Inhibitor of apoptosis proteins 1 and 2 are required for the homeostasis of murine RORγt$^+$ γδ T cells

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Abstract

RORγt$^+$ γδ T cells, known as γδT17, are an innate-like subset of T cells that produce interleukin (IL)-17A and initiate type 3 immune responses during infections or autoimmune pathologies. Herein we show that the cellular inhibitor of apoptosis proteins cIAP1 and 2 are required for the peripheral homeostasis of γδT17 but not for their thymic development. γδT17 cells that were deficient in both cIAP1 and 2 were profoundly reduced in the peripheral lymph nodes and skin. Likewise, both RORγt$^+$ innate lymphoid (ILC3) cells and RORγt$^+$ Tbet$^+$ γδ T cells were reduced in the lamina propria of adult mice. Further, cIAP1 and 2 were required for the expression of the transcription factors RORγt and cMAF in γδT17 during neonatal and adult life. Single deficiency of either cIAP1 or 2 did not affect the homeostasis or transcription factor profile of γδT17. Inhibition of the kinase RIPK1 partially reversed the deficiency of γδT17 cells but not the downregulation of RORγt in cIAP1 and cIAP2 double deficient animals. Further, bone marrow reconstitutions and transfer of neonatal γδ T cells to RAG1$^{-/-}$ hosts showed that

both intrinsic and extrinsic factors contribute to the loss in γδT17 cells lacking cIAP1 and cIAP2. Deficiency of γδT17 cells in cIAP1 and cIAP2 double deficient animals, or the presence of functionally defective γδT17 cells did not confer protection against IMQ-induced psoriasis. Collectively, our data reveal a previously undescribed role for cIAP1 and cIAP2 in the homeostasis of γδT17 and ILC3 cells.

Introduction

The interleukin (IL)-17 producing γδ T cells (γδT17) are an innate-like subset of T cells that is enriched at the mucosal barriers and peripheral lymph nodes (Chien *et al*, 2014). Like their IFNγ producing counterparts, they arise from thymic progenitors and egress the thymus during perinatal life (Prinz *et al*, 2013). Mouse γδT17 leave the thymus as pre-programmed effectors; thus, they only require cytokine stimulation by IL-7, IL-23 or IL-1β to produce copious amounts of IL-17A, IL-17F and IL-22. Hence, γδT17 are the main producers of IL-17A in the early stages of infections by *Mycobacterium tuberculosis*, *Candida albicans* or *Listeria monocytogenes* and are critical for initiating an immune response against such infections (Papotto *et al*, 2017). Conversely, γδT17 have also been implicated in augmenting autoimmune conditions as psoriasis and multiple sclerosis. Thus, IL-17A production by γδT17 cells is required for the progression of disease in EAE, which is a model of multiple sclerosis. Similarly, dermal γδT17 play a critical in the pathogenesis of psoriasis-like symptoms in imiquimod (IMQ)-induced psoriasis.(Sutton *et al*, 2009; van der Fits *et al*, 2009).

During perinatal thymic development, γδT17 cells undergo transcriptional and epigenetic programming that imprints their effector phenotypes(Schmolka *et al*, 2013). Thus, thymic development of γδT17 is dependent on the consecutive expression of the transcription factors Sox13, cMAF followed by the lineage defining expression of RORγt. Conversely, the IFNγ

46 producing γδT cells are characterized by the transcription factors Tbet and Tcf1 (Sagar *et al*,
47 2018; Zuberbuehler *et al*, 2019). Further, canonical and non-canonical NF-κB pathway signaling
48 in the thymic microenvironment are essential for the development of γδT17. For instance, the
49 non-canonical NF-κB inducing kinase (NIK) signaling in the thymic epithelia is essential for the
50 development of γδT17 (Mair *et al*, 2015). Moreover, γδT17 development requires lymphotoxin β
51 receptor (LTβR) signaling, through the non-canonical NF-κB transcription factor RelB.
52 Alternatively, the canonical NF-κB transcription factor RelA is required for the optimal
53 expression of LTβR ligands such as LIGHT and lymphotoxin(LT)α in thymocytes, which are
54 required for γδT17 development (Powolny-Budnicka *et al*, 2011; Silva-Santos *et al*, 2005).
55 However, the role of NF-κB signaling in the homeostasis of adult γδT17 is not well described.

56 Cellular inhibitor of apoptosis (cIAP)1 and 2 and conserved E3 ubiquitin ligases that control
57 the balance between canonical and non-canonical NF-κB activation downstream of TNF receptor
58 superfamily(Gyrd-Hansen & Meier, 2010). In unstimulated cells, cIAP1 and 2 associate in a
59 heterocomplex with TNF receptor associated factor (TRAF)2, TRAF3 and NIK whereby they
60 induce lysine(K)-48 ubiquitination of NIK, resulting in its proteasomal degradation (Zarnegar *et*
61 *al*, 2008; Varfolomeev *et al*, 2007). However, ligation of TNFRII, LTβR, CD40 or OX40
62 recruits the heterocomplex to the receptor, which results in dissociation of NIK followed by
63 activation of the non-canonical NF-κB pathway(Vallabhapurapu *et al*, 2008). Conversely, cIAP1
64 and 2 positively regulate the activation of canonical NF-κB downstream of TNFRI by K-63
65 stabilizing ubiquitination of receptor interacting kinase (RIPK)1(Bertrand *et al*, 2008).
66 Furthermore, cIAP1 and 2 inhibit apoptosis and necroptosis through degradative K-48
67 ubiquitination of RIPK1 in the ripoptosome complex (Annibaldi & Meier, 2018). cIAP1 and 2

68 have been frequently studied in the context of conventional αβ T cells as well as in cancers,
69 however their role in γδT cells is obscure.

70 Herein we investigated the functions of cIAP1 and cIAP2 in the development and homeostasis
71 of γδT17 and ILC3 cells. We show that mice that lacked cIAP2 and conditionally lacked cIAP1
72 in all RORγt expressing cells had significantly reduced frequencies and abundance of γδT17
73 cells in peripheral lymph nodes, skin of adult mice and lacked RORgt^{+} Tbet^{+} γδ T cells and ILC3
74 cells in the gut lamina propria. Consistently, production of IL-17 from γδT cells was severely
75 diminished in cIAP1 and cIAP2 double deficient mice. Single deficiency of either cIAP1 or
76 cIAP2 minimally impacted γδT17 development or homeostasis. Intriguingly, we found that
77 thymic development of γδT17 was not affected in cIAP1 and cIAP2 double deficient mice.
78 However, cIAP1 and 2 deficient γδT17 in peripheral lymph nodes downregulated the
79 transcription factors cMAF and RORγt from postnatal day 21 and were progressively decreased
80 in numbers culminating in marked reduction of γδT17 by week 12. Further, using chow that
81 contained RIPK1 inhibitor or control chow, we show the decline in frequency and abundance of
82 γδT17 was partially dependent on RIPK1 while the downregulation of RORγt was independent
83 of RIPK1. Also, we found that ectopic expression of the non-canonical NF-κB transcription
84 factor RelB in γδT17 cells downregulates the expression of cMAF. Using bone marrow
85 reconstitution and transfer of neonatal γδ T cells to RAG1 deficient hosts, we show that in mice
86 that lack cIAP2 and conditionally lack cIAP1, both cell extrinsic and intrinsic factors contribute
87 to the loss of γδT17 cells, while the loss of ILC3 cells were due to cell extrinsic signals only.
88 Finally, we show that the cIAP1 and cIAP2 -induced downregulation of cMAF and RORγt
89 renders γδT17 cells functionally defective and inhibits their response to IMQ-induced skin
90 inflammation but not manifestation of IMQ-induced skin inflammation.

91 **Results**

92 **cIAP1 and cIAP2 are redundantly required for the homeostasis of γδT17 cells**

93 In order to understand the functions of cIAP1 and cIAP2 in regulating γδT17 cells, we crossed
94 RORγt-Cre (Ebertl *et al*, 2004) to cIAP1 floxed (cIAP1$^{f/f}$)or cIAP1$^{f/f}$ cIAP2 deficient mice
95 (cIAP1$^{f/f}$ cIAP2$^{-/-}$). Thus, 4 strains (Fig 1A) were generated as follows: RORγt^{cre-} cIAP1$^{f/f}$
96 (hereafter referred to as littermate controls), RORγt^{cre+} cIAP1$^{f/f}$ (ΔcIAP1), RORγt^{cre-} cIAP1$^{f/f}$
97 cIAP2$^{-/-}$ (ΔcIAP2) and RORγt^{cre+} cIAP1$^{f/f}$ cIAP2$^{-/-}$ (ΔcIAP1/2). All 4 strains were viable and
98 showed no apparent defects in the development of peripheral lymph nodes (inguinal, brachial
99 and axillary; collectively referred to as pLNs) or peyer's patches (data not shown); this indicated
100 that there was no developmental effect on lymphoid tissue inducer (Lti) cells, which express
101 RORγt and are required for formation of lymph nodes and preyer's patches perinatally (Sawa *et*
102 *al*, 2010).

103 We then analyzed γδ T cell populations in the pLNs, skin and small or large intestinal lamina
104 propria of adult mice. In pLNs, we identified γδT17 cells as being TCRγδ^{+} CD27^{-} CD44^{hi}
105 CCR6^{+} cells. We found that ΔcIAP1/2 mice had significantly reduced proportion and numbers of
106 γδT17 compared to either littermate controls, ΔcIAP1 or ΔcIAP2 mice (Fig 1B). The reduction
107 in γδT17 in ΔcIAP1/2 mice affected both Vγ4^{+} and Vγ4^{-} γδT17 cells (Vγ chain nomenclature
108 following the Heilig & Tonegawa, 1986) (Fig S1C). We further noticed an increase in TCRγδ^{+}
109 CD27^{+} population in ΔcIAP1/2 compared to other strains (Fig S1A). Consistently, there was a
110 significant reduction in IL-17A and IL-22 production and a concomitant increase in IFNγ
111 production by γδ T cells in the peripheral lymph nodes, reflecting the skewed proportions of the
112 γδ T cell populations (Fig S1B). In the skin, we found that ΔcIAP1/2 mice had reduced numbers
113 of CD3^{+} Vγ5^{-} TCRγδ^{+} cells (γδT17), without a concomitant increase in the abundance of TCRβ^{+}

114 cells (Fig 1C) Further, both Vγ4$^+$ or Vγ4$^-$ skin γδT17 cells were reduced in ΔcIAP1/2 mice,
115 compared to other strains (Fig S1D).

