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 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. 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

25 IMQ-induced psoriasis. Collectively, our data reveal a previously undescribed role for cIAP1 and
26 cIAP2 in the homeostasis of γδT17 and ILC3 cells.

27 **Introduction**

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

41 During perinatal thymic development, γδT17 cells undergo transcriptional and epigenetic
42 programming that imprints their effector phenotypes(Schmolka *et al*, 2013). Thus, thymic
43 development of γδT17 is dependent on the consecutive expression of the transcription factors
44 Sox13, cMAF followed by the lineage defining expression of RORγt. Conversely, the IFNγ
45 producing γδT cells are characterized by the transcription factors Tbet and Tcf1 (Sagar *et al*,
46 2018; Zuberbuehler *et al*, 2019). Further, canonical and non-canonical NF-κB pathway signaling
47 in the thymic microenvironment are essential for the development of γδT17. For instance, the
48 non-canonical NF-κB inducing kinase (NIK) signaling in the thymic epithelia is essential for the
49 development of γδT17 (Mair *et al*, 2015). Moreover, γδT17 development requires lymphotoxin β

50 receptor (LTβR) signaling, through the non-canonical NF-κB transcription factor RelB.
51 Alternatively, the canonical NF-κB transcription factor RelA is required for the optimal
52 expression of LTβR ligands such as LIGHT and lymphotoxin(LT)α in thymocytes, which are
53 required for γδT17 development (Powolny-Budnicka *et al*, 2011; Silva-Santos *et al*, 2005).
54 However, the role of NF-κB signaling in the homeostasis of adult γδT17 is not well described.

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

69 Herein we investigated the functions of cIAP1 and cIAP2 in the development and
70 homeostasis of γδT17 and ILC3 cells. We show that mice that lacked cIAP2 and conditionally
71 lacked cIAP1 in all RORγt expressing cells had significantly reduced frequencies and
72 abundance of γδT17 cells in peripheral lymph nodes, skin of adult mice and lacked RORgt^{+}
73 Tbet^{+} γδ T cells and ILC3 cells in the gut lamina propria. Consistently, production of IL-17 from
74 γδT cells was severely diminished in cIAP1 and cIAP2 double deficient mice. Single deficiency

75 of either cIAP1 or cIAP2 minimally impacted γδT17 development or homeostasis. Intriguingly,
76 we found that thymic development of γδT17 was not affected in cIAP1 and cIAP2 double
77 deficient mice. However, cIAP1 and 2 deficient γδT17 in peripheral lymph nodes downregulated
78 the transcription factors cMAF and RORγt from postnatal day 21 and were progressively
79 reduced in numbers culminating in marked reduction of γδT17 by week 12. Also, we found that
80 ectopic expression of the non-canonical NF-κB transcription factor RelB in γδT17 cells
81 downregulates the expression of cMAF. Using bone marrow reconstitution and transfer of
82 neonatal γδ T cells to RAG1 deficient hosts, we show that in mice that lack cIAP2 and
83 conditionally lack cIAP1, both cell extrinsic and intrinsic factors contribute to the loss of γδT17
84 cells, while the loss of ILC3 cells were due to cell extrinsic signals only. Finally, we show that
85 the cIAP1 and cIAP2 -induced downregulation of cMAF and RORγt renders γδT17 cells
86 functionally defective and inhibits their response to IMQ-induced skin inflammation but does not
87 affect the development of IMQ-induced skin inflammation.

88 **Results**

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

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

99 We then analyzed γδ T cell populations in the pLNs, skin and small or large intestinal lamina
100 propria of adult mice. In pLNs, we identified γδT17 cells as being TCRγδ^{+} CD27^{-} CD44^{hi} CCR6^{+}
101 cells. We found that ΔcIAP1/2 mice had significantly reduced proportion and numbers of γδT17
102 compared to either littermate controls, ΔcIAP1 or ΔcIAP2 mice (Fig 1B). The reduction in γδT17
103 in ΔcIAP1/2 mice affected both Vγ4^{+} and Vγ4^{-} γδT17 cells (Vγ chain nomenclature following the
104 Heilig & Tonegawa, 1986) (Fig S1C). We further noticed an increase in TCRγδ^{+} CD27^{+}
105 population in ΔcIAP1/2 compared to other strains (Fig S1A). Consistently, there was a
106 significant reduction in IL-17A and IL-22 production and a concomitant increase in IFNγ
107 production by γδ T cells in the peripheral lymph nodes, reflecting the skewed proportions of the
108 γδ T cell populations (Fig S1B). In the skin, we found that ΔcIAP1/2 mice had reduced numbers
109 of CD3^{+} Vγ5^{-} TCRγδ^{+} cells (γδT17), without a concomitant increase in the abundance of TCRβ^{+}
110 cells (Fig 1C) Further, both Vγ4^{+} or Vγ4^{-} skin γδT17 cells were reduced in ΔcIAP1/2 mice,
111 compared to other strains (Fig S1D).

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

118 RORγt^{+} innate lymphoid cells (ILC3) cells are essential for the homeostasis of intestinal
119 mucosal tissue and for protection against pathogens. ILC3 cells are generally divided into Tbet^{-}
120 and Tbet^{+} subsets (Klose *et al*, 2013; Melo-Gonzalez & Hepworth, 2017). Akin to intestinal γδ T
121 cells, we found that ΔcIAP1/2 had reduced the frequency and numbers of ILC3 cells in siLP.
122 However, both Tbet^{+} and Tbet^{-} ILC3 cells were equally reduced in ΔcIAP1/2 (Fig 2C).
123 Collectively, this data show that both cIAP1 and 2 are required for the homeostasis of γδT17 in

124 the pLNs, skin of adult mice while single deficiency of either has no effect. It also shows that in
125 the gut lamina propria, cIAP1 and 2 are critical for RORγt^{+} Tbet^{+} γδ T and ILC3 cells.

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

128 The development of γδT17 cells occurs in the thymus at approximately embryonic day 16,
129 concomitant with the expression of the transcription factors *Rorc* and *Maf* (Haas *et al*, 2012;
130 Zuberbuehler *et al*, 2019). Thus, we aimed to track the perinatal and neonatal development and
131 homeostasis of γδT17 in ΔcIAP1/2, ΔcIAP1, ΔcIAP2 and littermate control mice. First, we
132 analyzed γδT17 in the thymi of 1-day old pups and found that the thymic output of γδT17 in
133 ΔcIAP1/2 pups was comparable to littermate controls. Thus, there was no difference in the
134 numbers of γδT17 cells nor in the expression of RORγt and cMaf by γδT cells between 1-day
135 old ΔcIAP1/2 pups and controls (Fig 3A-B). Further, the proliferation of γδT17 from 1-day old
136 pups, as measured by Ki67 staining, was also comparable to littermate controls (Fig S2A).

