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

John Rizk[1], Rasmus Agerholm[1], Darshana Kadekar[1,2], Monica Torrellas Viñals [1] and Vasileios Bekiaris*[1]

[1]Department of Health Technology, Technical University of Denmark, Kemitorvet, Bldg 202, 2800 Kgs Lyngby, Denmark.

[2]current address: Department of Bioscience, Aarhus University. Ny Munkegade 116, 8000 Aarhus C, Denmark.

*Corresponding author: Vasileios Bekiaris vasbek@dtu.dk

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 cells (ILC3) 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 cells during neonatal and adult life. Single deficiency of either cIAP1 or 2 did not affect the homeostasis or transcription factor profile of γδT17 cells. 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. Moreover, bone marrow reconstitutions and transfer of neonatal γδ T cells to RAG1$^{-/-}$ hosts

showed that both intrinsic and extrinsic factors contribute to the loss of γδT17 cells in cIAP1/2 double deficient mice, while only extrinsic signals were responsible for the decrease of ILC3 cells. 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

46 Sox13, cMAF followed by the lineage defining expression of RORγt. Conversely, the IFNγ
47 producing γδT cells are characterized by the transcription factors Tbet and Tcf1 (Sagar *et al*,
48 2018; Zuberbuehler *et al*, 2019). Further, canonical and non-canonical NF-κB pathway signaling
49 in the thymic microenvironment are essential for the development of γδT17. For instance, the
50 non-canonical NF-κB inducing kinase (NIK) signaling in the thymic epithelia is essential for the
51 development of γδT17 (Mair *et al*, 2015). Moreover, γδT17 development requires lymphotoxin β
52 receptor (LTβR) signaling, through the non-canonical NF-κB transcription factor RelB.
53 Alternatively, the canonical NF-κB transcription factor RelA is required for the optimal
54 expression of LTβR ligands such as LIGHT and lymphotoxin(LT)α in thymocytes, which are
55 required for γδT17 development (Powolny-Budnicka *et al*, 2011; Silva-Santos *et al*, 2005).
56 However, the role of NF-κB signaling in the homeostasis of adult γδT17 is not well described.

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

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

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

92 **Results**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

275 **Discussion**

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

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

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

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

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

319 **References**

320 Annibaldi A & Meier P (2018) Checkpoints in TNF-Induced Cell Death: Implications in
321 Inflammation and Cancer. *Trends Mol. Med.* **24:** 49–65

322 Bai L, Smith DC & Wang S (2014) Small-molecule SMAC mimetics as new cancer therapeutics.
323 *Pharmacol. Ther.* **144:** 82–95

324 Barros-Martins J, Schmolka N, Fontinha D, Pires de Miranda M, Simas JP, Brok I, Ferreira C,
325 Veldhoen M, Silva-Santos B & Serre K (2016) Effector γδ T Cell Differentiation Relies on
326 Master but Not Auxiliary Th Cell Transcription Factors. *J. Immunol.* **196:** 3642–3652

327 Bertrand MJM, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW,
328 Jaquith JB, Morris SJ & Barker PA (2008) cIAP1 and cIAP2 Facilitate Cancer Cell Survival
329 by Functioning as E3 Ligases that Promote RIP1 Ubiquitination. *Mol. Cell* **30:** 689–700

330 Chien Y-H, Meyer C & Bonneville M (2014) γδ T Cells: First Line of Defense and Beyond.
331 *Annu. Rev. Immunol.* **32:** 121–155

332 Ebertl G, Litman DR, Eberl G & Litman DR (2004) Thymic origin of intestinal αβ T cells
333 revealed by fate mapping of RORγt+ cells. *Science (80-.).* **305:** 248–251

334 van der Fits L, Mourits S, Voerman JSA, Kant M, Boon L, Laman JD, Cornelissen F, Mus A-M,
335 Florencia E, Prens EP & Lubberts E (2009) Imiquimod-Induced Psoriasis-Like Skin
336 Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. *J. Immunol.* **182:** 5836–5845

337 Gyrd-Hansen M & Meier P (2010) IAPs: from caspase inhibitors to modulators of NF-kappaB,
338 inflammation and cancer. *Nat. Rev. Cancer* **10:** 561–74

339 Haas JD, Ravens S, Düber S, Sandrock I, Oberdörfer L, Kashani E, Chennupati V, Föhse L,
340 Naumann R, Weiss S, Krueger A, Förster R & Prinz I (2012) Development of Interleukin-
341 17-Producing γδ T Cells Is Restricted to a Functional Embryonic Wave. *Immunity* **37:** 48–
342 59

343 Heilig JS & Tonegawa S (1986) Diversity of murine gamma genes and expression in fetal and
344 adult T lymphocytes. *Nature* **322:** 836–840

345 Kadekar D, Agerholm R, Rizk J, Neubauer H, Suske T, Maurer B, Viñals MT, Comelli E, Taibi
346 A, Moriggl R & Bekiaris V (2019) The neonatal microenvironment programs conventional
347 and intestinal Tbet+ γδT17 cells through the transcription factor STAT5. *bioRxiv***:** 658542

348 Klose CSN, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, D'Hargues Y, Göppert N, Croxford
349 AL, Waisman A, Tanriver Y & Diefenbach A (2013) A T-bet gradient controls the fate and
350 function of CCR6-RORγt + innate lymphoid cells. *Nature* **494:** 261–265

351 Mahoney DJ, Cheung HH, Mrad RL, Plenchette S, Simard C, Enwere E, Arora V, Mak TW,
352 Lacasse EC, Waring J & Korneluk RG (2008) Both cIAP1 and cIAP2 regulate TNF -
353 mediated NF- B activation. *Proc. Natl. Acad. Sci.* **105:** 11778–11783

354 Mair F, Joller S, Hoeppli R, Onder L, Hahn M, Ludewig B, Waisman A & Becher B (2015) The
355 NFκB-inducing kinase is essential for the developmental programming of skin- resident and
356 IL-17-producing Γδ Tδ cells. *Elife* **4:**

357 Melo-Gonzalez F & Hepworth MR (2017) Functional and phenotypic heterogeneity of group 3
358 innate lymphoid cells. *Immunology* **150:** 265–275