116 We have previously characterized a population of RORγt$^+$ Tbet$^+$ γδ T cells in the small
117 intestinal (siLP) and colonic lamina propria (cLP) (Kadekar *et al*, 2019). Thus, we investigated
118 the impact of cIAP1 and cIAP2 deficiency on the different RORγt$^+$ γδ T cells in gut lamina
119 propria. In both small intestinal and colonic lamina propria we found a significant reduction of
120 RORγt$^+$ Tbet$^+$ γδ T cells and a concomitant increase in Tbet$^+$ γδ T cells in cIAP1 and 2 double
121 deficient mice (Fig 2A-B).

122 RORγt$^+$ innate lymphoid cells (ILC3) cells are essential for the homeostasis of intestinal
123 mucosal tissue and for protection against pathogens. ILC3 cells are generally divided into Tbet$^-$
124 and Tbet$^+$ subsets (Klose *et al*, 2013; Melo-Gonzalez & Hepworth, 2017). Akin to intestinal γδ T
125 cells, we found that ΔcIAP1/2 had reduced the frequency and numbers of ILC3 cells in siLP.
126 However, both Tbet$^+$ and Tbet$^-$ ILC3 cells were equally reduced in ΔcIAP1/2 (Fig 2C).
127 Collectively, this data show that both cIAP1 and 2 are required for the homeostasis of γδT17 in
128 the pLNs, skin of adult mice while single deficiency of either has no effect. It also shows that in
129 the gut lamina propria, cIAP1 and 2 are critical for RORγt$^+$ Tbet$^+$ γδ T and ILC3 cells.

130 **cIAP1 and 2 maintain the expression of cMaf and RORγt in γδT17 during neonatal life**
131 **but not thymic development.**

132 The development of γδT17 cells occurs in the thymus at approximately embryonic day 16,
133 concomitant with the expression of the transcription factors *Rorc* and *Maf* (Haas *et al*, 2012;
134 Zuberbuehler *et al*, 2019). Thus, we aimed to track the perinatal and neonatal development and
135 homeostasis of γδT17 in ΔcIAP1/2, ΔcIAP1, ΔcIAP2 and littermate control mice. First, we

136 analyzed γδT17 in the thymi of 1-day old pups and found that the thymic output of γδT17 in
137 ΔcIAP1/2 pups was comparable to littermate controls. Thus, there was no difference in the
138 numbers of γδT17 cells nor in the expression of RORγt and cMaf by γδT cells between 1-day old
139 ΔcIAP1/2 pups and controls (Fig 3A-B). Further, the proliferation of γδT17 from 1-day old pups,
140 as measured by Ki67 staining, was also comparable to littermate controls (Fig S2A).

141 We found that in γδT17 from pLNs, there was a downregulation of both RORγt and cMaf that
142 started at postnatal day 21 and persisted afterwards, which occurred only ΔcIAP1/2 but not in
143 littermate controls (Fig 3D). However, the absolute numbers of γδT17 cells (identified as being
144 $CD44^{hi}$ $CD27^{-}$) in the pLNs of ΔcIAP1/2 remained comparable to those in littermate control
145 mice until approximately 5 weeks of age (Fig 3C). This culminated in a marked reduction of
146 γδT17 cells, identified by either surface markers or RORγt flow cytometry staining, in ΔcIAP1/2
147 mice at 12 weeks of age (Fig 3C & E). Collectively, this data suggests that cIAP1 and cIAP2 are
148 required for the adult homeostasis, but not perinatal development, of γδT17 cells in pLNs and
149 points to a role for cIAP1 and cIAP2 in regulating the expression of RORγt and cMaf in γδT17
150 cells.

151 **Loss of γδT17, but not RORγt expression, in ΔcIAP1/2 mice is partially dependent on**
152 **RIPK1**

153 cIAP1 and 2 regulate the stability and function of RIPK1 by either K63 stabilizing
154 ubiquitination or K48 ubiquitination that marks RIPK1 for proteasomal degradation; thus, in the
155 absence of cIAP1 and 2, RIPK1 accumulates in the cytoplasm and seeds the formation of the
156 ripoptosome complex thereby facilitating apoptosis or necroptosis (Silke & Meier, 2013).
157 Therefore, we asked whether the progressive reduction of γδT17 in ΔcIAP1/2 mice could be due
158 to increased RIPK1-dependent cell death. To test this possibility, we weaned mice from all 4

159 strains on either control chow or chow that contained a RIPK1 inhibitor compound (Ri). We then
160 analyzed γδT cell populations in pLNs and siLP and ILC3 cells in siLP at when the mice were at
161 least 11 weeks of age.

162 Based on surface phenotype, inhibition of RIPK1 in ΔcIAP1/2 partially rescued the frequency
163 and overall numbers of γδT17 in the pLNs, compared to littermate control mice that had received
164 control chow. There was no significant difference in frequency nor total numbers of γδT17 in the
165 pLNs between ΔcIAP1/2 and littermate controls that received Ri chow; this probably reflected
166 the tendency towards lower abundance of γδT17 in littermate controls on Ri chow compared to
167 controls receiving regular chow (Fig 4A). Surprisingly, transcription factor staining revealed that
168 RIPK1 inhibition did not affect the significant downregulation of RORγt in γδT17 cells that were
169 still present in the pLNs of ΔcIAP1/2 mice (Fig 4B-C). Further, RIPK1 inhibition had no
170 significant impact on the anti-apoptotic protein Bcl2 in γδT17 cells from ΔcIAP1/2 mice but we
171 noticed a mild decrease of Bcl2 in γδT17 from ΔcIAP2 mice (Fig. S2B). In siLP, the inhibition
172 of RIPK1 did not halt the reduction in RORγt^{+} Tbet^{+} γδ T cells in double deficient mice,
173 compared to control mice or mice that received control chow (Fig 4D). Taken together, our data
174 indicate that the decline in γδT17 frequency and abundance is partially dependent on RIPK1
175 while the concomitant downregulation in RORγt in γδT17 in ΔcIAP1/2 mice is RIPK1
176 independent.

177 **Activation of the non-canonical NF-κB in γδT17 cells downregulates cMAF expression.**

178 We have previously shown that the non-canonical NF-κB transcription factor RelB
179 downregulates the expression of the transcription factor cMAF in Th17 cells; this occurs
180 following the activation of the non-canonical NF-κB pathway through depletion of cIAP1 and 2
181 by SMAC mimetic compounds (Rizk *et al*, 2019). Thus, we hypothesized that the lack of cIAP1

182 and 2 expression in γδT17 activates the non-canonical NF-κB pathway and mobilizes RelB to
183 downregulate cMAF. To test this hypothesis, we purified γδT17 cells from thymi old 1-day old
184 wildtype mice and infected them with an empty retroviral vector or vector expressing RelB and
185 cultured the cells with IL-7. Subsequently, we FACS sorted infected γδT17 cells (TCRγδ^{+}
186 CD44^{hi} CD27^{-} GFP^{+}) and extracted RNA to assess the expression of key γδT17 transcription
187 factors. As expected, the RelB-RV infected cells expressed more RelB and NFκB2 compared to
188 empty-RV infected cells (Fig 5A). Furthermore, we found that RelB induced a downregulation
189 of *Maf*, but did not affect the expression of *Rorc* or *Sox13* (Fig 5B). This indicates that similar to
190 what happens in Th17 cells, activation of the non-canonical NFκB in γδT17 cells downregulates
191 cMAF.

192 **Intrinsic and extrinsic factors contribute to the loss of γδT17 in ΔcIAP1/2 mice.**

193 cIAP1 and cIAP2 regulate the cells response to extrinsic stimuli to TNF and other TNFRSF
194 ligands but are also important to inhibit cell death through control over the formation of the
195 ripoptosome. Therefore, we set to investigate whether cell extrinsic or intrinsic signals were
196 responsible for the loss of γδT17 cells in the ΔcIAP1/2 mice. To this end we set up mixed bone
197 marrow chimeras where wildtype CD45.1/CD45.2 hosts were sublethally irradiated and
198 reconstituted with a mixture of 1:1 wildtype (CD45.1) and ΔcIAP1/2 (CD45.2) bone marrow
199 cells (Fig 6A). We found that, under these conditions, γδT17 and CD27^{+} γδ T cells from
200 ΔcIAP1/2 bone marrow reconstituted the pLNs of the host as competitively as their wildtype
201 counterparts (Fig 6B). Further, only Tbet^{+} γδ T cells reconstituted the colonic lamina propria and
202 were slightly dominated by cells that originated from ΔcIAP1/2 bone marrow (BM) (Fig 6C).
203 Conversely, colonic ILC3 cells were effectively reconstituted and were encompassed
204 approximately equal proportions of CD45.1 (WT) and CD45.2 (ΔcIAP1/2) cells (Fig 6D). This

205 indicates that the presence of wildtype hematopoietic and stromal components prevents the loss
206 of γδT17 from ΔcIAP1/2 origin.