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

147

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

149 We have previously shown that the non-canonical NF-κB transcription factor RelB
150 downregulates the expression of the transcription factor cMAF in Th17 cells; this occurs
151 following the activation of the non-canonical NF-κB pathway through depletion of cIAP1 and 2
152 by SMAC mimetic compounds (Rizk *et al*, 2019). Thus, we hypothesized that the lack of cIAP1
153 and 2 expression in γδT17 activates the non-canonical NF-κB pathway and mobilizes RelB to
154 downregulate cMAF. To test this hypothesis, we purified γδT17 cells from thymi old 1-day old
155 wildtype mice and infected them with an empty retroviral vector or vector expressing RelB and
156 cultured the cells with IL-7. Subsequently, we FACS sorted infected γδT17 cells (TCRγδ^{+}
157 CD44^{hi} CD27^{-} GFP^{+}) and extracted RNA to assess the expression of key γδT17 transcription
158 factors. As expected, the RelB-RV infected cells expressed more RelB and NFκB2 compared to
159 empty-RV infected cells (Fig 4A). Furthermore, we found that RelB induced a downregulation of
160 *Maf*, but did not affect the expression of *Rorc* or *Sox13* (Fig 4B). This indicates that similar to
161 what happens in Th17 cells, activation of the non-canonical NFκB in γδT17 cells downregulates
162 cMAF.

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

164 cIAP1 and cIAP2 regulate the cells response to extrinsic stimuli to TNF and other TNFRSF
165 ligands but are also important to inhibit cell death through control over the formation of the
166 ripoptosome. Therefore, we set to investigate whether cell extrinsic or intrinsic signals were
167 responsible for the loss of γδT17 cells in the ΔcIAP1/2 mice. To this end we set up mixed bone
168 marrow chimeras where wildtype CD45.1/CD45.2 hosts were sublethally irradiated and
169 reconstituted with a mixture of 1:1 wildtype (CD45.1) and ΔcIAP1/2 (CD45.2) bone marrow
170 cells (Fig 5A). We found that, under these conditions, γδT17 and CD27^{+} γδ T cells from
171 ΔcIAP1/2 bone marrow reconstituted the pLNs of the host as competitively as their wildtype
172 counterparts (Fig 5B). Further, only Tbet^{+} γδ T cells reconstituted the colonic lamina propria and

173 were slightly dominated by cells that originated from ΔcIAP1/2 bone marrow (BM) (Fig 5C).
174 Conversely, colonic ILC3 cells were effectively reconstituted and were encompassed
175 approximately equal proportions of CD45.1 (WT) and CD45.2 (ΔcIAP1/2) cells (Fig 5D). This
176 indicates that the presence of wildtype hematopoietic and stromal components prevents the loss
177 of γδT17 from ΔcIAP1/2 origin.

178 We then asked whether the reconstitution of ΔcIAP1/2 mice with mixed BM chimeras would
179 be enough to stop the loss of γδT17 cells or not. To this end, we irradiated ΔcIAP1/2 mice that
180 were at least 12 weeks old, to ensure host mice lacked γδT17 cells, and reconstituted them with
181 either wildtype (CD45.1) or a mixture of 1:1 wildtype (CD45.1) and ΔcIAP1/2 (CD45.2) BM cells
182 (Fig 6A). In both instances we found that all γδT17 cells in the pLNs were from wildtype
183 (CD45.1) BM; conversely, CD27^{+} γδ T cells were either equally split into CD45.1 and CD45.2
184 components in mice that had received mixed BM chimeras or only CD45.1 cells in mice that had
185 received wildtype cells only (Fig 6B). In the siLP, almost all γδ T cells came from wildtype
186 (CD45.1) BM and expressed various levels of Tbet and some RORγt (Fig 6C). Although ILC3
187 that were of both wildtype (CD45.1) and ΔcIAP1/2 (CD45.2) origin could be found in the siLP,
188 wildtype ILC3 cells were more abundant than ILC3 cells from ΔcIAP1/2 BM (Fig 6D). Thus, the
189 presence of wildtype hematopoietic cells in ΔcIAP1/2 mice is not sufficient to reverse the loss of
190 γδT17 cells lacking cIAP1 and cIAP2 but is sufficient to reverse the loss of ILC3 cells.

191 As γδT17 cells are generated perinatally in the thymus, we thus reasoned that γδT17 cells
192 generated after BM chimera reconstitutions might be different from γδT17 cells that egress the
193 neonatal thymus. To address this issue, we purified γδ T cells from the thymi of 1-day old Ctrl,
194 ΔcIAP1, ΔcIAP2 or ΔcIAP1/2 mice and transferred them to RAG1$^{-/-}$ recipient mice (Fig 7A). After
195 12 weeks we analyzed the pLNs and cLP and found that γδT17 and CD27^{+} γδ T cells could be
196 recovered from the pLNs of RAG1$^{-/-}$ mice (Fig 7B) but again only Tbet^{+} γδ T cells could be found
197 in the cLP, regardless of the donor strain (Fig 7C).

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

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

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

218 We then sought to assess how the ΔcIAP1/2 mice would respond to IMQ-induced
219 inflammation in the presence of transcriptionally altered γδT17 cells. To this end, we applied
220 IMQ on the ear skin of 4 weeks old mice of all 4 genotypes and again assessed the
221 inflammatory response. We found that the epidermal thickening in the ear skin of ΔcIAP1/2 mice
222 was comparable to that of WT mice (Fig 8A). Further, we found that both in the skin and the

223 lymph nodes, γδT17 cells from ΔcIAP1/2 mice failed to expand in response to IMQ-inflammation
224 and were significantly less abundant than in the IMQ-treated control mice. Conversely, $CD4^+$ T
225 cells in skin of IMQ-treated ΔcIAP1/2 mice were significantly more abundant than in control mice
226 although there was no significant difference in the lymph nodes (Fig 8B). Functionally, we found
227 that γδT17 cells from IMQ-treated ΔcIAP1/2 produced significantly less IL-17A in the lymph
228 nodes or skin, compared to IMQ-treated control mice; instead, $CD4^+$ T cells from ΔcIAP1/2
229 produced copious amounts of IL-17A in both organs compared to controls (Fig 8C-D). Likewise,
230 lymph nodes γδT17 deficient in cIAP1 and cIAP2 failed to produce any IL-22 following IMQ-
231 psoriasis, while the $CD4^+$ T cells in the same mice produced significantly more IL-22 than in the
232 IMQ-treated control mice (Fig 8C); intriguingly, IL-22 production by γδT17 in the skin of IMQ-
233 treated ΔcIAP1/2 was comparable to that of control mice, yet still there was significantly more
234 IL-22 produced by the CD4+ T cells in the skin of ΔcIAP1/2 mice following IMQ-treatment,
235 compared to controls (Fig 8E).

236 Thus, our data show that although adult ΔcIAP1/2 mice are severely deficient in γδT17 cells
237 they are not protected from IMQ-induced psoriatic inflammation; similarly, reduced functionality
238 of γδT17 cells in young ΔcIAP1/2 mice did not protect the mice from IMQ-induced inflammation.
239 In both instances, the IMQ-induced skin inflammation appears to have been a function of
240 aggravated Th17 responses. These results are consistent with earlier report showing that $CD4^+$
241 T cells are responsible for the IMQ induced psoriasis following loss of γδ T cells during adult life
242 (Sandrock *et al*, 2018). Further, our data point out that during IMQ-induced inflammation not
243 only can $CD4^+$ T cells compensate for the lack of γδ T cells, but also for their compromised
244 functionality.