359 Moulin M, Anderton H, Voss AK, Thomas T, Wong WWL, Bankovacki A, Feltham R, Chau D,
360 Cook WD, Silke J & Vaux DL (2012) IAPs limit activation of RIP kinases by TNF receptor
361 1 during development. *EMBO J.* **31:** 1679–1691

362 Papotto PH, Ribot JC & Silva-Santos B (2017) IL-17 + γδ T cells as kick-starters of
363 inflammation. *Nat. Immunol.* **18:** 604–611

364 Powolny-Budnicka I, Riemann M, Tänzer S, Schmid RM, Hehlgans T & Weih F (2011) RelA
365 and RelB transcription factors in distinct thymocyte populations control lymphotoxin-
366 dependent interleukin-17 production in γδ T cells. *Immunity* **34:** 364–374

367 Prinz I, Silva-Santos B & Pennington DJ (2013) Functional development of γδ T cells. *Eur. J.*
368 *Immunol.* **43:** 1988–1994

369 Rizk J, Kaplinsky J, Agerholm R, Kadekar D, Ivars F, Agace WW, Wei-Lynn Wong W, Szucs
370 MJ, Myers SA, Carr SA, Waisman A & Bekiaris V (2019) SMAC mimetics promote NIK-
371 dependent inhibition of CD4+ TH17 cell differentiation. *Sci. Signal.* **12:**

372 Sagar, Pokrovskii M, Herman JS, Naik S, Sock E, Lausch U, Wegner M, Tanriver Y, Littman
373 DR & Grün D (2018) Deciphering the Regulatory Landscape of γδ T Cell Development by
374 Single-Cell RNA-Sequencing. *bioRxiv***:** 478529

375 Sandrock I, Reinhardt A, Ravens S, Binz C, Wilharm A, Martins J, Oberdörfer L, Tan L,
376 Lienenklaus S, Zhang B, Naumann R, Zhuang Y, Krueger A, Förster R & Prinz I (2018)
377 Genetic models reveal origin, persistence and non-redundant functions of IL-17–producing
378 γδ T cells. *J. Exp. Med.* **215:** 3006–3018

379 Sawa S, Cherrier M, Lochner M, Satoh-Takayama N, Fehling HJ, Langa F, Di Santo JP & Eberl
380 G (2010) Lineage Relationship Analysis of RORγt + Innate Lymphoid Cells. *Science (80-.*
381 *).* **330:** 665–669

382 Schmolka N, Serre K, Grosso AR, Rei M, Pennington DJ, Gomes AQ & Silva-Santos B (2013)
383 Epigenetic and transcriptional signatures of stable versus plastic differentiation of
384 proinflammatory γδ T cell subsets. *Nat. Immunol.* **14:** 1093–1100

385 Silke J & Meier P (2013) Inhibitor of apoptosis(IAP)proteins-modulators of cell death and
386 inflammation. *Cold Spring Harb. Perspect. Biol.* **5:** a008730

387 Silva-Santos B, Pennington DJ & Hayday AC (2005) Lymphotoxin-mediated regulation of γδ
388 cell differentiation by αβ T cell progenitors. *Science (80-.).* **307:** 925–928

389 Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC & Mills KHG (2009) Interleukin-1
390 and IL-23 Induce Innate IL-17 Production from γδ T Cells, Amplifying Th17 Responses
391 and Autoimmunity. *Immunity* **31:** 331–341

392 Tamm I, Kornblau SM, Segall H, Krajewski S, Welsh K, Kitada S, Scudiero DA, Tudor G, Qui
393 YH, Monks A, Andreeff M & Reed JC (2000) Expression and prognostic significance of
394 IAP-family genes in human cancers and myeloid leukemias. *Clin. Cancer Res.* **6:** 1796–803

395 Vallabhapurapu S, Matsuzawa A, Zhang WZ, Tseng PH, Keats JJ, Wang H, Vignali DAA,
396 Bergsagel PL & Karin M (2008) Nonredundant and complementary functions of TRAF2
397 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-κB
398 signaling. *Nat. Immunol.* **9:** 1364–1370

399 Varfolomeev E, Blankenship JW, Wayson SM, Fedorova A V., Kayagaki N, Garg P, Zobel K,
400 Dynek JN, Elliott LO, Wallweber HJA, Flygare JA, Fairbrother WJ, Deshayes K, Dixit VM
401 & Vucic D (2007) IAP Antagonists Induce Autoubiquitination of c-IAPs, NF-κB
402 Activation, and TNFα-Dependent Apoptosis. *Cell* **131:** 669–681

403 Wu HH, Wu JY, Cheng YW, Chen CY, Lee MC, Goan YG & Lee H (2010) cIAP2 upregulated
404 by E6 oncoprotein via epidermal growth factor receptor/phosphatidylinositol 3-kinase/AKT
405 pathway confers resistance to cisplatin in human papillomavirus 16/18-infected lung cancer.
406 *Clin. Cancer Res.* **16:** 5200–5210

407 Zarnegar BJ, Wang Y, Mahoney DJ, Dempsey PW, Cheung HH, He J, Shiba T, Yang X, Yeh
408 WC, Mak TW, Korneluk RG & Cheng G (2008) Noncanonical NF-κB activation requires
409 coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and
410 TRAF3 and the kinase NIK. *Nat. Immunol.* **9:** 1371–1378

411 Zuberbuehler MK, Parker ME, Wheaton JD, Espinosa JR, Salzler HR, Park E & Ciofani M
412 (2019) The transcription factor c-Maf is essential for the commitment of IL-17-producing
413 γδ T cells. *Nat. Immunol.* **20:** 73–85