207 We then asked whether the reconstitution of ΔcIAP1/2 mice with mixed BM chimeras would
208 be enough to stop the loss of γδT17 cells or not. To this end, we irradiated ΔcIAP1/2 mice that
209 were at least 12 weeks old, to ensure host mice lacked γδT17 cells, and reconstituted them with
210 either wildtype (CD45.1) or a mixture of 1:1 wildtype (CD45.1) and ΔcIAP1/2 (CD45.2) BM
211 cells (Fig 7A). In both instances we found that all γδT17 cells in the pLNs were from wildtype
212 (CD45.1) BM; conversely, $CD27^{+}$ γδ T cells were either equally split into CD45.1 and CD45.2
213 components in mice that had received mixed BM chimeras or only CD45.1 cells in mice that had
214 received wildtype cells only (Fig 8B). In the siLP, almost all γδ T cells came from wildtype
215 (CD45.1) BM and expressed various levels of Tbet and some RORγt (Fig 7C). Although ILC3
216 that were of both wildtype (CD45.1) and ΔcIAP1/2 (CD45.2) origin could be found in the siLP,
217 wildtype ILC3 cells were more abundant than ILC3 cells from ΔcIAP1/2 BM (Fig 7D). Thus, the
218 presence of wildtype hematopoietic cells in ΔcIAP1/2 mice is not sufficient to reverse the loss of
219 γδT17 cells lacking cIAP1 and cIAP2 but is sufficient to reverse the loss of ILC3 cells.

220 As γδT17 cells are generated perinatally in the thymus, we thus reasoned that γδT17 cells
221 generated after BM chimera reconstitutions might be different from γδT17 cells that egress the
222 neonatal thymus. To address this issue, we purified γδ T cells from the thymi of 1-day old Ctrl,
223 ΔcIAP1, ΔcIAP2 or ΔcIAP1/2 mice and transferred them to $RAG1^{-/-}$ recipient mice (Fig 8A).
224 After 12 weeks we analyzed the pLNs and cLP and found that γδT17 and $CD27^{+}$ γδ T cells could
225 be recovered from the pLNs of $RAG1^{-/-}$ mice (Fig 8B) but again only $Tbet^{+}$ γδ T cells could be
226 found in the cLP, regardless of the donor strain (Fig 8C).

227 Taken together our data show that γδT17 cells that lack cIAP1 and cIAP2 are intrinsically
228 defective in their response to an extrinsic stimulus that is altered in the ΔcIAP1/2 mice.
229 Conversely, ILC3 cells were intrinsically less dependent on the presence of cIAP1 and cIAP2 as
230 they could be rescued by the presence of wildtype hematopoietic, stromal compartment or both.

231 **RORγt^{cre} cIAP1$^{f/f}$ cIAP2$^{-/-}$ mice are not IMQ-psoriasis.**

232 The TLR7 ligand imiquimod (IMQ) induces a psoriasis-like pathology when applied to the
233 skin of mice; the development of disease symptoms is dependent on the presence of functional
234 γδT17 that produces IL-17A in response to increased IL-23 and IL-1β in the skin milieu (van der
235 Fits *et al*, 2009; Sandrock *et al*, 2018). As such, we aimed to test how the homeostatic reduction
236 in γδT17 in the skin of ΔcIAP1/2 affects the outcome of skin inflammatory conditions as
237 psoriasis. Thus, we applied IMQ on the ear skin of littermate controls and cIAP1 and 2 single or
238 double deficient mice and measured the inflammatory response in the ear skin, auricular and
239 cervical lymph nodes. Hematoxylin and Eosin (H&E) staining revealed comparable thickening
240 of the skin epidermis in all strains that received IMQ treatment (Fig S3A). Nevertheless, the
241 abundance of γδT17 cells in skin or lymph nodes of IMQ treated ΔcIAP1/2 remained
242 significantly reduced compared to treated or untreated littermate controls or cIAP1 or 2 deficient
243 mice (Fig S3B). Likewise, IL-17A production by lymph nodes γδ T cells was severely reduced in
244 both treated and untreated ΔcIAP1/2 mice compared to other strains (Fig S3C). Conversely, we
245 noticed an increase in IL-17^{+} CD4^{+} CD44^{+} T cells in the lymph nodes of IMQ treated ΔcIAP1/2
246 mice compared to treated or untreated littermate controls (Fig S3D)

247 We then sought to assess how the ΔcIAP1/2 mice would respond to IMQ-induced
248 inflammation in the presence of transcriptionally altered γδT17 cells. To this end, we applied
249 IMQ on the ear skin of 4 weeks old mice of all 4 genotypes and again assessed the inflammatory

250 response. We found that the epidermal thickening in the ear skin of ΔcIAP1/2 mice was
251 comparable to that of WT mice (Fig 9A). Further, we found that both in the skin and the lymph
252 nodes, γδT17 cells from ΔcIAP1/2 mice failed to expand in response to IMQ-inflammation and
253 were significantly less abundant than in the IMQ-treated control mice. Conversely, $CD4^+$ T cells
254 in skin of IMQ-treated ΔcIAP1/2 mice were significantly more abundant than in control mice
255 although there was no significant difference in the lymph nodes (Fig 9B). Functionally, we found
256 that γδT17 cells from IMQ-treated ΔcIAP1/2 produced significantly less IL-17A in the lymph
257 nodes or skin, compared to IMQ-treated control mice; instead, $CD4^+$ T cells from ΔcIAP1/2
258 produced copious amounts of IL-17A in both organs compared to controls (Fig 9C-D). Likewise,
259 lymph nodes γδT17 deficient in cIAP1 and cIAP2 failed to produce any IL-22 following IMQ-
260 psoriasis, while the $CD4^+$ T cells in the same mice produced significantly more IL-22 than in the
261 IMQ-treated control mice (Fig 9C); intriguingly, IL-22 production by γδT17 in the skin of IMQ-
262 treated ΔcIAP1/2 was comparable to that of control mice, yet still there was significantly more
263 IL-22 produced by the CD4+ T cells in the skin of ΔcIAP1/2 mice following IMQ-treatment,
264 compared to controls (Fig 9E).

265 Thus, our data show that although adult ΔcIAP1/2 mice are severely deficient in γδT17 cells
266 they are not protected from IMQ-induced psoriatic inflammation; similarly, reduced
267 functionality of γδT17 cells in young ΔcIAP1/2 mice did not protect the mice from IMQ-induced
268 inflammation. In both instances, the IMQ-induced skin inflammation appears to have been a
269 function of aggravated Th17 responses. These results are consistent with earlier report showing
270 that $CD4^+$ T cells are responsible for the IMQ induced psoriasis following loss of γδ T cells
271 during adult life (Sandrock *et al*, 2018). Further, our data point out that during IMQ-induced

272 inflammation not only can $CD4^+$ T cells compensate for the lack of γδ T cells, but also for their
273 compromised functionality.

274 **Discussion**

275 Herein we showed that cIAP1 and cIAP2 are required for the adult homeostasis of γδT17 cells
276 in peripheral lymph nodes, skin and gut lamina propria. Although cIAP1 and cIAP2 were not
277 required for the thymic development of γδT17 cells, mice that lacked cIAP2 and conditionally
278 lacked cIAP1 in RORγt expressing cells suffered progressive reduction in γδT17 cells in the
279 lymph nodes during late neonatal and adult life. Further, lack of cIAP1 and cIAP2 correlated
280 with a downregulation of RORγt and cMaf in γδT17 cells that preceded the loss of γδT17 cells.
281 Although inhibition of RIPK1 led to a partial rescue of γδT17 cells in the lymph nodes, it failed
282 to halt the downregulation of RORγt in cIAP1 and cIAP2 double deficient γδT17 cells; however,
283 RIPK1 inhibition did not affect the reduction in $ROR\gamma t^+$ $Tbet^+$ γδT cells in the siLP of cIAP1 and
284 cIAP2 double deficient mice. We further show that the both cell intrinsic and extrinsic factors
285 contribute to the decreased abundances of γδT17 cells in adult $ROR\gamma t^{cre}$ $cIAP1^{f/f}$ $cIAP2^{-/-}$ mice.
286 Finally, we show that adult $ROR\gamma t^{cre}$ $cIAP1^{f/f}$ $cIAP2^{-/-}$ mice were not protected against IMQ-
287 induced skin inflammation despite the severe reduction in γδT17 in these mice and that the
288 presence of functionally defective γδT17 cells in younger $ROR\gamma t^{cre}$ $cIAP1^{f/f}$ $cIAP2^{-/-}$ was not
289 sufficient to inhibit IMQ-induced psoriasis.

290 cIAP1 and cIAP2 are ubiquitously expressed proteins that are frequently upregulated in
291 cancers (Tamm *et al*, 2000; Wu *et al*, 2010). Downstream TNFR superfamily, they fine tune the
292 balance between NF-κB activation or induction of cell death. Thus, they are important targets in
293 the context of cancer therapy, whereby their antagonization can sensitize cancer cells to cell
294 death or modulate the functions of cytotoxic T cells (Gyrd-Hansen & Meier, 2010; Bai *et al*,

295 2014) . However, their role in γδ T cells has not been previously addressed. Consensus is that
296 cIAP1 and cIAP2 are functionally redundant (Mahoney *et al*, 2008); thus, mice deficient in either
297 proteins display no phenotypic changes. As the genetic knockout of both cIAP1 and cIAP2 is
298 embryonically lethal (Moulin *et al*, 2012), the RORγt driven deletion of cIAP1 combined with
299 full deletion of cIAP2 provides a targeted approach to probe their function in RORγt expressing
300 γδ T cells. The data presented herein reveals a critical, but redundant, role for cIAP1 and cIAP2
301 in the homeostasis of γδT17 cells.

