

Investigating the role of embryonic $\gamma\delta$ T cells and their T cell receptors at barrier sites

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*** $\alpha\beta$ T cells replacing dermal and epidermal $\gamma\delta$ T cells in *Tcrd*^{-/-} mice express an MHC-independent TCR-repertoire**

Christoph Binz, Anja Bubke, Inga Sandrock, Immo Prinz**

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The manuscript is peer reviewed and its current status at the time of submission of this thesis is accepted with minor alterations at European Journal of Immunology.

C.B. conducted the phenotypical flow cytometric analysis of newborn or adult WT and *Tcrd*^{-/-} mice, proofed the equality of TCR-expression pattern between epidermal and dermal cells of WT and *Tcrd*^{-/-} mice thus contributing to Fig.1, Fig.2 and Fig.3. C.B. performed the flow cytometric sorting of the different populations subjected to NGS TCR-repertoire analysis, the following sample-preparation by RNA-isolation and subsequent PCRs (except the sorting and preparation of CD103⁺ and CD103^{hi} dermal $\alpha\beta$ T cells from *Tcrd*^{-/-} mice) as well as the computational analysis of all sequencing-data thus contributing to Fig.4, Fig.5 and Fig.6. C.B. also interpreted the data and generated the manuscript.

***Interleukin-17 is disease promoting in early stages and protective in late stages of experimental periodontitis**

Anneke Wilharm*, **Christoph Binz***, Inga Sandrock, Francesca Rampoldi, Stefan Lienenklaus, Eva Blank, Andreas Winkel, Abdi Demera, Avi-Hai Hovav, Meike Stiesch, Immo Prinz**

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The manuscript is submitted for publication.

C.B. conducted the computational analysis of qPCR and μ CT-imaging, the generation of HE-stained sections and microscopic analysis of the HE-staining of the mice subjected to the experimental model of ligature-induced periodontitis. Therefore, C.B.

contributed to Fig.3, Fig.4 and Fig.5. C.B. also interpreted the data and generated the manuscript.

Genetic models reveal origin, persistence and nonredundant functions of IL-17–producing $\gamma\delta$ T cells

Inga Sandrock, Annika Reinhardt, Sarina Ravens, **Christoph Binz**, Anneke Wilharm, Joana Martins, Linda Oberdörfer, Likai Tan, Stefan Lienenklaus, Baojun Zhang, Ronald Naumann, Yuan Zhuang, Andreas Krueger, Reinhold Förster, and Immo Prinz**

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C.B. conducted the analysis of HE-stained ear-skin sections for evaluation of the epidermal thickness of the different mouse strains that were subjected to the experimental model of Imiquimod-induced psoriasis thus contributing to Fig.5.

Influenza A virus-induced thymus atrophy differentially affects dynamics of conventional and regulatory T-cell development in mice

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C.B. conducted the NGS-sequencing and computational analysis of TCR repertoire from T cells sorted from mice after IAV-induced thymic atrophy thus contributing to Fig.7.

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1. SUMMARY

$\gamma\delta$ T cells are the first lymphoid cells egressing the thymus as effector T cells, homing preferentially to various epithelial sites and establishing tissue-resident populations with a tissue-specific V γ -usage. In their tissues of residency, they exert functions in tissue integrity, infection, inflammation or cancer.

Analysing an $\alpha\beta$ TCR expressing replacement-population of the $\gamma\delta$ TCR expressing epidermis-resident dendritic epidermal T cells in *Tcrd*^{-/-} mice by flow-cytometry and NGS analysis of their TCR-repertoire, revealed the embryonic heritage of those replacement-population and their consequently MHC-independent TCR-repertoire. This in combination with other reports about influences onto the DETC TCR-repertoire suggests that the TCR-specificity might be irrelevant for the development of a DETC-population, but important for the correct functional development and maintenance of the original DETC in the skin. Identifying a second MHC-independent $\alpha\beta$ T cell population in the dermis, these assumptions may extend to other embryonic-derived T cells.

The function of the gingival IL-17 producing V γ 6⁺ $\gamma\delta$ T cells in periodontitis was investigated applying an experimental model of periodontitis to mice. While those cells are known as the major IL-17 producers in the gingiva, at this very specific site of the body-barrier, it seems that other immune cells play more important roles during the course of inflammation. Interestingly, IL-17 might be rather protective in the long-term perspective by allowing fast bacterial clearance.

In summary, this study gives insights into the role of the TCR for embryonic-derived $\gamma\delta$ T cells and into the function of an embryonic-derived V γ 6⁺ $\gamma\delta$ T cell population in the gingiva during inflammation.

2. INTRODUCTION

2.1 Generation of TCRs and their ligand recognition

T cells bear a T cell receptor (TCR), composed of two TCR-chains, of which each is built genetically by variable-diversity-joining- (VDJ)-recombination (Davis et al., 1988), generating $\alpha\beta$ T cells or $\gamma\delta$ T cells. A Variable (*V*)-segment is connected to a Constant (*C*)-segment via either a Joining (*J*)-segment (in case of the β and γ -chain) or a Diversity (*D*)-segment and a *J*-segment (in case of the α and δ -chain). During this recombination-activating-gene (RAG) mediated process, the terminal deoxynucleotidyl transferase (TdT) adds random N-nucleotides at the connections between two segments, enhancing the diversity of the resulting TCR-repertoire. Most of the diversity can be found in the so-called complementarity-determining region (CDR)-3, which is the connecting region between the *V*- and the *C*-segment (Cabaniols et al., 2001).

$\alpha\beta$ T cells express a TCR α and a TCR β chain, forming an $\alpha\beta$ TCR, that is known to bind major histocompatibility (MHC)-molecules (Grusby et al., 1993). Those MHC-molecules present peptides, that were processed by a cell, as a peptide-MHC (pMHC)-complex. It might be recognized by an $\alpha\beta$ TCR from a T cell. This recognition plays a role in the thymic selection of CD4/ CD8 double-positive (DP) cells during their development. The thymic selection of $\alpha\beta$ T cells favours certain biochemical and physical properties of the CDR3 amino acids, since unselected TCR-repertoires display shorter CDR3 containing more charged, hydrophobic or strongly interacting amino acids. Especially, cysteine is almost completely absent from CDR3 of selected MHC-restricted repertoires (J. Lu et al., 2019). The afore mentioned characteristics of unselected repertoires renders the respective TCRs stronger interacting with pMHC-complexes and thus self-reactive (Stadinski et al., 2016). Those self-reactive T cells are counter-selected. However, also MHC-independent repertoires have a certain inherent (Harsha Krovi et al., 2019) bias to recognize pMHC-complexes, to which especially a tryptophane at a certain position in the CDR3, the so-called P6-7 doublet (Stadinski et al., 2016), contributes.

The TCR-repertoire of $\gamma\delta$ T cells is generated in principle by the same mechanisms, using TCR γ and TCR δ segments (Davis et al., 1988). An important difference is that an important part of the $\gamma\delta$ T cells develops prior to onset of TdT, which is around

postnatal day 3 to 5 (Bogue et al., 1992), thus shows a very limited or no diversity and that $\gamma\delta$ T cells are not restricted to antigen presentation on MHC (Y. H. Chien et al., 1996; Deseke et al., 2020). Furthermore, it is not clear to which extent $\gamma\delta$ T cells are selected. Therefore, an important field of ongoing research is the search for direct $\gamma\delta$ TCR ligands. As known for $\alpha\beta$ TCRs, some MHC- or MHC-like molecules are reported to be ligands of $\gamma\delta$ TCRs in mice and humans, but the interaction seems to be independent from specific loaded peptides and thus potentially not via the same regions of classical pMHC-TCR interaction (Deseke et al., 2020). In mice, prominent examples are MHC-II I-E (Matis et al., 1989), MHC-I H-2 (Bluestone et al., 1988), MHC-related protein 1 (MR-1) (Le Nours et al., 2019) or T10/T22 (Crowley et al., 2000). Apart from that, a more IG-like recognition of antigens is observed, since several proteins and small molecules have been reported to be $\gamma\delta$ TCR ligands in mice and humans, as for example PE (Zeng et al., 2012) or the insulin peptide B:9-23 in mice (Deseke et al., 2020; Kemal Aydintug et al., 2014; L. Zhang et al., 2010). A more complex way of antigen recognition is the sensing of phosphoantigens intracellularly bound to BTN3A1, that interacts with BTN2A1, which in turn directly binds to the predominant human blood $\gamma\delta$ T cells (Karunakaran et al., 2020; Rigau et al., 2020), expressing semi-invariant V γ 9V δ 2 TCRs. Moreover, heterodimerising butyrophilin-like (BTNL) 3 and BTNL8 were shown to be relevant for the activation of human intestinal V γ 4⁺ $\gamma\delta$ T cells, analogously, heterodimerising Btl1 and Btl6 were shown to be important for the development of mouse V γ 7⁺ intestinal IELs (Di Marco Barros et al., 2016; Vantourout et al., 2018) and Skint1, a butyrophilin-homologue in mice, has gained attention as a potential ligand of mouse dendritic epidermal T cells (DETC), since it was reported to be indispensable for establishment of their characteristic almost clonal TCR-repertoire (Lewis et al., 2006).

2.2 $\gamma\delta$ T cells and their development in embryonic waves

It is well known, that the immune system of humans and animals tries to distinguish between foreign and self, dangerous and non-dangerous. The most prominent interface of foreign and self is the outer body barrier, covered usually by epithelia. In mice those epithelia are populated by a majority of $\gamma\delta$ T cells (Vantourout et al., 2013), while in secondary lymphoid organs $\gamma\delta$ T cells represent only a minority of all T cells. The mouse γ -locus contains seven V-segments (Clark et al., 1995; Garman et al., 1986; Hayday, 2000; Heilig et al., 1986). $\gamma\delta$ T cells are known to develop as the first

lymphocyte lineage subsets in the embryonic phase (Havran et al., 1988), egress from thymus in waves expressing discrete V γ -segments and tend to display a tissue-specific V γ -segment usage (table 1).

