells[12] but recent studies have suggested that IL-4 may also have potent and context dependent roles in CD8 T cells, that have implications for T cell-based immunotherapy success[13,14] or failure[15].

One model known to be dependent on IL-4 is intravenous immunisation with RAS[16,17]. RAS injection induces protective immunity due to activation of CD8 T cells in the spleen by cDC1[18]. These activated CD8 T cells then expand and a proportion of them differentiate into memory phenotype cells including liver resident memory T cells (T_{RM}) that can protect against re-infection[19]. Strikingly, in both animal models and human vaccination, γδ T cells are associated with the success of this response[20], though just how these cells contribute is unclear.

γδ T cells are innate sensors[21] that respond to infection, stress, or damage[21,22]. Once activated, γδ T cells exert diverse effector functions including the rapid release of cytokines[23-25]. In mice, $V\gamma1^+$, and $V\gamma4^+$ populations[26] are found in lymphoid tissues including the spleen[22]. In humans, the predominant γδ T cell subsets express either $V\delta1^+$, $V\delta2^+$ or $V\delta3^+$, with the majority of $V\delta2^+$ cells preferentially pairing with Vγ9[27]. $V\gamma9^+V\delta2^+$ T cells are the most abundant γδ T cell population in human peripheral blood and are also found in the spleen[28]. Strong correlations between γδ T cell functions and the generation of protective immunity following RAS vaccination have been established in humans and in mice[20,29,30]. However, the molecular pathways driving these associations, the specific γδ T cell populations involved, and the molecular signals they contribute to protective immunity remain unclear.

Here, we show that in mice the $V\gamma1^+$ subset of γδ T cells initiate the CD8 T cell response to liver stage malaria parasites via IL-4 production. Our data have implications for our understanding of how CD8 T cell responses to poor immunogens can be strengthened early in the priming phase and reveal a role for $V\gamma1^+$ γδ T cells and IL-4 in enhancing CD8 T cell responses.

Results

γδ T cells affect early expansion of CD8 and CD4 T cells

99 The T cell response to intravenously injected RAS is initiated largely in the spleen with priming
100 and expansion occurring in this site, followed by recirculation and resultant rapid accumulation
101 of activated T cells in the liver[18]. A proportion of these cells will then differentiate into memory
102 T cell subsets, including protective liver T_{RM} cells[19]. To understand if the previously described
103 role for γδ T cells in the generation of memory T cell responses in RAS vaccinated mice[20] was
104 due to a role for γδ T cells in T cell priming, or in the generation of memory T cells, we used
105 PbT-I TCR transgenic T cells to study the response at an antigen-specific level. These T cells
106 recognise an H-$2K^b$-restricted epitope ($PbRPL6_{120-127}$) from the *P. berghei*-derived RPL6
107 protein[31,18,32]. PbT-I cells were adoptively transferred into B6 or $\delta^{-/-}$ mice and responses
108 assessed 6 days after vaccination with RAS (**Figure 1a, b**). This revealed a defect in the
109 accumulation of PbT-I cells in the spleen and consequently, the liver of $\delta^{-/-}$ mice. As a result
110 of this early failure to respond to RAS, fewer memory PbT-I cells were found in either organ
111 after 3 weeks (**Figure 1c, d**), with impaired formation of memory T cells subsets, including
112 liver T_{RM} cells (**Figure 1e and f**).
113
114 CD8 T cells within the endogenous repertoire specific for $PbRPL6_{120-127}$[31] also showed
115 impaired accumulation in the spleen and liver of $\delta^{-/-}$ mice 6 days after RAS vaccination
116 (**Supplementary Figure 1a-c)** and, analogous to the PbT-I cells, did not form memory in the
117 spleen or liver (**Supplementary 1d-g**). The response of PbT-I cells therefore phenocopied the
118 response of *Plasmodium*-specific T cells within the endogenous repertoire. Together these
119 findings showed that γδ T cells were required for the initiation phase of the CD8 response to
120 RAS, explaining why memory responses were blunted.
121
122 Of note, the poor initial response in $\delta^{-/-}$ mice only extended to vaccination with the sporozoite
123 form of this parasite as responses to blood-stage parasites (irradiated infected red blood cells)
124 were unaffected (**Supplementary Figure 1h**), despite this response being categorised as a
125 relatively weak response based on its dependence on CD4 T cell help[33].
126
127 We next assessed whether the initiation of the CD4 T cell response to RAS vaccination was
128 also γδ T cell dependent. Here we utilized PbT-II cells[33] from the MHC-II restricted P. *berghei*-
129 specific TCR transgenic line[34]. Adoptive transfer of both PbT-I and PbT-II cells into B6 or $\delta^{-/-}$
130 mice (**Figure 1g**) showed that splenic accumulation of PbT-II cells was dependent upon γδ T
131 cell-mediated help in response to RAS vaccination (**Figure 1h**). As a result, fewer PbT-II cells

132 were present in the livers of $\delta^{-/-}$ mice (**Supplementary Figure 1i**). PbT-I cell accumulation in
133 the spleen (**Figure 1i**) and liver (**Supplementary Figure 1j**) of the $\delta^{-/-}$ mice was also lower
134 than the controls, demonstrating that the addition of large numbers of naïve antigen-specific
135 CD4 T cells could not rescue the response of CD8 PbT-I cells when γδ T cells were absent.
136
137 **Vγ1^{+} γδ T cells initiate immunity to RAS**
138 To ascertain when γδ T cells affect CD4 and CD8 T cell responses, we utilised the pan-TCRδ
139 blocking antibody (α-γδ, clone GL3)[35] to block γδ T cell function at the time of, or 24 hr after
140 RAS vaccination (**Figure 2a**). Blocking γδ T cell function prior to RAS vaccination impaired
141 the PbT-I response comparably to that previously observed in $\delta^{-/-}$ mice (**Figure 2b**). In contrast,
142 blocking γδ T cell function from 24 hr post-RAS injection had a milder effect (**Figure 2b**).
143 These data suggest that the first 24 hr are the crucial window of γδ T cell activation.
144
145 Knowing that the first 24 hours were critical, we next examined early γδ T cell activation.
146 Splenic γδ T cells were split into the two major populations found in murine spleen, Vγ1^{+} or
147 Vγ1^{-} populations (**Supplementary Figure 2a**). Up-regulation of the canonical T cell activation
148 markers CD69, CD25 and CD11a in the Vγ1^{+}, but not the Vγ1^{-} γδ T cell population, indicated
149 that Vγ1^{+} γδ T cells were preferentially activated by RAS vaccination (**Figure 2c, d and**
150 **Supplementary Figure 2b**). Blocking the Vγ1^{+} γδ T cell population via injection of a Vγ1
151 blocking antibody (clone 2.11)[36] (**Figure 2e, f**) had the same effect on PbT-I accumulation as
152 blockade of the entire γδ T cell population (α-γδ). Vγ1^{+} γδ T cells therefore initiate the CD8 T
153 cell response to RAS in mice.
154
155 **Antigen presentation of *Plasmodium*-derived peptides is intact in the absence of γδ T cells**
156 Due to the very early timing of γδ T cell activation, and the effect of Vγ1^{+} γδ T cells on CD8 T
157 cell accumulation in the spleen, we hypothesised that the Vγ1^{+} γδ T cells may impact the ability
158 of cDC1 to present antigen to CD8 T cells in response to RAS vaccination. To investigate this
159 possibility, we examined initial up-regulation of CD69 within OT-I T cells following injection
160 of SIINFEKL expressing CS5M sporozoites (CS5M-RAS). OT-I T cells recapitulated the
161 phenotype of PbT-I cells in $\delta^{-/-}$ mice, with impaired responses in the spleen and liver 6 days
162 after vaccination with CS5M-RAS (**Supplementary Figure 3a and b**). At early time points
163 post-CS5M-RAS vaccination, equal proportions (**Figure 3a and b**) and numbers (**Figure 3c**)

164 of cell trace violet (CTV) labelled OT-I T cells up-regulated cell surface expression of CD69
165 in the spleens of B6 and $\delta^{-/-}$ mice. These data indicated that responding T cells had access to
166 antigenic signals capable of up-regulating CD69 and initiating some proliferation even when
167 γδ T cells were absent. Failure to accumulate on day 6 (**Supplementary Figure 3a and b**),
168 however, suggested a lack of signals for extended T cell expansion, differentiation and/or
169 survival. This conclusion strongly suggested a lack of co-stimulation or cytokines in the T cell
170 priming process, raising the intriguing possibility that γδ T cells are crucial for triggering
171 upregulation of co-stimulation-like signals on cDC1.
172
173 **γδ T cells are not required to supply the CD40L signal to cDC1**
174 As a deficiency in co-stimulation was implicated, we hypothesised that γδ T cells may provide
175 CD40L for signalling CD40 on cDC1, a signal known to be essential in the response to RAS[37].
176 To investigate this possibility, PbT-I cells were transferred into WT, $\delta^{-/-}$ or *Cd40l*$^{-/-}$ mice. CD8
177 T cell expansion is dependent on help from CD4 T cells in the RAS model[9,10], and these CD4
178 T cells were previously presumed to provide the CD40L signal. To test this assumption, one
179 group of *Cd40l*$^{-/-}$ mice were also given CD40L sufficient PbT-II cells (**Figure 3d**). PbT-I cell
180 accumulation in the spleen 6 days after RAS injection was severely diminished in *Cd40l*$^{-/-}$ mice
181 (**Figure 3e**), confirming that CD8 T cell accumulation in this model is dependent on CD40L
182 signalling. Addition of CD40L sufficient PbT-II cells, however, rescued the PbT-I cell response,
183 even though γδ T cells lacked expression of CD40L in these mice (**Figure 3e**). Therefore, CD4
184 T cells can provide the CD40L signal required for the response to RAS suggesting that γδ T
185 cells are not required to provide this co-stimulatory function. Furthermore, rescue by CD40L
186 sufficient PbT-II cells showed that this signal is normally provided by CD4 T cells but is
187 insufficient to ensure an appropriate CD8 T cell response if γδ T cells are absent (**Figure 3f**).
188
189 **IL-4 is required for the CD8 T cell response to RAS vaccination**
190 As CD40L was not the crucial signal provided by γδ T cells, we rationalised that they may
191 provide 'signal 3' i.e. a cytokine signal, for differentiation and expansion of responding T cells.
192 Previous studies suggested IL-4 is a crucial factor in the generation of memory CD8 T cells in
193 the liver following RAS vaccination[16,38]. Given that splenic $V\gamma1^{+}$ γδ T cells have been reported
194 to secrete IL-4[24], we next investigated the role of IL-4 in the PbT-I cell response either via
195 transfer into *Il4*$^{-/-}$ hosts (**Figure 4a and b**) or by antibody-mediated blockade (**Supplementary**