302 We have previously shown that antagonization of cIAP1 and cIAP2 in Th17 cells
303 downregulates *Il17a* and *Maf* through the non-canonical NF-κB transcription factors RelB and
304 p52 (Rizk *et al*, 2019). Thus, it is plausible that a similar mechanism exists in γδT17 cells; as
305 such, the lack of cIAP1 and cIAP2 in γδT17 activates the non-canonical NF-κB, which
306 downregulates *Maf* expression. Consequently, *Rorc* is downregulated as cMaf is required to
307 maintain its expression in γδT17. Eventually, such cells would progressively perish as cMaf and
308 RORγt are required for the homeostasis of γδT17 (Barros-Martins *et al*, 2016; Zuberbuehler *et*
309 *al*, 2019). The partial rescue of γδT17 in the cIAP1/2 double deficient mice by RIPK1 could be
310 explained by the fact that cMaf deficiency in γδT17 results in increased apoptosis(Zuberbuehler
311 *et al*, 2019), which could be dependent upon RIPK1 .

312 Nonetheless, several outstanding questions remain to be resolved in such model. Specifically,
313 the persistence of expression of γδT17 surface markers ($CCR6^{+}$ $CD44^{hi}$) despite the significant
314 downregulation of RORγt and cMaf in cIAP1 and 2 deficient γδT17 cells is intriguing.
315 Furthermore, such downregulation of RORγt and cMaf occurred in the periphery but not in the
316 thymus and started at approximately 3 weeks of age. Likewise, the intrinsic and extrinsic factors
317 contributing to the loss of γδT17 in the cIAP1/2 double deficient mice remain unresolved.

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413

414

415 **Materials and methods**

416 **Mice**

417 All animals were bred and maintained in-house at DTU health tech with the approval of the
418 Danish animal experiments inspectorate. cIAP1$^{f/f}$ and cIAP1$^{f/f}$ cIAP2$^{-/-}$ mice were provided by
419 Prof. W. Wei-Lynn Wong at the University of Zurich, Switzerland with the permission of Prof.
420 John Silke, VIC Australia. RORγt^{CRE} mice were provided by Prof. Gerard Eberl at Pasteur
421 Institute, Paris, France.

422 **Cell culture media and buffers**

423 For all preparations of single cell suspensions and cell cultures RPMI 1460 (Invitrogen)
424 supplemented with 10% heat inactivated FBS (GIBCO), 20mM Hepes pH 7.4 (Gibco), 50 µM 2-
425 mercaptoethanol, 2 mM L-glutamine (Gibco) and 10,000 U/ mL penicillin-streptomycin (Gibco),
426 was used. FACS buffer was prepared by supplementing PBS with 3% heat inactivated FBS.

427 **Lymphocyte isolation from mouse organs**

428 Lymphocytes were isolated from peripheral lymph nodes (axial, brachial and inguinal), thymus,
429 ear skin, small intestinal and colonic lamina propria following the previously described protocols
430 (Kadekar *et al*, 2019). Lymphocytes were isolated from cervical and auricular lymph nodes in
431 case of IMQ-induced psoriasis.

432 **IMQ-induced psoriasis**

433 Psoriasis was induced in mice by applying 7 mg of Aldara cream (containing 5% imiquimod) to
434 the dorsal side of each ear for 7 days. Histological sections were prepared by fixing ear tissue in
435 10% formalin overnight and then paraffin embedded. The paraffin embedded section were
436 stained by H&E.

437 **Flow cytometry staining**

438 Surface antigens, intracellular cytokines and transcription factors were stained for flow
439 cytometry as previously described (Rizk *et al*, 2019). The following antibodies were used herein
440 at 1:200 dilution unless otherwise indicated:

441 anti-IL-17A (TC11-18H10; BV786 and PE), anti-IFNγ (XMG1.2; PE-Cy7 and APC), anti-IL-22
442 (1H8PWSR; PE), anti-cMAF (symOF1; PE and Percp-Cy5.5; 5 µL/test), anti-CD4 (GK1.5;
443 BUV395), anti-TCRγδ (GL3; BV421), anti-CD27 (LG.3A10; PE-Cy7), anti-CCR6 (140706;
444 Alexa Fluor 647), anti-CD44 (1M7; V500), anti-CD19 (6D5; FITC), anti-TCRβ (H57-597; APC-
445 eflour780), anti-CD3e (BM10-37, PeCF594 and PE), anti-Tbet(4B10; PeCy7), anti- CD8 (53-
446 6.7;FITC), anti-Vγ5 (536; FITC), anti-Vγ4(UC3-10A6; Percp-eflour710), anti-GATA3(TWAJ;
447 Percp-eFlour710; 1:30), anti-CD45(30-F11;PE and V500) and anti-RORγt (B2D; APC).

448 **Retroviral infection of neonatal γδT17 cells.**

449 First, pooled thymi from one-day old wildtype mice were isolated and crushed against 70 µm
450 filter to prepare single cell solution. Subsequently, γδT17 cells were enriched by magnetic
451 depletion of $CD4^+$, $CD8^+$, $TCR\beta^+$ and $CD27^+$ cells as follows: total thymocytes were re-
452 suspended in MACS buffer at 1e8 cells/ml containing 50 µL/ml normal rat serum and 1:200
453 biotin labelled anti- $CD4^+$, $CD8^+$, $TCR\beta^+$ and $CD27^+$ antibodies; the cells were incubated for 10
454 minutes at room temperature and then incubated with 75 µL/ml EasySep RaphidSphere
455 streptavidin beads (#50001) for 2.5 minutes then transferred to EasySep magnet for 2.5 minutes.
456 The non-bound fraction was collected by decantation and centrifugated for 5 minutes at 400g at
457 4ºC. Approximately 2e5 of the enriched γδT17 cells were then cultured overnight with 20 ng/ml
458 recombinant IL-7 in round-bottom 96-well tissue culture plate. The cells were then spin-infected

459 by adding 4 μL of the desired retrovirus and 5 μg Retronectin (TAKARA) and centrifugation at
460 1800 rpm for 30 minutes at 32ºC with deceleration set to 0. Subsequently, the cells were
461 incubated for 90 minutes at 32ºC before transfer to 37ºC incubator. Recombinant IL-7 at 20
462 ng/ml was again added to the cultures after 48 hours and the cells were FACS sorted 24 hours
463 later.

464 **Transfer of neonatal γδ T cells to RAG1$^{-/-}$ hosts.**

465 First, thymi from 1-2 days old mice, from all 4 strains, were isolated and crushed individually
466 against 70 μm filter to prepare single cell solutions. Subsequently, total γδ T cells were enriched
467 by magnetic depletion of CD4^{+}, CD8^{+}, TCRβ^{+} cells as aforementioned. The enriched γδ T cells
468 from each donor mouse were re-suspended in PBS and then i.v. injected into the tail vein of a
469 RAG1$^{-/-}$ host. The RAG1$^{-/-}$ hosts were euthanized for organs after 12 weeks.

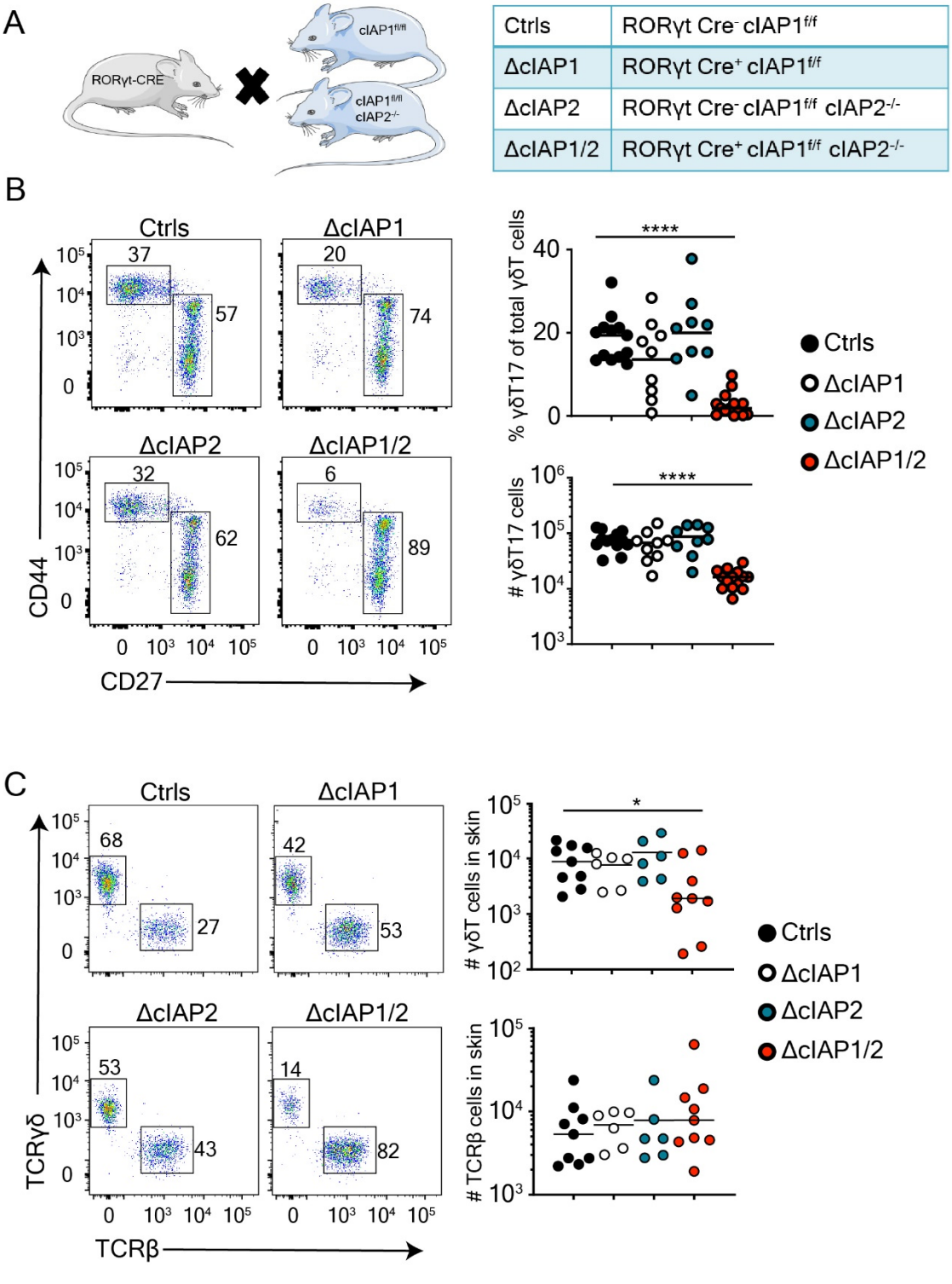
470 **Bone marrow chimeras**

471 The bone marrow cells for reconstitution were isolated by flushing the tibia and femur, which
472 were dissected from donor mice, with culture media. Total bone marrow cells were then
473 centrifugated at 400g for 5 minutes at 4ºC. The cells were then re-suspended and passed through
474 70 μm filter. Subsequently, red blood cells were then lysed using RBC lysis buffer (Biolegend)
475 and a single cell suspension of bone marrow cells was the prepared by passing the cells through
476 40 μm filter. The prepared cells were then counted and mixed as appropriate.