414

415

416 **Materials and methods**

417 **Mice**

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

423 **Cell culture media and buffers**

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

428 **Lymphocyte isolation from mouse organs**

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

433 **IMQ-induced psoriasis**

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

438 **Flow cytometry staining**

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

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

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

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

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

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

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

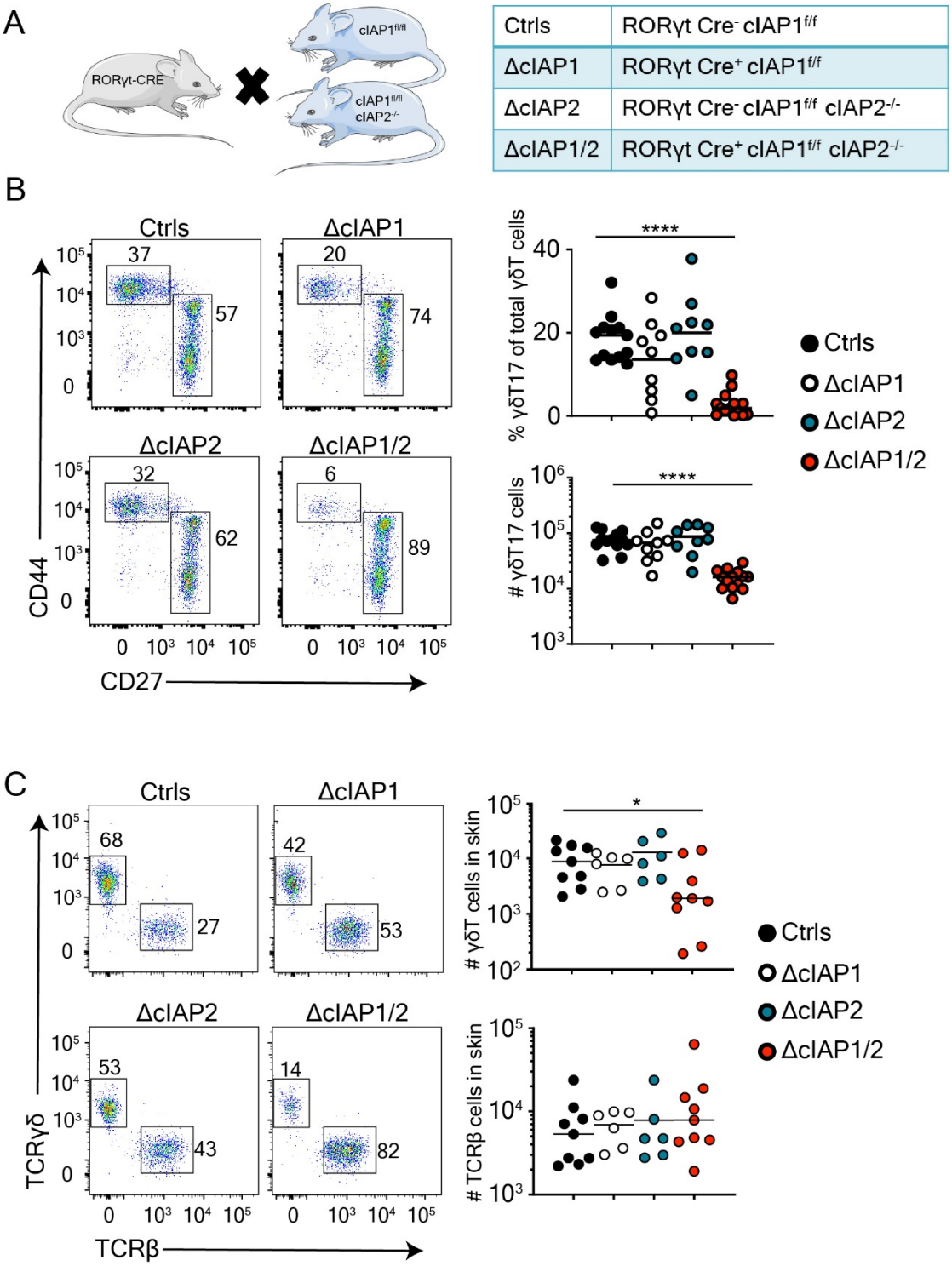
471 **Bone marrow chimeras**

472 The bone marrow cells for reconstitution were isolated by flushing the tibia and femur, which
473 were dissected from donor mice, with culture media. Total bone marrow cells were then
474 centrifugated at 400g for 5 minutes at 4ºC. The cells were then re-suspended and passed through
475 70 μm filter. Subsequently, red blood cells were then lysed using RBC lysis buffer (Biolegend)
476 and a single cell suspension of bone marrow cells was the prepared by passing the cells through
477 40 μm filter. The prepared cells were then counted and mixed as appropriate.

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

482 **Figures**

483 **Figure 1**



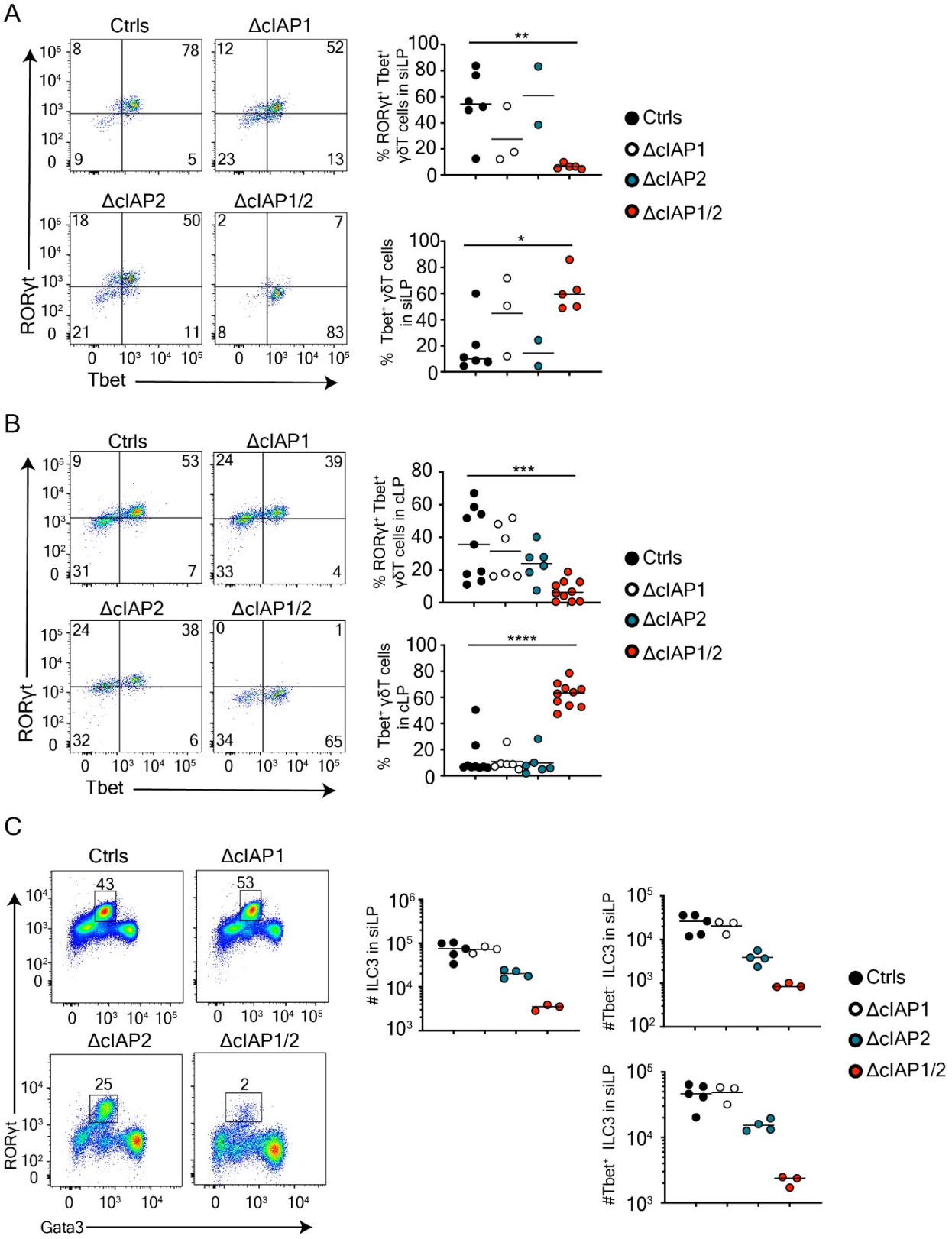
Ctrls	RORγt Cre⁻ cIAP1f/f
ΔcIAP1	RORγt Cre⁺ cIAP1f/f
ΔcIAP2	RORγt Cre⁻ cIAP1f/f cIAP2-/-
ΔcIAP1/2	RORγt Cre⁺ cIAP1f/f cIAP2-/-