245 **Discussion**

246 Herein we showed that cIAP1 and cIAP2 are required for the adult homeostasis of γδT17
247 cells in peripheral lymph nodes, skin and gut lamina propria. Although cIAP1 and cIAP2 were

248 not required for the thymic development of γδT17 cells, mice that lacked cIAP2 and conditionally
249 lacked cIAP1 in RORγt expressing cells suffered progressive reduction in γδT17 cells in the
250 lymph nodes during late neonatal and adult life. Further, lack of cIAP1 and cIAP2 correlated with
251 a downregulation of RORγt and cMaf in γδT17 cells that preceded the loss of γδT17 cells. We
252 further show that the both cell intrinsic and extrinsic factors contribute to the decreased
253 abundances of γδT17 cells in adult RORγt^{cre} cIAP1$^{f/f}$ cIAP2$^{-/-}$ mice. Finally, we show that adult
254 RORγt^{cre} cIAP1$^{f/f}$ cIAP2$^{-/-}$ mice were not protected against IMQ-induced skin inflammation
255 despite the severe reduction in γδT17 in these mice and that the presence of functionally
256 defective γδT17 cells in younger RORγt^{cre} cIAP1$^{f/f}$ cIAP2$^{-/-}$ was not sufficient to inhibit IMQ-
257 induced psoriasis.

258 cIAP1 and cIAP2 are ubiquitously expressed proteins that are frequently upregulated in
259 cancers (Tamm *et al*, 2000; Wu *et al*, 2010). Downstream TNFR superfamily, they fine tune the
260 balance between NF-κB activation or induction of cell death. Thus, they are important targets in
261 the context of cancer therapy, whereby their antagonization can sensitize cancer cells to cell
262 death or modulate the functions of cytotoxic T cells (Gyrd-Hansen & Meier, 2010; Bai *et al*,
263 2014) . However, their role in γδ T cells has not been previously addressed. Consensus is that
264 cIAP1 and cIAP2 are functionally redundant (Mahoney *et al*, 2008); thus, mice deficient in either
265 proteins display no phenotypic changes. As the genetic knockout of both cIAP1 and cIAP2 is
266 embryonically lethal (Moulin *et al*, 2012), the RORγt driven deletion of cIAP1 combined with full
267 deletion of cIAP2 provides a targeted approach to probe their function in RORγt expressing γδ T
268 cells. The data presented herein reveals a critical, but redundant, role for cIAP1 and cIAP2 in
269 the homeostasis of γδT17 cells.

270 We have previously shown that antagonization of cIAP1 and cIAP2 in Th17 cells
271 downregulates *Il17a* and *Maf* through the non-canonical NF-κB transcription factors RelB and
272 p52 (Rizk *et al*, 2019). Thus, it is plausible that a similar mechanism exists in γδT17 cells; as

273 such, the lack of cIAP1 and cIAP2 in γδT17 activates the non-canonical NF-κB, which
274 downregulates *Maf* expression. Consequently, *Rorc* is downregulated as cMaf is required to
275 maintain its expression in γδT17. Eventually, such cells would progressively perish as cMaf and
276 RORγt are required for the homeostasis of γδT17 (Barros-Martins *et al*, 2016; Zuberbuehler *et*
277 *al*, 2019).

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

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381

382

383 **Materials and methods**

384 **Mice**

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

390 **Cell culture media and buffers**

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

395 **Lymphocyte isolation from mouse organs**

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

400 **IMQ-induced psoriasis**

401 Psoriasis was induced in mice by applying 7 mg of Aldara cream (containing 5% imiquimod) to
402 the dorsal side of each ear for 7 days. Histological sections were prepared by fixing ear tissue in
403 10% formalin overnight and then paraffin embedded. The paraffin embedded section were
404 stained by H&E.

405 **Flow cytometry staining**

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

409 anti-IL-17A (TC11-18H10; BV786 and PE), anti-IFNγ (XMG1.2; PE-Cy7 and APC), anti-IL-22
410 (1H8PWSR; PE), anti-cMAF (symOF1; PE and Percp-Cy5.5; 5 µL/test), anti-CD4 (GK1.5;
411 BUV395), anti-TCRγδ (GL3; BV421), anti-CD27 (LG.3A10; PE-Cy7), anti-CCR6 (140706; Alexa
412 Fluor 647), anti-CD44 (1M7; V500), anti-CD19 (6D5; FITC), anti-TCRβ (H57-597; APC-
413 eflour780), anti-CD3e (BM10-37, PeCF594 and PE), anti-Tbet(4B10; PeCy7), anti- CD8 (53-
414 6.7;FITC), anti-Vγ5 (536; FITC), anti-Vγ4(UC3-10A6; Percp-eflour710), anti-GATA3(TWAJ;
415 Percp-eFlour710; 1:30), anti-CD45(30-F11;PE and V500) and anti-RORγt (B2D; APC).

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

417 First, pooled thymi from one-day old wildtype mice were isolated and crushed against 70 µm
418 filter to prepare single cell solution. Subsequently, γδT17 cells were enriched by magnetic
419 depletion of $CD4^+$, $CD8^+$, $TCR\beta^+$ and $CD27^+$ cells as follows: total thymocytes were re-
420 suspended in MACS buffer at 1e8 cells/ml containing 50 µL/ml normal rat serum and 1:200
421 biotin labelled anti- $CD4^+$, $CD8^+$, $TCR\beta^+$ and $CD27^+$ antibodies; the cells were incubated for 10
422 minutes at room temperature and then incubated with 75 µL/ml EasySep RaphidSphere
423 streptavidin beads (#50001) for 2.5 minutes then transferred to EasySep magnet for 2.5
424 minutes. The non-bound fraction was collected by decantation and centrifugated for 5 minutes
425 at 400g at 4°C. Approximately 2e5 of the enriched γδT17 cells were then cultured overnight with
426 20 ng/ml recombinant IL-7 in round-bottom 96-well tissue culture plate. The cells were then
427 spin-infected by adding 4 µL of the desired retrovirus and 5 µg Retronectin (TAKARA) and
428 centrifugation at 1800 rpm for 30 minutes at 32°C with deceleration set to 0. Subsequently, the
429 cells were incubated for 90 minutes at 32°C before transfer to 37°C incubator. Recombinant IL-7

430 at 20 ng/ml was again added to the cultures after 48 hours and the cells were FACS sorted 24
431 hours later.