477 Conversely, host mice were sub-lethally irradiated by 2 doses of 4.5 Gy that were at least 4 hours
478 apart. After 24 hours, the hosts were reconstituted with 10e6 bone marrow cells that were i.v.
479 injected into the tail vein of the host mice. The hosts were euthanized for organs after at least 12
480 weeks.

481 **Figures**

482 **Figure 1**



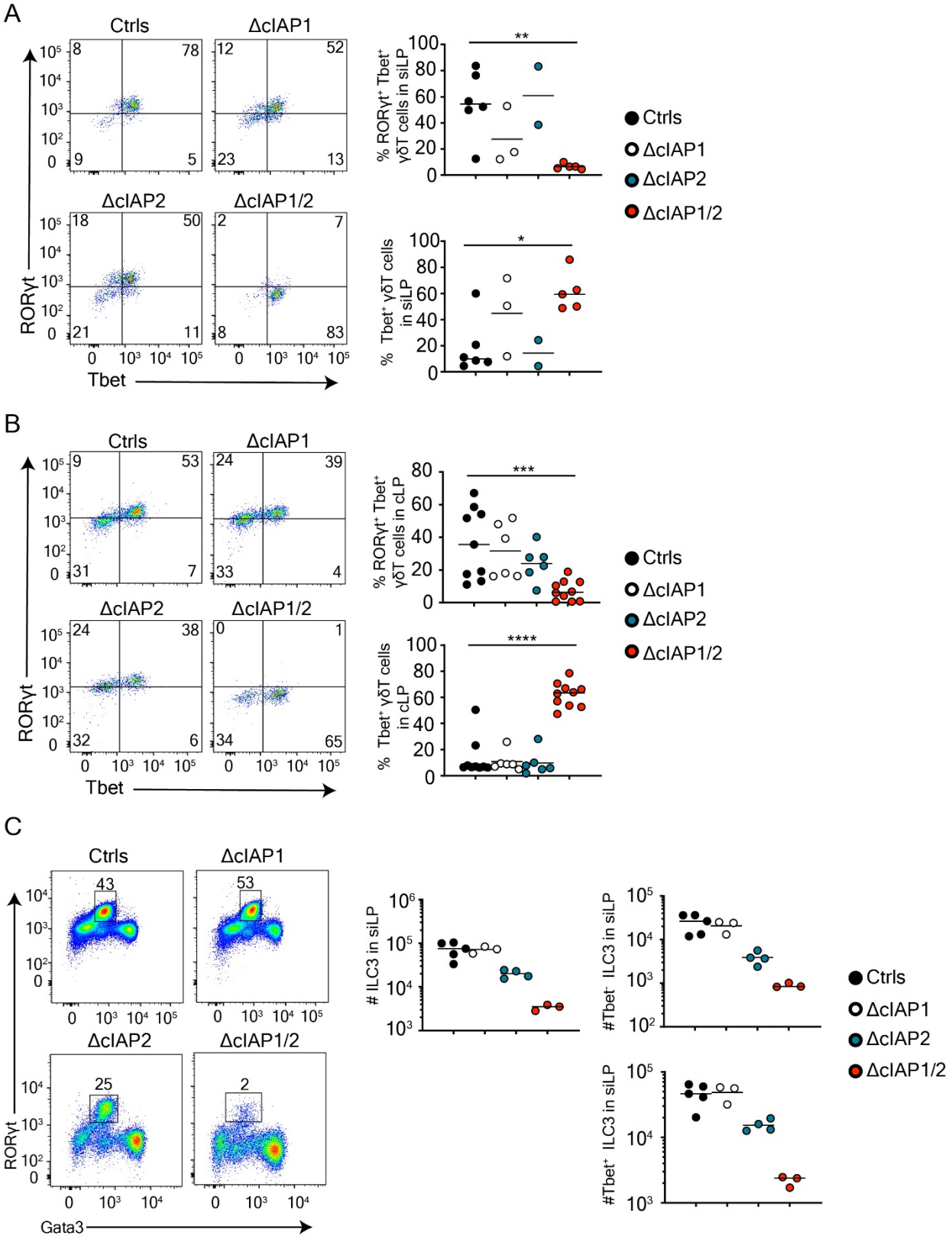
Ctrls	RORγt Cre⁻ cIAP1f/f
ΔcIAP1	RORγt Cre⁺ cIAP1f/f
ΔcIAP2	RORγt Cre⁻ cIAP1f/f cIAP2-/-
ΔcIAP1/2	RORγt Cre⁺ cIAP1f/f cIAP2-/-

483

Figure 1. cIAP1 and cIAP2 are required for the homeostasis of γδT17 cells in pLNs and skin.

(A) Mice strains used herein were generated by crossing RORγt^{CRE} strain with cIAP1$^{f/f}$ or cIAP1$^{f/f}$cIAP2$^{-/-}$ strains; the resulting 4 strains were as follows RORγt^{CRE} cIAP1$^{f/f}$ (Cre^{-}: ctrls, Cre^{+}: ΔcIAP1) and RORγt^{CRE} cIAP1$^{f/f}$cIAP2$^{-/-}$ (Cre^{-}: ΔcIAP2, Cre^{+}: ΔcIAP1/2). (B) Flow cytometric analysis plots (left) and quantification (right) of TCRγδ^{+} populations in the pLNs of all 4 strains. γδT17 cells were identified as CD44^{hi} CD27^{-} TCR γδ^{+}. (C) Flow cytometric analysis plots (left) and quantification (right) of TCRγδ^{+} and TCRβ^{+} in the skin of all 4 strains. In the skin, γδT17 cells were identified as CD45^{+} Vγ5^{-} CD3^{+} TCRγδ^{+}. In graphs, each symbol represents a mouse and line the mean. **$p < 0.01$, ****$p < 0.0001$ using Mann-Whitney test.

496 **Figure 2**



497

498 **Figure 2. cIAP1 and cIAP2 are required for the homeostasis of intestinal RORγt$^+$ Tbet$^+$ γδ**
499 **T and ILC3 cells.**
500 Flow cytometric analysis plots (left) and quantification (right) of TCRγδ$^+$ populations in the siLP
501 (A) or cLP(B) of all 4 strains. γδT cell populations were identified as CD45$^+$ CD3$^+$ TCRβ$^-$
502 TCRγδ$^+$. (C) Flow cytometric analysis plots of (left) and quantification (right) of ILC3 cells in
503 the siLP of all 4 strains. ILC cells were identified as CD45^{int} CD3$^-$ CD127$^+$ TCRγδ$^-$ TCRβ$^-$ cells.
504 In graphs, each symbol represents a mouse and line the mean. *$p < 0.05$, **$p < 0.01$, ***$p <$
505 0.001, ****$p < 0.0001$ using Mann-Whitney test.
506