Table 1: Developmental waves of thymus egressing mouse $\gamma\delta$ T cells

V γ -usage	egress	tissue	cytokine profile	TCR-repertoire
V γ 5	embryonic	epidermis	IFN γ or IL-17	invariant
V γ 6	embryonic	oral epithelia, dermis, uterus, lymph nodes, uterus	IL-17	semi-invariant
V γ 7	embryonic	intestinal epithelium	IFN γ	semi-invariant
V γ 1V δ 6.3	perinatal	spleen, liver	IFN γ and IL-4	invariant
V γ 1	perinatal	blood, lymphoid organs	IFN γ	diverse
V γ 4	perinatal	dermis lymphoid organs, blood	IFN γ or IL-17	diverse
V γ 1, V γ 4	postnatal	periphery	naïve	diverse

The first wave are the dendritic epidermal T cells (DETC), that egress from thymus around day E15, express an almost clonal V γ 5V δ 1-TCR and populate the mouse epidermis (Asarnow et al., 1988; McConell et al., 1989; Steiner et al., 1988). Around day E17, V γ 6V δ 1-TCR expressing $\gamma\delta$ T cells, that appear in the skin dermis (O'Brien et al., 2015), the entheses (Reinhardt et al., 2016), the oral epithelia (Wilharm et al., 2019), tongue, the testis (Wilharm et al., 2021) or the uterus (Itohara et al., 1990) of mice, leave the thymus. Also, the V γ 6V δ 1-TCR expressing cells show a low-diversity TCR-repertoire. V γ 7⁺ $\gamma\delta$ T cells exit the thymus around birth to home especially to the intestinal epithelium (Chennupati et al., 2010; T. Lin et al., 1993; Taguchi et al., 1991). V γ 1⁺ and V γ 4⁺ $\gamma\delta$ T cells exit the thymus around birth and afterwards. While most V γ 1⁺ and V γ 4⁺ $\gamma\delta$ T cells have a diverse TCR-repertoire and locate to the blood or lymphoid tissues (Carding et al., 2002; Itohara et al., 1990; Vermijlen et al., 2014) and are known to be circulating between epithelia and lymphoid organs (McKenzie et al., 2017; Nakamizo et al., 2015), a subset of invariant V γ 1⁺V δ 6.3⁺ cells homes especially to spleen and liver (Azuara et al., 1997; Grigoriadou et al., 2003; Vicari et al., 1996).

The first waves of $\gamma\delta$ T cells, that develop solely in the embryonic thymus (Carding et al., 2002; Prinz et al., 2013) seem to show a genetically pre-committed developmental Behavior (Fiala et al., 2020) in that they develop prior to postnatal onset of TdT (Bogue et al., 1992) and thus display no N-nucleotide insertions and no or very low junctional diversity and have an intrinsic IL-17-production ($\gamma\delta$ T17) fate. This initial $\gamma\delta$ T17 fate can

be switched towards an interferon (IFN)- γ -production ($\gamma\delta$ T1) fate by encountering strong TCR signals (Jensen et al., 2008; Muñoz-Ruiz et al., 2016; Spidale et al., 2018; Sumaria et al., 2017). Accordingly, $\gamma\delta$ T17 have been reported to retain a limited amount of plasticity for expression of both IL-17 and IFN- γ (Schmolka et al., 2013, 2018). The interaction of medullary thymic epithelial cells (mTEC) with $\gamma\delta$ T cell precursors seems to be important for the development of $\gamma\delta$ T cells, since $\gamma\delta$ T cell precursors drive expression of autoimmune regulator (Aire) and Skint1 by mTEC (Inglesfield et al., 2019) and vice versa, defects in maturation of mTEC influence (Briseño et al., 2017; Mair et al., 2015; Riemann et al., 2017; Roberts et al., 2012) $\gamma\delta$ T cell development. Deficiency in filaggrin, which is an epidermal structural protein that is also expressed in the thymic medulla, lead to increased development of $\gamma\delta$ T17 cells (Jee et al., 2018, 2020).

Since $\gamma\delta$ T cells egress the thymus as effector T cells, they were shown to diverge transcriptomically early during their development. Three clusters that have been identified represent DETC precursor, $V\gamma 4^+$ and $V\gamma 6^+$ $\gamma\delta$ T17 cells and $V\gamma 1^+$, $V\gamma 4^+$, $V\gamma 7^+$, and invariant $V\gamma 1^+V\delta 6.3^+$ $\gamma\delta$ T1 (Narayan et al., 2012). $\gamma\delta$ T17 cells and $\gamma\delta$ T1 can be distinguished by their CD27 expression, since $\gamma\delta$ T1 are characterized as CD27 $^+$, and $\gamma\delta$ T17 as CD27 $^-$ (Ribot et al., 2009). Apart from the embryonic waves of $\gamma\delta$ T cells, less differentiated, naïve $\gamma\delta$ T cells egress the thymus postnatally until adulthood. Both embryonic and postnatal $\gamma\delta$ T cells are thought to derive from different hematopoietic precursors (Buus et al., 2017; Ramond et al., 2014).

Since IL-17 is known as a highly pro-inflammatory cytokine and $\gamma\delta$ T cells were reported to express natural killer (NK) cell receptors and natural cytotoxicity receptors (NCR) (Hudspeth et al., 2013; Stewart et al., 2007; Von Lilienfeld-Toal et al., 2006), $\gamma\delta$ T17 cells can be described as the first players in place in the epithelia, the first facing the pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) and reacting immediately (Hamada et al., 2008; O'Brien et al., 2009; Shibata et al., 2007; Zeng et al., 2012) by inducing an inflammation by IL-17 production. Therefore, and due to their preferential location at epithelial tissues, they seem to be linkers of innate and adaptive immunity and were termed to be the “first line of defence” (Y. H. Chien et al., 2014).

2.3 Dendritic epidermal T cells

The main epidermal mouse T cell population was called “dendritic epidermal T cells” (DETC) due to their dendritic morphology (Steiner et al., 1988). The whole population expresses an almost clonal V γ 5V δ 1 TCR with no N-nucleotide additions and thus no junctional diversity (Itohara et al., 1990). This TCR of the DETC is known to be concentrated in so-called phosphotyrosine-rich aggregates located on projections (PALP) at the tips of their dendrites, via which they contact the tight-junctions between the keratinocytes (Chodaczek et al., 2012). These contacts immobilize the DETC, whose cell-body locates to the basal layers of the epidermis. The DETC, as well as epidermal Langerhans-cells (LC), were reported to maintain a non-random distribution in the epidermis leading to a narrowed range of minimum-distances to their neighbours, which they actively establish, maintain and re-establish after skin-injury (S. Park et al., 2021).

The DETC arise from yolk sac haematopoiesis (Gentek et al., 2018). Therefore, their hematopoietic precursors invade the thymus between embryonic day E7 and E10. Five days later, around E15, DETC appear in the embryonic skin (Haas et al., 2012; Prinz et al., 2013). At that timepoint V γ 5V δ 1 TCR expressing $\gamma\delta$ T cells make up more than 70% of all $\gamma\delta$ T cells in the embryonic thymus (Lewis et al., 2006), since the embryonic TCR rearrangement is known to take place time-restricted and sequentially according to the genomic position of the different TCR gene segments (Asarnow et al., 1993; Itohara et al., 1993). Already until day E17, the fraction of V γ 5V δ 1 TCR expressing T cells goes down to 50%, since the rearrangement of other gene-segments, as V γ 6, begins (Lewis et al., 2006). However, the V γ 6⁺ T cells are known to express exactly the same δ -chain as the V γ 5V δ 1 TCR. Moreover, the CDR3 γ and CDR3 δ of the V γ 6V δ 1 TCR is the same as the one from V γ 5V δ 1 TCR (Lewis et al., 2006).

The thymic precursors of DETC display an activated phenotype, including the expression of CD122 (Kawai et al., 1998; Van Beneden et al., 2002) and CD45RB and downregulation of CD62L and CD24 (Lewis et al., 2006). Upon activation, those cells start to express thymus-egress receptors as sphingosine-1-phosphate receptor 1 (S1P1) and important skin-homing receptors of DETC as C-C chemokine receptor type (CCR)-10 (Y. Jin et al., 2010; Xiong et al., 2004).

The DETC-development, as the overall development of $\gamma\delta$ T cells, is dependent on IL-7 (S. Y. Park et al., 1995; Ye et al., 2001). Apart from this, they are dependent on IL-15 (De Creus et al., 2002; Edelbaum et al., 1995; Wang et al., 2017) and IL-2 (Kawai et al., 1998). Developmentally, DETC rely on the transcription factor Runx3, since Runx3 deficiency leads to a complete lack of DETC (Woolf et al., 2007). Runx3 regulates the expression of IL2R β and CD103, known to be important for the DETC skin homing (Schön et al., 2002) and for the structure of their PALPs (Chodaczek et al., 2018).

A molecule that was reported to be indispensable for the establishment of an almost clonal DETC-population in the epidermis is “selection and upkeep of intraepithelial T cells protein” (Skint)-1 (Skint)-1 (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006). Skint1 is a butyrophilin-homologue in mice, that is expressed in the thymus and in the epidermis of mice. In humans or chimpanzees, the Skint1 is non-functional due to in-frame stop-codons and both do not show comparable $\gamma\delta$ T cell populations in the skin. Rats and cows in contrast, as mice, express Skint1 orthologs and interestingly show highly focussed epidermal $\gamma\delta$ T cell populations (Boyden et al., 2008). The absence of Skint1 in mice induces a diversification of the DETC TCR-repertoire (Lewis et al., 2006). A diversification of the DETC repertoire is also observed upon mTEC-specific deficiency of NF- κ B inducing kinase (NIK) (Mair et al., 2015), since NF- κ B signalling is required for mTEC maturation and Skint1-expression (Briseño et al., 2017; Riemann et al., 2017; Roberts et al., 2012). Therefore, it was proposed that Skint1 might positively select the V γ 5V δ 1 TCR expressing DETC by interacting with this TCR or another surface molecule on the DETC-precursors (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006; Xiong et al., 2004). Skint1 was also reported to influence the functional development of DETC by suppressing their IL-17 production capacity and promoting their IFN- γ production phenotype (Turchinovich et al., 2011). However, the mechanism leading to the appearance of DETC is restricted to the embryonic phase, since no DETC development after tamoxifen-induced expression of RAG in adult *Rag*-inducible mice (Almeida et al., 2015), that do not express RAG during their embryonic phase, is observed.