196 **Figure 4a and b**). PbT-I cells did not accumulate in the spleen or liver in the absence of IL-4,
197 showing that IL-4 was crucial, and the absence of IL-4 mirrors the effect of γδ T cell deficiency.
198
199 To further investigate if, as previously reported, CD4 T cells are the relevant source of IL-4[16],
200 we transferred IL-4 sufficient PbT-I cells with, or without, IL-4 sufficient PbT-II cells into B6
201 or *Il4*$^{-/-}$ mice and measured T cell accumulation as the output (**Figure 4a**). Accumulation of
202 PbT-I cells was impaired in the *Il4*$^{-/-}$ mice even in the presence of antigen-specific CD4 T cells
203 that could make IL-4 (**Figure 4c**). In contrast, antigen-specific CD4 PbT-II cells expanded in
204 the spleen in both B6 and *Il4*$^{-/-}$ mice (**Figure 4d**) demonstrating two phenomena; 1) the CD4 T
205 cell response was not IL-4 dependent and 2) IL-4 sufficient CD4 T cells could not rescue the
206 antigen-specific CD8 T cell response. CD4 T cells, therefore, are not the crucial source of IL-
207 4 for CD8 T cell accumulation following RAS vaccination.
208
209 **γδ T cells produce IL-4 in experimental malaria**
210 To determine if γδ T cells provide IL-4 in response to RAS vaccination in mice, we generated
211 several groups of mixed bone marrow (BM) chimeras (**Figure 4e, f**). Eight weeks after
212 reconstitution, the γδ T cell compartment was reconstituted as expected (**Figure 4g**).
213 Assessment of the PbT-I cell response 6 days after RAS vaccination demonstrated impaired
214 PbT-I cell accumulation in the spleens of *Il4*$^{-/-}$+δ$^{-/-}$→B6 chimeras (group 5) when compared to
215 B6+δ$^{-/-}$→B6 chimeras (group 4) (**Figure 4h**). These data demonstrated that γδ T cells were the
216 source of IL-4, since B6+δ$^{-/-}$→B6 chimeras contained γδ T cells that could produce IL-4,
217 whereas the *Il4*$^{-/-}$+δ$^{-/-}$→B6 chimeras lacked these cells. These data strongly suggest that γδ T
218 cells are the essential source of IL-4 for the initiation of an effective CD8 T cell response to
219 RAS vaccination.
220
221 To further confirm that γδ T cells are the crucial source of IL-4, we reconstituted δ$^{-/-}$ mice with
222 splenic γδ T cells from either WT *or Il4*$^{-/-}$ donors and tested whether these cells could rescue
223 the PbT-I cell accumulation following RAS vaccination. TCRδ-deficient mice received either
224 splenic γδ T cells or no γδ T cell transfer (**Figure 4i**). Six days after RAS vaccination (8 days
225 after γδ T cell transfer), γδ T cells were recovered from the spleens demonstrating that the
226 transfer was effective (**Figure 4j**). As hypothesised, B6-derived but not *Il4*$^{-/-}$-derived γδ T cells
227 supported PbT-I cell accumulation in the spleen in response to RAS vaccination, confirming
228 that these cells provide IL-4 in this vaccination setting (**Figure 4k**).

229

230 **IL-4 has a dual mechanism of action by signalling both CD8 T cells and cDC1 to initiate**
231 **the CD8 response to RAS**

232 IL-4 is a prototypical T helper 2 cytokine that has potent effects on CD4 T cell differentiation
233 and function[12], but less well studied impacts on CD8 T cells. We therefore asked if the RAS-
234 induced γδ T cell-derived IL-4 acted directly on the responding CD8 and/or CD4 T cells. To
235 address this question, we used CRISPR-Cas9 to delete the IL-4Rα gene (*Il4ra*) in T cells.
236 Purified naïve PbT-I and/or PbT-II cells had *Il4ra* (or *Cd19* as a control) ablated prior to transfer
237 and immunisation with RAS (**Figure 5a and Supplementary Figure 5a)**. Comparison of the
238 number of PbT-I cells six days after vaccination showed that direct IL-4Rα signalling in PbT-
239 I cells was important for PbT-I cell accumulation in the spleen (**Figure 5b**). These data show
240 that in the context of RAS vaccination direct IL-4 signals to CD8 T cells affect their expansion.

241

242 PbT-I cells lacking the IL-4R still retained a modest capability to accumulate when compared
243 to a complete failure in the absence of γδ T cells (**Figure 1a**), or IL-4 itself (**Figure 4b**). This
244 suggested that IL-4 has an additional activity that contributes to CD8 T cell accumulation. To
245 investigate whether this involved direct signalling of cDC1 by IL-4, we developed an *in vivo*
246 model for cDC1 manipulation. Donor cDC1 were expanded *in vivo* in B6 mice[39] prior to
247 enrichment, CRISPR-Cas9-mediated deletion of either *Cd19* or *Il4ra* and then transfer into
248 *Batf3*$^{-/-}$ mice for replenishment of the cDC1 pool (**Figure 5c**). Two days later, PbT-I cells were
249 transferred, and the mice vaccinated with RAS for assessment of PbT-I cell numbers 6 days
250 later. Splenic PbT-I cell accumulation was impaired when cDC1 did not express IL-4Ra
251 (sg*Il4ra*) (**Figure 5d**), suggesting that direct IL-4 signalling in cDC1 enhances the CD8 T cell
252 response to RAS vaccination. Numbers of cDC1 recovered 6 days post-RAS vaccination were
253 equivalent between groups (**Figure 5e and Supplementary Figure 5b**). Collectively, these
254 experiments demonstrate that IL-4 acts directly on DC and on CD8 T cells for optimal CD8 T
255 cell expansion in the context of RAS vaccination (**Figure 5f**).

256

257 **IL-4 synergises with CD40 to drive IL-12 production by cDC1**

258 To identify the molecules induced by IL-4 in cDC1 that may contribute to the response to RAS
259 vaccination, cDC1 gene expression was assessed *in vitro* following stimulation with IL-4 and
260 αCD40. Expanded cDC1 were sort-purified and then cultured in each condition before

261 isolation of RNA at 4 hours[40]. Sorted cDC1 were assessed after exposure to the following
262 conditions: media alone, αCD40, IL-4, or αCD40+IL-4 (**Figure 6a**).
263
264 Analysis of sequenced RNA revealed a marked transcriptomic shift in the presence of IL-4 that
265 was augmented by the addition of αCD40 (**Figure 6b and c**). Specifically, there were 841 up-
266 regulated and 762 down-regulated genes in response to αCD40+IL-4 when compared to media
267 alone (**Figure 6b**). *Il12a*, was the most upregulated gene when comparing media with
268 αCD40+IL-4 (LFC 11.17, FDR $1.5x10^{-59}$) and the second most upregulated gene when
269 comparing αCD40+IL-4 with αCD40 alone (LFC 11.69, FDR $1.48x10^{-52}$) (**Figure 6d and e**).
270 This gene encodes the p35 subunit of IL-12 which, when combined with the p40 subunit (which
271 is constitutively expressed by cDC1), makes bioactive IL-12, a key stimulator of CD8 T cells.
272 As it was only weakly upregulated in cDC1 exposed to IL-4 alone, efficient upregulation of
273 *Il12a* also appeared dependent on the combination of both CD40 and IL-4 signals (**Figure 6e**).
274
275 To determine the contribution of bioactive IL-12 *in vivo* in the context of RAS vaccination,
276 PbT-I cell accumulation was assessed after IL-12 blockade (**Figure 6f**). Assessment of PbT-I
277 accumulation on day 6 showed significant impairment when IL-12 was blocked (**Figure 6g**).
278 This supports a model where CD40 signalling synergises with IL-4 in cDC1 to induce IL-12
279 that is crucial for the accumulation of CD8 T cells in response to RAS vaccination (**Figure 6h**).
280
281 **IL-4 promotes CD8 T cell expansion by increasing IL12R expression in CD8 T cells.**
282 IL-4 and IL-12 were both essential for the enhanced accumulation of CD8 T cells in response
283 to RAS vaccination. This led us to investigate whether these cytokines work synergistically to
284 promote CD8 T cell expansion. To isolate the effects of the cytokines, we first examined the
285 impact of IL-4 and IL-12 on CD8 T cell expansion *in vitro*.
286
287 PbT-I cells were activated by peptide coated antigen presenting cells in the presence of, no
288 added cytokine, IL-4, IL-12, or both, and the number of cells retrieved from the cultures
289 enumerated on day 4 and day 6. Limited cell growth was observed in the absence of exogenous
290 cytokine (**Figure 7a**) and addition of either IL-4 or IL-12 only modestly impacted the number
291 of cells recovered between day 4 and day 6 (**Figure 7b and c).** In contrast, addition of both IL-
292 4 and IL-12 resulted in significantly higher cell recovery from the culture on day 6 (**Figure**

293 **7d**). These data suggested that the combination of IL-4 and IL-12 act synergistically in
294 increasing the T cell response *in vitro*.
295
296 To explore whether the synergy between IL-4 and IL-12 was mediated by changes in cytokine
297 sensitivity resulting from alterations in receptor expression, we assessed IL-4R and IL-12R
298 expression on T cells at day 4 of culture. Compared to addition of IL-4 alone, a combination
299 of IL-4 and IL-12 induced a small increase in IL-4R (**Figure 7e**). More substantially, the
300 addition of IL-4 + IL-12 increased expression of IL-12R over either cytokine alone (**Figure**
301 **7f**). These data suggested that the combination of IL-4 and IL-12 amplifies the sensitivity of
302 CD8 T cells to IL-12 signalling through increased expression of IL-12R, thereby enhancing the
303 CD8 T cell response. Further, *in vitro* activated OT-I T cells stimulated with IL-4 and IL-12
304 were maintained in greater numbers when transferred into mice (**Figure 7g**).
305
306 **γδ T cell-derived IL-4 and cDC1-derived IL-12 synergise to promote the generation of**
307 **protective liver T_{RM} cells.**
308 Our data showed that in the absence of γδ T cells there is a defect in the initiation of the CD8
309 T cell response leading to a failure in the formation of T cell memory and defective generation
310 of protective liver T_{RM} cells (**Figure 1e and f and Supplementary Figure 1f and g**). The
311 important factor in this response was γδ T cell-derived IL-4, which induced cDC1 IL-12
312 production, and the two cytokines then synergised to promote CD8 T cell expansion. To
313 confirm that removal of both IL-4 and IL-12 *in vivo* would recapitulate the lack of γδ T cells
314 and therefore inhibit the formation of protective liver T_{RM} cells, we blocked IL-4 or IL-12, or
315 both, in the context of RAS vaccination, and then examined T_{RM} numbers in the liver 29 days
316 later (**Figure 7h**). Enumeration of the number of liver T_{RM} cells showed that IL-4 had a
317 dominant effect on liver T_{RM} formation, but blockade of both cytokines phenocopied blockade
318 of γδ T cells (with α-γδ Ab) in the spleen (**Figure 7i and j)** and in the liver (**Figure 7k and l**).
319 These data showed that γδ T cells drive the response to RAS via delivery of IL-4 to DC, which
320 in turn drives IL-12 production. The γδ T cell derived IL-4 and DC-derived IL-12 then
321 synergise to enhance the expansion of CD8 T cells (**Figure 7m**), a proportion of which will
322 differentiate into protective liver T_{RM} cells.
323
324 **Discussion**

For full immunogenicity, DC must be effectively activated, either via strong PRR signalling or through CD4 T cell help via CD40L[1]. Here, we showed that the priming of CD8 T cells to *Plasmodium* sporozoite antigens depends on an additional layer of DC activation via IL-4, which is produced by γδ T cells. Here, IL-4 has a dual role: 1) when synergised with CD40 signals in DC, it promotes enhanced production of bioactive IL-12 and 2) by directly signalling CD8 T cells to increase IL-12R, it increases their sensitivity to IL-12 and thereby promotes expansion. In this vaccination setting, IL-4 is produced by $V\gamma1^+$ γδ T cells in the spleen, which become activated within the first 24 hours of injection of sporozoites.