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

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

438 **Bone marrow chimeras**

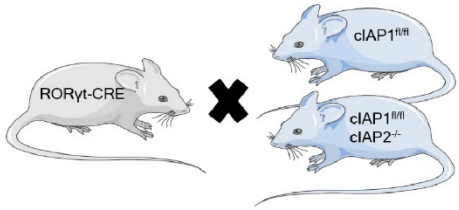
439 The bone marrow cells for reconstitution were isolated by flushing the tibia and femur, which
440 were dissected from donor mice, with culture media. Total bone marrow cells were then
441 centrifugated at 400g for 5 minutes at 4°C. The cells were then re-suspended and passed
442 through 70 μm filter.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

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

449 **Figures**

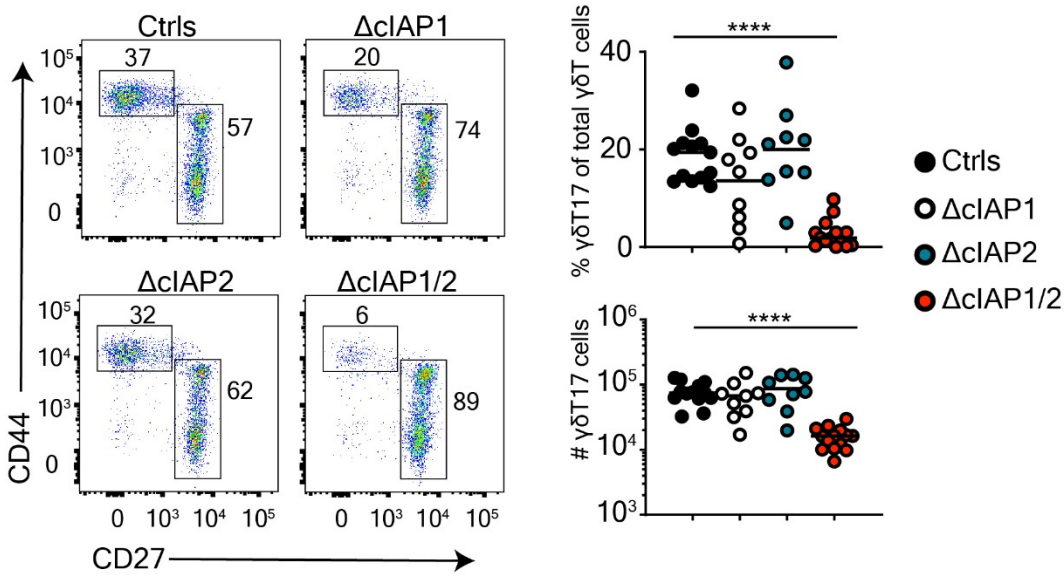
450 **Figure 1**

A



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

B



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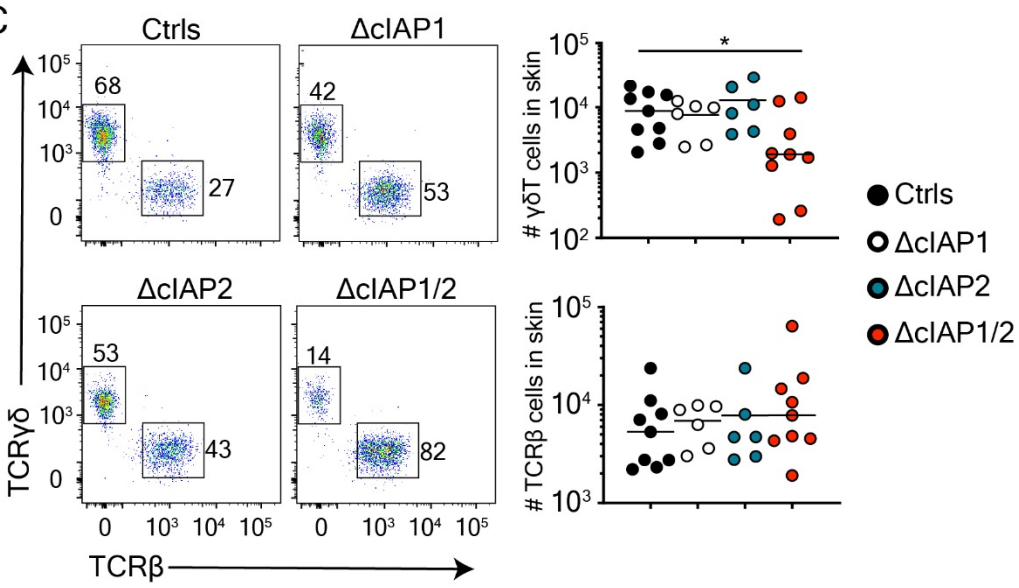
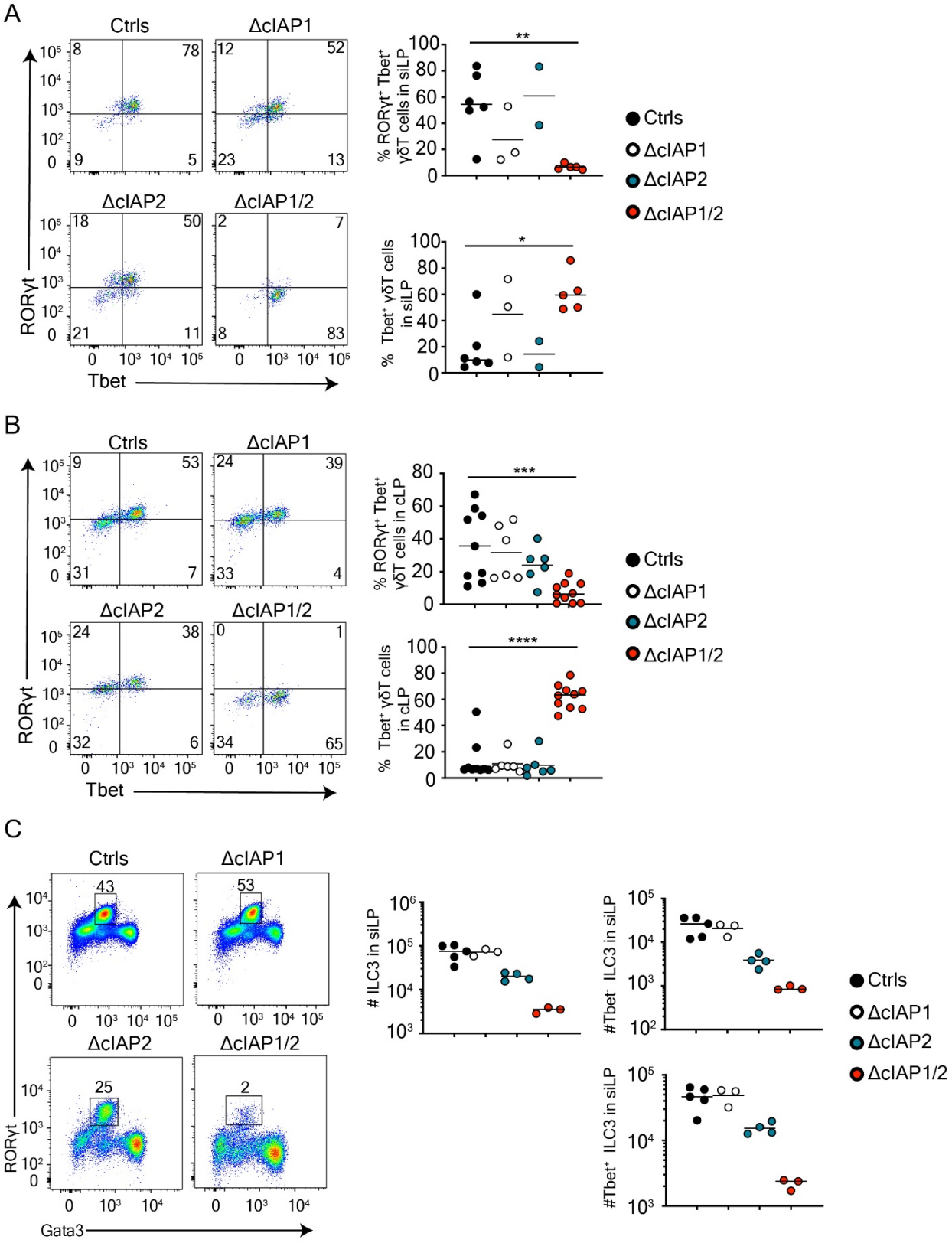


451

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.