Starting from day E15 the DETC precursors egress from the thymus during a short time-window to home to the epidermis. The mechanism of functional development and

skin-homing was reported to be dependent on TCR-activation (Kadlecek et al., 1998; Kawai et al., 1998; Lauritsen et al., 2009; Mallick-Wood et al., 1996). However, still CD3⁺ cells were detected in the epidermis of Spleen-tyrosine-kinase (SYK)-deficient mice (Mallick-Wood et al., 1996). Similarly, deficiency in IL-2-inducible T cell kinase (ITK), an important downstream target of TCR-signaling (Andreotti et al., 2010), leads to a block in thymic egress of DETC by inhibiting S1P1 and CCR10 expression (Xia et al., 2010). However, ITK is also a target downstream of cytokine receptor signalling (Berg et al., 2005; Fischer et al., 2004; Sahu et al., 2008; Takesono et al., 2004; Woods et al., 2001).

In the epidermis, the DETC are maintained by clonal self-renewal during adulthood (Gentek et al., 2018; Jameson et al., 2004) in a TCR-dependent manner (B. Zhang et al., 2015). They play a role in wound-healing (Jameson et al., 2002). Upon skin-injury, the ligand of the DETC-TCR was reported to become transiently expressed by keratinocytes at the wound-edges and the interaction of the DETC with this ligand was shown to be indispensable for their activation (Komori et al., 2012). DETC are known to produce insulin-like growth factor (IGF)-1 (Sharp et al., 2005), fibroblast growth factor (FGF)-7 (Boismenu et al., 1994), FGF10 (Jameson et al., 2002) and to round up in response to activation via their TCR (Jameson et al., 2002). Apart from providing growth-factors for wound-healing and ensuring tissue integrity, the activation induces proliferation of the DETC, so that the DETC-network re-establishes (S. Park et al., 2021). Vice versa, keratinocytes provide IL-15 (Edelbaum et al., 1995), on which the DETC rely for physiological function, development and IGF-1 production (Wang et al., 2017). Several co-stimulatory molecules of DETC have been described. Upon injury, Plexin-B2 is expressed by keratinocytes and interacts with CD100 (also Semaphorin-4D) on DETC. This leads to rounding-up and IL-2 secretion of DETC and to IL1 β -production by keratinocytes (Witherden et al., 2012). Furthermore, DETC express junctional-adhesion-molecule-like (JAML) (Witherden et al., 2010), that was reported to show important co-stimulatory functions on DETC. An important interaction is the one of NKG2D on DETC (Whang et al., 2009) with one of its keratinocyte expressed ligands as histocompatibility 60c (H60C), retinoic acid early inducible-1 (Rae-1) or mouse UL16-binding protein-like transcript-1 (Mult-1) (Nielsen et al., 2015; Yoshida et al., 2012) upon injury, which leads to IL-2 and IL-13 production by DETC (Strid et al., 2011), that induces keratinocyte proliferation. NKG2D also directly triggers the

cytotoxicity of DETC, since it can induce degranulation (Ibusuki et al., 2014). Thus, DETC show NKG2D and 2B4 dependent functions in anti-tumor responses (Girardi et al., 2003; Schuhmachers et al., 1995) and contact dermatitis (Mraz et al., 2020; Nielsen et al., 2015). Although DETC are mainly IFN- γ producing $\gamma\delta$ T cells (Turchinovich et al., 2011), a subset of the DETC was reported to produce IL-17 upon skin-injury (Nielsen et al., 2014). IL-17 deficiency in DETC leads to delayed wound-healing (MacLeod et al., 2013). Thus, DETC contribute to body-barrier integrity.

How the high clonality in the TCR-repertoire of the DETC is achieved was addressed in a variety of studies. It was reported, that the presence of the *Cy1* cluster in the DETC-precursors is indispensable for the appearance of a DETC population, since mice lacking the *Cy1* cluster were devoid of DETC, which could be restored by introducing a *Vy2-Jy1Cy1*-construct (Xiong et al., 2004). However, *Vy5*^{-/-} mice show DETC populations employing other *C*-segments than *Cy1* (Mallick-Wood et al., 1998). In both *Vy5*^{-/-} (Mallick-Wood et al., 1998) and *V δ 1*^{-/-} (Hara et al., 2000) mice, diversified DETC populations expressing other *Vy*- or *V δ* -segments appear. Those populations are of smaller size than the original one, but retain a certain degree of structural identity to the original DETC and are partially activatable by keratinocytes. Early induction of TdT leads to an initial diversification of the DETC TCR-repertoire, which refocuses with time towards the original *Vy5V δ 1* TCR (Aono et al., 2000). Similarly, *Skint1* deficiency, as described above, leads to a diversification (Lewis et al., 2006). In *Tcrd*^{-/-} mice, the epidermal niche is occupied by a diverse $\alpha\beta$ DETC population of smaller size than the original one (Jameson et al., 2004), that is not functional, since those mice display a delay in wound healing due to inability to be activated by keratinocytes (Jameson et al., 2004) and their dendrites cannot immobilize at the keratinocyte tight-junctions (Chodaczek et al., 2012).

No ligand of the DETC-TCR was identified so far. As described above, *Skint1* is indispensable for establishment of the characteristic almost monoclonal TCR-repertoire (Lewis et al., 2006) of the DETC and is thus discussed as a positive-selecting ligand. However, it was reported, that the activating DETC-TCR ligand is expressed transiently by keratinocytes upon injury (Komori et al., 2012). At the same time, the PALP-concentrated *Vy5V δ 1* TCR was reported to be necessary for immobilisation of the DETC-dendrites at the tight junctions (Chodaczek et al., 2012), suggesting that

they interact with a ligand here in steady state. Moreover, the PALPs show structural similarities to immunological synapses (IS), contain high levels of CD103 and lymphocyte function-associated antigen (LFA)-1 (Chodaczek et al., 2012) and, reminiscent of directional intracellular trafficking induced by IS, the dendrites were reported to serve as bidirectional conduits for intracellular cargo (Chodaczek et al., 2018). Overall, it seems that $\gamma\delta$ TCRs do not recognize a single ligand that activates them, as suggested by overall development in $\gamma\delta$ TCR ligand research (Deseke et al., 2020), but that they might have multiple ligands, of which potentially not all must be necessarily activating or fully activating, but might have functions in driving the $\gamma\delta$ T cells to their site of destination or, as proposed for the DETC-TCR (Sutoh et al., 2018), in homeostatic maintenance of the cells in the tissue (B. Zhang et al., 2015).

2.4 $\gamma\delta$ T17 cells

Around day E16 more and more embryonic $\gamma\delta$ T cell precursors rearrange $V\gamma$ -gene segments (Lewis et al., 2006; Prinz et al., 2013), of which the most frequent is the $V\gamma 6$ segment (Haas et al., 2012; Prinz et al., 2013). The resulting $\gamma\delta$ T cells are $\gamma\delta$ T17 cells that express an invariant $V\gamma 6V\delta 1$ TCR (Itohara et al., 1990). They egress from the thymus before birth (Haas et al., 2012) and home to diverse epithelial tissues (Itohara et al., 1990; O'Brien et al., 2015; Reinhardt et al., 2016; Wilharm et al., 2019, 2021). As described for DETC, their development is restricted to the embryonic phase (Haas et al., 2012; Prinz et al., 2013; Reinhardt et al., 2016). In the skin, they specifically home to the dermis, where they constitute a $V\gamma 6^+$ long-lived resident population that undergoes self-renewal (Y. hsiu Chien et al., 2013; Haas et al., 2012), as reported for several other epithelial sites (Tan et al., 2019; Wilharm et al., 2019). Those cells can be distinguished from the other large $\gamma\delta$ T17 population in the dermis, expressing more diverse $V\gamma 4^+$ TCRs, by their expression of SCART1 (Tan et al., 2019). The $V\gamma 4^+$ $\gamma\delta$ T cells mainly express SCART2 (Tan et al., 2019) and develop mainly postnatally. However, transcriptomically both populations are by far identical (Tan et al., 2019). A main difference of the $V\gamma 6^+$ $\gamma\delta$ T cells is that those express high levels of anti-apoptotic proteins, that ensure their persistence in the tissue, since they cannot be replenished during adulthood. Nothing is known about ligands of the $V\gamma 6V\delta 1$ TCR, albeit it was shown that the thymic cortical epithelium plays a role during their development and TCR-repertoire determination (Nitta et al., 2015) and it is commonly thought that it probably recognizes stress-induced self-antigens (Mukasa et al., 1999).

Phenotypically $\gamma\delta$ T17 cells are characterized by expression of high levels of CD44 as well as CCR6 and IL23R, distinguishing them from CD27, NK1.1 and CD122 expressing $\gamma\delta$ T1 cells (Haas et al., 2009; Jensen et al., 2008; Kisielow et al., 2008; Ribot et al., 2009; Shibata et al., 2008). Apart from IL-17A and IL17F (Sutton et al., 2012; Veldhoen, 2017) $\gamma\delta$ T17 cells were reported to express IL-22 (Blink et al., 2014; Sutton et al., 2009), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Blink et al., 2014; Lukens et al., 2012) or amphiregulin (Areg) (Krishnan et al., 2018).