The absolute requirement for γδ T cells for successful RAS vaccination in mice[20] and the correlations with effective RAS vaccination in humans[20,29,41,42] suggests that although *Plasmodium* sporozoites are reported to trigger toll like receptor 2 signalling[43], this stimulation is not sufficient to push DC over a required activation threshold and that an additional IL-4 signal in combination with CD40 is required. Our findings likely extend beyond the context of RAS vaccination and may be applicable to other scenarios involving poor immunogens. In such cases, the sensing of infection or cellular damage by innate or innate-like cells may trigger IL-4 production, thereby enabling DC to effectively prime protective immune responses. One such innate-like T cell population are invariant natural killer T (NKT) cells, which are known to produce IL-4 in response to lipid ligands[44]. These ligands can be used as adjuvants to boost the response to RAS[45] and to heat killed sporozoites[46], likely via enhanced IL-4 production. However, the need for NKT cell-derived cytokines on top of CD4 T cell help in a four-cell paradigm, as we have shown here for γδ T cells, has not been described.

Here we identified that in mice, the $V\gamma1^+$ subset of γδ T cells are activated by RAS vaccination and these $V\gamma1^+$ cells provide a crucial function by supplying IL-4 for initiation of the CD8 T cell response. Previous studies demonstrated that provision of IL-4 by $V\gamma1^+$ γδ T cells had potent effects on B cells[47] but $V\gamma1^+$ cells have not previously been directly linked to CD8 T cell responses *in vivo.* Despite identifying the specific subset involved and resolving the timing of activation, we have not yet successfully identified the trigger for $V\gamma1^+$ γδ T cell activation in response to RAS vaccination. Identification of the activation triggers for γδ T cells remain a challenge for the field[48,49] but we hypothesise that the cells are responding to a parasite or parasite induced self-ligand exposed by the infectious process. In mice, γδ T cell-derived IL-17[50] and IFN-γ[51], induced by liver-stage parasites have been shown to play important roles in

358 the pathology of *Plasmodium* blood stage infection. Here, we have shown that the initiation of
359 the CD4 response to liver stage parasites is also dependent on a factor provided by γδ T cells
360 but have not yet identified the nature of this factor. These concepts highlight that γδ T cells
361 play a varied and substantial role in malaria disease.
362
363 Previous studies that described a role for γδ T cells in RAS vaccination proposed that a
364 population of CD8 DC (now referred to as cDC1) that accumulated in the liver of RAS
365 vaccinated mice when γδ T cells were present also correlated with the generation of hepatic
366 CD8 T cell memory[20]. Although we have not directly investigated or ruled out a role for these
367 hepatic DC in the memory component of the response to RAS, our data show that splenic cDC1
368 collaborate with γδ T cells to drive efficient priming of splenic CD8 T cells. It is possible that
369 the inflammation induced by the combination of IL-4 and IL-12 observed when γδ T cells are
370 present also leads to inflammation in the liver and the accumulation of CD8 cDC,
371 commensurate with the accumulation of CD8 T cells in the liver. Given that the DC
372 accumulation in the liver was observed late in the response (>60 days post-immunisation), this
373 effect is unlikely to contribute to the crucial γδ T cell-dependent priming observed in the current
374 study.
375
376 Prior to the discovery of T_{RM} cells, a role for IL-4 in RAS vaccination (for *P. yoelii* in BALB/c
377 mice[16,17]) was identified. CD4 T cells, rather than γδ T cells, were implicated as the source of
378 IL-4[16,17]. We speculate that a lack of CD40L signalling in the absence of CD4 T cells, rather
379 than a lack of CD4 T cell-derived IL-4, was likely responsible for the phenotype observed.
380 However, we currently cannot exclude the possibility that γδ T cell-derived IL-4 drives
381 production of IL-4 by CD4 T cells. Nevertheless, we have shown that the initial and most
382 important source of IL-4 is γδ T cell-derived.
383
384 Notably, we have shown that IL-4 and IL-12 are synergistic for CD8 T cell expansion due to
385 enhanced expression of IL-12R, likely making the T cells more sensitive to bioactive IL-12.
386 This is counterintuitive to published literature examining CD4 T cell responses, which states
387 that IL-4-induced GATA3 activation suppresses IL-12 signalling[52]. However, synergistic
388 effects of IL-4 and IL-12 have previously been reported for CD4 T cells[53] and human T cells[54],
389 although the precise molecular mechanisms driving this synergy are currently unknown and
390 require further investigation. Here, we show that IL-4 plays a key role in promoting the

391 expansion of antigen-specific T cells not only by stimulating IL-12 production in DC but also
392 enhancing CD8$^+$ T cell sensitivity to the IL-12 produced. In the absence of IL-4 the entire
393 response fails, making IL-4, and the triggers that induce its secretion by γδ T cells or other
394 innate or innate-like cells, of major relevance to weakly stimulating pathogens (or tumours)
395 that cannot intrinsically drive strong CD8 responses.
396
397 Importantly, a newly appreciated role for IL-4 in the context of immunotherapy has recently
398 been highlighted. IL-4 production by B cells was shown to be one mechanism for successful
399 PD-1 immunotherapy[55] and two recent studies demonstrated the importance of an IL-4 driven
400 type-2 response for effective and long-lived CAR T cell or anti-tumour responses[13,14]. These
401 studies demonstrate that IL-4 has therapeutic implications beyond infectious diseases and
402 indicate that γδ T cells should be investigated as a source of IL-4 in these models.
403
404 In conclusion, our study reveals that by providing IL-4, Vγ1$^+$ γδ T cells drive the initiation of
405 the response to a poorly immunogenic pathogen, transforming it into a robust trigger for CD8$^+$
406 T cell immunity and tissue-tropic memory.
407
408 **Methods**
409 **Mice, mosquitoes, and parasites**
410 C57BL/6, B6.SJL-PtprcaPep3b/BoyJ (CD45.1), PbT-I GFP, PbT-II GFP, *Tcrd*$^{-/-}$[56],
411 Trdc$^{tm1(EGFP/HBEGF/luc)Impr}$ (TCRδ-GDL)[57], *Il4*$^{-/-}$, *Batf3*$^{-/-}$, *Cd40l (Cd154)*$^{-/-}$, *Il17a*$^{-/-}$ mice were
412 bred and held at the Department of Microbiology and Immunology, The University of
413 Melbourne, Australia. All experimental work and animal handling was conducted in strict
414 accordance with the standards approved by the Animal Ethics Committee at the University of
415 Melbourne (ethic project ID: 2015168, 20088). Experimental mice were sex matched and used
416 at 6-16 wks of age. Both male and female mice were used. TCRδ-GDL[57] mice were kindly
417 provided by I. Prinz (University Medical Center Hamburg-Eppendorf).
418
419 *Plasmodium berghei* ANKA (PbA) wild-type Cl15cy1 (BEI Resources, NIAID, NIH: MRA-
420 871) or *P. berghei* CS5M, which express the cognate antigen for OT-I T cells[58], parasites were
421 used. Sporozoites were generated in 4-5-week old male Swiss Webster mice purchased from
422 the Monash Animal Service and held at the School of Botany, The University of Melbourne,
423 Australia. *Anopheles stephensi* mosquitoes (strain STE2/MRA-128 from BEI Resources, The

424 Malaria Research and Reference Reagent Resource Centre) were reared and infected as
425 described previously[59]. Sporozoites were hand-dissected from mosquito salivary glands,
426 resuspended in cold PBS then irradiated with 20 K rads from a gamma ^{60}Co source (WEHI
427 irradiation facility). Irradiated sporozoites were diluted to a total number of 5 x10^4 per mouse
428 and injected via the tail vein using 26G insulin syringes.
429
430 In blood-stage malaria experiments, donor mice were injected with frozen stabilates of blood-
431 stage P.*berghei* ANKA (PbA) wild-type Cl15cy1 (BEI Resources, NIAID, NIH: MRA-871).
432 Three to seven days later, mice were bled for parasitemia, the RBCs irradiated with 20 K rads
433 from a gamma ^{60}Co source (WEHI irradiation facility), and recipient mice were injected with
434 5 x10^4 irradiated PbA-infected RBCs diluted in 0.2 mL of sterile PBS.
435
436 **Cell Enrichments**
437 **T cells**
438 Naïve $CD8^+$ PbT-I or $CD4^+$ PbT-II cells were negatively enriched from the spleens and lymph
439 nodes of PbT-I/uGFP, OT-I/uGFP or PbT-II/uGFP transgenic mice. Briefly, cells were passed
440 through a 70 µM filter sieve to obtain a single-cell suspension and red blood cells lysed. Single-
441 cell suspensions were labelled with rat monoclonal antibodies specific for MHC Class II (M5-
442 114), Mac-1 (M1/70, F4/80), Gr-1 (RB6-8C5) and either CD4 (GK1.4) (for PbT-I and OT-I
443 CD8 T cells) or CD8 (53-6.7) (for PbT-II CD4 T cells) prior to incubation with BioMag goat
444 anti-rat IgG coupled magnetic beads (Qiagen) and magnetic separation. Enriched T cells were
445 typically 80-95% pure. 1 x10^6 purified T cells in 0.2 mL of sterile PBS were injected via the
446 tail vein into recipient mice. Where co-transfers were performed, a total number of 2 x10^6
447 purified T cells were injected via the tail vein where cell types were transferred at a ratio of 1:1.
448
449 **γδ T cells**
450 Naïve γδ T cells were negatively enriched from spleens of B6 or *Il4*$^{-/-}$ mice. Single cell-
451 suspensions were achieved by finely mincing and digesting spleens in 1 mg/mL Collagenase
452 Type 3, 20 µg/mL DNase I supplemented with a competitive antagonist of $P2X_7R$ (A-438079
453 hydrochloride; Santa Cruz Biotechnology) with intermittent agitation. Undigested fragments
454 were removed by filtering through a 70 µM mesh followed by red blood cell lysis. Single cell
455 suspensions were labelled with rat monoclonal antibodies specific for MHC Class II (M5-114),
456 Mac-1 (M1/70, F4/80), CD4 (GK1.4), CD8 (53-6.7) and B220 (RA3-6B2) prior to incubation

with BioMag goat anti-rat IgG coupled magnetic beads (Qiagen) and magnetic separation. $2x10^6$ enriched γδ T cells in 0.2 mL of sterile PBS were injected via the tail vein into recipient mice.

Dendritic cells

Dendritic cells were isolated from spleens of B16.Flt3L tumour-bearing mice. Briefly, spleens were finely minced in 1 mg/ml Collagenase Type 3, 20 µg/ml DNase I and continuously agitated for 15 minutes. DC-T cell complexes were disrupted with the addition of 0.1M EDTA (pH7.2). Undigested fragments were removed by filtering through a 70 µM mesh before light-density separation with 1.077g/cm^3 Nycodenz medium via centrifugation at 1700rpm at 4°C for 12min. To increase the purity of cDC1 precursors, the light density fraction was collected, and cDC1s/pre-cDC1s were negatively enriched for through incubation with rat mAb against CD3ε (KT3), Thy1.1 (T24/31.7), Gr-1 (RB6-8C5), B220 (RA3-6B2), Ter-119 and CD11b (M1/70) prior to incubation with BioMag goat anti-rat IgG coupled magnetic beads (Qiagen) and magnetic separation. Target cells were characterised as CD11c$^+$, MHC-II$^{int/+}$, CD11b$^-$, CD24^{hi}, CD8α$^+$ with purities ranging between 60-80%. 2-5 x 10^6 purified cDC1s in 0.2 mL of sterile PBS were injected via the tail vein into recipient mice, 2 days prior to PbT-I cell transfer.