464 **Figure 2**



465

466 **Figure 2. cIAP1 and cIAP2 are required for the homeostasis of intestinal RORγt$^+$ Tbet$^+$ γδ**
467 **T and ILC3 cells.**
468 Flow cytometric analysis plots (left) and quantification (right) of TCRγδ$^+$ populations in the siLP
469 (A) or cLP(B) of all 4 strains. γδT cell populations were identified as CD45$^+$ CD3$^+$ TCRβ$^-$
470 TCRγδ$^+$. (C) Flow cytometric analysis plots of (left) and quantification (right) of ILC3 cells in the
471 siLP of all 4 strains. ILC cells were identified as CD45^{int} CD3$^-$ CD127$^+$ TCRγδ$^-$ TCRβ$^-$ cells. In
472 graphs, each symbol represents a mouse and line the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,
473 ****$p < 0.0001$ using Mann-Whitney test.
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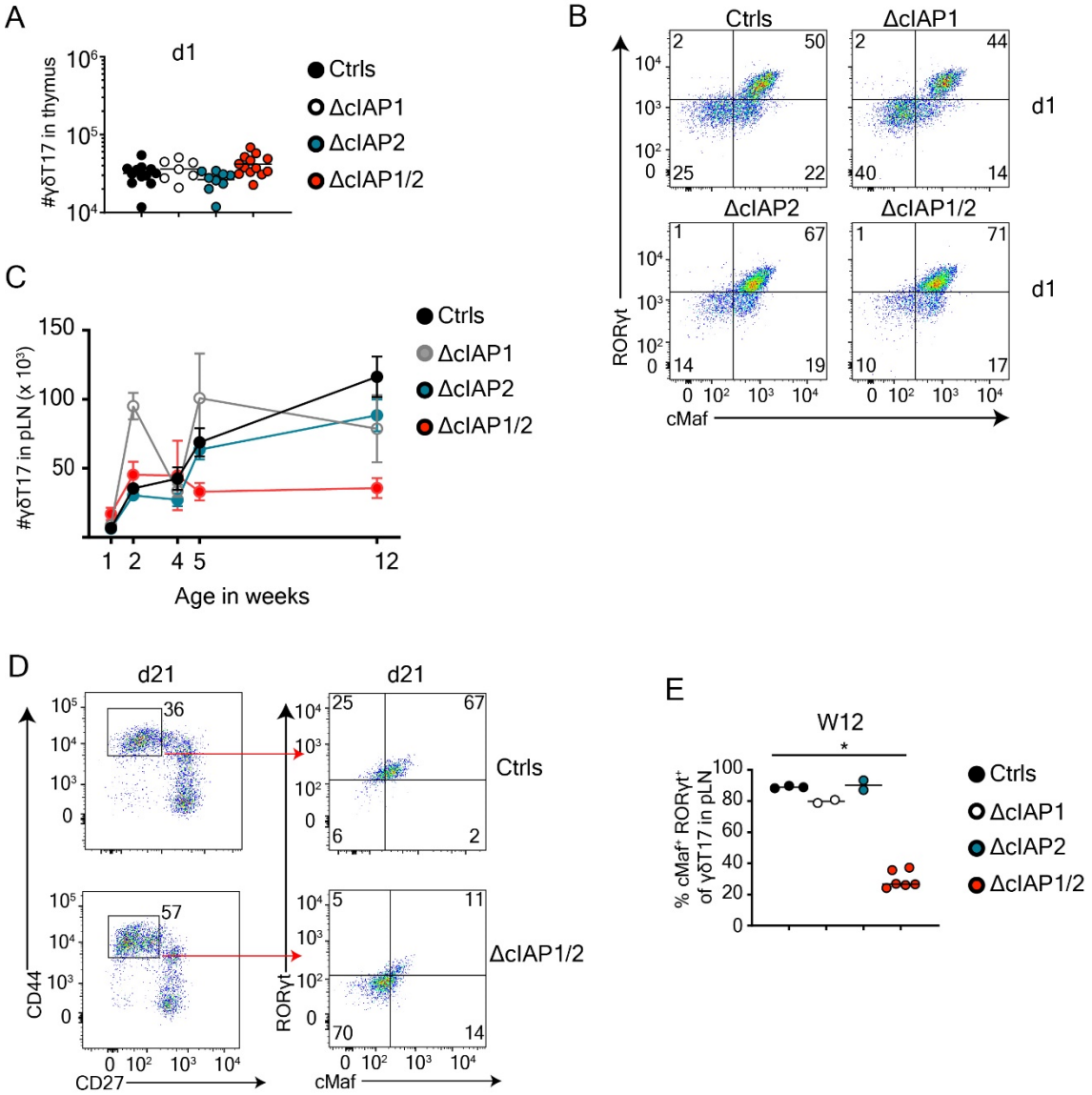
476