The central transcription factor allowing IL-17 production by $\gamma\delta$ T cells, as well as Th17 $\alpha\beta$ T cells, is retinoic-acid-receptor-related orphan nuclear receptor (ROR)- γ t (Barros-Martins et al., 2016; X. Chen et al., 2019; Ribot et al., 2009). The transcription factor c-Maf has been reported to sustain ROR- γ t driven IL-17 expression (Zuberbuehler et al., 2019) and also HEB is known to be important for IL-17 effector fate (In et al., 2017; Ivanov et al., 2006; Lochner et al., 2008; Xu et al., 2009; F. Zhang et al., 2014; Zuberbuehler et al., 2019). Auxiliary transcription factors of Th17 cells, as Basic leucine zipper transcriptional factor ATF-like (BATF) and ROR- α (Ciofani et al., 2012), are not required in $\gamma\delta$ T17 cells (Barros-Martins et al., 2016), which rely developmentally on IL-7 and transforming-growth-factor (TGF)- β signalling (Do et al., 2010; Michel et al., 2012). In contrast Th17 cells require TGF β and IL-6 (Bettelli et al., 2006; Veldhoen et al., 2006) signalling. An important link between cytokine-receptor signalling from IL1R or IL23R and IL-17 production by $\gamma\delta$ T17 and Th17 (Ribot et al., 2010; Sutton et al., 2009; Veldhoen et al., 2006) is interferon-regulatory factor (IRF)-4 (Cai, Xue, Qin, et al., 2019), although it has been shown that IRF4 is not absolutely necessary for IL-17 production by $\gamma\delta$ T17 (Raifer et al., 2012). Apart from those master transcription factors for $\gamma\delta$ T17 cells, different subpopulations rely developmentally and/or functionally on specific other transcription factors. While V γ 4⁺ $\gamma\delta$ T17 cells were shown to rely on Sex-determining region Y-box (SOX)-4 (Malhotra et al., 2013) and SOX13 (Gray et al., 2013) developmentally and to engage signal-transducer-and-activator-of-transcription (STAT)-3 (Cai, Xue, Qin, et al., 2019) in the signalling for IL-17 production, V γ 6⁺ $\gamma\delta$ T17 cells engage the promyelocytic leukemia zink finger (PLZF) transcription factor (Y. Lu et al., 2015).

How exactly $\gamma\delta$ T cells are activated to produce IL-17 or IFN- γ in vivo is not fully clear, since the role of the TCR in this context is unclear. Besides CD27 (Ribot et al., 2010)

as a necessary costimulatory molecule on $\gamma\delta$ T1, CD137 (Lee et al., 2013) was reported to sustain expansion of $V\gamma 1^+$ $\gamma\delta$ T1. Molecules as CD30 (Sun et al., 2013), B- and T-lymphocyte attenuator (BTLA) (Bekiaris et al., 2013) and Programmed cell death protein (PD)-1 (Imai et al., 2015) are co-stimulatory for $\gamma\delta$ T17. The well-known co-stimulatory molecule CD28 is important for activation of both $\gamma\delta$ T17 and $\gamma\delta$ T1 (Ribot et al., 2012). Apart from that, $\gamma\delta$ T cells can also be activated for IL-17 production solely by cytokines, as IL-23 or IL-1 β (Cai, Xue, Quan, et al., 2019; Sutton et al., 2009; Wencker et al., 2014), produced by myeloid cells, and IL-7 (Michel et al., 2012) has been shown to be indispensable for $\gamma\delta$ T cell development. Moreover, IL-23 or IL-1 β have been reported to induce $\gamma\delta$ T17 maturation in adult peripheral $\gamma\delta$ T cells (Muschaweckh et al., 2017; Papotto et al., 2017). Additionally, several pattern-recognition receptors can be involved in rapid (Hamada et al., 2008; O'Brien et al., 2009; Shibata et al., 2007; Zeng et al., 2012) IL-17 production by those cells in place in their tissue of residency. Some toll-like receptors (TLR) or dectin-1 can be expressed by $\gamma\delta$ T17 and induce IL-17 expression (Martin et al., 2009).

As described above for the DETC, the exact role of the TCR in development and/or function of the $\gamma\delta$ T17 is not clear. Very strong TCR-signalling was reported to reduce c-Maf expression, thus inhibiting IL-17 production (Zuberbuehler et al., 2019). TCR-signalling via SYK/PI3K was shown to be important for $\gamma\delta$ T17 development (Muro et al., 2018) and IL-17 production. IL-17 itself is a strong negative regulator of IL-17 production by $\gamma\delta$ T17, since IL17R deficiency leads to enhanced IL-17 production (Fleming et al., 2017; Haas et al., 2012).

The IL-17 receptor is expressed by immune cells as macrophages or dendritic cells (DC), as well as by fibroblasts or epithelial cells. Its engagement leads to multiple effects as the secretion of cytokines, matrix-metalloproteinases or antimicrobial peptides (Amatya et al., 2017; Gaffen, 2009). While the IL-17 is a pro-inflammatory cytokine, that induces inflammation by attracting especially neutrophils that in turn guide more immune cells to the site of infection, $\gamma\delta$ T17 cells have been reported context-dependent as beneficial or detrimental (Cai et al., 2011; Matsuzaki et al., 2018; van der Fits et al., 2009).

Rapid IL-17 production by $\gamma\delta$ T17 is important for effective bacterial clearance (Hamada et al., 2008; Lockhart et al., 2006; McMenamin et al., 1994; Misiak et al., 2017; Mokuno

et al., 2000; Murphy et al., 2014; O'Brien et al., 2009; Romagnoli et al., 2016; Sheridan et al., 2013; Shibata et al., 2007; Umemura et al., 2007) or control of fungal burden (Dejima et al., 2011). In the uterus they are protective for the fetus against blood-borne pathogens (Heyborne et al., 1992; Itohara et al., 1990; Pinget et al., 2016; Polese et al., 2018; Song et al., 2016). Moreover, $\gamma\delta$ T17 play a role in wound healing and maintenance of tissue-integrity in the intestine (Dalton et al., 2006; Ismail et al., 2011; Wei et al., 2015). In the oral cavity, $V\gamma 6^+$ $\gamma\delta$ T17 play a protective role in immunity against *Candida albicans* in the tongue epithelium (Conti et al., 2014) and in orthodontic tooth-movement they were described as somehow beneficial in the sense that their presence allows bone-remodelling in the tooth-supporting tissue (Wald et al., 2021).

However, in a variety of tissues $\gamma\delta$ T17 cells are associated with detrimental actions in the context of autoimmune-diseases, but also with physiological functions in inducing an inflammation, when necessary. In the context of an experimental model of psoriasis, the $\gamma\delta$ T17 cells, especially the $V\gamma 4^+$ ones, have been described as important and potent pro-inflammatory populations (Cai et al., 2011; Yoshiki et al., 2014). Their depletion lead to amelioration of the symptoms (Sandrock et al., 2018). Comparably, in models of spondylo- and rheumatoid arthritis $\gamma\delta$ T17 cells were reported as IL-23 dependent major source of IL-17 thus aggravating the enthesitis (Akitsu et al., 2015; Reinhardt et al., 2016; Roark et al., 2007). Additionally to their accumulation at the Achilles tendon and the spinal entheses, IL-23 overexpression lead to mild symptoms of psoriasis in mice, aortic valve inflammation, infiltration of $V\gamma 6^+$ $\gamma\delta$ T cells into the aortic valve and root and into the ciliary body of the uvea (Reinhardt et al., 2016). Although Th17 cells are also important producers of IL-17 in experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis, the critical role of $\gamma\delta$ T17 cells in induction of the disease has become clear (Malik et al., 2016; Papotto et al., 2017; Petermann et al., 2010; Sutton et al., 2009). Also, in the gingiva and the periodontal ligament (PDL) $V\gamma 6^+$ $\gamma\delta$ T17 were reported as the major source of IL-17 (Wald et al., 2021; Wilharm et al., 2019).

In a variety of tissues host-commensal interactions were described to involve $\gamma\delta$ T17 cells. The presence of liver $\gamma\delta$ T cells depends on the gut microbiota (Li et al., 2017), the lung microbiota mediates IL-23 and IL-1 β dependent accumulation of $\gamma\delta$ T17 cells

(C. Jin et al., 2019), eye-commensals promote $\gamma\delta$ T17 activation (St. Leger et al., 2017), commensals were reported to promote CD103⁺ DC contacts to $\gamma\delta$ T17 thus inducing their proliferation (Fleming et al., 2017). The presence of $\gamma\delta$ T17 cells in the gingiva is influenced by the oral microbiota (Wald et al., 2021; Wilharm et al., 2019) and vice-versa the composition of the oral microbiota is dysregulated upon depletion of $\gamma\delta$ T17 cells (Wilharm et al., 2019). Moreover, the functional differentiation of the oral epithelia is dependent on the oral microbiota (Koren et al., 2021).

2.5 Anatomical structure of the gingiva

The oral mucosa is a stratified squamous epithelium, underlaid by its lamina propria. Areas that are heavily prone to face mechanical stress due to mastication, as the tongue mucosa and the mucosa of the palate are keratinized and termed “masticatory epithelia”. Most other oral mucosal surfaces are non-keratinized, as the buccal mucosa, and termed “lining epithelia”. The gingiva, which surrounds the tooth and covers the alveolar bone, comprises both masticatory and lining epithelia (Moutsopoulos et al., 2018).

The tooth is anchored in the alveolar bone (AB, fig.1) by its roots and consists of dentin, a mineralized collagen-rich substance. The crown of the tooth, directly exposed into the oral cavity, is covered with enamel, which is an even harder mineralized substance, even harder than bones, while the roots are covered by a thin layer of cementum, a third mineralized substance, that protects the underneath and less mineralized dentin. In the center of the tooth there is a cavity, the so-called pulp chamber, filled with blood vessels and nerves, that are connected to the bodies circulation and nervous system via the root-tips. The tooth is connected to the alveolar bone via the periodontal ligament (PDL, fig.1), which is a collagen-rich matrix allowing a certain degree of elasticity. Mice differ from humans in that the enamelogenesis in humans ends as soon as the tooth is readily developed, while in mice the teeth continue growing throughout their life and thus enamelogenesis also continues.

The outward-oriented epithelium of the gingiva, the oral epithelium (OE, fig.1), is keratinized. The gingival sulcus is covered by the non-keratinized sulcular epithelium (SE, fig.1). At its deepest point, the gingival sulcus contacts the tooth at the anatomical site where the dentin starts to be covered by enamel, the so-called cemento-enamel-junction (CEJ, fig.1). This part of the sulcular epithelium is termed junctional epithelium (JE, fig.1). Under physiological conditions, the alveolar-bone-crest (ABC, fig.1) covers the roots of the tooth up to the CEJ. The JE is particularly thin and due to the mineralization of the tooth only loosely attached to the tooth via hemidesmosomes. Via the JE the gingival crevicular fluid (GCF, fig.1), containing immunoglobulins, plasma proteins and cells, especially neutrophils, constantly passes the epithelium. The JE, but also the rest of the gingiva, is in direct contact to the bacterial biofilm that is attached to the tooth and easily accumulates in the gingival sulcus. Therefore, the gingiva can be seen as one of the anatomical sites allowing most direct interaction between immune cells and commensals or potential pathogens (Lacruz et al., 2017; Moutsopoulos et al., 2018).