CRISPR/Cas9 gene-editing

T cells

Purified CD8$^+$ PbT-I or CD4$^+$ PbT-II cells were gene-edited via electroporation with single guide (sg) RNA ribonucleoproteins (RNPs) (sgRNA/Cas9 RNPs). Briefly, sgRNA/Cas9 RNPs were formed in Nuclease-free H_2O using 0.6 µl of Alt-R S.*pyogenes* Cas9 Nuclease V3 (10 mg/mL, Integrated DNA Technologies) incubated with 0.3 nmol of sgRNA targeting either *Il4rα* (5'-AGUGGAGUCCUAGCAUCACG-3', 3'-AUCCAGGAACCACUCACACG-5') or *Cd19* (5'-AAUGUCUCAGACCAUAUGGG-3') purchased from Synthego (CRISPRevolution sgRNA EZ Kit) for 10 mins at room temperature. For each sgRNA/Cas9 electroporation reaction, 10 x 10^6 target cells were required. In each reaction, target cells were resuspended in 20 µl of reconstituted P3 buffer then mixed with the sgRNA/Cas9 RNP complex and electroporated using a Lonza 4D-Nucleofector (program code: DN100). Electroporated cells were then rested in a 96-well plate for at least 10 minutes at 37°C. For injection into mice, 10^6 cells were injected into mice via the tail vein in 0.2 mL of PBS, one day prior to RAS vaccination.

490

491 **Dendritic Cells**

492 Purified pre-cDC1s were gene-edited via electroporation with single guide (sg) RNA
493 ribonucleoproteins (RNPs) (sgRNA/Cas9 RNPs) using an optimised protocol. sgRNA/Cas9
494 RNPs were formed as described for T cell gene-editing. For each sgRNA/Cas9 electroporation
495 reaction, 10 x 10^6 target cells were required. In each reaction, target cells were resuspended in
496 20 µl of reconstituted P3 buffer then mixed with the sgRNA/Cas9 RNP complex and
497 electroporated using a Lonza 4D-Nucleofector (program code: CM137). Electroporated cells
498 were then rested in a 96-well plate for at least 20 minutes at 37°C then injected intravenously
499 into *Batf3*$^{-/-}$ mice three days prior to immunisation. All mice received PbT-I cells one day prior
500 to RAS immunisation.

501

502 ***In vitro* cDC1 gene-expression assay**

503 Dendritic cells were isolated through the protocol outlined above. CD11c$^+$, MHC-II$^{int/+}$,
504 CD11b$^-$, CD24^{hi}, F4/80$^-$, B220$^-$, CD172α$^-$ cells were then sorted using a BD FACSAria^{TM} III
505 Cell Sorter into filtered RPMI 1640 supplemented with 50% heat-inactivated FCS and pelleted.
506 In a 24-well plate, 5 x 10^5 cells were plated in complete Kenneth D Shortman RPMI (KDS-
507 RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/mL streptomycin and
508 50 mM 2-mercaptoethanol). To assess cDC1 gene expression in response to IL-4, cells were
509 incubated in the presence of α-CD40 (10 µg/ml), recombinant mouse IL-4 (60 ng/mL), IFN-γ
510 (20 ng/mL), or LPS (1 µg/ml). Plates were then incubated at 37°C and 5% CO_2 for 4 hours. For
511 RNA isolation, cells were washed and resuspended using TRIzol (Life Technologies), snap
512 frozen and stored at -80°C.

513

514 **RNA Isolation and sequencing**

515 To determine gene expression in cDC1s, mRNA was extracted using a Direct-zol RNA
516 MicroPrep kit (Zymo Research) as per manufacturer's protocol. Libraries were prepared using
517 Illumina stranded mRNA library kits and the outputs sequenced to a depth of 20M reads per
518 sample on a 150PE on Illumina NovaSeq X Plus 10B flow cell. Library preparation and
519 sequencing was conducted at the Australian Genome Research Facility.

520

521 **Bioinformatics Analyses**

522 Sequencing reads were aligned to the GRCm39 reference genome and transcriptome (version
523 105) using the STAR aligner (version 2.7.8a)[60] and transcript counts established using
524 featureCounts from the subread package (version 2.0.0)[61]. The data was then analysed using R
525 (version 4.2.0), limma (version 4.54.1)[62] and plots generated with ggplot2[63]. We first merged
526 technical replicates from separate sequencing lanes into their respective biological replicates
527 by summing up the counts. To obtain differentially expressed genes between the experimental
528 groups, the data were filtered for lowly or non-expressed genes (filterByExpr function) and
529 normalised counts by Trimmed Mean of M-values (TMM). Mean-variance trends were
530 estimated with the voom function, and a linear model was fit using lmFit. The model
531 parameters were moderated using eBayes, p values corrected for multiple testing using the
532 Benjamini-Hochberg method and differential genes obtained for each of the pairwise contrasts
533 between the experimental groups. The heatmap of expression values of top differential genes
534 was generated with the pheatmap package (version 1.0.12) on log2 normalised CPM values.
535

536 **Bone-marrow chimeras**

537 Mice were irradiated with two doses of 550 rad 3 hours apart. Mice were reconstituted with 3-
538 5 x 10^6 bone marrow (BM) cells from either B6, *Il4*$^{-/-}$ or *Tcrd*$^{-/-}$ mice. In the case of mixed bone-
539 marrow chimeras, mice were reconstituted with a 1:1 ratio of bone-marrow cells from B6, *Il4*$^{-}$
540 $^{/-}$ and/or *Tcrd*$^{-/-}$ mice totalling to 3-5 x 10^6 BM cells per mouse. BM cells were prepared by
541 removing the tibia and femur of donor mice. Bones were flushed with complete RPMI using a
542 23G needle and a 10 mL syringe then passed through a 70 µM mesh filter to collect bone
543 marrow. T cells were removed by resuspending the pellet with Abs against CD4 (RL172),
544 CD8α (3.168), and Thy1.1 (Jlj) for 30 minutes at 4°C followed by incubation with rabbit
545 complement for 20 minutes at 37°C to remove bound cells. All irradiated mice were injected
546 via the tail vein with 3-5 x 10^6 bone marrow cells in 0.2 mL of PBS. The following day, chimeras
547 were injected intraperitoneally with 0.1 mL of T24 (α-Thy1.1). Irradiated mice received
548 antibiotic water containing 2.5 g/L Neomycin Sulphate and 0.94 g/L Polymyxin B sulphate for
549 the first 4 weeks after irradiation followed by normal water for at least another 4 weeks prior
550 to experimental work.
551

552 ***In vivo* antibody depletion/blockade**

553 Mice were treated intraperitoneally with purified α-IL-4 (clone 11B11) for 6 days starting one
554 day prior to RAS vaccination. For the first 3 days, mice were treated with 500 µg then 200 µg

555 for the final 3 days. Control mice were treated with α-horseradish peroxidase (clone HRPN)
556 using the same dose and regimen[16].
557 For IL-12p40 neutralisation, mice were treated intraperitoneally with 250 μg of purified α-IL-
558 12p40 (clone C17.8) for 2 days beginning one day prior to RAS vaccination with an additional
559 dose the day prior to the end point. Control mice were treated with α-trinitrophenol (clone 2A3)
560 at the same dose and schedule[64].
561 To block γδ T cell function *in vivo*, mice received a single 200 μg dose of α-pan-γδTCR (clone
562 GL3) or α-Vγ1 (clone 2.11)[65] intravenously on the day prior to immunisation with RAS. As a
563 control, separate mice were treated with 200 μg of Purified Armenian Hamster IgG (clone
564 HTK888)[20].
565
566 **Organ processing and flow cytometry**
567 Single cell suspensions were prepared from spleen or livers by passing the tissues through 70
568 μM mesh filters. For experiments in which splenic DCs were enumerated, spleens were finely
569 minced in 1mg/ml Collagenase Type 3, 20 μg/ml DNase I and continuously agitated for 15
570 minutes. DC-T cell complexes were disrupted with the addition of 0.1M EDTA (pH7.2) prior
571 to filtration through a 70 μM mesh. Liver preparation required an additional step of
572 resuspending cells in 35% isotonic Percoll and centrifugation at 500 rpm for 20 minutes at room
573 temperature. Red blood cells were removed from spleen and liver pellets and labelled with a
574 near-IR fixable live-dead dye to exclude dead cells. Fluorochrome-conjugated monoclonal
575 antibodies specific for TCRδ, CD3ε, NK1.1, CD8α, CD4, B220, IL-4R, TCRβ, CD69, CD62L,
576 CD44, CD27, Vγ1, Vγ4, CD11a, CXCR6, CD11c, CD11b, XCR1, CD24, CD172a and MHC-
577 II were used for surface staining and performed for an hour at 4°C. H2-K^b-PbRPL6_{120-}
578 $_{127,}$ tetramers were provided by Professor Stephanie Gras. Sphero Blank Calibration beads were
579 used to generate cell counts. Cells were acquired on a Cytek Aurora using the SpectroFlo v3.0
580 system (Cytek) and further analysed on FlowJo (TreeStar, Inc).
581
582 ***In-vitro* activation and culture of T cells**
583 PbT-I cells were activated in complete RPMI (RPMI 1640, 10% FCS, 2mM L-glutamine,
584 100 U/mL penicillin, 100 mg/mL streptomycin and 50 mM 2-mercaptoethanol) for up to 6 days
585 with PbRPL$6_{120-127}$ (NVFDFNNL) peptide-pulsed splenocytes and in the presence of LPS (1
586 μg/mL; Sigma-Aldrich) and either: no added cytokine, recombinant mouse IL-12 (10 ng/mL[66]),
587 recombinant mouse IL-4 (60 ng/mL[34]; BioLegend) or both IL-12 and IL-4. Activated cells were

fed with complete RPMI on day 3 and split on day 4. To assess phenotype, cells were washed and labelled with a near-IR fixable live-dead dye to exclude dead cells and fluorochrome-conjugated monoclonal antibodies specific for TCRβ, IL-4R, IL-12R, CD44, Vα8.3, and CD8α.

Statistical Analyses

Statistical analyses of graphed data were performed using GraphPad Prism software. Data are shown as mean values ± SEM. The statistical tests used, and p values are indicated in each figure legend. All statistical tests were performed on log-transformed data unless otherwise specified.

Acknowledgments

The work was supported by The Australian National Health and Medical Research Council (Grant #1105817 and 2002682) and The Australian Research Council (Grant #DP220103545). S.G. is supported by an NHMRC SRF fellowship (#1159272). We wish to acknowledge the University of Melbourne Bioresources platform and the Melbourne Cytometry platform for support and expertise. We also wish to thank Melanie Damtsis and Gayle Davey for all the indirect contributions that make great research possible and Dr Yannick Alexandre for critical reading of the manuscript.

Author Contributions

SL performed the experiments with help from DM, SLiu, ZG, RM and LB and wrote the draft of the paper. AC provided RAS and CJ both under supervision by GM. CX and TB contributed methodology supervised by FK or LM. SG and IP provided resources. JS conducted bioinformatic analysis of the RNAseq data. AB, SB, IC and DFR helped with conceptualization and methodology. WRH and LB conceptualized, funded and supervised the study and wrote the paper.

Declaration of Interests

The authors declare no competing interests.

