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

**Introduction**

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

During perinatal thymic development, γδT17 cells undergo transcriptional and epigenetic programming that imprints their effector phenotypes (Schmolka *et al.*, 2013). Thus, thymic development of γδT17 is dependent on the consecutive expression of the transcription factors Sox13, cMAF followed by the lineage defining expression of RORγt. Conversely, the IFNγ producing γδT cells are characterized by the transcription factors Tbet and Tcf1 (Sagar *et al.*, 2018; Zuberbuehler *et al.*, 2019). Further, canonical and non-canonical NF-κB pathway signaling in the thymic microenvironment are essential for the development of γδT17. For instance, the non-canonical NF-κB inducing kinase (NIK) signaling in the thymic epithelia is essential for the development of γδT17 (Mair *et al.*, 2015). Moreover, γδT17 development requires lymphotoxin β
receptor (LTβR) signaling, through the non-canonical NF-κB transcription factor RelB. Alternatively, the canonical NF-κB transcription factor RelA is required for the optimal expression of LTβR ligands such as LIGHT and lymphotoxin(LT)α in thymocytes, which are required for γδT17 development (Powolny-Budnicka et al, 2011; Silva-Santos et al, 2005). However, the role of NF-κB signaling in the homeostasis of adult γδT17 is not well described.

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

Herein we investigated the functions of cIAP1 and cIAP2 in the development and homeostasis of γδT17 and ILC3 cells. We show that mice that lacked cIAP2 and conditionally lacked cIAP1 in all RORγt expressing cells had significantly reduced frequencies and abundance of γδT17 cells in peripheral lymph nodes, skin of adult mice and lacked RORgt+ Tbet+ γδ T cells and ILC3 cells in the gut lamina propria. Consistently, production of IL-17 from γδT cells was severely diminished in cIAP1 and cIAP2 double deficient mice. Single deficiency
of either cIAP1 or cIAP2 minimally impacted γδT17 development or homeostasis. Intriguingly, we found that thymic development of γδT17 was not affected in cIAP1 and cIAP2 double deficient mice. However, cIAP1 and 2 deficient γδT17 in peripheral lymph nodes downregulated the transcription factors cMAF and RORγt from postnatal day 21 and were progressively reduced in numbers culminating in marked reduction of γδT17 by week 12. Also, we found that ectopic expression of the non-canonical NF-κB transcription factor RelB in γδT17 cells downregulates the expression of cMAF. Using bone marrow reconstitution and transfer of neonatal γδ T cells to RAG1 deficient hosts, we show that in mice that lack cIAP2 and conditionally lack cIAP1, both cell extrinsic and intrinsic factors contribute to the loss of γδT17 cells, while the loss of ILC3 cells were due to cell extrinsic signals only. Finally, we show that the cIAP1 and cIAP2 -induced downregulation of cMAF and RORγt renders γδT17 cells functionally defective and inhibits their response to IMQ-induced skin inflammation but does not affect the development of IMQ-induced skin inflammation.

**Results**

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

In order to understand the functions of cIAP1 and cIAP2 in regulating γδT17 cells, we crossed RORγt-Cre (Ebertl *et al*, 2004) to cIAP1 floxed (cIAP1^f/f^)or cIAP1^f/f^ cIAP2 deficient mice (cIAP1^f/f^ cIAP2^-/-^). Thus, 4 strains (Fig 1A) were generated as follows: RORγt^cre-^ cIAP1^f/f^ (hereafter referred to as littermate controls), RORγt^cre+^ cIAP1^f/f^ (ΔcIAP1), RORγt^cre+^ cIAP1^f/f^ cIAP2^-/-^ (ΔcIAP2) and RORγt^cre+^ cIAP1^f/f^ cIAP2^-/-^ (ΔcIAP1/2). All 4 strains were viable and showed no apparent defects in the development of peripheral lymph nodes (inguinal, brachial and axillary; collectively referred to as pLN) or peyer’s patches (data not shown); this indicated that there was no developmental effect on lymphoid tissue inducer (Lti) cells, which express RORγt and are required for formation of lymph nodes and peyer’s patches perinatally (Sawa *et al*, 2010).
We then analyzed γδ T cell populations in the pLNs, skin and small or large intestinal lamina propria of adult mice. In pLNs, we identified γδT17 cells as being TCRγδ+ CD27− CD44hi CCR6+ cells. We found that ΔcIAP1/2 mice had significantly reduced proportion and numbers of γδT17 compared to either littermate controls, ΔcIAP1 or ΔcIAP2 mice (Fig 1B). The reduction in γδT17 in ΔcIAP1/2 mice affected both Vγ4+ and Vγ4− γδT17 cells (Vγ chain nomenclature following the Heilig & Tonegawa, 1986) (Fig S1C). We further noticed an increase in TCRγδ+ CD27+ population in ΔcIAP1/2 compared to other strains (Fig S1A). Consistently, there was a significant reduction in IL-17A and IL-22 production and a concomitant increase in IFNγ production by γδ T cells in the peripheral lymph nodes, reflecting the skewed proportions of the γδ T cell populations (Fig S1B). In the skin, we found that ΔcIAP1/2 mice had reduced numbers of CD3+ Vγ5− TCRγδ+ cells (γδT17), without a concomitant increase in the abundance of TCRβ+ cells (Fig 1C) Further, both Vγ4+ or Vγ4− skin γδT17 cells were reduced in ΔcIAP1/2 mice, compared to other strains (Fig S1D).