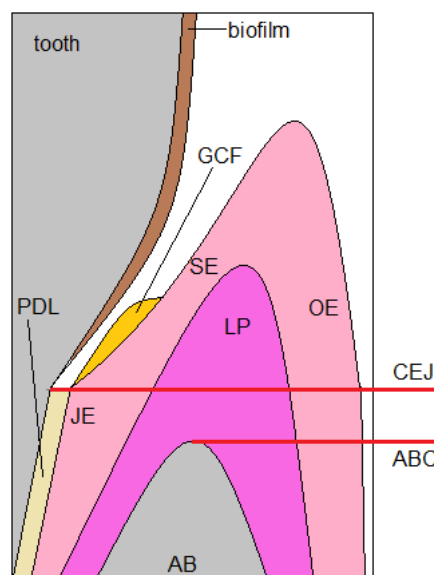


Fig.1: Simplifying scheme of the tooth-supporting tissue

The gingiva covers the alveolar bone (AB), consists of the outward-oriented oral epithelium (OE), the sulcular epithelium (SE) and the junctional epithelium (JE) and is underlaid by its lamina propria (LP). The highest point of the AB is the alveolar bone crest (ABC). The gingival crevicular fluid (GCF) constantly passes the JE. The tooth crown, superior to the cemento-enamel-junction (CEJ), is covered by a biofilm. The tooth root, inferior to the CEJ, is connected to the tooth-supporting tissue via the periodontal ligament (PDL).

2.6 $\gamma\delta$ T cells in the gingival immune system

The anatomical structure of the gingiva causes a very close interaction of the immune cells with the rich and diverse oral microbiota, comprising in humans more than 700 species (Verma et al., 2018). Thus, the gingiva contains elevated presence of immune cells compared to other oral mucosal sites (Dutzan et al., 2016) and gingival immune cells tolerate commensals, but simultaneously fight pathogens (Darveau, 2010).

More than 95% of all leukocytes in the gingival crevice are neutrophils (Cortés-Vieyra et al., 2016; Delima et al., 2003), that are under non-inflammatory conditions constantly

attracted to the gingiva by IL-8, ICAM and E-selectin expression from epithelial cells (Darveau, 2010; Tonetti et al., 1998). Apart from their functions in microbial surveillance/killing under homeostatic conditions (Barros et al., 2016; Eskin et al., 2012; Hanna et al., 2012; Zenobia et al., 2013), by releasing granules (Deas et al., 2003; E. Hajishengallis et al., 2014) they are important for immune homeostasis, since their efferocytosis by macrophages limits macrophage-produced IL-23, that otherwise leads to an excessive IL-17 response inducing periodontal inflammation. CD11b⁺ CD11c⁺ gingival dendritic cells (DC) localize primarily to the lamina propria (Banchereau et al., 1998), while Epithelial-cell-adhesion-molecule (EpCAM), CD207 and Langerin expressing Langerhans-cells (LC) localize to the epithelia and have prominent roles in regulating immune-surveillance and tolerance by promoting T cell activation (Allam et al., 2008; Hovav, 2014). Although innate lymphoid cells (ILC), NK cells, T_{reg} (Dutzan et al., 2016) and other gingival immune cells have been described, their exact function has not yet been described in detail.

Regarding gingival T cells, tissue-resident T cells have been shown to be relevant for the pathology of periodontitis and to produce IL-17 or IFN- γ . Mechanical damage due to mastication leads especially to an accumulation of Th17 cells via IL-6 production by epithelial cells (Dutzan et al., 2017). However, this accumulation is controlled by the microbiota, since the microbiota-dependent expression of growth-arrest-specific (GAS)-6 after birth controls the expression of IL-6 (Nassar et al., 2017). Apart from $\alpha\beta$ T cells, also $\gamma\delta$ T cells are known to reside in the gingiva (Koren et al., 2021; Krishnan et al., 2018; Tsukasaki et al., 2018; Wald et al., 2021; Wilharm et al., 2019). V γ 4⁺ and V γ 1⁺ $\gamma\delta$ T cells have been described as protective during periodontal inflammation by providing Amphiregulin (Krishnan et al., 2018), which is beneficial for wound healing and tissue integrity (Arpaia et al., 2015; F. Chen et al., 2018; Hall et al., 2016; Monticelli et al., 2011, 2015; D. M. Zaiss et al., 2006; D. M. W. Zaiss et al., 2013, 2015). However, other groups found V γ 6⁺ $\gamma\delta$ T cells to represent the major $\gamma\delta$ T cell subsets in the gingiva (Koren et al., 2021; Wald et al., 2021; Wilharm et al., 2019). The V γ 6⁺ $\gamma\delta$ T cells were shown to be embryonic-derived, partially tissue resident $\gamma\delta$ T17 cells (Wilharm et al., 2019) and have an activated phenotype characterized by CD44 and CD69 expression and low levels of CD62L and CD24 (Wilharm et al., 2019). They represent the major IL-17 producing population in the gingiva (Koren et al., 2021; Wald et al., 2021; Wilharm et al., 2019). V γ 6⁺ $\gamma\delta$ T cells locate especially to the JE (Wilharm

et al., 2019), but also to the PDL (Wald et al., 2021), demonstrating their close interaction with the microbiota.

Further evidence for interdependencies between V γ 6⁺ $\gamma\delta$ T cells and the microbiota arises from analysis of germ-free (GF) mice. In GF mice, the presence of V γ 6⁺ $\gamma\delta$ T cells, neutrophils and monocytes in the gingiva is reduced (Wald et al., 2021; Wilharm et al., 2019). Both microbiota and IL-17 are involved in recruiting neutrophils to the gingiva and PDL, especially during the first three weeks of the mouse life. In IL-17 deficient mice this recruitment was not observed, while in GF mice it is (Koren et al., 2021). In line with this, the microbiota is an important driver of final immunological functionalization and terminally differentiation of the oral epithelium (Koren et al., 2021). Vice versa, in *Tcrd*^{-/-} mice, the richness and diversity of the microbiota is enhanced as well as the pro-inflammatory milieu. However, this does not translate into enhanced osteoclast-activity or bone-loss (Wilharm et al., 2019), although the neutrophil and monocyte recruiting by $\gamma\delta$ T cells was shown to allow bone-remodelling processes via regulation of osteoclasts (Wald et al., 2021). Furthermore, in steady state some innate defence mechanisms in the oral cavity were described associated with the saliva, that contains an array of anti-microbial substances (Koren et al., 2021).

However, reduced oral hygiene is associated with increased and dysbiotic microbial load and leads to a certain degree of gingival inflammation, that can evolve to the relatively common periodontal inflammatory disease periodontitis.

2.7 Periodontitis

Periodontitis is one of the most common inflammatory diseases of the tooth-supporting tissue, with a prevalence of almost 50% in adults (Darveau, 2010; Eke et al., 2015; G. Hajishengallis, 2014; White et al., 2012). It is induced by oral bacteria as well as by the subsequent reaction of the host immune system (Hernández et al., 2011). Untreated, the chronic inflammation is accompanied by the loss of periodontal attachment and retraction of the alveolar bone, which can result in tooth loss (Tonetti et al., 2018).

The development of gingivitis into its chronic and much more tissue-destructive form periodontitis (Bascones et al., 2005; Pihlstrom et al., 2005) involves a dysbiotic microbiota (Kumar et al., 2006). *Porphyromonas gingivalis*, together with *Tennerella*

forsythia and *Treponema denticola*, belongs to the “red complex” of bacteria, which are strongly associated with periodontal disease (Silva et al., 2008; Socransky et al., 1998). Invading oral bacteria can disseminate throughout the body (Tsukasaki et al., 2018) and drive inflammation there (Atarashi et al., 2017; Y. W. Han et al., 2013), which was already proposed in 1891 in the aptly named article “The human mouth as a focus of infection” (Miller, 1891). Furthermore, periodontitis is linked to diseases like cardiovascular diseases (Bahekar et al., 2007) and rheumatoid arthritis (Farquharson et al., 2012; Konig et al., 2016). Apart from the microbiota itself, the hosts immune reaction triggers the periodontal tissue destruction (Hernández et al., 2011), since the dysbiotic microbiota induces an aggravation of the inflammatory response (Bartold et al., 2013; Graves, 2008; Graves et al., 2011; Silva et al., 2015) and infiltrating lymphocytes secrete matrix-metalloproteinases (MMP) (Sapna et al., 2014). Two mechanisms for terminally differentiation and activation of bone-resorption by osteoclasts are known in periodontitis. During the course of periodontitis, osteoclasts are activated by receptor activator of NF κ B ligand (RANKL) (Cochran, 2008) and secreted osteoclastogenic factor of activated T cells (SOFAT) (Jarry et al., 2013) resulting in the destruction of alveolar bone.