621 **Figure Legends**

622

623 **Figure 1 - γδ T cells are essential for liver T_{RM} cell generation.**

624 10^6 RPL6-specific transgenic $CD8^+$ T cells (PbT-I) were transferred into mice one day prior to
625 vaccination with 5 x10^4 RAS. PbT-I cells were analysed either 6 or 23 days later.

626 (**a and b)** Enumeration of PbT-I cells in the (**a**) spleen or (**b**) liver of B6 or *Tcrd*$^{-/-}$ mice, 6 days
627 after immunisation.

628

629 (**c and d)** Numbers of PbT-I cells in the (**c)** spleen or (**d**) liver of B6 or *Tcrd*$^{-/-}$ mice, 23 days
630 after vaccination.

631

632 **(e and f**) Quantified memory T cell (T_{MEM}) subsets; central memory T_{CM} ($CD62L^+$, $CD69^-$),
633 effector memory T_{EM} ($CD62L^-$, $CD69^-$) and resident memory T_{RM} ($CD62L^-$, $CD69^+$) within the
634 CD44+ PbT-I cell compartment of the (**e**) spleen or (**f**) liver at day 23.

635

636 **(g)** Experimental design for (**h and i**). 10^6 transgenic $CD8^+$ (PbT-I) and $CD4^+$ (PbT-II) T cells
637 were transferred into mice one day prior to vaccination with 5x10^4 RAS

638

639 (**h**) PbT-II or (**i**) PbT-I cell counts in the spleen on day 6.

640

641 Data pooled from (**a-b**) 2 or (**c-i**) 3 independent experiments with n = 9-15 mice per group.
642 Error bars indicate mean + SEM. Data were log-transformed and compared using an unpaired
643 t test. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns=p>0.05.

644

645 **Figure 2 - Splenic Vγ1$^+$ γδ T cells initiate immunity to RAS**

646 (**a**) Experimental design for (**b**). 10^6 PbT-I cells were transferred to recipient mice one day prior
647 to RAS vaccination. Mice were treated with an α-pan-γδ-TCR (α-γδ, clone GL3) or an isotype
648 control Ab, "CTRL", either (i) before RAS, or (ii) 24 hours after RAS.

649

650 (**b**) Numbers of PbT-I cells in the spleen were assessed on day 6 post-vaccination.

651

652 (**c**) Representative flow cytometry plots of Vγ1$^+$ CD11a^{hi} γδ T cells in naïve (left) or RAS-
653 vaccinated (right) TCRδ-GDL mice 24 hours after injection.

654

655 (**d**) Frequency of activated ($CD69^+$ $CD11a^{hi}$ $CD25^+$) $V\gamma1^+$ or $V\gamma1^-$ γδ T cells in naïve or RAS-
656 vaccinated TCRδ-GDL mice 24 hours post-injection.
657
658 (**e**) Experimental design for (**f**)
659
660 (**f**) Normalised splenic PbT-I cell numbers 6 days post-vaccination in isotype control Ab treated
661 "CTRL", α-Vγ1-treated (clone 2.11) or α-pan-γδ-TCR treated (clone GL3) mice. Numbers
662 were normalised by expressing them as a fraction of the mean of the "CTRL" group.
663
664 Data pooled from (**a-d**) 3 or (**e, f**) 4 independent experiments with n= 9-20mice per group. Error
665 bars indicate mean + SEM. Data were log-transformed and compared using an (**b, f**) ordinary
666 one-way or (**d**) two-way ANOVA. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$,
667 ns=$p>0.05$.
668
669 **Figure 3 - Antigen presentation and CD40-CD40L signalling is intact in the absence of γδ**
670 **T cells.**
671 10^6 CTV-labelled OVA-specific $CD8^+$ (OT-I) T cells were transferred into B6 or *Tcrd*$^{-/-}$ recipient
672 mice, which were subsequently vaccinated one day later with 5 x10^4 CS5M-RAS. OT-I cell
673 numbers were assessed on day 1, day 2 and day 3 after transfer.
674 (**a**) Representative flow cytometry plots of $CD44^+$ OT-I cells in the spleen at the indicated time
675 points.
676
677 (**b, c**) (**b**) Frequency and (**c**) number of $CD69^+$ CTV^{hi} OT-I cells in the spleen of either B6 or
678 *Tcrd*$^{-/-}$ mice.
679
680 (**d**) Experimental design for (**e**). B6, *Tcrd*$^{-/-}$ and *Cd40l*(*Cd154*)$^{-/-}$ mice received PbT-I cells with
681 or without PbT-II cells co-transferred one day prior to vaccination with 5x10^4 RAS.
682
683 (**e**) PbT-I cell counts in the spleen at day 6 post-vaccination.
684
685 (**f**) CD40 signalling to DC is intact when CD40L sufficient T cells are provided, showing that
686 CD4 T cells, not γδ T cells provide CD40L in this system.

687

688 Data pooled from 2 independent experiments with (**a-d**) n = 8 or (**f**) n= 6 mice. Error bars
689 indicate mean + SEM. Data were log-transformed data and compared using an unpaired t test
690 (**d**) or an ordinary one-way ANOVA (**f**). ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05,
691 ns=p>0.05.

692

693 **Figure 4 - IL-4 is required for the CD8 T cell response to RAS vaccination.**
694 10^6 PbT-I cells were transferred into B6, *Il4*$^{-/-}$ or *Tcrd*$^{-/-}$ mice one day prior to immunisation. An
695 additional cohort of *Il4*$^{-/-}$ mice received both 10^6 PbT-I cells and 10^6 PbT-II cells.
696 (**a**) Experimental design for (**b-d**).
697 (**b**) Number of PbT-I cells in the spleen of B6, *Il4*$^{-/-}$ or *Tcrd*$^{-/-}$ mice at 6 days post-vaccination.

698

699 (**c and d**) Numbers of (**c**) PbT-I cells and (**d**) PbT-II cells in the spleen of B6, *Il4*$^{-/-}$ or *Tcrd*$^{-/-}$
700 mice that received both PbT-I and PbT-II cells.

701

702 (**e**) Experimental design for (**f-h**). Chimeras were given 10^6 PbT-I cells one day prior to RAS
703 vaccination then analysed 6 days later.

704

705 **(f)** Single and mixed bone marrow chimeras were prepared using bone marrow from B6, *Tcrd*$^{-}$
706 $^{/-}$, *Il4*$^{-/-}$ donors as indicated.

707

708 (**g**) Enumerated splenic γδ T cells in single and mixed chimeras separated by Vγ1 expression.

709

710 (**h**) Splenic PbT-I cell numbers were assessed in chimeras, 6 days after RAS vaccination.

711

712 (**i**) Experimental design for (**j and k**). *Tcrd*$^{-/-}$ mice received γδ T cells enriched from the spleens
713 of either B6 or *Il4*$^{-/-}$ donors one day prior to transfer of PbT-I cells. Recipient mice were
714 subsequently vaccinated with RAS and analysed 6 days later.

715

716 (**j**) Quantified splenic γδ T cells 6 days after RAS vaccination in *Tcrd*$^{-/-}$ mice that received γδ T
717 cells from B6 (+γδ B6), or *Il4*$^{-/-}$ mice (+γδ *Il4*$^{-}$) or no γδ T cells (no transfer).

718

719 (**k**) Normalised PbT-I cell counts in the spleen 6 days after RAS vaccination. Counts were
720 normalised by expressing them as a fraction of the mean of the "+ γδ (B6)" group.
721
722 Data pooled from **(b, g and h**) 4 or **(c, d, j and k**) 3 independent experiments where n=5-23
723 mice per group. Error bars indicate mean + SEM. Data in (**b-d, g, h, j, k**) were log-transformed
724 and compared using an ordinary one-way ANOVA. ****p<0.0001, ***p<0.001, **p<0.01,
725 *p<0.05, ns=p>0.05
726
727 **Figure 5 - CD8 T cells and dendritic cells respond directly to IL-4.**
728 (**a**) Experimental design for (**b**). 10^6 CRISPR-Cas9 ablated sg*Cd19* (control) or sg*Il4ra* $CD8^+$
729 PbT-I cells were transferred into recipients one day prior to vaccination with RAS.(**b**)
730 Numbers of PbT-I cells in the spleen on day 6 post-vaccination.
731
732 (**c**) Experimental design for (**d, e**). $2x10^6$ B16.Flt3L-elicted sg*Cd19* (control) or sg*Il4ra*
733 CRISPR/Cas9-edited CD24+ cDC1 were transferred into *Batf3*$^{-/-}$ recipients then given 10^6
734 $CD8^+$ PbT-I cells two days later. Recipient mice were vaccinated with $5x10^4$ RAS and
735 analysed on day 6 post-RAS.
736
737 (**f**) Number of PbT-I cells in the spleens of *Batf3*$^{-/-}$ mice that received CRISPR-Cas9 edited
738 cDC1s, or no cDC1s (- transfer).
739
740 (**d**) Splenic cDC1 counts in *Batf3*$^{-/-}$ mice that received CRISPR-Cas9 edited cDC1s (+sg*Cd19*
741 or +sg*Il4ra*), or no cDC1s (- transfer).
742
743 (**g**) IL-4 acts directly on CD8 T cells and cDC1s for CD8 T cell accumulation.
744
745 Data pooled from 2 independent experiments where n=6-16 mice per group. Error bars indicate
746 mean + SEM. Data were log-transformed and compared using an ordinary one-way ANOVA.
747 ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns=p>0.05
748
749
750 **Figure 6 - CD40 and IL-4 drive a unique gene expression profile in cDC1**.
751 (**a**) Experimental design for (**b-e**).

752

753 (**b**) Heatmap showing the expression of differentially expressed genes (DEG) between media
754 alone and αCD40+IL-4, across the different stimulation conditions.
755
756 (**c**) Venn diagram showing DEGs between cDC1s cultured in media alone and cDC1s cultured
757 with αCD40 only or IL-4 only or αCD40+IL-4 stimuli.
758
759 (**d**) Volcano plot highlighting DEGs between αCD40 only and αCD40+IL-4 stimuli.
760
761 (**e**) Gene expression of *Il12a* shown as counts per million (CPM).
762
763 (**f**) Experimental design for (**g**). Mice received PbT-I cells one day prior to RAS vaccination
764 and were treated with either α-IL-12p40 or an isotype control Ab on days –1, d0 and day 5.
765
766 (**g**) Number of PbT-I cells in the spleen on day 6 post-RAS vaccination.
767
768 (**h**) γδ T cell-derived IL-4 acts on cDC1s to promote IL-12 production which, along with IL-4,
769 is required for $CD8^+$ T cell accumulation.
770
771 Data pooled from (**a-d**) n= 3-5 sequencing replicates derived from individual cDC1 cultures or
772 (**f**) n=10 mice from 2 independent experiments. Error bars indicate mean + SEM. Data were
773 log-transformed and compared using an ordinary one-way ANOVA. ****$p<0.0001$,
774 ***$p<0.001$, **$p<0.01$, *$p<0.05$, ns=$p>0.05$.
775
776 **Figure 7 - IL-4 and IL-12 synergise to promote expansion of CD8 T cells.**
777 PbT-I cells were peptide-activated *in vitro* in (**a**) media alone or in the presence of (**b**) 60 ng/mL
778 rmIL-4, (**c**) 10 ng/mL rmIL-12 or (**d**) both rmIL-4 and rmIL-12.
779
780 (**e**) IL-4R and (**f**) IL-12R surface expression on activated T cells 4 days after culture in the
781 presence of different cytokines.
782
783 OT-I T cells were activated *in vitro* in the presence of 60 ng/mL rmIL-4, 10 ng/mL rmIL-12 or
784 both rmIL-4 and rmIL-12 then transferred into recipient mice at day 4 of culture.