484

485 **Figure 1. cIAP1 and cIAP2 are required for the homeostasis of γδT17 cells in pLNs and**
486 **skin.**
487 (A) Mice strains used herein were generated by crossing RORγt^{CRE} strain with cIAP1$^{f/f}$ or
488 cIAP1$^{f/f}$cIAP2$^{-/-}$ strains; the resulting 4 strains were as follows RORγt^{CRE} cIAP1$^{f/f}$ (Cre^{-}: ctrls,
489 Cre^{+}: ΔcIAP1) and RORγt^{CRE} cIAP1$^{f/f}$cIAP2$^{-/-}$ (Cre^{-}: ΔcIAP2, Cre^{+}: ΔcIAP1/2). (B) Flow
490 cytometric analysis plots (left) and quantification (right) of TCRγδ^{+} populations in the pLNs of
491 all 4 strains. γδT17 cells were identified as CD44^{hi} CD27^{-} TCR γδ^{+}. (C) Flow cytometric analysis
492 plots (left) and quantification (right) of TCRγδ^{+} and TCRβ^{+} in the skin of all 4 strains. In the
493 skin, γδT17 cells were identified as CD45^{+} Vγ5^{-} CD3^{+} TCRγδ^{+}. In graphs, each symbol
494 represents a mouse and line the mean. **$p < 0.01$, ****$p < 0.0001$ using Mann-Whitney test.
495