T cells induce RANKL expression by osteoblastic and ligament cells via IL-17 signalling (D. Lin et al., 2015; Sato et al., 2006; Yago et al., 2009) and also express RANKL themselves, both also true for periodontitis (Tsukasaki et al., 2018). The binding of RANKL to RANK, expressed by osteoclastic progenitors, can be inhibited by osteoprotegerin (OPG), which binds and thus blocks RANKL (Nagasawa et al., 2007). SOFAT is a RANKL independent activator of osteoclastogenesis, directly secreted from T cells and increased in periodontitis patients (Jarry et al., 2013). DC can aggravate bone loss by initiation of a Th1 response, associated with increased levels of IFN- γ and IL-2, as well as Th17 responses associated with enhanced production of IFN- γ , IL-17, TNF, IL-6, and IL-1 β by Th17 cells (Beklen et al., 2007; Cardoso et al., 2009; Ribeiro Souto et al., 2014), thus providing a pro-inflammatory cocktail inducing RANKL expression (Teng et al., 2005). However, bone-protective functions of DC were described via initiation of a Th2 response, since IL-4 yields a shift of the RANKL/ OPG ratio towards OPG (Ihn et al., 2002; Saidenberg-Kermanac’h et al., 2004; Su et al., 2015). While the RANKL/ OPG ratio describes how osteoclastogenic the milieu is, the expression of tartrate-resistant acid-phosphatase (TRAP) directly reflects osteoclast

activity (Halleen et al., 1999). LC have bone-protective functions by promoting T_{reg} infiltration into the tissue, which produce immunosuppressive cytokines as IL-10 and TGF β (Araujo-Pires et al., 2015; Arizon et al., 2012; Glowacki et al., 2013). However, RANKL expressing B cells were attracted by IL-10 and IL-6 to infiltrate the tissue, thus aggravating bone loss during periodontitis (Abe et al., 2015; Dutzan et al., 2016; X. Han et al., 2006; Mahanonda et al., 2016; Nakajima et al., 2008; Oliver-Bell et al., 2015).

Neutrophils, the most abundant immune cells in the gingival crevice, are important regulators of oral health. IL-17 is indispensable for the recruitment of neutrophils to the gingiva (Yu et al., 2007) during infection, whose efferocytosis by macrophages in turn limits the production of IL-17 (Ley, 2017; Serhan et al., 2008; Stark et al., 2005). IL-17 also triggers their degranulation (Pelletier et al., 2010; Sato et al., 2006; von Vietinghoff et al., 2008). In IL-17RA knockout mice, this recruitment is not possible and the alveolar bone loss is enhanced during periodontitis (Yu et al., 2007). Leukocyte adhesion deficiency type 1 (LAD1), leading to strong reduction in neutrophil number, and LAD2, leading to inability of neutrophils to transmigrate, develop spontaneously periodontitis (Deas et al., 2003; Hanna et al., 2012). Conversely, in periodontitis patients an accumulation of neutrophils is observed (Aboodi et al., 2011; Eskin et al., 2012; Landzberg et al., 2015). Thus IL-17 is a key cytokine during the development and course of periodontitis (Bittner-Eddy et al., 2016; Cheng et al., 2014, 2016; Dutzan et al., 2017, 2018). Therefore, the protective role of $\gamma\delta$ T cells (Krishnan et al., 2018) and bone-loss inducing role of Th17 $\alpha\beta$ T cells described in periodontitis (Tsukasaki et al., 2018) seems to be in contrast to their predominant $\gamma\delta$ T17 phenotype.

3. OBJECTIVES

It is known that $\alpha\beta$ DETC are present in *Tcrd*^{+/-} mice and that they are a non-functional replacement-population (Chodaczek et al., 2012; Jameson et al., 2004). However, it can be hypothesized that they might develop during the same embryonic time-window as $\gamma\delta$ DETC do (Almeida et al., 2015), thus challenging the idea of a selection for $\gamma\delta$ DETC with a certain TCR-repertoire. Thus, one objective of this study is to

compare the $\alpha\beta$ DETC in *Tcrd*^{+/-} mice phenotypically and regarding their TCR-repertoire to the original $\gamma\delta$ DETC from WT mice (manuscript I).

Regarding the function of $\gamma\delta$ T17 cells there are conflicting reports about their role in the gingiva. While bone-protective functions during periodontal inflammation have been reported (Krishnan et al., 2018), that others could not detect (Tsukasaki et al., 2018), gingival $\gamma\delta$ T cells were reported to be the major IL-17 producers in steady-state (Wilharm et al., 2019). IL-17, known as a pro-inflammatory cytokine, was shown to be bone-destructive in early periodontitis (Tsukasaki et al., 2018) but bone-protective by attracting neutrophils (Yu et al., 2007). Thus, a second objective of this study is to

address the function of $\gamma\delta$ T17 cells and the function of IL-17 itself in the inflammatory disease periodontitis (manuscript II).

4. RESULTS

4.1 Summary publication I: $\alpha\beta$ T cells replacing dermal and epidermal $\gamma\delta$ T cells in *Tcrd*^{-/-} mice express an MHC-independent TCR-repertoire

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DETC bear an almost clonal TCR-repertoire. While this repertoire is thought to be potentially selected during thymic development of the DETC, to which the putative ligand of the V γ 5V δ 1 TCR, Skint1, might contribute, a predominant role of the TCR in the function and maintenance of DETC in the mouse epidermis was proposed. Here, the $\alpha\beta$ DETC, reported to be present in the epidermis of *Tcrd*^{-/-} mice, are compared phenotypically to the WT $\gamma\delta$ DETC and their TCR-repertoire is analyzed in detail by NGS-sequencing.

The phenotypical analysis of age- and sex-matched *Tcrd*^{-/-} and WT mouse ear skin by flow-cytometry confirmed the complete absence of $\gamma\delta$ T cells from *Tcrd*^{-/-} skin and the appearance of $\alpha\beta$ DETC instead. The typical TCR-complex expression-pattern, comprising a CD3^{hi}TCR^{hi} population and a second CD3⁺TCR⁺ population, as known for WT skin, was observed in *Tcrd*^{-/-} mice. Also, in *Tcrd*^{-/-} mice, the location of the CD3^{hi}TCR^{hi} population to the epidermis and the location of the CD3⁺TCR⁺ population to the dermis, as known for WT mice, was shown by mechanical separation of epidermis and dermis prior to flow-cytometric analysis. The $\alpha\beta$ DETC were reduced by number and frequency in the *Tcrd*^{-/-} mice compared to WT. Further phenotypical comparison of prominent $\gamma\delta$ DETC surface proteins revealed that the $\alpha\beta$ DETC express CD103, CD5 and the DETC-costimulatory molecules NKG2D and JAML to comparable levels as $\gamma\delta$ DETC. Also, CD122 (IL2R β) was similarly expressed by both genotypes. However, CD127 (IL7R α) and CD215 (IL15R α) were lower expressed by $\alpha\beta$ DETC. Additionally, the activation-marker CD69 was lower expressed by $\alpha\beta$ DETC. The $\alpha\beta$ DETC displayed a CD4/ CD8 double-negative phenotype, that was also found to be enhanced in the dermal $\alpha\beta$ T cell population in *Tcrd*^{-/-} mice compared to dermal WT

$\alpha\beta$ T cells. Apart from this, the double-negative dermal $\alpha\beta$ T cell population (as well as the $\alpha\beta$ DETC population) displayed a CD103^{hi} phenotype, resembling the skin $\gamma\delta$ T cells, while the CD4/ CD8 expressing cells did not. No population from *Tcrd*^{-/-} skin was to be bound by the 17D1-antibody, which is reactive to both $\gamma\delta$ DETC and V γ 6⁺ WT dermal T cells. NGS-sequencing of the TCR-repertoire from sorted $\gamma\delta$ DETC, $\alpha\beta$ DETC, dermal WT $\alpha\beta$ T cells, dermal *Tcrd*^{-/-} $\alpha\beta$ T cells, *Tcrd*^{-/-} pLN $\alpha\beta$ T cells as well as CD103⁺ and CD103^{hi} dermal *Tcrd*^{-/-} $\alpha\beta$ T cells was conducted. The TCR β -repertoire of the *Tcrd*^{-/-} skin $\alpha\beta$ T cells showed a diminished diversity compared to WT dermal $\alpha\beta$ T cells. However, their repertoire was not as highly focused as the one from WT $\gamma\delta$ DETC. The TCR-rearrangements of $\alpha\beta$ DETC as well as CD103^{hi} dermal $\alpha\beta$ T cells showed a reduction in their CDR3-length of about one amino acid and an enhanced rearrangement-probability. Furthermore, $\alpha\beta$ DETC and CD103^{hi} dermal *Tcrd*^{-/-} $\alpha\beta$ T cells displayed alterations in their CDR3-amino acid composition. Especially, the cysteine and tryptophane usage was enhanced. Interestingly, the tryptophane usage was exceptionally high inside the P6-7 doublet, known to usually interact directly with the pMHC-complex. Comparison of the different analysed TCR-repertoires to published (J. Lu et al., 2019) TCR-repertoires of MHC-independent $\alpha\beta$ T cells revealed MHC-independent characteristics of $\alpha\beta$ DETC and CD103^{hi} dermal *Tcrd*^{-/-} $\alpha\beta$ T cells in that they use rather large, charged or hydrophobic amino acids and thus strong interacting ones that display a high ΔG -energy, low degree of disorder and prefer locations inside the protein. Flow-cytometric analysis of newborn *Tcrd*^{-/-} mice revealed the presence of $\alpha\beta$ T cells already before onset of TdT in the skin.

In summary, this study identified $\alpha\beta$ T cell populations in the *Tcrd*^{-/-} mouse dermis and epidermis, that replace the missing $\gamma\delta$ T cells in those tissues and express MHC-independent $\alpha\beta$ TCR-repertoires. Those replacement-populations resemble the original $\gamma\delta$ T cells especially in their CD103^{hi} phenotype and are present in the skin already in newborn *Tcrd*^{-/-} mice.

4.2 Summary publication II: Interleukin-17 is disease promoting in early stages and protective in late stages of experimental periodontitis

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The manuscript is submitted for publication.

Periodontitis is a very common infectious disease in humans. However, it is unclear which role IL-17 plays during the course of periodontitis and whether Th17 $\alpha\beta$ T cells or $\gamma\delta$ T cells are decisive for the outcome of the inflammation. In this study, several genetic mouse models deficient for either $\gamma\delta$ T cells or IL-17 cytokines were employed in an experimental model of periodontitis to elucidate the contribution of $\gamma\delta$ T cells.