785 (**g**) Number of OT-I T cells in the spleen 3 days after effector transfer.
786
787 (**h**) Experimental design for (**i-l**). Mice received 10^6 PbT-I cells one day prior to RAS
788 vaccination and were treated with α-IL-12p40 or α-IL-4 according to the indicated regimen.
789
790 (**i and k**) Number of PbT-I cells in the (**i**) spleen and (**k**) liver, 29 days after vaccination.
791
792 (**j and l**) Frequency of T_{CM}, T_{EM} and T_{RM} within the $CD44^+$ PbT-I cell compartment in the (**j**)
793 spleen and (**l**) liver 29 days after RAS vaccination.
794
795 (**m**) γδ T cell-derived IL-4 act on cDC1s to promote IL-12 production which, along with IL-4,
796 signals directly on $CD8^+$ T cells to drive proliferation and therefore enhance liver T_{RM}
797 formation.
798
799 Data pooled from (**a**) 5 (**e and f**) 4 (**g**) 3 or (**i-k**) 2 independent experiments where (**a-f**) has n
800 = 4-5 individual cultures per group and (**g-l**) has n = 6-9 mice per group. Error bars indicate
801 mean + SEM. Data were log-transformed then compared using (**a-d**) a paired t test or (**e-l**) an
802 ordinary one-way ANOVA. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$, ns=$p>0.05$
803
804 **Figure S1 – Related to Figure 1; γδ T cells are essential for liver T_{RM} cell generation.**
805 Mice were vaccinated with 5 x10^4 RAS then assessed **(a, b**) 6 or **(c-g)** 35 days later.
806 (**a**) Representative FACS plots showing RPL6 tetramer staining in the liver in B6 or *Tcrd*$^{-/-}$ mice
807 6 days after RAS.
808 (**b**) Numbers of RPL6 tetramer positive $CD8^+$ T cells in the (**b**) spleen and (**c**) liver of B6 or
809 *Tcrd*$^{-/-}$ mice, 6 days after immunisation with 5 x10^4 RAS.
810
811 (**d and e**) Numbers of RPL6 tetramer positive $CD8^+$ T cells in the (**d**) spleen or (**e**) liver of B6
812 or *Tcrd*$^{-/-}$ mice, 35 days after RAS vaccination.
813
814 (**f and g**) Frequency of memory T cell (T_{MEM}) subsets; central memory T_{CM} ($CD62L^+$, $CD69^-$),
815 effector memory T_{EM} ($CD62L^-$, $CD69^-$) and resident memory T_{RM} ($CD62L^-$, $CD69^+$) within the
816 RPL6 tetramer positive $CD44^+$ $CD8^+$ T cell compartment in the (**f**) spleen or (**g**) liver of B6 or
817 *Tcrd*$^{-/-}$ mice.

818

819 (**h**) Numbers of PbT-I cells in the spleen of B6 or *Tcrd*$^{-/-}$ mice, 6 days after injection of irradiated
820 *P. berghei* infected red blood cells.
821
822 Relating to Figure 1(**g-i**), mice received 10^6 PbT-I and II cells one day prior to 5 x10^4 RAS and
823 were analysed at day 6 post-immunisation. Numbers of (**i**) PbT-II or (**j**) PbT-I cells recovered
824 in the liver of B6 or *Tcrd*$^{-/-}$ mice.
825
826 Data pooled from (**a, d-g, i-j**) 3 or (**b, h**) 2 independent experiments where n = 5-14 mice per
827 group. Error bars indicate mean + SEM. Data were log-transformed and compared using an
828 unpaired t test. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns=p>0.05.
829
830 **Figure S2 – Related to Figure 2; Splenic Vγ1$^+$ γδ T cells initiate immunity to RAS**
831 TCRδ-GDL mice were vaccinated with 10^5 RAS then analysed 24 hours later.
832 (**a**) Representative flow cytometry plots indicating gating strategy used to generate cell numbers
833 in Figure S2b and frequencies in Figure 2D.
834
835 (**b**) Number of activated (CD11a^{hi}, CD69$^+$, CD25$^+$) Vγ1$^+$ or Vγ1$^-$ γδ T cells.
836
837 Data pooled from 4 independent experiments where n= 16 mice per group. Error bars indicate
838 mean + SEM. Data were log-transformed and compared using a two-way ANOVA.
839 ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns=p>0.05.
840
841
842 **Figure S3 – Related to Figure 3; Antigen presentation and CD40-CD40L signalling is**
843 **intact in the absence of γδ T cells.**
844 10^6 CTV-labelled OVA-specific CD8$^+$ T (OT-I) cells were transferred into B6 or *Tcrd*$^{-/-}$ recipient
845 mice one day prior to vaccination with 5 x10^4 RAS. Mice were analysed at day 6 after transfer.
846
847 (**a and b)** Number of OT-I T cells in the (**a)** spleen or (**b)** liver of B6 or *Tcrd*$^{-/-}$ mice, 6 days after
848 immunisation with 5 x10^4 RAS
849

850 Data pooled from 2 independent experiments with n= 3-8 mice. Error bars indicate mean +
851 SEM. Data were log-transformed and compared using an unpaired t test. **p<0.01, *p<0.05
852
853 **Figure S4 – Related to Figure 4; IL-4 is required for the CD8 T cell response to RAS**
854 **vaccination.**
855 (**a**) Experimental design for (**b**). Wild-type mice received 10^6 PbT-I cells then were vaccinated
856 one day later with 5 x10^4 RAS. To determine the effect of blocking IL-4, mice were immunised
857 with α-IL-4 (or isotype control) across 6 days, beginning one day prior to RAS immunisation.
858 Mice were culled 6 days after RAS vaccination.
859
860 (**b**) Numbers of PbT-I cells in the spleen of isotype control and α-IL-4 treated mice 6 days after
861 RAS vaccination.
862
863 Data pooled from 4 independent experiments where n=8-14 mice per group. Error bars indicate
864 mean + SEM. Data in (**b**) were log-transformed and compared using an unpaired t test.
865 **p<0.01.
866
867 **Figure S5 – Related to Figure 5; $CD8^+$ T cells and dendritic cells respond directly to IL-**
868 **4.**
869
870 (**a**) Histogram of surface IL-4Ra expression on CRISPR-Cas9 ablated sg*Cd19* (control) or
871 sg*Il4ra* PbT-I cells**,** day 6 post-RAS.
872
873 (**b**) Representative flow cytometry plots showing cDC1 gating on live, MHC-II^+ $CD11c^+$ in the
874 spleen of *Batf3*$^{-/-}$ mice that received CRISPR-Cas9 edited pre-cDC1s (+sg*Cd19*, +sg*Il4ra*), or
875 no pre-cDC1s (- transfer)
876
877 Data pooled from 2 independent experiments where n=9 mice per group.
878
879
880

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Figure 1

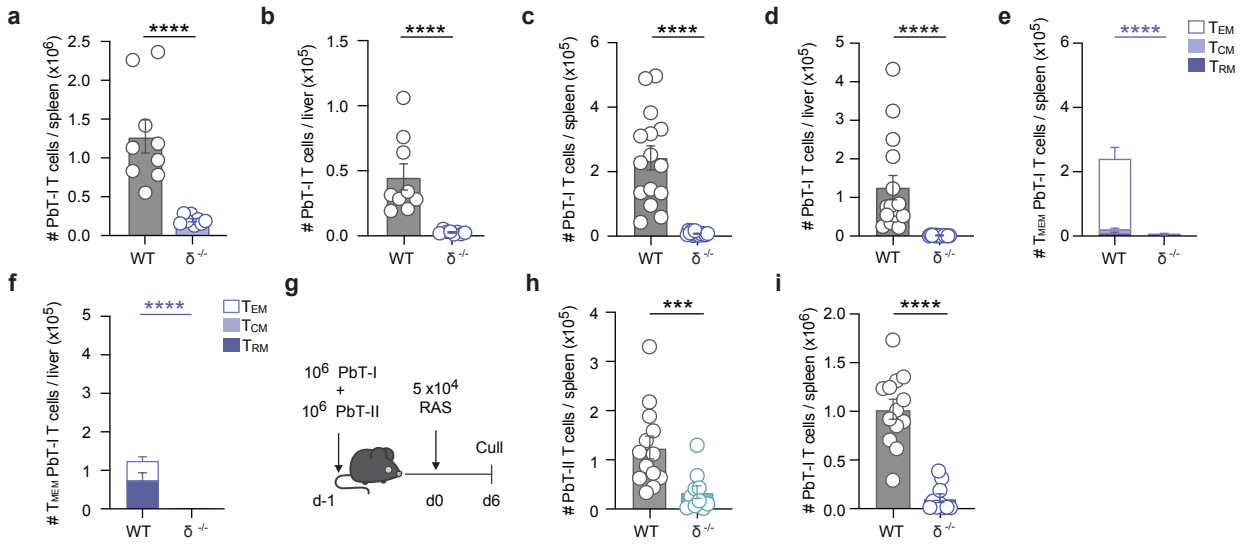

a
PbT-I T cells / spleen (x10^6)
****
WT
δ -/-
b
PbT-I T cells / liver (x10^5)
****
c
PbT-I T cells / spleen (x10^5)
****
d
PbT-I T cells / liver (x10^5)
****
e
TMEM PbT-I T cells / spleen (x10^5)
****
TEM
TCM
TRM
f
TMEM PbT-I T cells / liver (x10^5)
****
g
10^6 PbT-I
+
10^6 PbT-II
5 x10^4
RAS
Cull
d-1
d0
d6
h
PbT-II T cells / spleen (x10^5)
***
i
PbT-I T cells / spleen (x10^6)
****

Figure 2

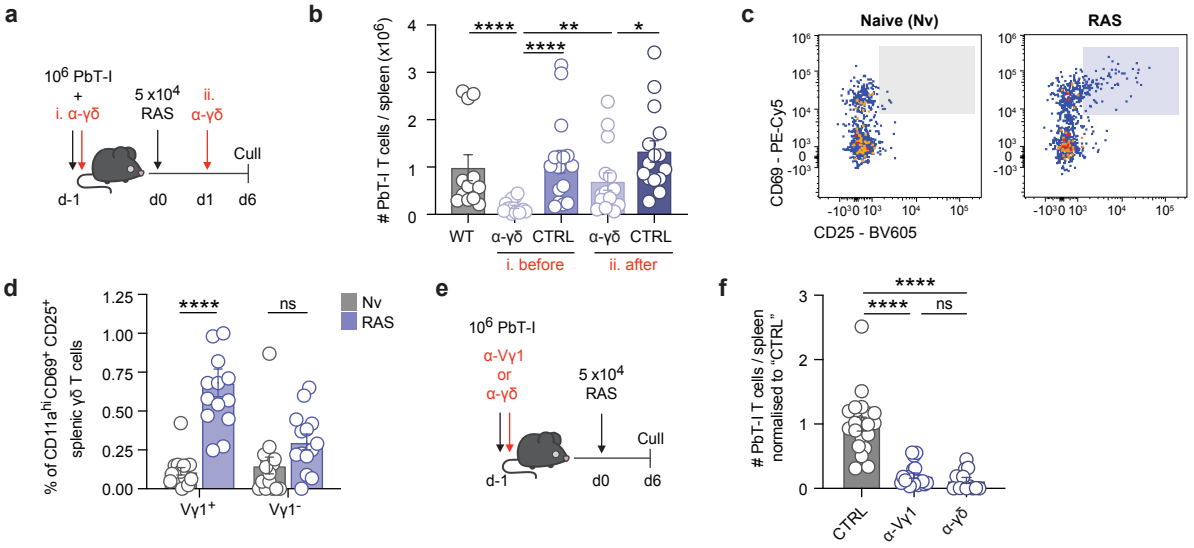

a
10^6 PbT-I
+
i. α-γδ
5 x10^4
RAS
ii.
α-γδ
Cull
d-1
d0
d1
d6
b
PbT-I T cells / spleen (x10^6)
****
**
*
****
WT
α-γδ
CTRL
α-γδ
CTRL
i. before
ii. after
c
Naive (Nv)
RAS
CD69 - PE-Cy5
CD25 - BV605
d
% of CD11a^hi CD69^+ CD25^+ splenic γδ T cells
****
ns
Nv
RAS
Vγ1^+
Vγ1^-
e
10^6 PbT-I
α-Vγ1
or
α-γδ
5 x10^4
RAS
Cull
d-1
d0
d6
f
PbT-I T cells / spleen normalised to "CTRL"
****
****
ns
CTRL
α-Vγ1
α-γδ