496

497 **Figure 2**



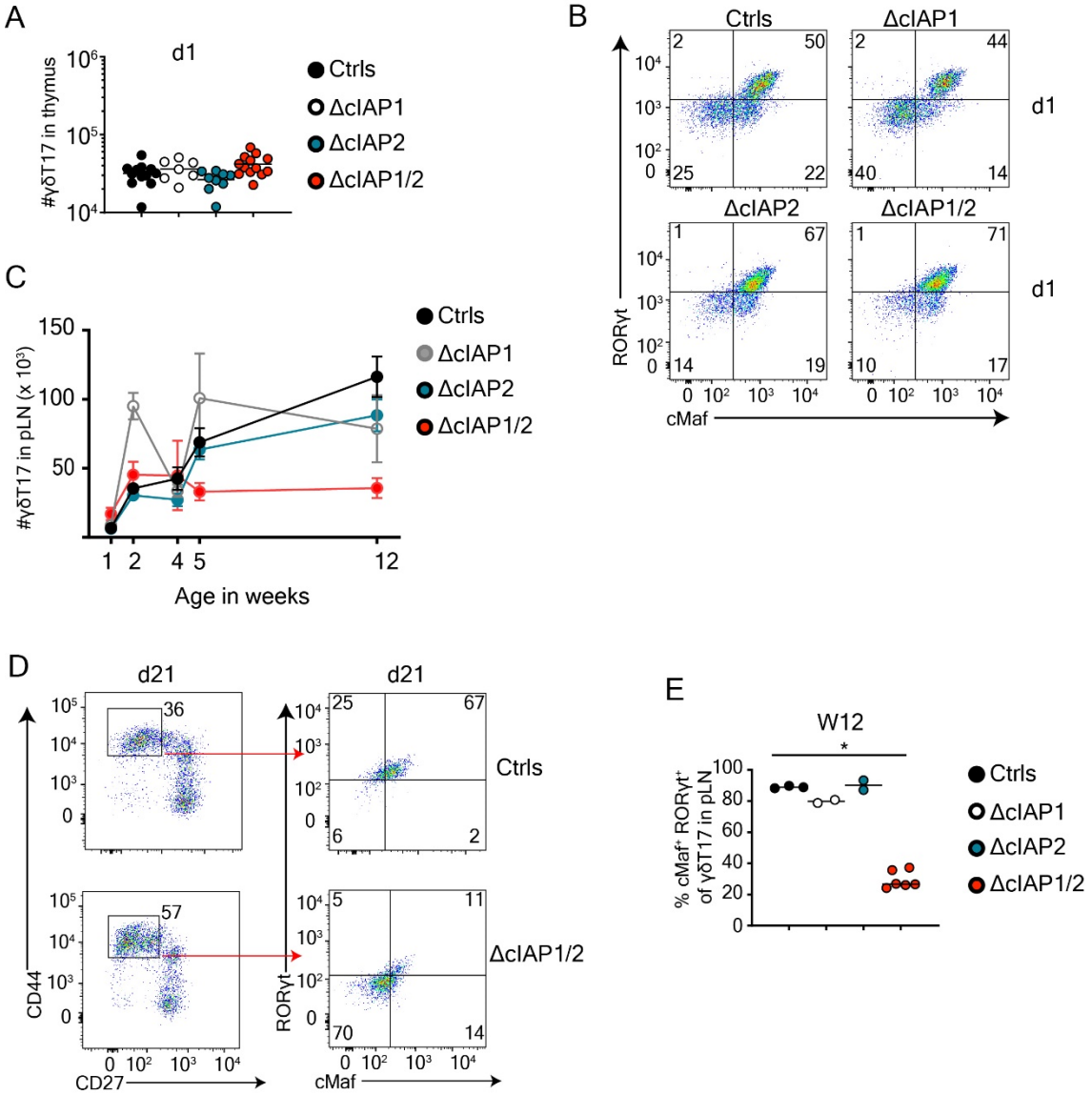
498

499 **Figure 2. cIAP1 and cIAP2 are required for the homeostasis of intestinal RORγt$^+$ Tbet$^+$ γδ**
500 **T and ILC3 cells.**
501 Flow cytometric analysis plots (left) and quantification (right) of TCRγδ$^+$ populations in the siLP
502 (A) or cLP(B) of all 4 strains. γδT cell populations were identified as CD45$^+$ CD3$^+$ TCRβ$^-$
503 TCRγδ$^+$. (C) Flow cytometric analysis plots of (left) and quantification (right) of ILC3 cells in
504 the siLP of all 4 strains. ILC cells were identified as CD45^{int} CD3$^-$ CD127$^+$ TCRγδ$^-$ TCRβ$^-$ cells.
505 In graphs, each symbol represents a mouse and line the mean. *$p < 0.05$, **$p < 0.01$, ***$p <$
506 0.001, ****$p < 0.0001$ using Mann-Whitney test.
507

508

509

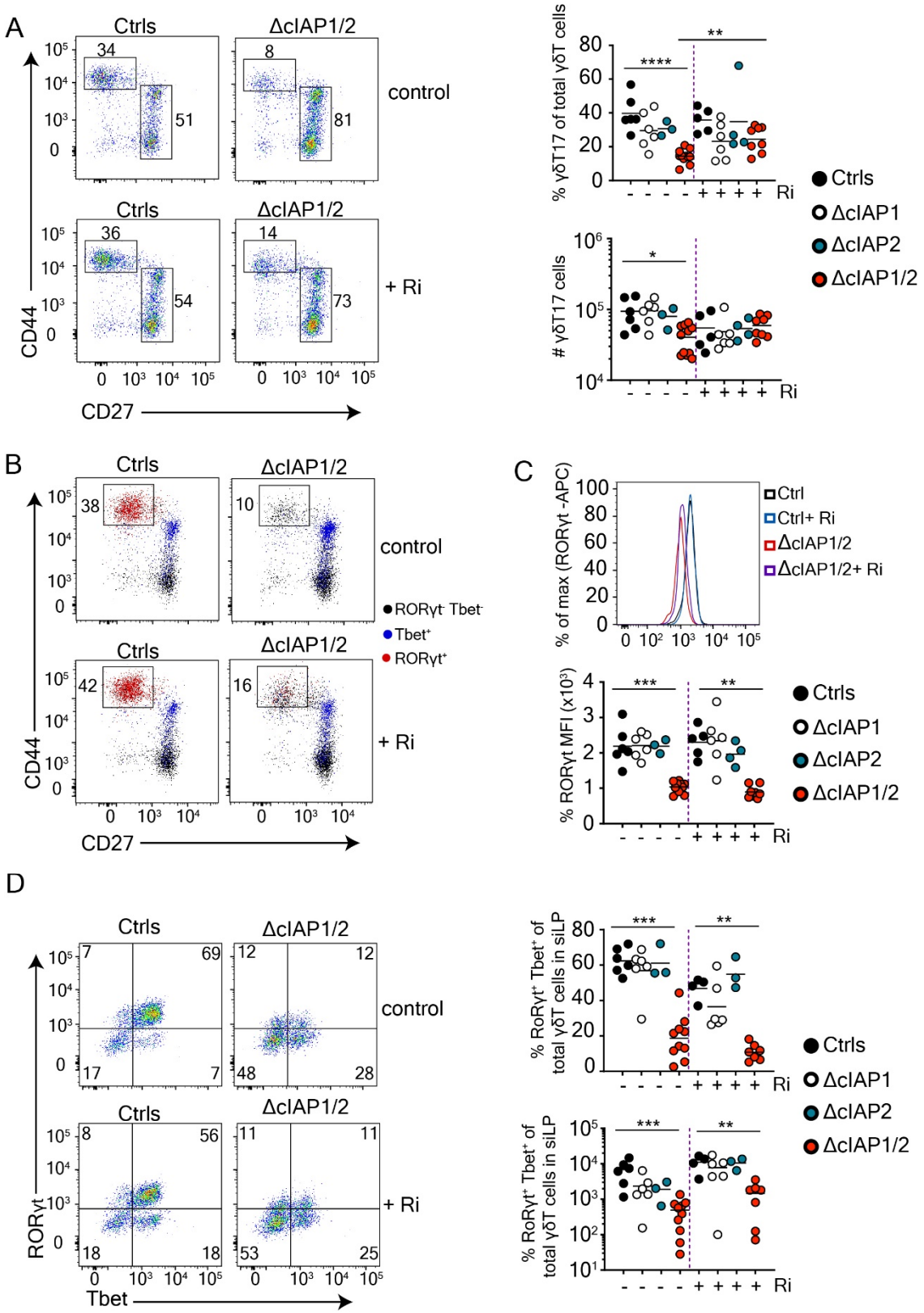
510 **Figure 3**



511

512 **Figure 3. cIAP1 and cIAP2 are not required for thymic development of γδT17 cells but are**
513 **required for cMaf and RORγt expression during neonatal and adult life.**
514 (A) Numbers of γδT17 cells in the day1 (d1) thymi of all 4 strains (gated as in Fig1). (B) Flow
515 cytometric analysis of RORγt and cMaf expression in γδ T cells from d1 thymi of all 4 strains.
516 (C) Numbers of γδT17 cells in the pLNs at the indicated timepoints. (D) analysis of γδ T cell
517 populations from d21 pLNs of the indicated strains, while arrows show RORγt and cMaf
518 expression within γδT17 cells compartment. (E) frequency of RORγt and cMaf expression in
519 γδT17 in the pLNs of 12-weeks old from all strains. In graphs each symbol represents a mouse
520 and line the mean. *$p < 0.05$ using Mann-Whitney test.
521
522