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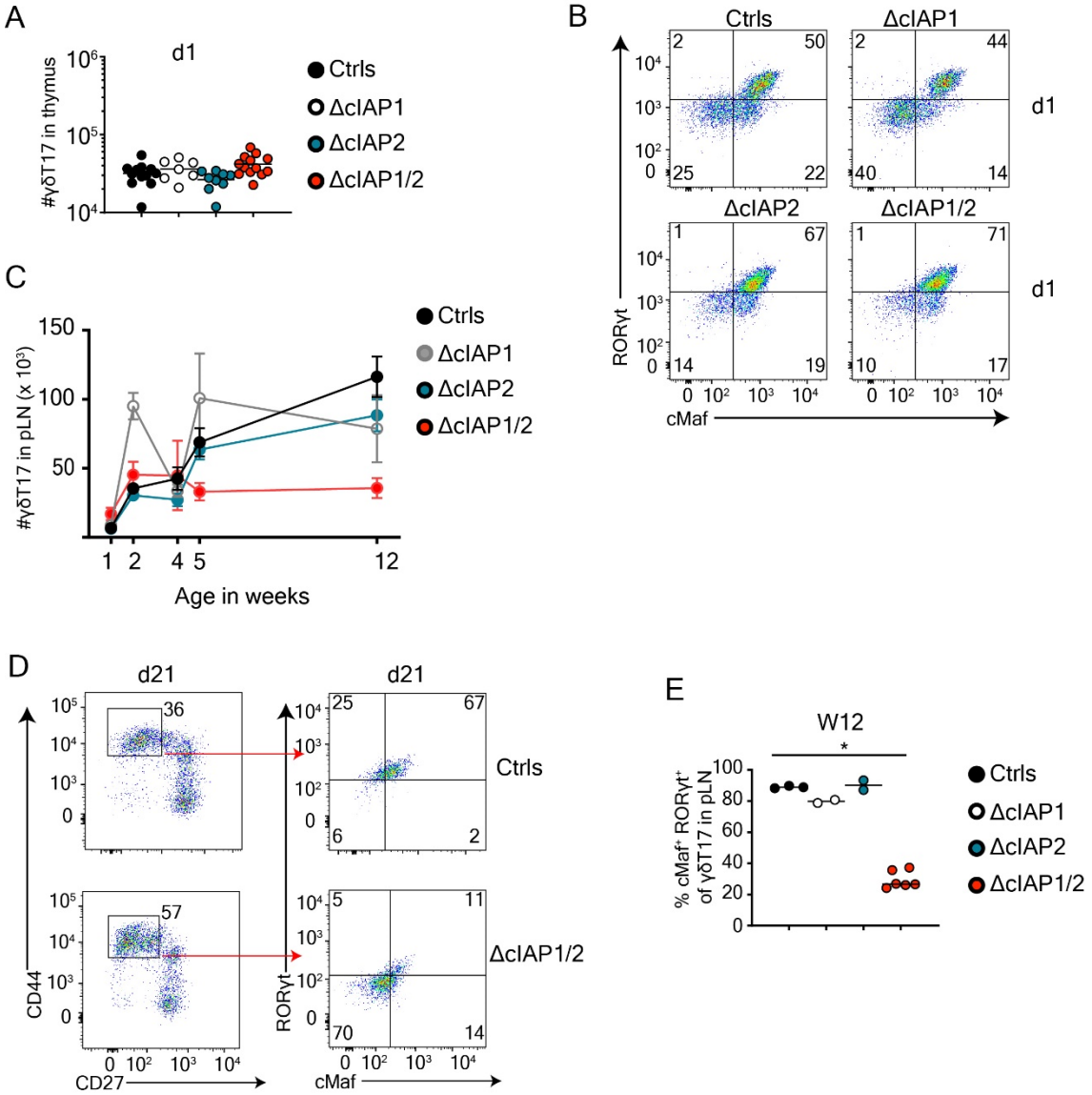
508

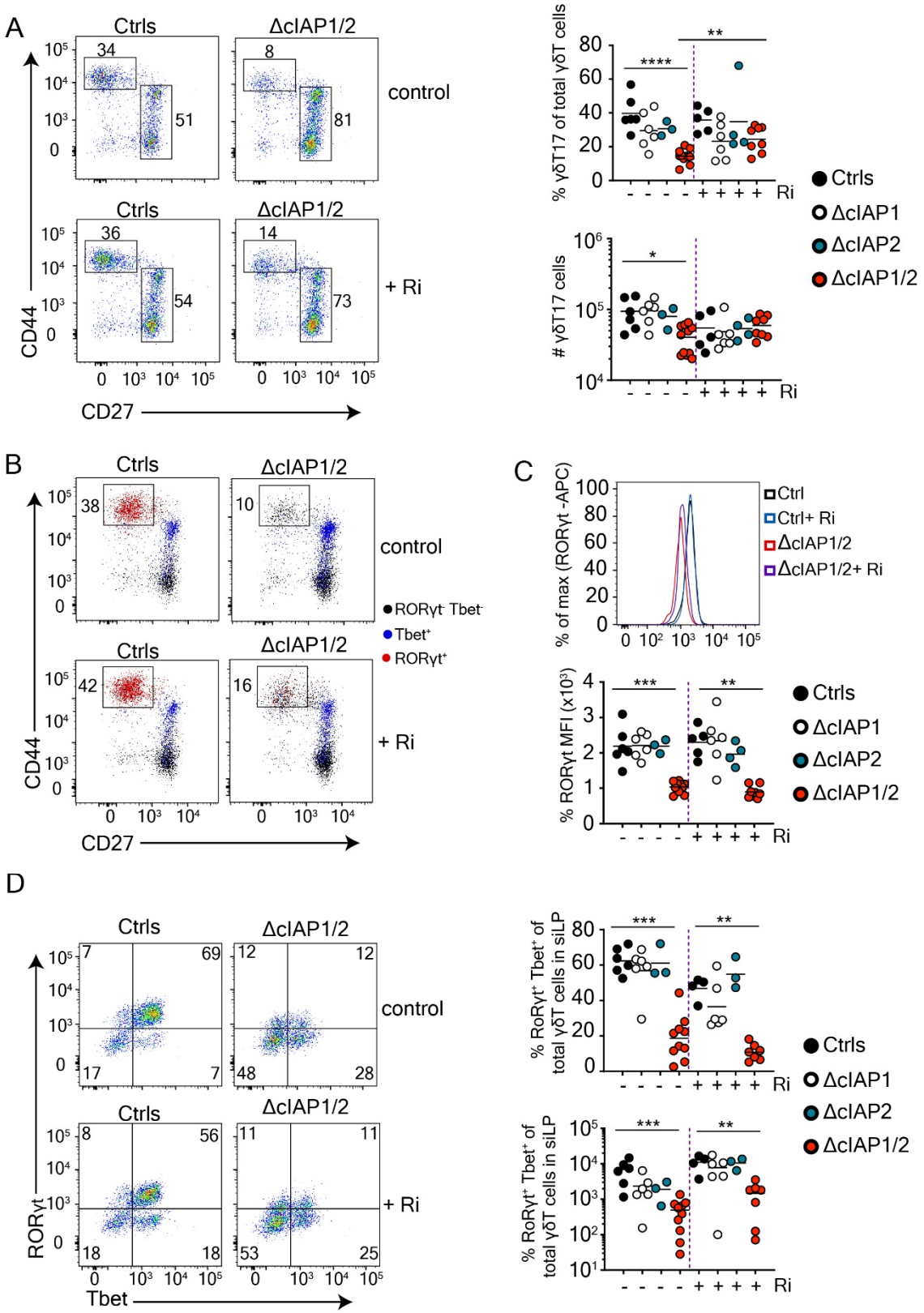
509 **Figure 3**



510

511 **Figure 3. cIAP1 and cIAP2 are not required for thymic development of γδT17 cells but are**
512 **required for cMaf and RORγt expression during neonatal and adult life.**
513 (A) Numbers of γδT17 cells in the day1 (d1) thymi of all 4 strains (gated as in Fig1). (B) Flow
514 cytometric analysis of RORγt and cMaf expression in γδ T cells from d1 thymi of all 4 strains.
515 (C) Numbers of γδT17 cells in the pLNs at the indicated timepoints. (D) analysis of γδ T cell
516 populations from d21 pLNs of the indicated strains, while arrows show RORγt and cMaf
517 expression within γδT17 cells compartment. (E) frequency of RORγt and cMaf expression in
518 γδT17 in the pLNs of 12-weeks old from all strains. In graphs each symbol represents a mouse
519 and line the mean. *p < 0.05 using Mann-Whitney test.
520
521

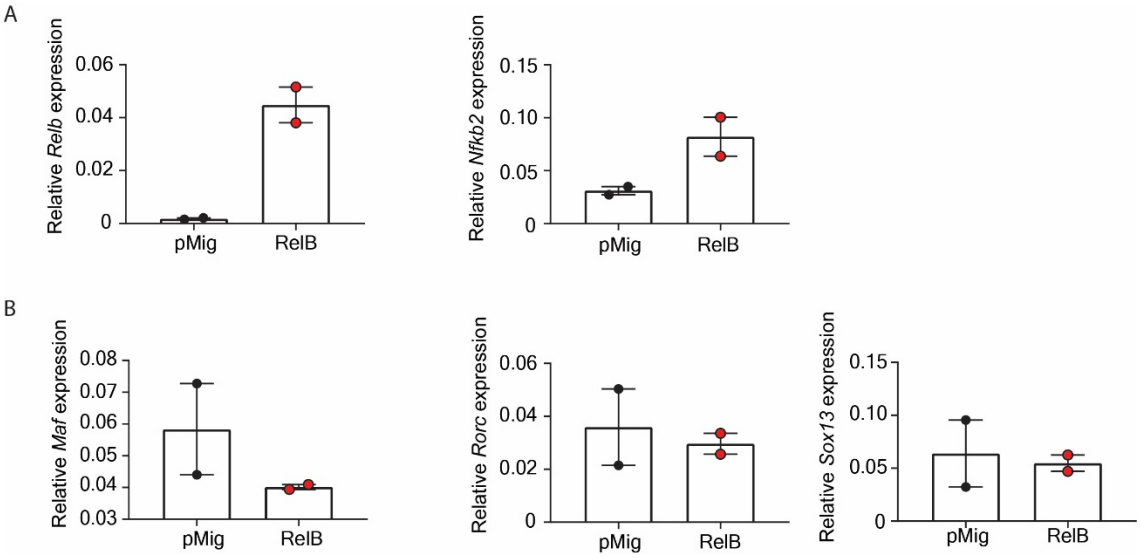
522 **Figure 4**



523

524 **Figure 4. Inhibition of RIPK1 partially rescues the reduction in γδT17 in ΔcIAP1/2 mice**
525 **but not the downregulation of RORγt**
526 (A) Flow cytometric analysis plots (left) and quantification (right) of TCRγδ^{+} populations in the
527 pLNs of indicated strains that received regular chow (control) or chow that contained RIPK1
528 inhibitor (Ri). γδT17 cells were identified by surface phenotyping as CD44^{hi} CD27^{-} TCRγδ^{+}. (B)
529 Flow cytometric analysis plots showing TCRγδ^{+} populations in the pLNs of littermate controls
530 and ΔcIAP1/2 that received control chow or Ri chow; overlaid is RORγt^{+} (red), Tbet^{+} (blue) or
531 RORγt^{-} Tbet^{-} (black) cells within the γδT cell compartment. (C) Histogram (upper) and
532 quantification (lower) of the mean fluorescence intensity (MFI) of RORγt in CD44^{hi} CD27^{-}
533 TCRγδ^{+} in the pLNs of the indicated strains that received control or Ri chow. (D) Flow
534 cytometric analysis plots (left) and quantification (right) of TCRγδ^{+} populations in the siLP of
535 the indicated strains that received control or Ri chow. In graphs each symbol represents a mouse
536 and line the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ using Mann-Whitney
537 test.