Figure 3

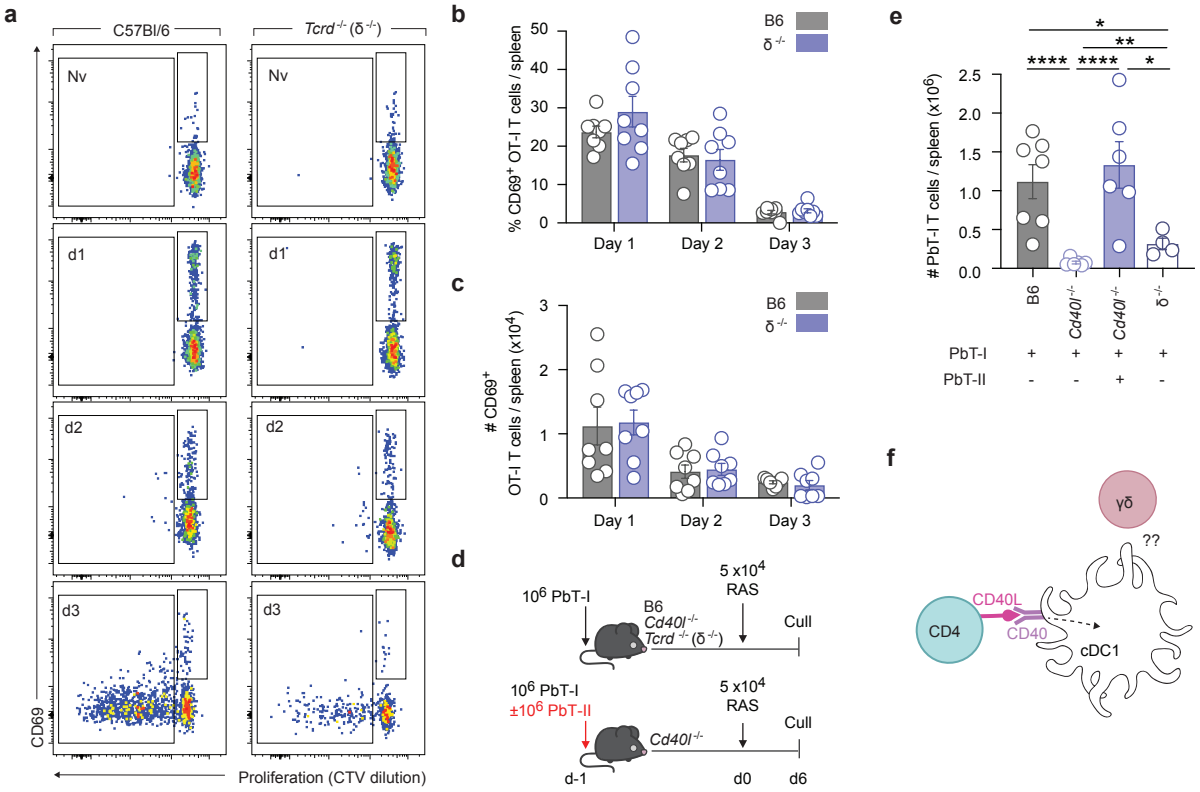
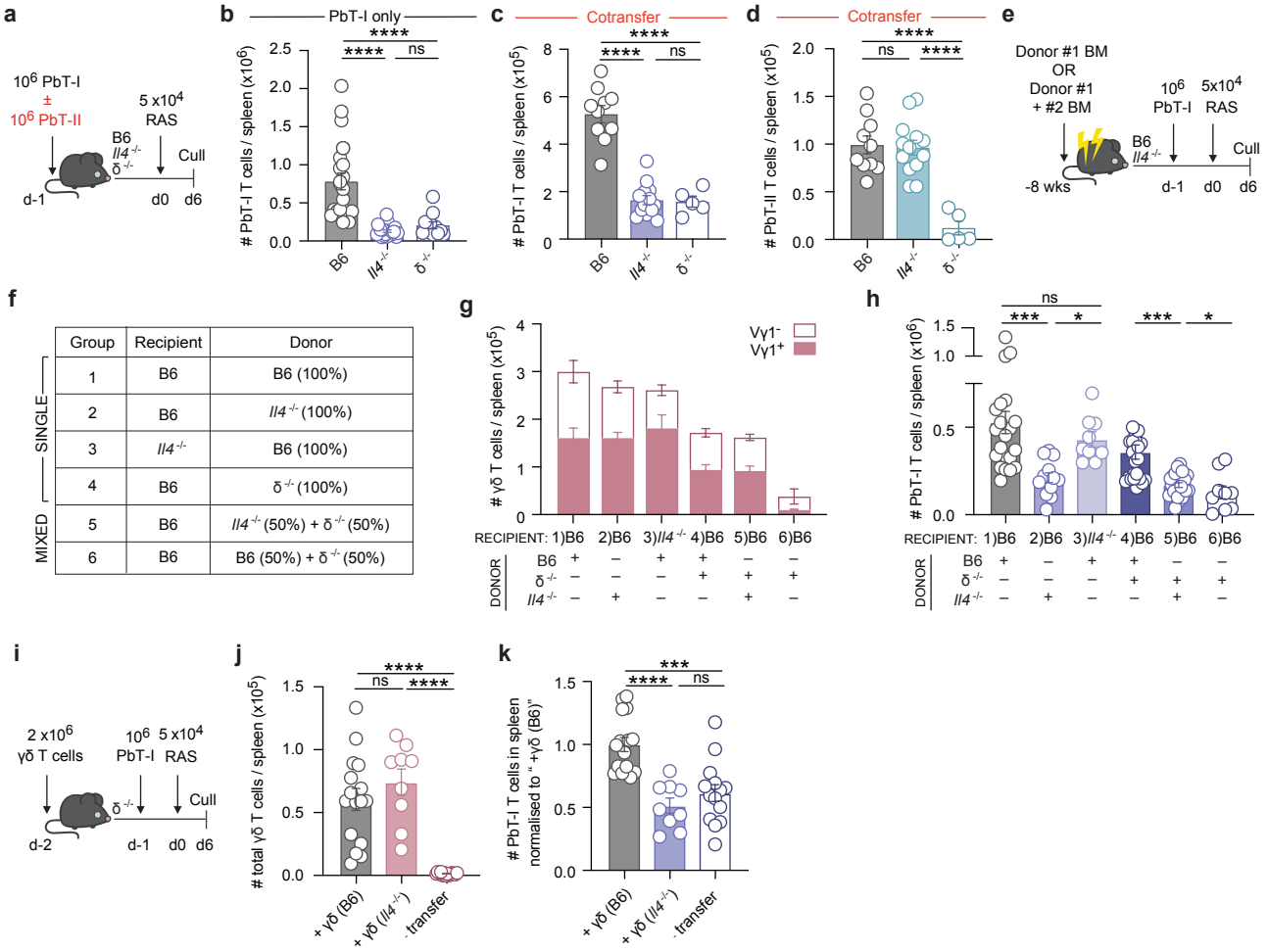

a
C57Bl/6
Tcrd-/- (δ-/-)
Nv
d1
d2
d3
CD69
Proliferation (CTV dilution)
b
% CD69+ OT-I T cells / spleen
B6
δ-/-
Day 1
Day 2
Day 3
c
CD69+ OT-I T cells / spleen (x10^4)
d
10^6 PbT-I
B6
Cd40l-/-
Tcrd-/- (δ-/-)
5 x10^4 RAS
Cull
±10^6 PbT-II
d-1
d0
d6
e
PbT-I T cells / spleen (x10^6)
PbT-I
PbT-II
f
γδ
??
CD4
CD40L
CD40
cDC1

Figure 4



	Group	Recipient	Donor
SINGLE	1	B6	B6 (100%)
SINGLE	2	B6	*Il4* $^{-/-}$ (100%)
SINGLE	3	*Il4* $^{-/-}$	B6 (100%)
SINGLE	4	B6	δ $^{-/-}$ (100%)
MIXED	5	B6	*Il4* $^{-/-}$ (50%) + δ $^{-/-}$ (50%)
MIXED	6	B6	B6 (50%) + δ $^{-/-}$ (50%)

Figure 5

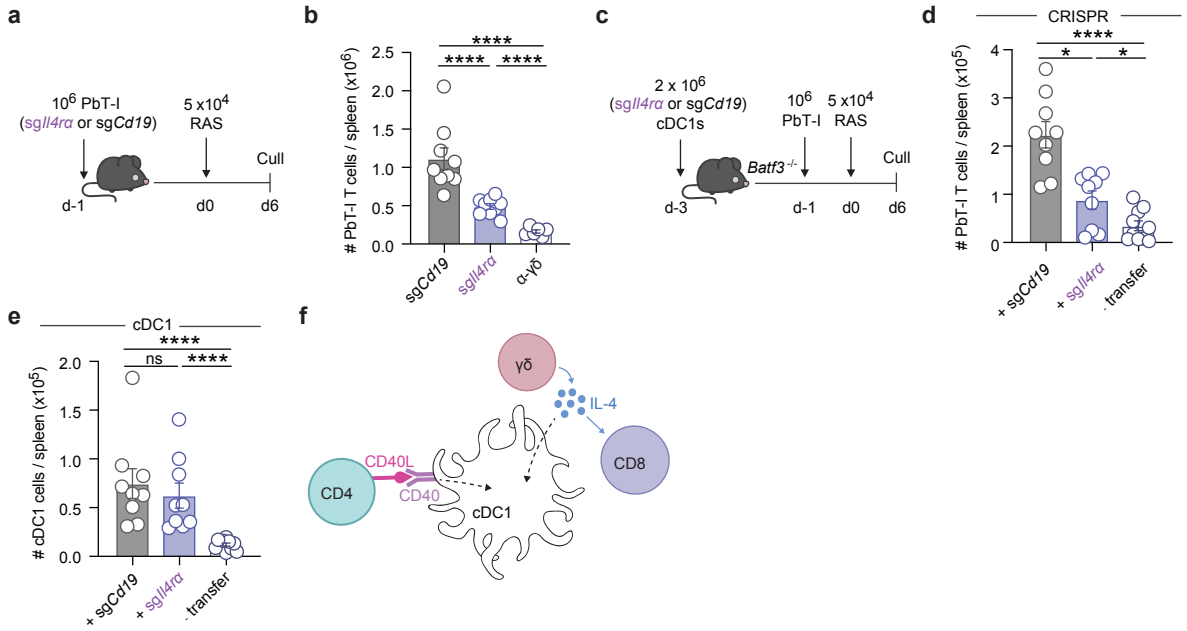

a
10^6 PbT-I
(sgIl4rα or sgCd19)
5 x10^4
RAS
Cull
d-1
d0
d6
b
PbT-I T cells / spleen (x10^6)
2.5
2.0
1.5
1.0
0.5
0.0
****
****
****
sgCd19
sgIl4rα
α-γδ
c
2 x 10^6
(sgIl4rα or sgCd19)
cDC1s
10^6
PbT-I
5 x10^4
RAS
Batf3 -/-
Cull
d-3
d-1
d0
d6
d
CRISPR
PbT-I T cells / spleen (x10^5)
4
3
2
1
0
****
*
*
+ sgCd19
+ sgIl4rα
- transfer
e
cDC1
cDC1 cells / spleen (x10^5)
2.0
1.5
1.0
0.5
0.0
****
ns
****
+ sgCd19
+ sgIl4rα
- transfer
f
γδ
IL-4
CD8
CD4
CD40L
CD40
cDC1