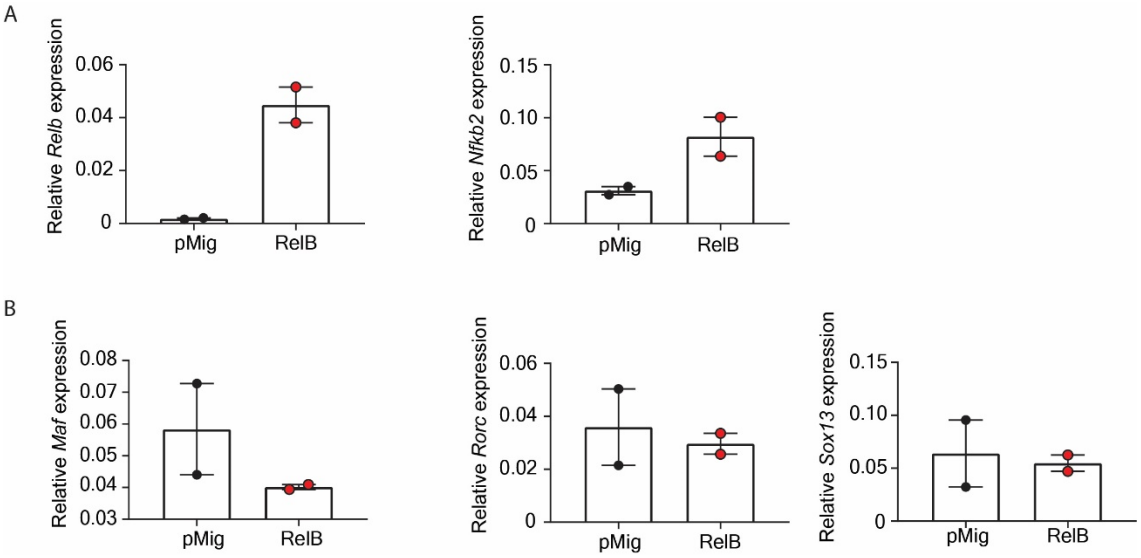
523 **Figure 4**



524

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

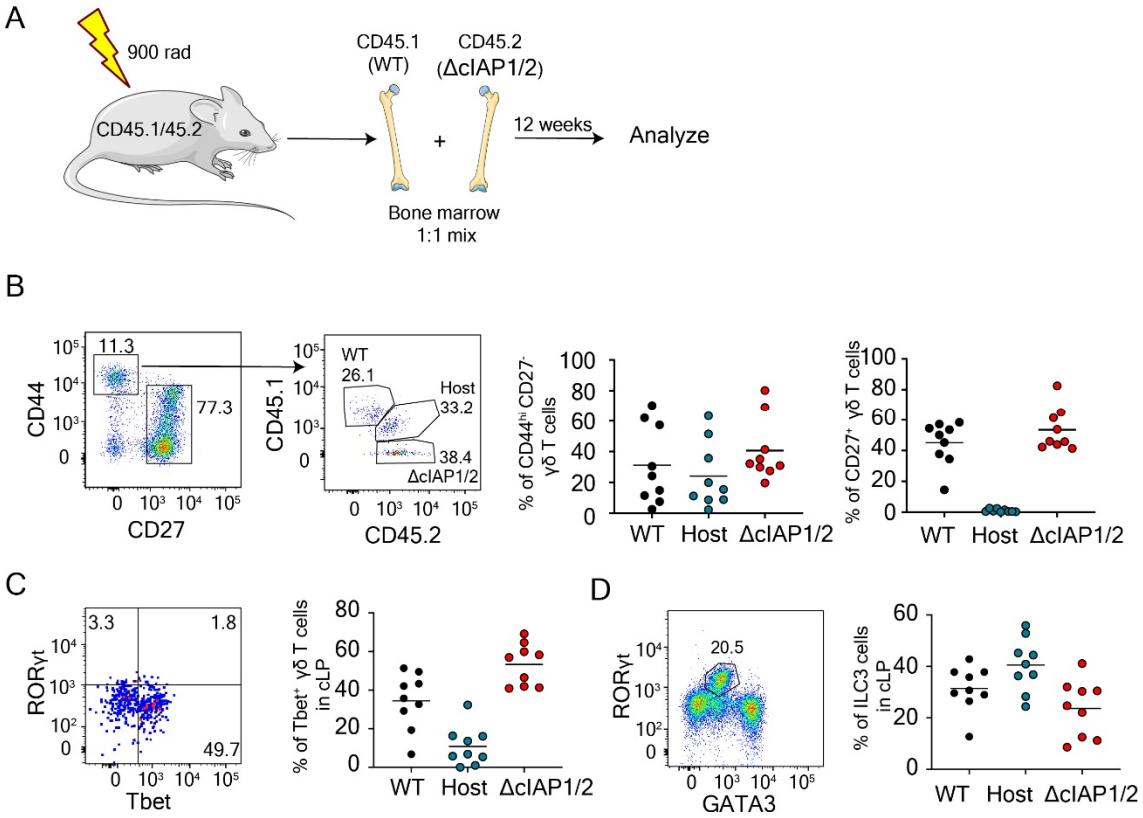
539 **Figure 5**



540

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

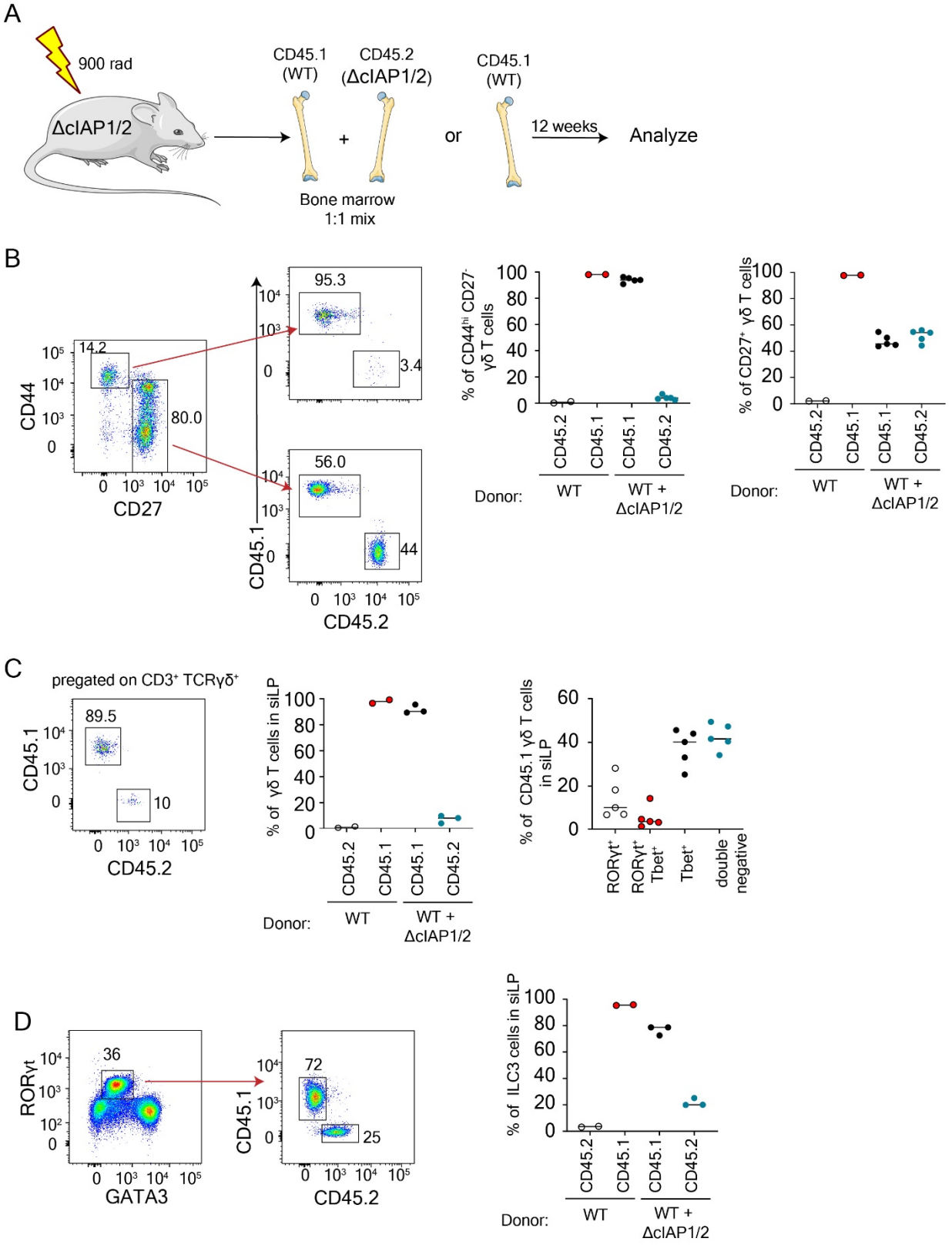
546 **Figure 6**



547

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

559 **Figure 7**

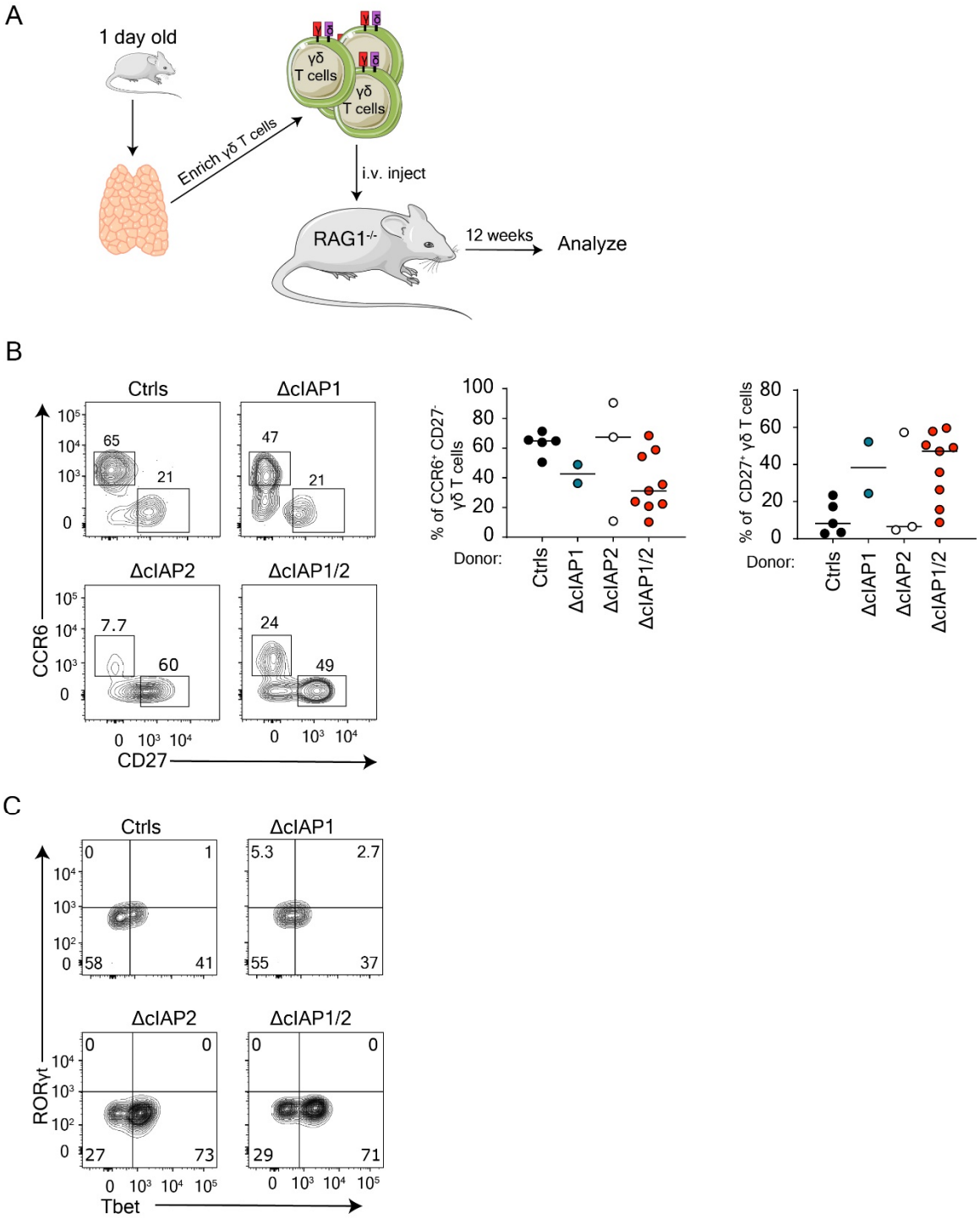


560

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

571

572 **Figure 8**



573

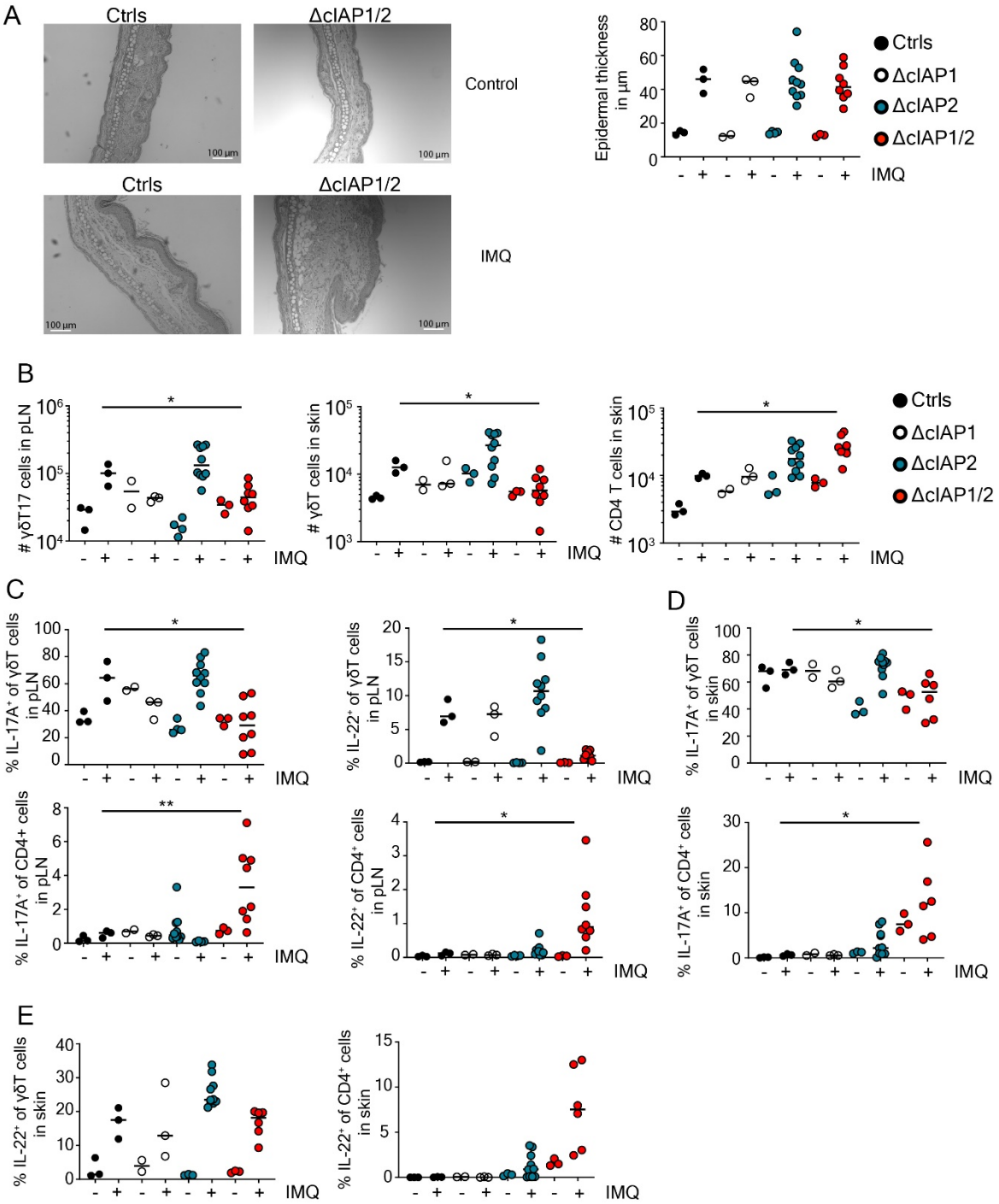
574 **Figure 8. Neonatal γδT17 lacking cIAP1 and cIAP2 can reconstitute RAG$^{-/-}$ hosts.**
575 (A) schematic representation of the experimental design whereby γδ T cells were enriched from
576 the thymi from one-day old mice of all 4 strains and i.v. injected into RAG$^{-/-}$ hosts. (B) Flow
577 cytometry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

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

582

583

584 **Figure 9**



585

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

590 thickening from H&E stained sections. (B) Numbers of γδT17 and $CD4^+$ $TCR\beta^+$ cells in the pLN
591 or skin of all strains of IMQ treated or control mice. In the pLN, γδT17 were identified as $CD44^{hi}$
592 $CD27^-$ $TCR\gamma\delta^+$, while in the skin, they were identified as $CD45^+$ $V\gamma5^-$ $CD3^+$ $TCR\gamma\delta^+$.
593 Quantification of IL-17A (C, D) or IL-22 (C, E) production by γδ T and CD4 T cells in the pLN
594 (C) or skin (E) in control or IMQ-treated mice of all strains. In graphs, each symbol represents a
595 mouse and line the mean. Data is pool of 3 experiments. *$p < 0.05$, **$p < 0.01$using Mann-
596 Whitney test.
597