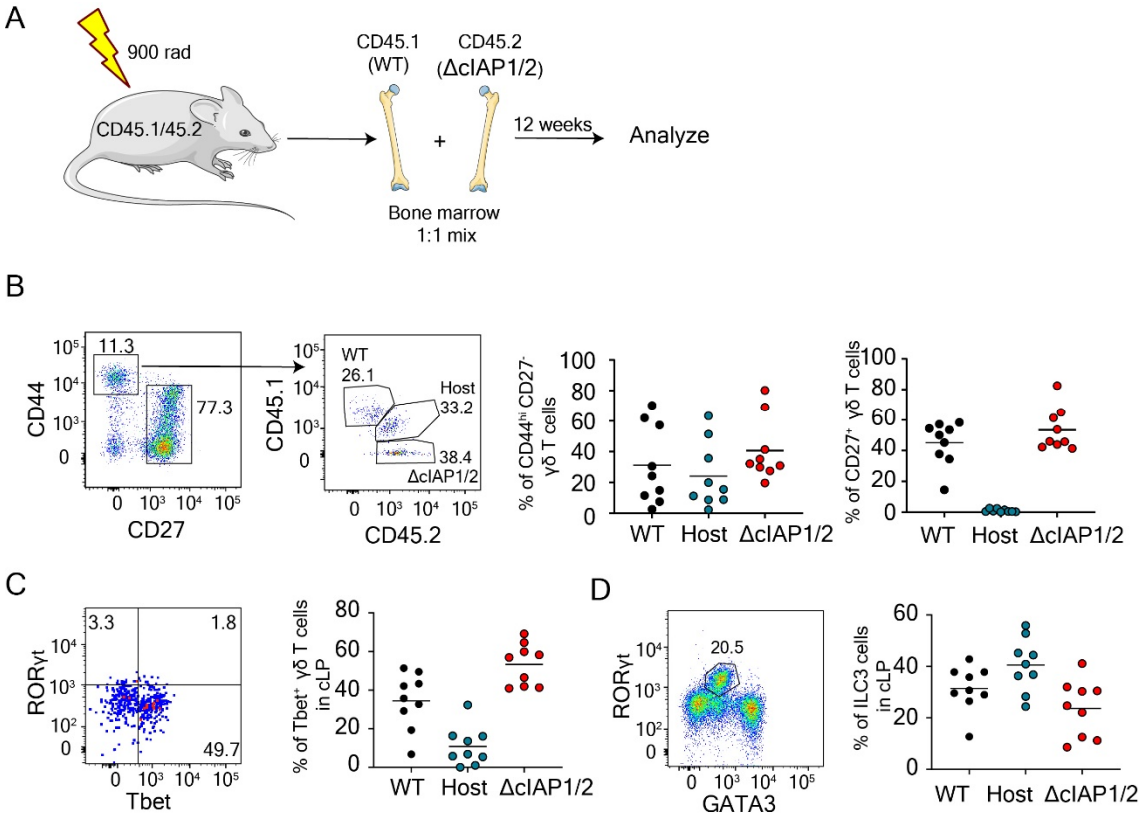
538 **Figure 5**



539

540 **Figure 5. Activation of the non-canonical NF-κB pathway downregulates the expression of**
541 ***Maf* in γδT17 cells.**
542 (A-B) RT-qPCR analysis of the indicated genes in γδT17 cells that were infected with empty
543 retrovirus (pMIG) or retrovirus expressing RelB. only GFP^+ cells (retrovirus infected cells) were
544 FACS sorted and analyzed. In graphs each symbol represents an independent experiment.

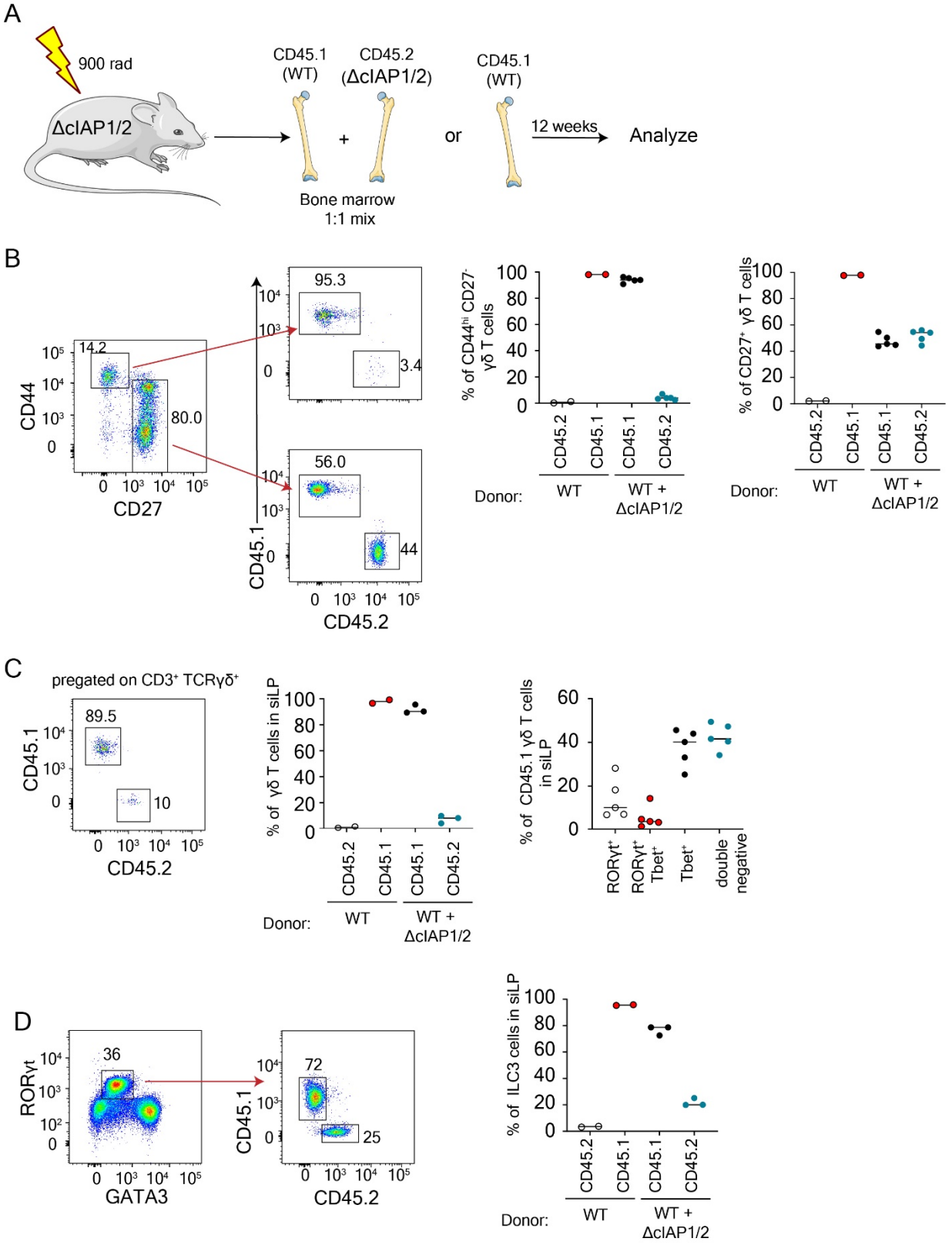
545 **Figure 6**



546

547 **Figure 6. Cell extrinsic signals mediate the loss of cIAP1 and cIAP2 double deficient γδT17**
548 **and ILC3 cells in ΔcIAP1/2 mice.**
549 (A) schematic representation of the experimental design whereby sub-lethally irradiated wildtype
550 hosts (CD45.1/45.2) were reconstituted with 1:1 mixture of WT (CD45.1) and ΔcIAP1/2
551 (CD45.2) bone marrow. (B-C) Flow cytometry analysis of γδT cells in the pLNs and cLP of host
552 mice after 12 weeks of reconstitution and indicating the frequency of WT, host, or ΔcIAP1/2
553 γδT17 and CD27^{+} γδ T cells. Graphs show frequency of WT, host, or ΔcIAP1/2 in γδ T cell
554 populations in pLN of host mice amalgamated from 2 independent experiments. (D) Flow
555 cytometry analysis of ILC3 cells in the cLP of host mice. Graph show frequency of WT, host, or
556 ΔcIAP1/2 of all ILC3 cells in cLP of host mice amalgamated from 2 independent experiments. In
557 graphs each symbol represents a mouse and line the mean.