Figure 6

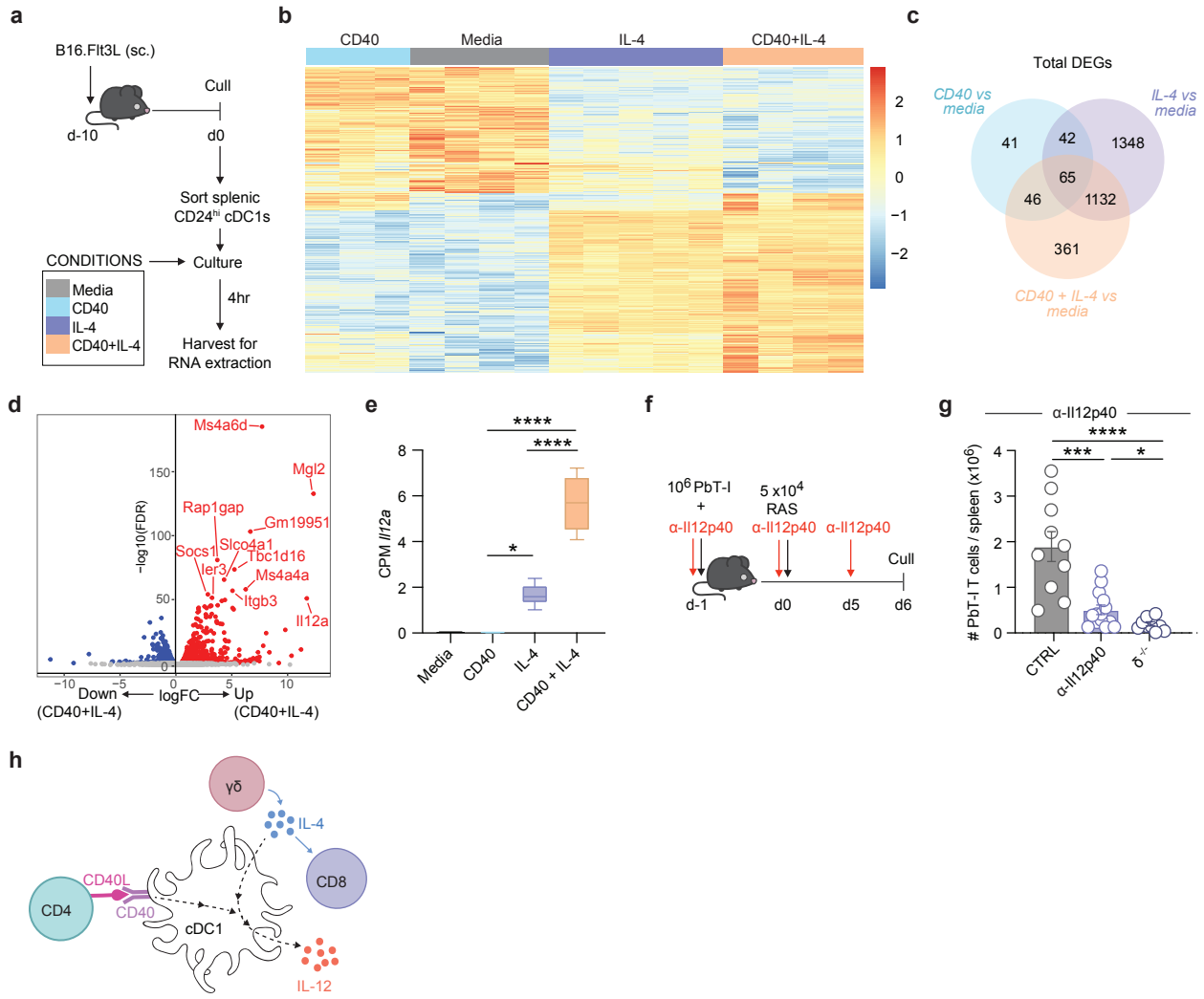
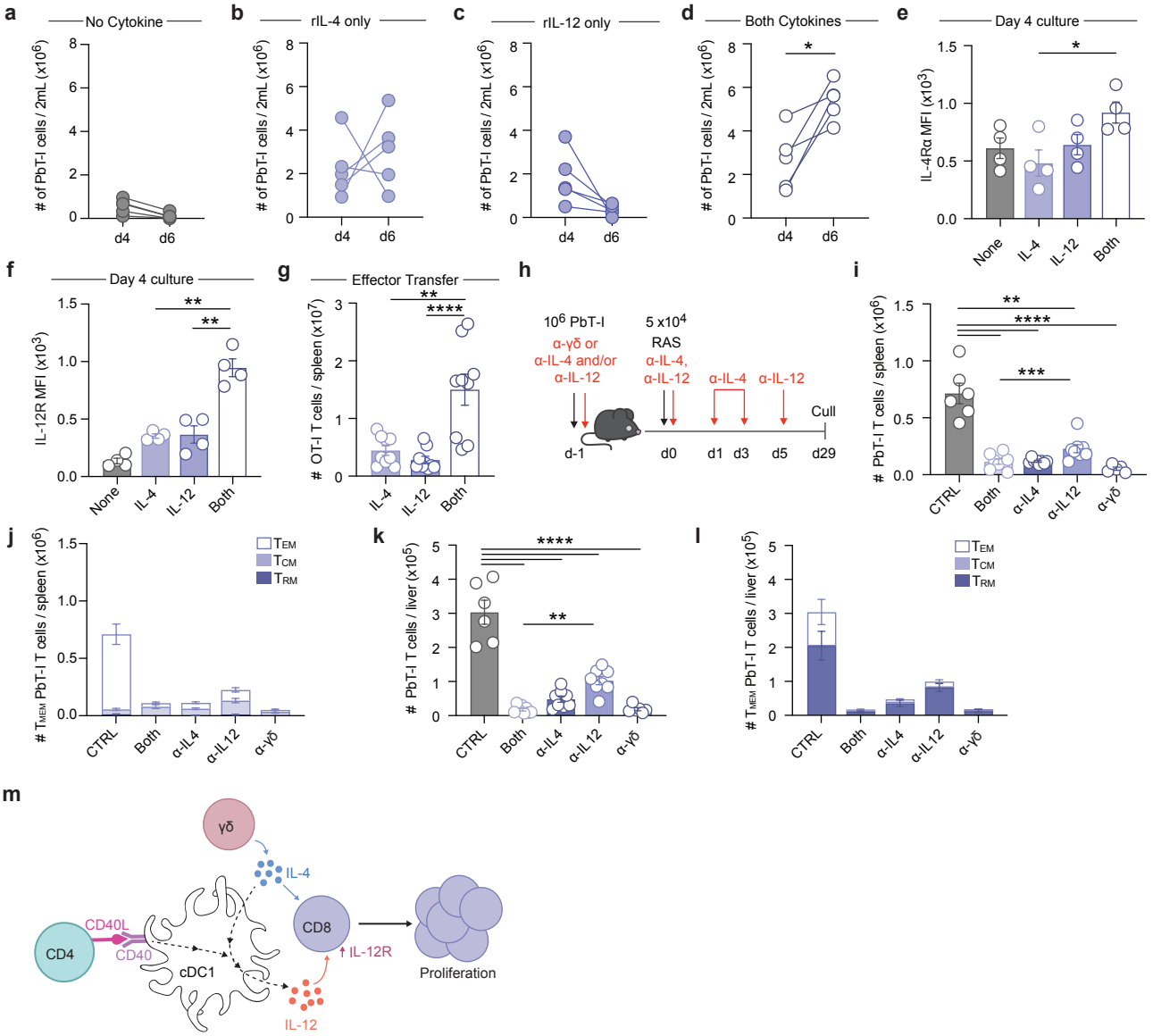

a
B16.Flt3L (sc.)
d-10
Cull
d0
Sort splenic CD24hi cDC1s
CONDITIONS
Culture
Media
CD40
IL-4
CD40+IL-4
4hr
Harvest for RNA extraction
b
CD40
Media
IL-4
CD40+IL-4
2
1
0
−1
−2
c
Total DEGs
CD40 vs media
IL-4 vs media
CD40 + IL-4 vs media
41
42
1348
65
46
1132
361
d
Ms4a6d
Mgl2
Rap1gap
Gm19951
Slco4a1
Socs1
Tbc1d16
Ier3
Ms4a4a
Itgb3
Il12a
−log10(FDR)
logFC
Down (CD40+IL-4)
Up (CD40+IL-4)
e
CPM Il12a
Media
CD40
IL-4
CD40 + IL-4
f
10^6 PbT-I + α-Il12p40
5 x10^4 RAS
α-Il12p40
α-Il12p40
Cull
d-1
d0
d5
d6
g
α-Il12p40
PbT-I T cells / spleen (x10^6)
CTRL
α-Il12p40
δ-/-
h
γδ
IL-4
CD8
CD4
CD40L
CD40
cDC1
IL-12

Figure 7


a
No Cytokine
# of PbT-I cells / 2mL (x10^6)
d4
d6
b
rIL-4 only
c
rIL-12 only
d
Both Cytokines
*
e
Day 4 culture
IL-4Rα MFI (x10^3)
None
IL-4
IL-12
Both
f
Day 4 culture
IL-12R MFI (x10^3)
**
g
Effector Transfer
OT-I T cells / spleen (x10^7)
****
h
10^6 PbT-I
α-γδ or
α-IL-4 and/or
α-IL-12
5 x10^4
RAS
α-IL-4,
α-IL-12
α-IL-4
α-IL-12
Cull
d-1
d0
d1
d3
d5
d29
i
PbT-I T cells / spleen (x10^6)
***
CTRL
α-IL4
α-IL12
α-γδ
j
TMEM PbT-I T cells / spleen (x10^6)
TEM
TCM
TRM
k
PbT-I T cells / liver (x10^5)
l
TMEM PbT-I T cells / liver (x10^5)
m
γδ
IL-4
CD8
CD4
CD40L
CD40
cDC1
IL-12R
IL-12
Proliferation