Application of an experimental model of ligature-induced periodontitis, during which the periodontal inflammation is induced by placing a ligature from silk around the second molar tooth of the mouse, did not reveal any differences in *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17a*^{-/-} mice regarding their bone-loss during the inflammation, judged by the CEJ-ABC distance as well as the area between CEJ and ABC from a palatal perspective. These experiments were additionally performed in a second slightly different experimental model, which includes the administration of *P.gingivalis* bacteria into the oral cavity of the mouse. Also here, no significant differences were to be observed. A flow cytometric analysis of the gingival immune cells and comparison of both models revealed no significant differences between both. Overall, the presence of immune cells (CD3⁺ cells and CD45⁺ cells) slightly increased during the course of the 4 weeks infection. Especially, Ly6G⁺Ly6C⁺ neutrophils infiltrated the tissue. The main $\gamma\delta$ T cell subset that was detected expressed a V γ 6⁺ TCR. Further experiments were therefore restricted to the model without application of bacteria. Analysing further experiments by μ CT-imaging of the jaws and CEJ-ABC distance measurement, again no differences could be detected between either genotype from neither palatal nor buccal perspective. Similar, HE-staining and microscopic measurement of the CEJ-ABC distance did not reveal any differences between the genotypes. An analysis of

the gingiva after ligature-induced periodontal inflammation by qPCR for expression of IL-17 revealed comparable induction in *Tcrd*-H2BeGFP and *Tcrd*^{-/-}, while no induction was detectable in *IL17af*^{-/-} mice. Also, IL-23 induction was diminished in *IL17af*^{-/-} mice. No differences regarding the induction of GAS6 or TRAP were found in either genotype. However, the RANKL/OPG ratio was significantly higher in *IL17af*^{-/-} mice. Finally, the ligature-induced periodontitis model was applied to *Tcrd*-GDL mice that were either depleted of $\gamma\delta$ T cells by diphtheria-toxin-treatment or not. Here, no difference in bone-loss assessed by measuring the CEJ-ABC distance on HE-stained sections could be detected.

In summary, this study suggests that $\gamma\delta$ T cells are not the decisive IL-17 producers in periodontitis and that this is not observed due to a functional replacement of gingival $\gamma\delta$ T cells. Since in this study the mice were analysed after four weeks of inflammation and thus in later stages of periodontitis than in other studies, the data suggest that despite their reported $\gamma\delta$ T17 phenotype, the tissue-damage with or without $\gamma\delta$ T cells equalizes in late stages of periodontitis. Thus, other inflammatory processes seem to drive IL-17 independent osteoclast activity and thus tissue-damage. Therefore, IL-17 might be protective in late stages by having induced an inflammation in the early phase to eradicate the pathogens, before the overall tissue damage gets out of control.

5. DISCUSSION

By investigating the TCR-repertoire and phenotypical properties of $\alpha\beta$ DETC in *Tcrd*^{-/-} mice, it was emphasized to get insight into the effects of ligation of the unique V γ 5V δ 1 TCR of $\gamma\delta$ DETC for those cells. There are several potential effects, apart from the ones described for activation of the DETC (Chodaczek et al., 2012, 2018; Jameson et al., 2002; Komori et al., 2012).

To assume that the TCR is important for the development of this population, the population must be dependent on it. The differences between $\gamma\delta$ DETC and $\alpha\beta$ DETC that were described so far are related to their function, as their defective wound-healing capacities (Jameson et al., 2004), their reduced activation state (Chodaczek et al., 2012; Jameson et al., 2004), their inability to immobilize themselves at the keratinocyte tight junctions (Chodaczek et al., 2012), their lower presence and age-decline (Jameson et al., 2004), their inability to react to the keratinocyte-expressed TCR ligand (Komori et al., 2012) and the reduced IL-15 and IL-7 receptor expression, that we described. Also, the putative ligand Skint1 was shown to influence the functional development of DETC (Turchinovich et al., 2011), not their presence (Lewis et al., 2006). Although it was reported that SYK-deficiency “disrupts” the epithelial $\gamma\delta$ T cell populations, still CD3⁺ cells are detected in those mice epidermis in lower numbers (Mallick-Wood et al., 1996), as also true for ZAP70 (Kadlecek et al., 1998). Circumstances under which mice are devoid of DETC are by inhibition of the TCR-rearrangement in RAG deficiency (Almeida et al., 2015) and by deleting an important developmental transcription factor Runx3 (Woolf et al., 2007). Therefore, it is reasonable to assume that the V γ 5V δ 1 TCR itself does not play an important role for the development of a DETC population. Interestingly, also DETC-comparable cells devoid of a TCR have been described in lampreys (Sutoh et al., 2018). Nonetheless, a functional V γ 5V δ 1 TCR is critical for their functional development (Turchinovich et al., 2011) in the thymus and function and maintenance in the skin (Chodaczek et al., 2012).

However, this goes hand-in-hand with the second potential function of the DETC-TCR, which might be to ensure the appearance of the DETC in the epidermis after a “positive selection” in the thymus, potentially by Skint1. Researchers have collected numerous pieces of a puzzle, to which my work adds another one. Something that was clear

already from literature is that the bare appearance of dendritic shaped epithelial cells does not necessarily relies on the expression of the V γ 5V δ 1 TCR. DETC-populations expressing different TCR-repertoires are present in V γ 5^{-/-} (Mallick-Wood et al., 1998) and V δ 1^{-/-} (Hara et al., 2000) mice. Moreover, early induction of TdT leads to a diversified TCR-repertoire of DETC (Aono et al., 2000), showing that the DETC appearing in the epidermis are initially not filtered in the thymus. In combination with the observation that Skint1 deficiency leads to a diversified DETC-TCR repertoire (Lewis et al., 2006), one might come to the conclusion that also non-V γ 5V δ 1 TCRs can be driven to the epidermis, irrespective of presence or absence of Skint1 or other, non-V γ 5V δ 1 TCRs. Interestingly, introducing a TCR α transgene into WT or *Tcrd*^{-/-} mice leads to the development of CD4/ CD8 DN $\alpha\beta$ T cells expressing the TCR α transgene that were suggested to derive from $\gamma\delta$ T cell precursors (Fritsch et al., 1998) and shown to establish a TCR α -transgene DETC population (Leandersson et al., 2006) alongside the original $\gamma\delta$ DETC, when the transgene is introduced into WT mice, as well as alone, when the transgene is introduced in *Tcrd*^{-/-} mice. This suggests that the V γ 5V δ 1 TCR does not play an important role for a “selective” homing. My work suggests that also without introduction of a pre-rearranged TCR chain, $\alpha\beta$ DETC, that likely derive from the same embryonic precursors as the original DETC and therefore express an MHC-independent TCR-repertoire, can be driven to the epidermis. Together it seems that whatever TCR-rearrangement is ready in the DETC-precursors at the relevant embryonic timepoint around E15, will be driven to the epidermis. Apart from this, the unique V γ 5V δ 1 TCR that is highly clonally expressed in WT mice, is known to be the genetically imprinted preferred rearrangement at the relevant timepoint (Asarnow et al., 1993; Itohara et al., 1993), which allows the interpretation that the V γ 5V δ 1 TCR and its specificity plays a role in place in the epidermis for the physiological functionality of the cells and their maintenance and not before. Interestingly, the high CD103 expression, that we observed in the replacement-populations, suggests that other, TCR independent mechanisms might be able to drive the tissue-homing of those cells, as suggested also by reduction of the DETC-population in CD103 deficient mice (Schön et al., 2002) and the complete absence of DETC from the skin in Runx3 deficient mice (Woolf et al., 2007). Runx3 is reported to control CD103-expression (Grueter et al., 2005). This mechanism might be comparable for the second wave of $\gamma\delta$ T cells leaving the thymus, although more research is necessary to answer this. However, we could show that also a part of the

dermal T cell population of *Tcrd*^{-/-} mice displays an MHC-independent TCR-repertoire. This suggests that potentially overall during the embryonic $\gamma\delta$ T cell development whatever present TCR rearrangements get driven to certain sites of destination. Assuming this, the site of destination must be defined independent from the TCR.

Accepting that the TCR-specificity of embryonically developing $\gamma\delta$ T cells is primarily genetically predetermined, since changes on DNA-level directly translate into the TCR-repertoire of the $\gamma\delta$ T cells in their different tissues, the TCR might play an important role in the tissue, as proposed before (Chodaczek et al., 2012; Sutoh et al., 2018). We could confirm earlier reports about the lower population-size of $\alpha\beta$ DETC and their lower activation-state, which both suggest insufficient functionality of the TCR. Furthermore, $\alpha\beta$ DETC have been shown to be incapable of recognizing a transiently expressed stress-induced ligand on keratinocytes (Komori et al., 2012), which means that indeed the main effect of deleting the V γ 5V δ 1 TCR from the DETC is not a developmental defect or a homing/tissue-specificity related defect, but a functional defect. Based on our data we cannot say anything about functional defects of the dermal replacement-population. This requires further analysis. However, it is known that $\gamma\delta$ T17 cells do not necessarily require TCR-signals to be activated for IL-17 production. Therefore, one might speculate that this replacement-population potentially might be functionally not as impaired as the DETC are, which would be in line with reports about functional replacement-capacities in an experimental model of psoriasis (Sandrock et al., 2018). However, the V γ 6V δ 1 TCR will most likely have functions that can be impaired.