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

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

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

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

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

We have previously shown that the non-canonical NF-κB transcription factor RelB downregulates the expression of the transcription factor cMAF in Th17 cells; this occurs following the activation of the non-canonical NF-κB pathway through depletion of cIAP1 and 2 by SMAC mimetic compounds (Rizk et al., 2019). Thus, we hypothesized that the lack of cIAP1 and 2 expression in γδT17 activates the non-canonical NF-κB pathway and mobilizes RelB to downregulate cMAF. To test this hypothesis, we purified γδT17 cells from thymi old 1-day old wildtype mice and infected them with an empty retroviral vector or vector expressing RelB and cultured the cells with IL-7. Subsequently, we FACS sorted infected γδT17 cells (TCRγδ+CD44hi CD27- GFP+) and extracted RNA to assess the expression of key γδT17 transcription factors. As expected, the RelB-RV infected cells expressed more RelB and NFκB2 compared to empty-RV infected cells (Fig 4A). Furthermore, we found that RelB induced a downregulation of Maf, but did not affect the expression of Rorc or Sox13 (Fig 4B). This indicates that similar to what happens in Th17 cells, activation of the non-canonical NFκB in γδT17 cells downregulates cMAF.

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

cIAP1 and cIAP2 regulate the cells response to extrinsic stimuli to TNF and other TNFRSF ligands but are also important to inhibit cell death through control over the formation of the ripoptosome. Therefore, we set to investigate whether cell extrinsic or intrinsic signals were responsible for the loss of γδT17 cells in the ΔcIAP1/2 mice. To this end we set up mixed bone marrow chimeras where wildtype CD45.1/CD45.2 hosts were sublethally irradiated and reconstituted with a mixture of 1:1 wildtype (CD45.1) and ΔcIAP1/2 (CD45.2 ) bone marrow cells (Fig 5A). We found that, under these conditions, γδT17 and CD27+ γδ T cells from ΔcIAP1/2 bone marrow reconstituted the pLN of the host as competitively as their wildtype counterparts (Fig 5B). Further, only Tbet+ γδ T cells reconstituted the colonic lamina propria and
were slightly dominated by cells that originated from ΔclAP1/2 bone marrow (BM) (Fig 5C).

Conversely, colonic ILC3 cells were effectively reconstituted and were encompassed approximately equal proportions of CD45.1 (WT) and CD45.2 (ΔclAP1/2) cells (Fig 5D). This indicates that the presence of wildtype hematopoietic and stromal components prevents the loss of γδT17 from ΔclAP1/2 origin.

We then asked whether the reconstitution of ΔclAP1/2 mice with mixed BM chimeras would be enough to stop the loss of γδT17 cells or not. To this end, we irradiated ΔclAP1/2 mice that were at least 12 weeks old, to ensure host mice lacked γδT17 cells, and reconstituted them with either wildtype (CD45.1) or a mixture of 1:1 wildtype (CD45.1) and ΔclAP1/2 (CD45.2) BM cells (Fig 6A). In both instances we found that all γδT17 cells in the pLNs were from wildtype (CD45.1) BM; conversely, CD27+ γδ T cells were either equally split into CD45.1 and CD45.2 components in mice that had received mixed BM chimeras or only CD45.1 cells in mice that had received wildtype cells only (Fig 6B). In the siLP, almost all γδ T cells came from wildtype (CD45.1) BM and expressed various levels of Tbet and some RORγt (Fig 6C). Although ILC3 that were of both wildtype (CD45.1) and ΔclAP1/2 (CD45.2) origin could be found in the siLP, wildtype ILC3 cells were more abundant than ILC3 cells from ΔclAP1/2 BM (Fig 6D). Thus, the presence of wildtype hematopoietic cells in ΔclAP1/2 mice is not sufficient to reverse the loss of γδT17 cells lacking clAP1 and clAP2 but is sufficient to reverse the loss of ILC3 cells.