477 **Figure 3**

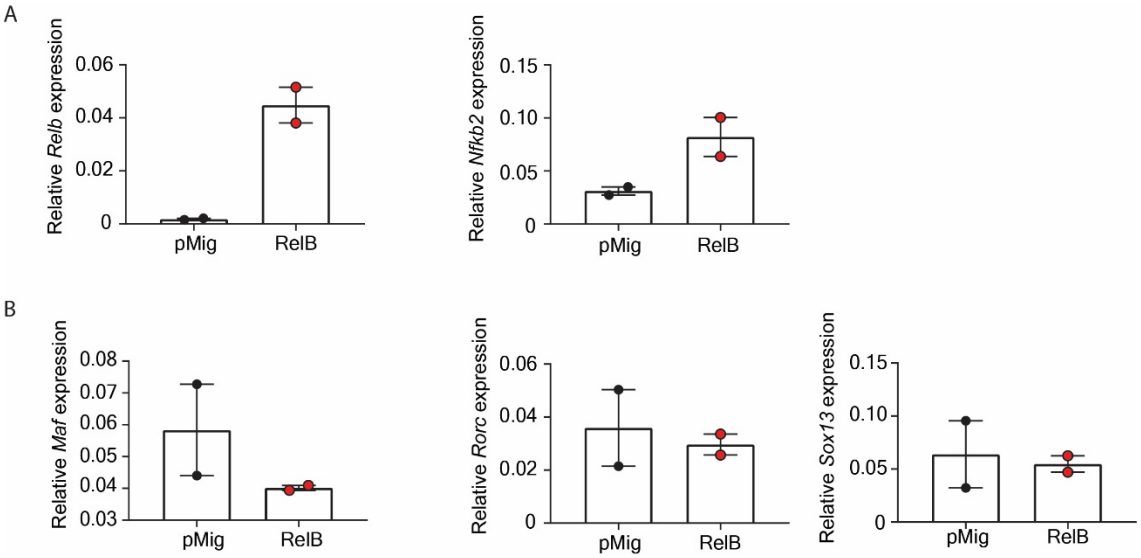


478

479 **Figure 3. cIAP1 and cIAP2 are not required for thymic development of γδT17 cells but are**
480 **required for cMaf and RORγt expression during neonatal and adult life.**
481 (A) Numbers of γδT17 cells in the day1 (d1) thymi of all 4 strains (gated as in Fig1). (B) Flow
482 cytometric analysis of RORγt and cMaf expression in γδ T cells from d1 thymi of all 4 strains.
483 (C) Numbers of γδT17 cells in the pLNs at the indicated timepoints. (D) analysis of γδ T cell
484 populations from d21 pLNs of the indicated strains, while arrows show RORγt and cMaf
485 expression within γδT17 cells compartment. (E) frequency of RORγt and cMaf expression in
486 γδT17 in the pLNs of 12-weeks old from all strains. In graphs each symbol represents a mouse
487 and line the mean. *p < 0.05 using Mann-Whitney test.
488
489

490

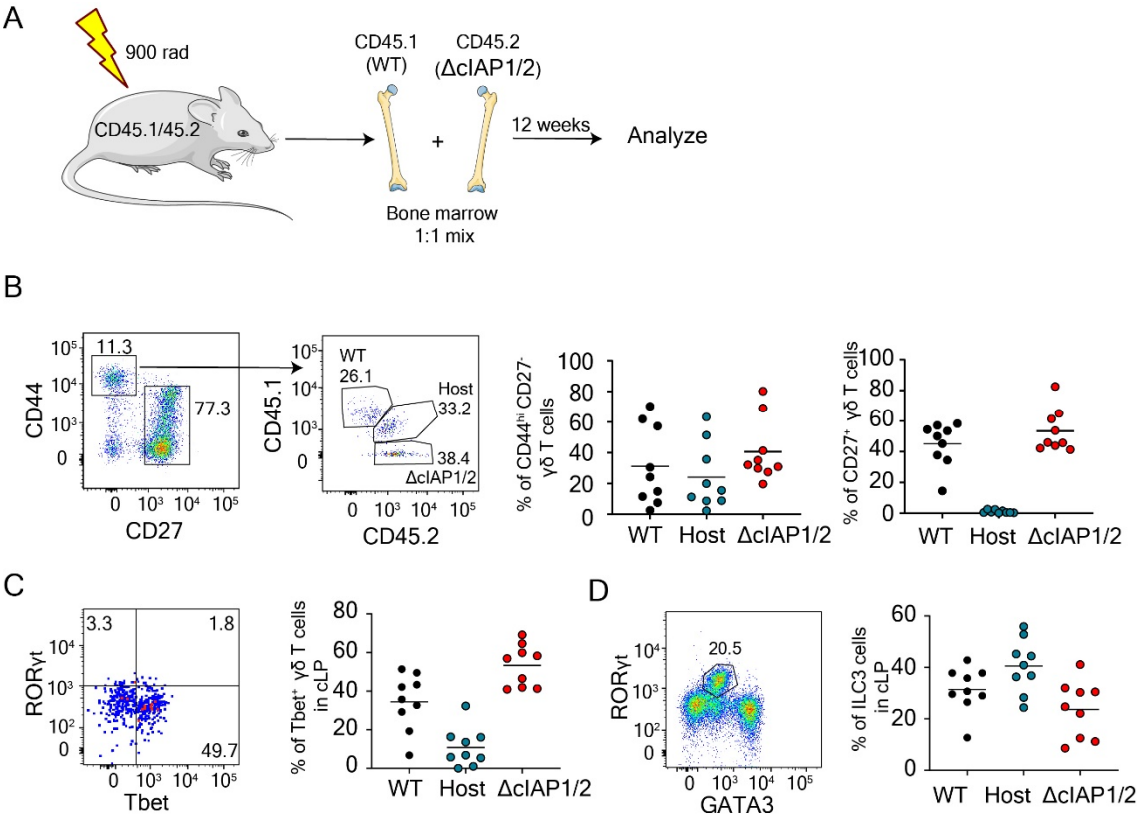
491 **Figure 4**



492

493 **Figure 4. Activation of the non-canonical NF-κB pathway downregulates the expression**
494 **of *Maf* in γδT17 cells.**
495 (A-B) RT-qPCR analysis of the indicated genes in γδT17 cells that were infected with empty
496 retrovirus (pMIG) or retrovirus expressing RelB. only GFP^+ cells (retrovirus infected cells) were
497 FACS sorted and analyzed. In graphs each symbol represents an independent experiment.