558 **Figure 7**

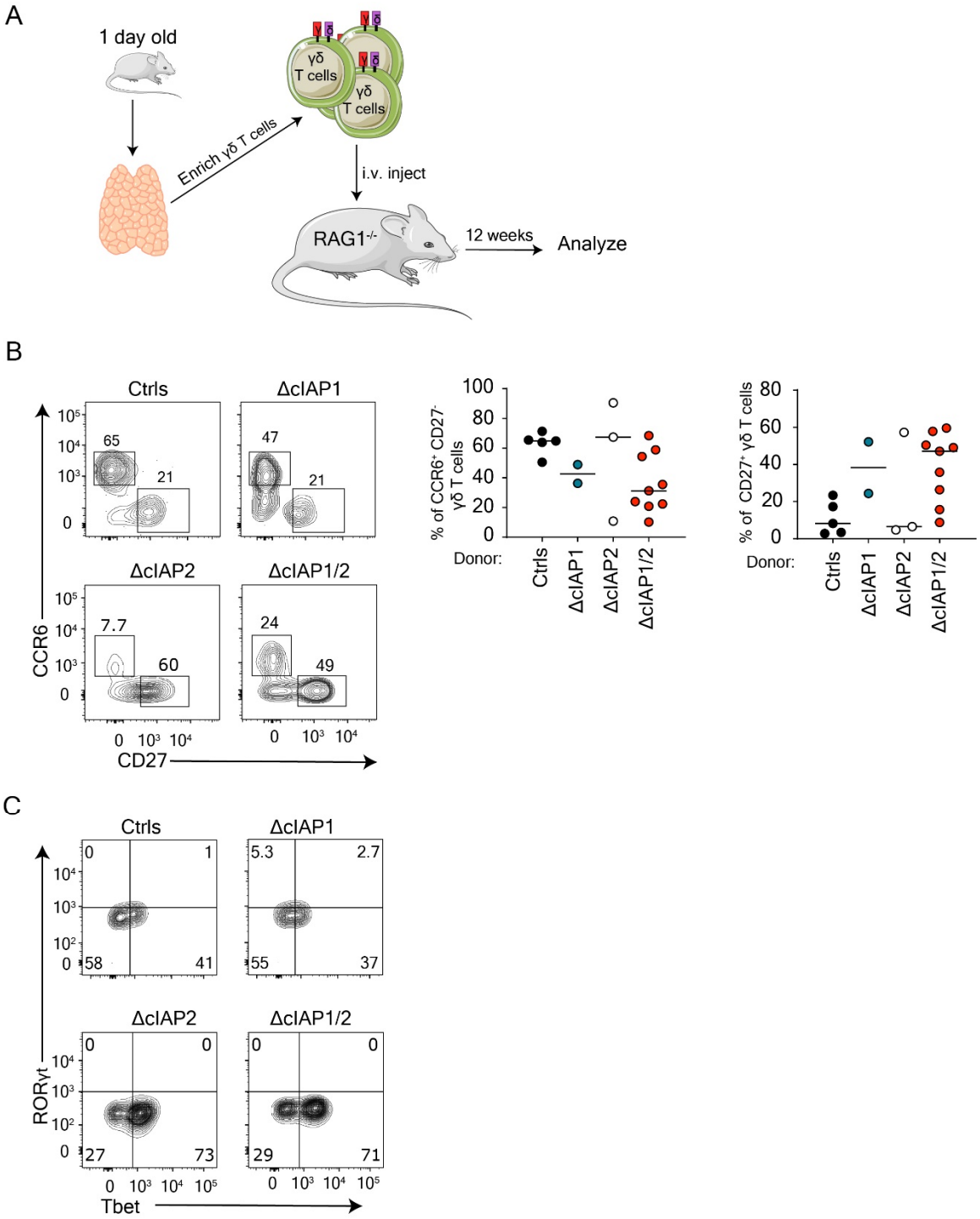


559

560 **Figure 7. Reconstitution of ΔcIAP1/2 mice with wildtype hematopoietic system rescues the**
561 **loss of ILC3 cells but not γδT17 cells.**
562 (A) schematic representation of the experimental design whereby sub-lethally irradiated
563 ΔcIAP1/2 (CD45.2) were reconstituted with either WT (CD45.1) or 1:1 mixture of WT (CD45.1)
564 and ΔcIAP1/2 (CD45.2) bone marrow. Flow cytometry analysis of γδT cells in the pLNs (B) and
565 siLP (C) of host mice after 12 weeks of reconstitution and indicating the frequency of WT or
566 ΔcIAP1/2 γδT17 and $CD27^{+}$ γδT cells. Graphs show frequency of WT or ΔcIAP1/2 in γδ T cell
567 populations in pLN and siLP. (D) Flow cytometry analysis of ILC3 cells in the cLP of host mice.
568 Graph show frequency of WT or ΔcIAP1/2 of all ILC3 cells in siLP. In graphs each symbol
569 represents a mouse and line the mean.

570

571 **Figure 8**



572

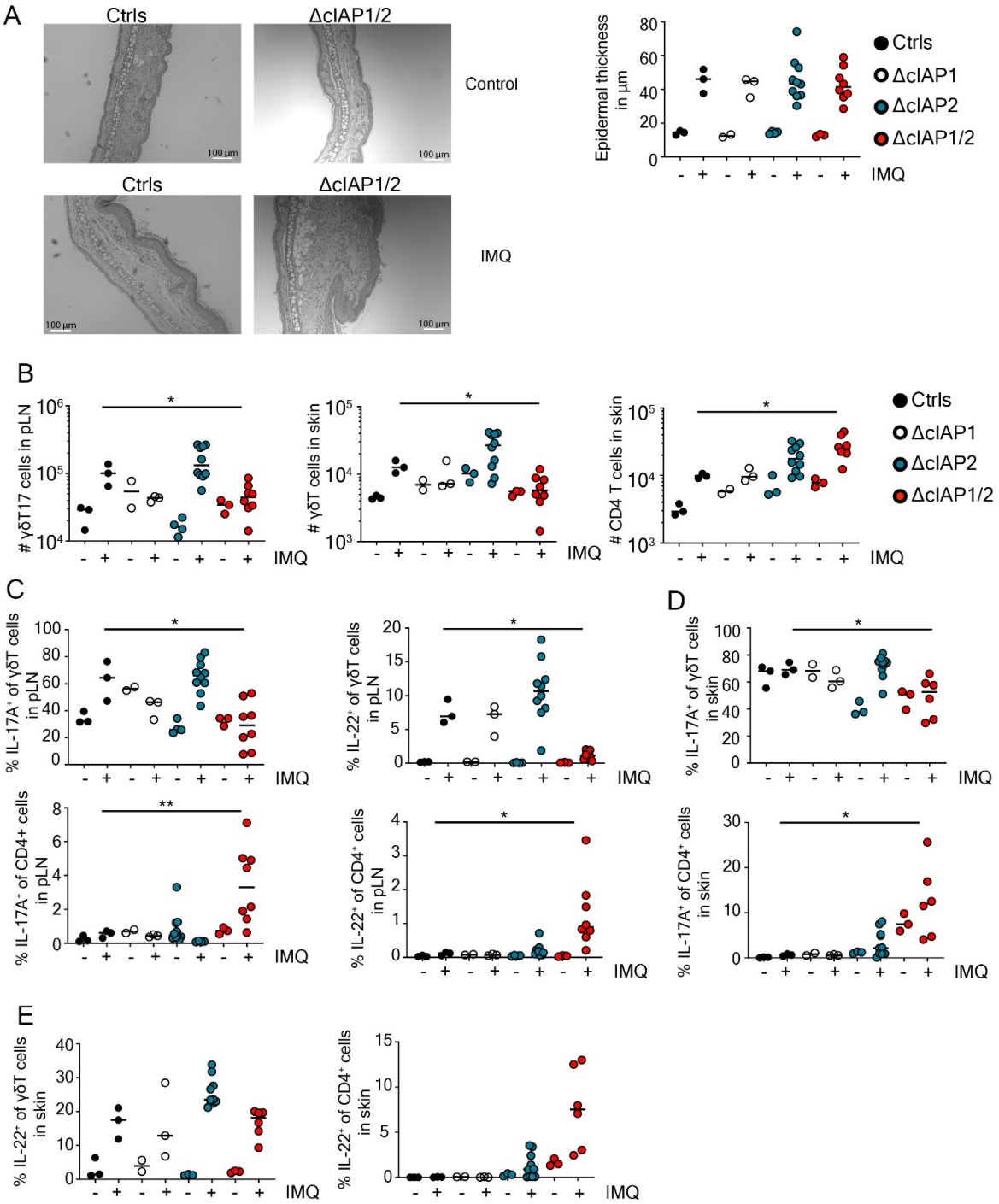
573 **Figure 8. Neonatal γδT17 lacking cIAP1 and cIAP2 can reconstitute RAG$^{-/-}$ hosts.**
574 (A) schematic representation of the experimental design whereby γδ T cells were enriched from
575 the thymi from one-day old mice of all 4 strains and i.v. injected into RAG$^{-/-}$ hosts. (B) Flow
576 cytometry analysis of γδT cells in the pLNs of host RAG$^{-/-}$ mice at 12 weeks after transfer of the

577 γδT cells from the indicated strain. Graphs show frequency of recovered γδT17 or $CD27^{+}$ γδT
578 cells of total γδT cells. In graphs each symbol represents a donor mouse and line the median.
579 Data is amalgamated from 3 independent experiments. (C) Flow cytometry analysis of γδT cells
580 in cLP of host $RAG^{-/-}$ mice at 12 weeks after transfer of the γδT cells from the indicated strain.

581

582

583 **Figure 9**



584

585 **Figure 9. cIAP1 and 2 double deficient γδT17 cells are functionally defective and do not**
586 **respond to IMQ-induced psoriasis.**
587 (A) Representative light microscopy images (right) indicating ear skin sections stained for H&E
588 before and after IMQ-induced psoriasis in the indicated strains (left) Quantification of epidermal

589 thickening from H&E stained sections. (B) Numbers of γδT17 and $CD4^+$ $TCR\beta^+$ cells in the pLN
590 or skin of all strains of IMQ treated or control mice. In the pLN, γδT17 were identified as $CD44^{hi}$
591 $CD27^-$ $TCR\gamma\delta^+$, while in the skin, they were identified as $CD45^+$ $V\gamma5^-$ $CD3^+$ $TCR\gamma\delta^+$.
592 Quantification of IL-17A (C, D) or IL-22 (C, E) production by γδ T and CD4 T cells in the pLN
593 (C) or skin (E) in control or IMQ-treated mice of all strains. In graphs, each symbol represents a
594 mouse and line the mean. Data is pool of 3 experiments. *$p < 0.05$, **$p < 0.01$using Mann-
595 Whitney test.
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