As γδT17 cells are generated perinatally in the thymus, we thus reasoned that γδT17 cells generated after BM chimera reconstitutions might be different from γδT17 cells that egress the neonatal thymus. To address this issue, we purified γδ T cells from the thymi of 1-day old Ctrl, ΔclAP1, ΔclAP2 or ΔclAP1/2 mice and transferred them to RAG1−/− recipient mice (Fig 7A). After 12 weeks we analyzed the pLNs and cLP and found that γδT17 and CD27+ γδ T cells could be recovered from the pLNs of RAG1−/− mice (Fig 7B) but again only Tbet+ γδ T cells could be found in the cLP, regardless of the donor strain (Fig 7C).
Taken together our data show that γδT17 cells that lack cIAP1 and cIAP2 are intrinsically
defective in their response to an extrinsic stimulus that is altered in the ΔcIAP1/2 mice.

Conversely, ILC3 cells were intrinsically less dependent on the presence of cIAP1 and cIAP2 as
they could be rescued by the presence of wildtype hematopoietic, stromal compartment or both.

**RORγtcre cIAP1f/f cIAP2−/− mice are not resistant to IMQ-psoriasis.**

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

We then sought to assess how the ΔcIAP1/2 mice would respond to IMQ-induced
inflammation in the presence of transcriptionally altered γδT17 cells. To this end, we applied
IMQ on the ear skin of 4 weeks old mice of all 4 genotypes and again assessed the
inflammatory response. We found that the epidermal thickening in the ear skin of ΔcIAP1/2 mice
was comparable to that of WT mice (Fig 8A). Further, we found that both in the skin and the
lymph nodes, γδT17 cells from ΔcIAP1/2 mice failed to expand in response to IMQ-inflammation
and were significantly less abundant than in the IMQ-treated control mice. Conversely, CD4+ T
cells in skin of IMQ-treated ΔcIAP1/2 mice were significantly more abundant than in control mice
although there was no significant difference in the lymph nodes (Fig 8B). Functionally, we found
that γδT17 cells from IMQ-treated ΔcIAP1/2 produced significantly less IL-17A in the lymph
nodes or skin, compared to IMQ-treated control mice; instead, CD4+ T cells from ΔcIAP1/2
produced copious amounts of IL-17A in both organs compared to controls (Fig 8C-D). Likewise,
lymph nodes γδT17 deficient in cIAP1 and cIAP2 failed to produce any IL-22 following IMQ-
psoriasis, while the CD4+ T cells in the same mice produced significantly more IL-22 than in the
IMQ-treated control mice (Fig 8C); intriguingly, IL-22 production by γδT17 in the skin of IMQ-
treated ΔcIAP1/2 was comparable to that of control mice, yet still there was significantly more
IL-22 produced by the CD4+ T cells in the skin of ΔcIAP1/2 mice following IMQ-treatment,
compared to controls (Fig 8E).

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

Discussion

Herein we showed that cIAP1 and cIAP2 are required for the adult homeostasis of γδT17
cells in peripheral lymph nodes, skin and gut lamina propria. Although cIAP1 and cIAP2 were
not required for the thymic development of γδT17 cells, mice that lacked cIAP2 and conditionally
lacked cIAP1 in RORγt expressing cells suffered progressive reduction in γδT17 cells in the
lymph nodes during late neonatal and adult life. Further, lack of cIAP1 and cIAP2 correlated with
a downregulation of RORγt and cMaf in γδT17 cells that preceded the loss of γδT17 cells. We
further show that the both cell intrinsic and extrinsic factors contribute to the decreased
abundances of γδT17 cells in adult RORγt cre cIAP1^{f/f} cIAP2^{−/−} mice. Finally, we show that adult
RORγt cre cIAP1^{f/f} cIAP2^{−/−} mice were not protected against IMQ-induced skin inflammation
despite the severe reduction in γδT17 in these mice and that the presence of functionally
defective γδT17 cells in younger RORγt cre cIAP1^{f/f} cIAP2^{−/−} was not sufficient to inhibit IMQ-
induced psoriasis.