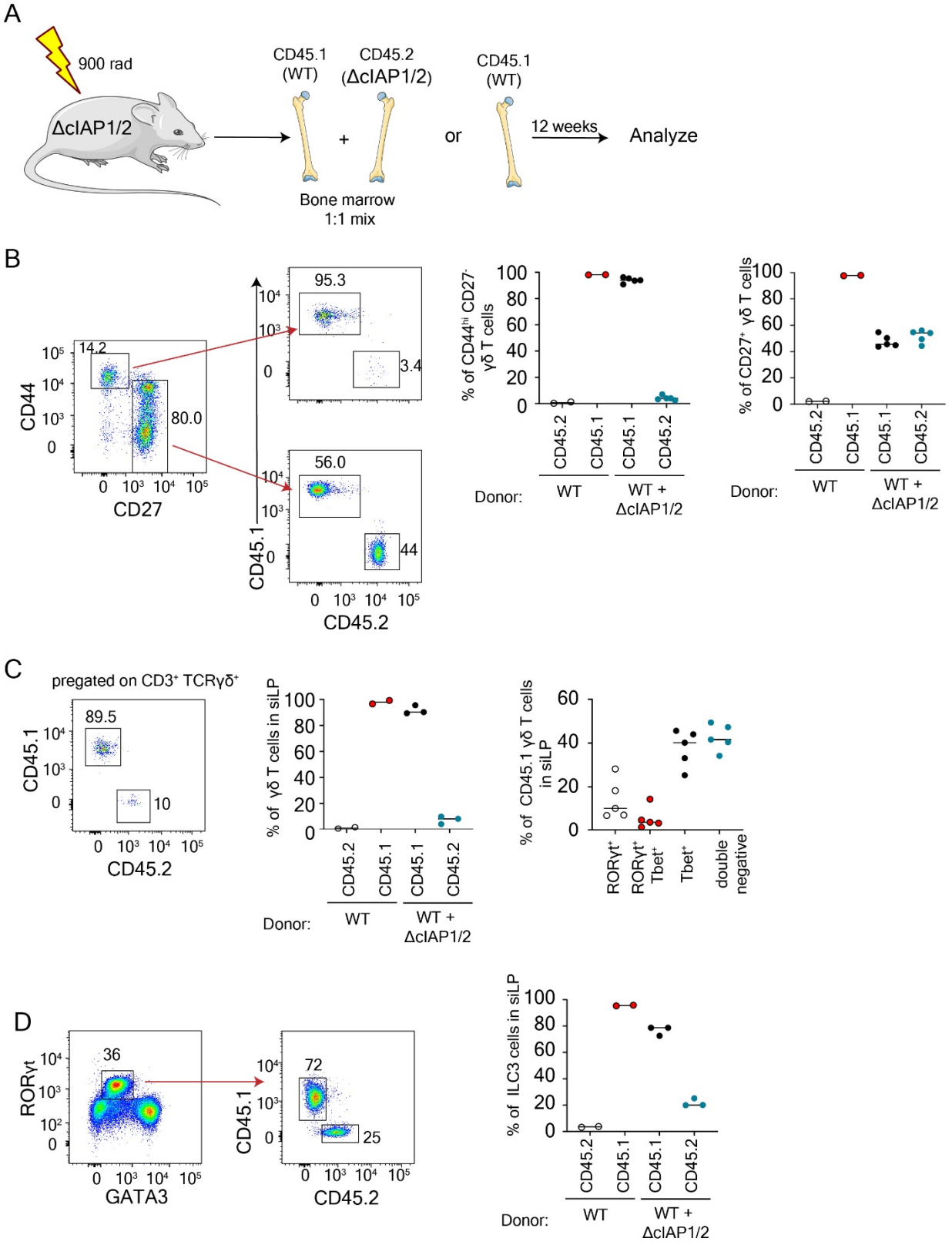
498 **Figure 5**



499

500 **Figure 5. Cell extrinsic signals mediate the loss of cIAP1 and cIAP2 double deficient**
501 **γδT17 and ILC3 cells in ΔcIAP1/2 mice.**
502 (A) schematic representation of the experimental design whereby sub-lethally irradiated wildtype
503 hosts (CD45.1/45.2) were reconstituted with 1:1 mixture of WT (CD45.1) and ΔcIAP1/2
504 (CD45.2) bone marrow. (B-C) Flow cytometry analysis of γδT cells in the pLNs and cLP of host
505 mice after 12 weeks of reconstitution and indicating the frequency of WT, host, or ΔcIAP1/2
506 γδT17 and CD27^{+} γδ T cells. Graphs show frequency of WT, host, or ΔcIAP1/2 in γδ T cell
507 populations in pLN of host mice amalgamated from 2 independent experiments. (D) Flow
508 cytometry analysis of ILC3 cells in the cLP of host mice. Graph show frequency of WT, host, or
509 ΔcIAP1/2 of all ILC3 cells in cLP of host mice amalgamated from 2 independent experiments. In
510 graphs each symbol represents a mouse and line the mean.