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

We have previously shown that antagonization of cIAP1 and cIAP2 in Th17 cells
downregulates Il17a and Maf through the non-canonical NF-κB transcription factors RelB and
p52 (Rizk et al, 2019). Thus, it is plausible that a similar mechanism exists in γδT17 cells; as
such, the lack of cIAP1 and cIAP2 in γδT17 activates the non-canonical NF-κB, which
downregulates Maf expression. Consequently, Rorc is downregulated as cMaf is required to
maintain its expression in γδT17. Eventually, such cells would progressively perish as cMaf and
RORγt are required for the homeostasis of γδT17 (Barros-Martins et al., 2016; Zuberbuehler et
al., 2019).

Nonetheless, several outstanding questions remain to be resolved in such model.
Specifically, the persistence of expression of γδT17 surface markers (CCR6+ CD44hi) despite
the significant downregulation of RORγt and cMaf in cIAP1 and 2 deficient γδT17 cells is
intriguing. Furthermore, such downregulation of RORγt and cMaf occurred in the periphery but
not in the thymus and started at approximately 3 weeks of age. Likewise, the intrinsic and
extrinsic factors contributing to the loss of γδT17 in the cIAP1/2 double deficient mice remain
unresolved.

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Materials and methods

Mice

All animals were bred and maintained in-house at DTU health tech with the approval of the Danish animal experiments inspectorate. cIAP1<sup>−/−</sup> and cIAP1<sup>−/−</sup> cIAP2<sup>−/−</sup> mice were provided by Prof. W. Wei-Lynn Wong at the University of Zurich, Switzerland with the permission of Prof. John Silke, VIC Australia. RORγt<sup>CRE</sup> mice were provided by Prof. Gerard Eberl at Pasteur Institute, Paris, France.

Cell culture media and buffers

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

Lymphocyte isolation from mouse organs

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

IMQ-induced psoriasis

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

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

- anti-IL-17A (TC11-18H10; BV786 and PE), anti-IFNγ (XMG1.2; PE-Cy7 and APC), anti-IL-22 (1H8PWSR; PE), anti-cMAF (symOF1; PE and Percp-Cy5.5; 5 μL/test), anti-CD4 (GK1.5; BUV395), anti-TCRγδ (GL3; BV421), anti-CD27 (LG.3A10; PE-Cy7), anti-CCR6 (140706; Alexa Fluor 647), anti-CD44 (1M7; V500), anti-CD19 (6D5; FITC), anti-TCRβ (H57-597; APC-eFlour780), anti-CD3e (BM10-37, PeCF594 and PE), anti-Tbet(4B10; PeCy7), anti-CD8 (53-6.7; FITC), anti-Vγ5 (536; FITC), anti-Vγ4(UC3-10A6; Percp-eFlour710), anti-GATA3(TWAJ; Percp-eFlour710; 1:30), anti-CD45(30-F11;PE and V500) and anti-RORγt (B2D; APC).

**Retroviral infection of neonatal γδT17 cells.**

First, pooled thymi from one-day old wildtype mice were isolated and crushed against 70 μm filter to prepare single cell solution. Subsequently, γδT17 cells were enriched by magnetic depletion of CD4+ , CD8+ , TCRβ+ and CD27+ cells as follows: total thymocytes were re-suspended in MACS buffer at 1e8 cells/ml containing 50 μL/ml normal rat serum and 1:200 biotin labelled anti- CD4+, CD8+, TCRβ+ and CD27+ antibodies; the cells were incubated for 10 minutes at room temperature and then incubated with 75 μL/ml EasySep RaphidSphere streptavidin beads (#50001) for 2.5 minutes then transferred to EasySep magnet for 2.5 minutes. The non-bound fraction was collected by decantation and centrifugated for 5 minutes at 400g at 4°C. Approximately 2e5 of the enriched γδT17 cells were then cultured overnight with 20 ng/ml recombinant IL-7 in round-bottom 96-well tissue culture plate. The cells were then spin-infected by adding 4 μL of the desired retrovirus and 5 μg Retronectin (TAKARA) and centrifugation at 1800 rpm for 30 minutes at 32°C with deceleration set to 0. Subsequently, the cells were incubated for 90 minutes at 32°C before transfer to 37°C incubator. Recombinant IL-7
at 20 ng/ml was again added to the cultures after 48 hours and the cells were FACS sorted 24 hours later.

Transfer of neonatal γδ T cells to RAG1<sup>−/−</sup> hosts.

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

Bone marrow chimeras

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

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

Figures
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 RORγtCRE strain with cIAP1<sup>fl/fl</sup> or cIAP1<sup>fl/fl</sup>cIAP2<sup>−/−</sup> strains; the resulting 4 strains were as follows RORγtCRE cIAP1<sup>fl/fl</sup> (Cre−: ctrls, Cre+: ΔcIAP1) and RORγtCRE cIAP1<sup>fl/fl</sup>cIAP2<sup>−/−</sup> (Cre−: ΔcIAP2, Cre+: ΔcIAP1/2). (B) Flow cytometric analysis plots (left) and quantification (right) of TCRγδ<sup>+</sup> populations in the pLNs of all 4 strains. γδT17 cells were identified as CD44<sup>hi</sup> CD27<sup>−</sup> TCR γδ<sup>+</sup>. (C) Flow cytometric analysis plots (left) and quantification (right) of TCRγδ<sup>+</sup> and TCRβ<sup>+</sup> in the skin of all 4 strains. In the skin, γδT17 cells were identified as CD45<sup>+</sup> Vγ5<sup>−</sup> CD3<sup>+</sup> TCRγδ<sup>+</sup>. In graphs, each symbol represents a mouse and line the mean. **p < 0.01, ****p < 0.0001 using Mann-Whitney test.
Figure 2

A

B

C
Figure 2. cIAP1 and cIAP2 are required for the homeostasis of intestinal RORγt+ Tbet+ γδ T and ILC3 cells.

Flow cytometric analysis plots (left) and quantification (right) of TCRγδ+ populations in the siLP (A) or cLP (B) of all 4 strains. γδT cell populations were identified as CD45+ CD3+ TCRβ− TCRγδ+. (C) Flow cytometric analysis plots of (left) and quantification (right) of ILC3 cells in the siLP of all 4 strains. ILC cells were identified as CD45int CD3− CD127+ TCRγδ− TCRβ− cells. In graphs, each symbol represents a mouse and line the mean. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 using Mann-Whitney test.
Figure 3. cIAP1 and cIAP2 are not required for thymic development of γδT17 cells but are required for cMaf and RORγt expression during neonatal and adult life.

(A) Numbers of γδT17 cells in the day1 (d1) thymi of all 4 strains (gated as in Fig1). (B) Flow cytometric analysis of RORγt and cMaf expression in γδ T cells from d1 thymi of all 4 strains. (C) Numbers of γδT17 cells in the pLNs at the indicated timepoints. (D) analysis of γδ T cell populations from d21 pLNs of the indicated strains, while arrows show RORγt and cMaf expression within γδT17 cells compartment. (E) frequency of RORγt and cMaf expression in γδT17 in the pLNs of 12-weeks old from all strains. In graphs each symbol represents a mouse and line the mean. *p < 0.05 using Mann-Whitney test.
Figure 4. Activation of the non-canonical NF-κB pathway downregulates the expression of Maf in γδT17 cells.

(A-B) RT-qPCR analysis of the indicated genes in γδT17 cells that were infected with empty retrovirus (pMIG) or retrovirus expressing RelB. Only GFP+ cells (retrovirus infected cells) were FACS sorted and analyzed. In graphs each symbol represents an independent experiment.
Figure 5

(A) schematic representation of the experimental design whereby sub-lethally irradiated wildtype hosts (CD45.1/45.2) were reconstituted with 1:1 mixture of WT (CD45.1) and ΔcIAP1/2 (CD45.2) bone marrow. (B-C) Flow cytometry analysis of γδT cells in the pLNs and cLP of host mice after 12 weeks of reconstitution and indicating the frequency of WT, host, or ΔcIAP1/2 γδT17 and CD27+ γδ T cells. Graphs show frequency of WT, host, or ΔcIAP1/2 in γδ T cell populations in pLN of host mice amalgamated from 2 independent experiments. (D) Flow cytometry analysis of ILC3 cells in the cLP of host mice. Graph show frequency of WT, host, or ΔcIAP1/2 of all ILC3 cells in cLP of host mice amalgamated from 2 independent experiments. In graphs each symbol represents a mouse and line the mean.