511 **Figure 6**

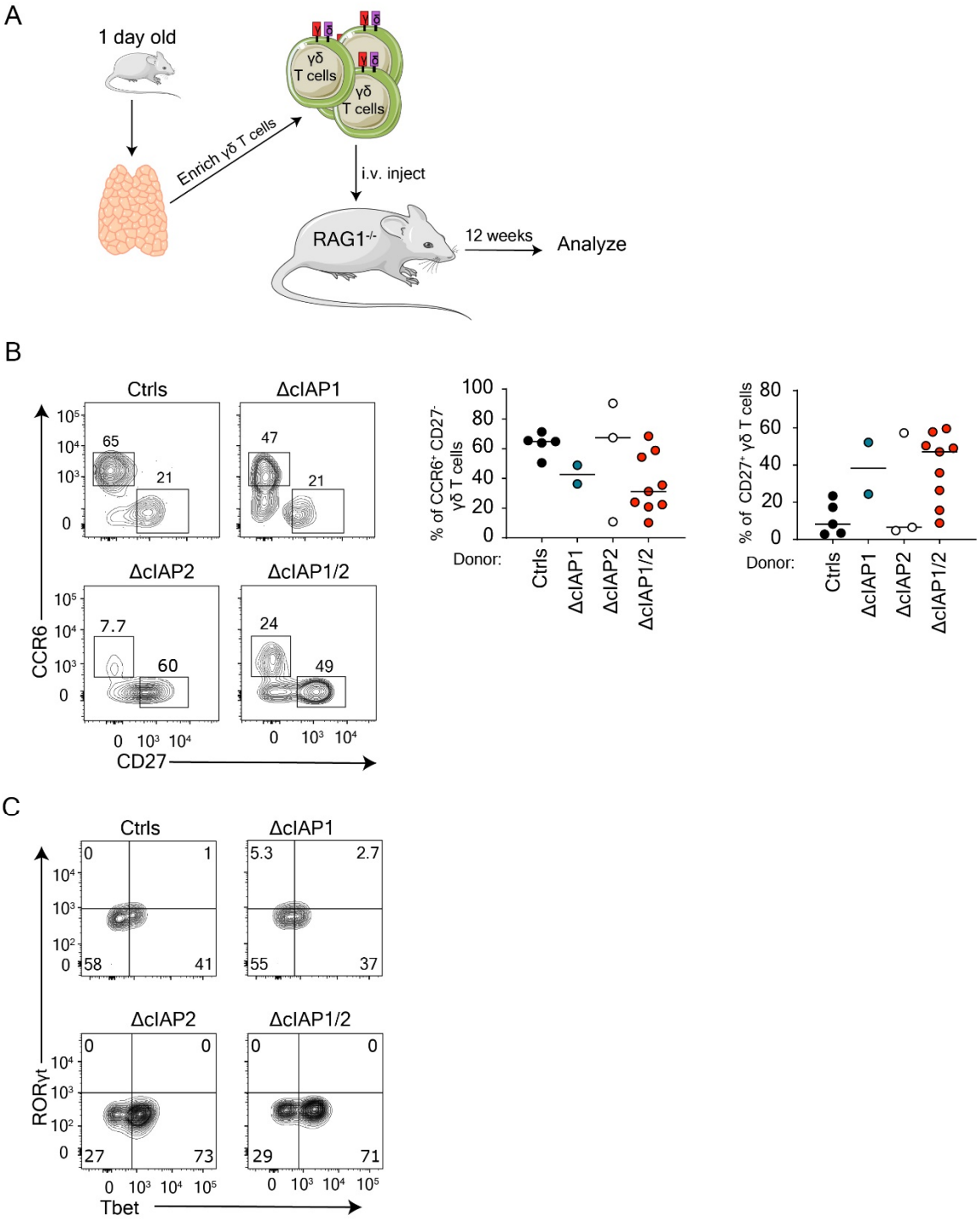


512

513 **Figure 6. Reconstitution of ΔcIAP1/2 mice with wildtype hematopoietic system rescues**
514 **the loss of ILC3 cells but not γδT17 cells.**
515 (A) schematic representation of the experimental design whereby sub-lethally irradiated
516 ΔcIAP1/2 (CD45.2) were reconstituted with either WT (CD45.1) or 1:1 mixture of WT (CD45.1)
517 and ΔcIAP1/2 (CD45.2) bone marrow. Flow cytometry analysis of γδT cells in the pLNs (B) and
518 siLP (C) of host mice after 12 weeks of reconstitution and indicating the frequency of WT or
519 ΔcIAP1/2 γδT17 and CD27^{+} γδT cells. Graphs show frequency of WT or ΔcIAP1/2 in γδ T cell
520 populations in pLN and siLP. (D) Flow cytometry analysis of ILC3 cells in the cLP of host mice.
521 Graph show frequency of WT or ΔcIAP1/2 of all ILC3 cells in siLP. In graphs each symbol
522 represents a mouse and line the mean.

523

524 **Figure 7**



525

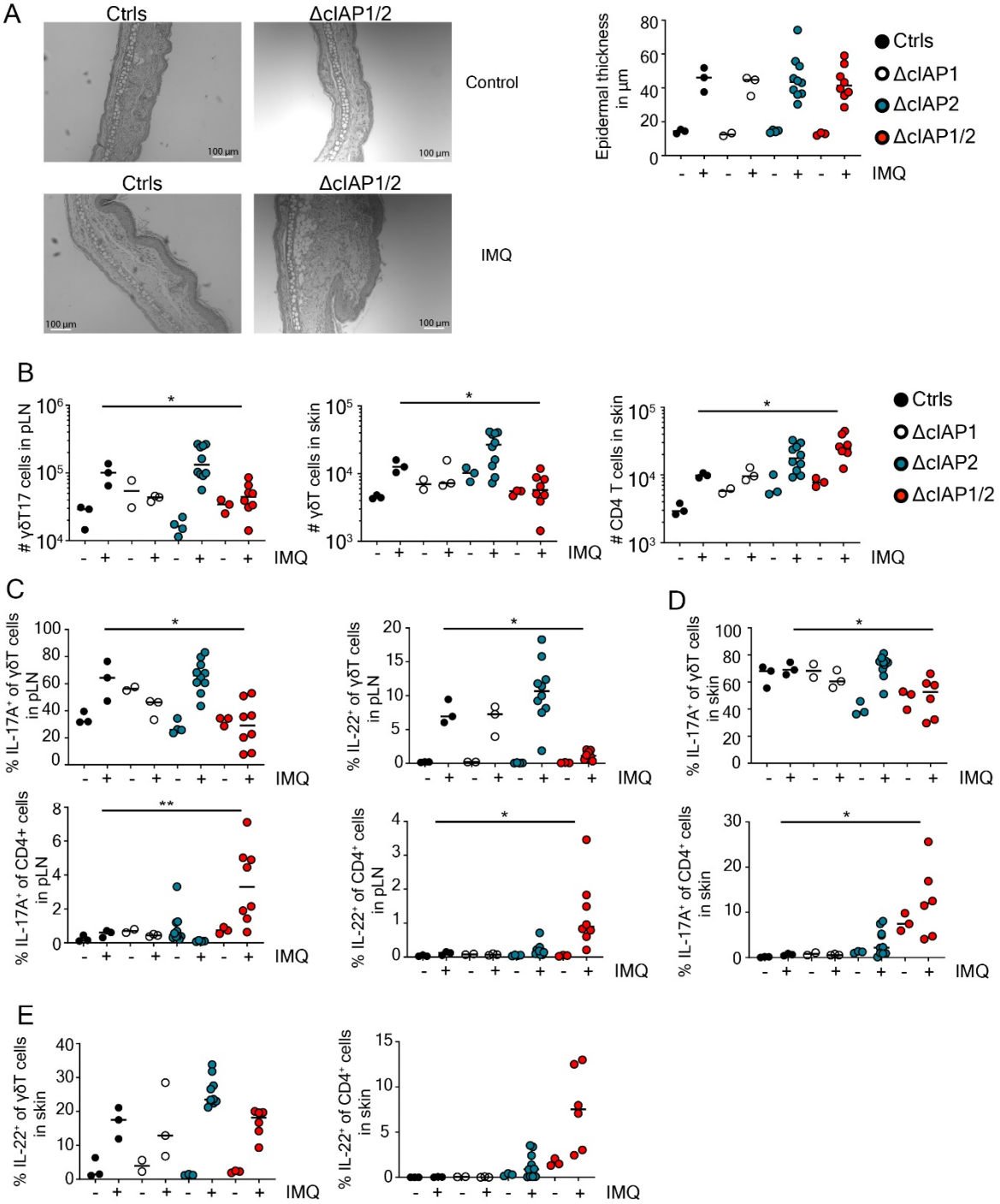
526 **Figure 7. Neonatal γδT17 lacking cIAP1 and cIAP2 can reconstitute RAG$^{-/-}$ hosts.**
527 (A) schematic representation of the experimental design whereby γδ T cells were enriched from
528 the thymi from one-day old mice of all 4 strains and i.v. injected into RAG$^{-/-}$ hosts. (B) Flow
529 cytometry analysis of γδT cells in the pLNs of host RAG$^{-/-}$ mice at 12 weeks after transfer of the
530 γδT cells from the indicated strain. Graphs show frequency of recovered γδT17 or CD27^{+} γδT

531 cells of total γδT cells. In graphs each symbol represents a donor mouse and line the median.
532 Data is amalgamated from 3 independent experiments. (C) Flow cytometry analysis of γδT cells
533 in cLP of host RAG$^{-/-}$ mice at 12 weeks after transfer of the γδT cells from the indicated strain.

534

535

536 **Figure 8**



537

538 **Figure 8. cIAP1 and 2 double deficient γδT17 cells are functionally defective and do not**
539 **respond to IMQ-induced psoriasis.**
540 (A) Representative light microscopy images (right) indicating ear skin sections stained for H&E
541 before and after IMQ-induced psoriasis in the indicated strains (left) Quantification of epidermal

542 thickening from H&E stained sections. (B) Numbers of γδT17 and $CD4^+$ $TCR\beta^+$ cells in the pLN
543 or skin of all strains of IMQ treated or control mice. In the pLN, γδT17 were identified as $CD44^{hi}$
544 $CD27^-$ $TCR\gamma\delta^+$, while in the skin, they were identified as $CD45^+$ $V\gamma5^-$ $CD3^+$ $TCR\gamma\delta^+$.
545 Quantification of IL-17A (C, D) or IL-22 (C, E) production by γδ T and CD4 T cells in the pLN (C)
546 or skin (E) in control or IMQ-treated mice of all strains. In graphs, each symbol represents a
547 mouse and line the mean. Data is pool of 3 experiments. *$p < 0.05$, **$p < 0.01$using Mann-
548 Whitney test.
549