Figure 5. Cell extrinsic signals mediate the loss of cIAP1 and cIAP2 double deficient γδT17 and ILC3 cells in ΔcIAP1/2 mice.
Figure 6

A

ΔclAP1/2

900 rad

CD45.1 (WT)

CD45.2 (ΔclAP1/2)

12 weeks

Analyzer

Bone marrow 1:1 mix

B

CD44

95.3

CD27

14.2

80.0

56.0

44

CD45.1

CD45.2

% of CD44+CD27- γδ T cells

Donor: WT

WT + ΔclAP1/2

Donor: WT

WT + ΔclAP1/2

C

pregated on CD3+ TCRyβ+

89.5

10

CD45.1

CD45.2

% of γδ T cells in sLP

Donor: WT

WT + ΔclAP1/2

Donor: WT

WT + ΔclAP1/2

D

RORγt

GATA3

36

72

25

CD45.1

CD45.2

% of ILC3 cells in sLP

Donor: WT

WT + ΔclAP1/2

Donor: WT

WT + ΔclAP1/2
Figure 6. Reconstitution of ΔcIAP1/2 mice with wildtype hematopoietic system rescues the loss of ILC3 cells but not γδT17 cells.

(A) schematic representation of the experimental design whereby sub-lethally irradiated ΔcIAP1/2 (CD45.2) were reconstituted with either WT (CD45.1) or 1:1 mixture of WT (CD45.1) and ΔcIAP1/2 (CD45.2) bone marrow. Flow cytometry analysis of γδT cells in the pLNs (B) and siLP (C) of host mice after 12 weeks of reconstitution and indicating the frequency of WT or ΔcIAP1/2 γδT17 and CD27+ γδT cells. Graphs show frequency of WT or ΔcIAP1/2 in γδ T cell populations in pLN and siLP. (D) Flow cytometry analysis of ILC3 cells in the cLP of host mice. Graph show frequency of WT or ΔcIAP1/2 of all ILC3 cells in siLP. In graphs each symbol represents a mouse and line the mean.
Figure 7

(A) schematic representation of the experimental design whereby γδ T cells were enriched from the thymi from one-day old mice of all 4 strains and i.v. injected into RAG-/- hosts. (B) Flow cytometry analysis of γδT cells in the pLNs of host RAG-/- mice at 12 weeks after transfer of the γδT cells from the indicated strain. Graphs show frequency of recovered γδT17 or CD27+ γδT cells.

Figure 7. Neonatal γδT17 lacking cIAP1 and cIAP2 can reconstitute RAG-/- hosts. (A) schematic representation of the experimental design whereby γδ T cells were enriched from the thymi from one-day old mice of all 4 strains and i.v. injected into RAG-/- hosts. (B) Flow cytometry analysis of γδT cells in the pLNs of host RAG-/- mice at 12 weeks after transfer of the γδT cells from the indicated strain. Graphs show frequency of recovered γδT17 or CD27+ γδT cells.
cells of total γδT cells. In graphs each symbol represents a donor mouse and line the median.

Data is amalgamated from 3 independent experiments. (C) Flow cytometry analysis of γδT cells in cLP of host RAG-/- mice at 12 weeks after transfer of the γδT cells from the indicated strain.
Figure 8. cIAP1 and 2 double deficient γδT17 cells are functionally defective and do not respond to IMQ-induced psoriasis.

(A) Representative light microscopy images (right) indicating ear skin sections stained for H&E before and after IMQ-induced psoriasis in the indicated strains (left). Quantification of epidermal thickness in μm.

B. Number of γδT17 cells in pLN.

C. Percentage of IL-17A+ γδT17 cells.

D. Percentage of IL-22+ γδT17 cells.

E. Percentage of IL-22+ CD4+ T cells.
thickening from H&E stained sections. (B) Numbers of γδT17 and CD4+ TCRβ+ cells in the pLN or skin of all strains of IMQ treated or control mice. In the pLN, γδT17 were identified as CD44hi CD27- TCRγδ+, while in the skin, they were identified as CD45+ Vγ5− CD3+ TCRγδ+.

Quantification of IL-17A (C, D) or IL-22 (C, E) production by γδ T and CD4 T cells in the pLN (C) or skin (E) in control or IMQ-treated mice of all strains. In graphs, each symbol represents a mouse and line the mean. Data is pool of 3 experiments. *p < 0.05, **p < 0.01 using Mann-Whitney test.