Regarding the function of the V γ 6⁺ $\gamma\delta$ T17 in the gingiva, A. Wilharms and my work underlines that the timepoint could be important for judging the function of $\gamma\delta$ T17 cells. In most epithelial tissues, where $\gamma\delta$ T17 cells reside, they have been reported to be the main source of IL-17. As known for models of psoriasis (Cai et al., 2011; Yoshiki et al., 2014), EAE (Malik et al., 2016; Papotto et al., 2017; Petermann et al., 2010; Sutton et al., 2009) and enthesitis (Reinhardt et al., 2016), $\gamma\delta$ T17 cells might initially be damaging for the tissue, in this case the tooth-supporting tissue, via rapid (Hamada et al., 2008; O'Brien et al., 2009; Shibata et al., 2007; Zeng et al., 2012) IL-17 secretion. Especially in the long-term perspective, this might allow an efficient resolving of the initial inflammation-inducer. Accordingly, $\gamma\delta$ T17 cells should be quite decisive for the fast development of an inflammation and thus, as reported for psoriasis, for the

magnitude of tissue-damages (Cai et al., 2011; Sandrock et al., 2018). However, a truly destructive role for $\gamma\delta$ T cells was not shown (Tsukasaki et al., 2018). It seems rather that they have protective functions (Krishnan et al., 2018). However, IL-17 was reported to be tissue damaging in early stages of periodontitis (Tsukasaki et al., 2018) and $V\gamma 6^+$ $\gamma\delta$ T cells were reported to be the major IL-17 producing population in the non-inflamed gingiva (Koren et al., 2021; Wald et al., 2021; Wilharm et al., 2019). Thus, later during the course of the inflammation, the immune cells infiltrating the tissue might gain influence onto the inflammatory process and thus also onto the severity of resulting tissue-damages. This might explain the equal tissue damages we observed in late stages of periodontitis in *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17a*^{-/-} mice. Also, it is imaginable that IL-17-independent mechanisms, potentially pathogen-induced or related to SOFAT, might influence the tissue-destruction in periodontitis, suggested by our finding of an enhanced RANKL/OPG ratio in *IL17a*^{-/-} mice after ligature-induced periodontitis and the enhanced tissue-damage in IL17RA deficient mice (Yu et al., 2007). Interestingly, a similar early inflammation-stages controlling function of $\gamma\delta$ T17 cells in the lung has been described (Dejima et al., 2011), suggesting that this might be a general phenomenon.

The evolutionary perspective suggests that $\gamma\delta$ T17 cells might be overall protective in periodontitis, but representing a “double-edged sword”, as proposed earlier (Tsukasaki et al., 2018). The teeth are of course relevant for mastication of the nutrition. On the other hand, the body barrier at the JE is uniquely thin, subjected to forces during mastication and in direct contact to a microbiota. Furthermore, the mouth is known as a focus of infection by disseminating oral bacteria to other sites of the organism. Thus, in the short-term perspective it might be evolutionary senseful to risk a certain degree of tissue-damage induced as a collateral damage in fighting invading pathogens. This might also avoid spreading of the periodontal inflammation to other teeth. However, in the long-term perspective, this bone-loss might be beneficial, since it finally allows to lose the tooth and thus to re-seal the outer body barrier and stop bacterial dissemination (Tsukasaki et al., 2018). Losing the tooth would be the lesser evil compared to ongoing dissemination of bacteria to the whole organism.

6. CONCLUDING REMARKS

The finding of replacement-populations for $\gamma\delta$ T cells in more than one tissue opens several questions. It needs to be elucidated in future studies, whether also in other tissues comparable replacement-populations are present in *Tcrd*^{-/-} mice. Furthermore, one might ask, whether the non-embryonic-derived $\gamma\delta$ T cells are replaced by any mechanism. Also from the functional perspective, the replacement-populations should be further investigated, since this could help to understand the functions of $\gamma\delta$ TCRs. Considering the wide conservation of $\gamma\delta$ T cells in vertebrates, a wide functionality of the replacement-populations seems rather unlikely. Potentially $\gamma\delta$ T cells mimic certain basic rough-and-ready functions, which could also be fulfilled by conventional $\alpha\beta$ T cells without much specialisation.

The study further investigated the function on $\gamma\delta$ T cells in periodontal inflammation. We could surprisingly not find a pivotal effect of $\gamma\delta$ T cell deficiency or IL-17 deficiency in late stages of periodontitis, despite the reported $\gamma\delta$ T17 phenotype of gingival $\gamma\delta$ T cells. Also, the gingival $\gamma\delta$ T cells in *Tcrd*^{-/-} mice seem not to be functionally replaced by other cells, as suggested for other sites. IL-17 seems to have an overall protective role, as described earlier, in late periodontitis. Thus, longitudinal studies observing the full course of periodontitis might be senseful to get a full picture.

7. APPENDIX

7.1 References

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7.2 Abbreviations

Areg	amphiregulin
AB	alveolar bone
ABC	alveolar-bone-crest
CD	cluster of differentiation
CCR	C-C chemokine receptor type
CDR3	complementarity determining region 3
CEJ	cemento-enamel-junction
DAMP	danger associated molecular pattern
DC	dendritic cell
DETC	dendritic epidermal T cell
DP	double positive
E	embryonic day
EAE	experimental autoimmune encephalitis
FGF	fibroblast growth factor
GAS6	growth arrest specific 6
GCF	gingival crevicular fluid
GF	germ free
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN- γ	interferon- γ
IG	immunoglobulin
IGF-1	insulin-like growth factor 1
IL	interleukin
ILC	innate lymphoid cell
IRF4	interferon-regulatory factor
IS	immune synapse
ITK	IL-2-inducible T cell kinase
JE	junctional epithelium
LC	Langerhans cell
LFA-1	lymphocyte function associated antigen 1
LAD	leukocyte adhesion deficiency
LP	lamina propria
Maf	musculoaponeurotic fibrosarcoma
MHC	major histocompatibility complex

MMP	matrix metalloproteinase
MR-1	MHC-related protein 1
mTEC	medullary thymic epithelial cells
NCR	natural cytotoxicity receptor
NIK	NF- κ B inducing kinase
NK cell	natural killer cell
OE	oral epithelium
OPG	osteoprotegerin
PALP	phosphotyrosine-rich aggregates located on projections
PAMP	pathogen associated molecular pattern
PDL	periodontal ligament
PE	phycoerythrine
pLN	peripheral lymph nodes
PLZF	promyelocytic leukemia zinc finger
pMHC	peptide major histocompatibility complex
S1P1	sphingosine-1-phosphate receptor 1
SCART	scavenger receptor
SE	sulcular epithelium
Skint	selection and upkeep of intraepithelial T cells protein
SOFAT	secreted osteoclastogenic factor of activated T cells
SOX	Sex-determining region Y-box
STAT	signal transducer and activator of transcription
SYK	spleen-tyrosine-kinase
TGF	transforming growth factor
TRAP	tartrate-resistant acid-phosphatase
RAG	recombination activating gene
RANKL	receptor activator of nuclear factor- κ B ligand
ROR	retinoic-acid-receptor-related orphan nuclear receptor
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TLR	toll-like receptor
V	variable gene segment
WT	wild type

7.3 Publications

7.3.1 Manuscript I

$\alpha\beta$ T cells replacing dermal and epidermal $\gamma\delta$ T cells in *Tcrd*^{-/-} mice express an MHC-independent TCR-repertoire

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Key words: DETC, skin T cells, *Tcrd*^{-/-} mouse, TCR-repertoire, MHC-independent $\alpha\beta$ TCR, NGS

Abbreviations: DETC, dendritic epidermal T cell; $\gamma\delta$ DETC, dendritic epidermal T cell expressing a $\gamma\delta$ TCR; $\alpha\beta$ DETC, dendritic epidermal T cell expressing an $\alpha\beta$ TCR; CDR3, complementarity-determining region 3; PALP, phosphotyrosine-rich aggregates located on projections; TdT, terminal deoxynucleotidyl transferase; WT, wild-type; DN, double-negative for CD4 and CD8; SP, single-positive for either CD4 or CD8; RAG, recombination-activating gene; AA, amino acid; pLN, peripheral lymph-node

Abstract

The epidermis of mouse skin is usually populated by dendritic epidermal T cells ($\gamma\delta$ DETC) expressing an invariant $V\gamma 5V\delta 1^+$ TCR. In *Tcrd*^{-/-} mice, skin-resident $\gamma\delta$ DETC are replaced by $\alpha\beta$ DETC carrying polyclonal $\alpha\beta$ TCRs. Although they exhibit a dendritic morphology, $\alpha\beta$ DETC were reported to be less functional than genuine $\gamma\delta$ DETC, likely because their TCR is unable to interact with the original TCR ligands of $\gamma\delta$ DETC. However, the TCR repertoire of those replacement DETC in *Tcrd*^{-/-} mice might provide clues for understanding the development and selection of canonical $\gamma\delta$ DETC. Here, we compare the phenotype and TCR repertoires of wild-type (WT) and *Tcrd*^{-/-} mouse skin T cells. Our data reveal that $\alpha\beta$ DETC are CD4/CD8 double negative and express an MHC-independent TCR-repertoire. Furthermore, we identify a second MHC-independent population of CD103⁺ CD4/CD8 double-negative $\alpha\beta$ T cells in the dermis of *Tcrd*^{-/-} mice.

Introduction

$\gamma\delta$ T cells are innate T lymphocytes that develop already early in the fetal thymus [1] and populate the periphery in several waves [2] of T cells expressing T cell receptors (TCR) using distinct $V\gamma$ and $V\delta$ segments. In mice, they constitute the majority of T cells in a variety of epithelial tissues [3]. In epidermal dendritic $\gamma\delta$ T cells ($\gamma\delta$ DETC), the entire $\gamma\delta$ TCR, including the complementarity-determining region 3 (CDR3), is identical [4]. Indeed, the skin harbours several prominent examples for $\gamma\delta$ T cells displaying so-called canonical TCRs. Whereas the dermis is settled mainly by $\gamma\delta$ T cells expressing either polyclonal $V\gamma 4^+$ TCR or a canonical $V\gamma 6V\delta 1^+$ TCR [3],[5],[6], the epidermis is populated by a unique T cell population expressing an almost clonal $V\gamma 5V\delta 1^+$ TCR [7],[8]. These cells exclusively reside in the epidermis and exhibit a dendritic morphology [9]. They have been reported to play a role in wound-healing of the skin [10],[11]. It is also thought that they are relevant for the body-barrier-surveillance [12], since they continuously scan keratinocytes for the antigen [13],[14] of their TCR, which is unknown. Nevertheless, TCR-signalling has been shown to be necessary for maintaining clonal self-renewal and wound-healing capacities [15]. Moreover, they ensure epidermal integrity of the body-barrier by secreting insulin-like growth factor (IGF)-1 [16] in response to interleukin (IL)-15 signalling [17], that is produced by keratinocytes [18].

The ontogeny of DETC has been tracked back to the yolk sac haematopoiesis [19]. In line with this evolutionary very old developmental background, they make up the first wave of $\gamma\delta$ T cells leaving the thymus around day E15 [1],[6] to migrate to the epidermis. Here, the population persists and undergoes continuous self-renewal [19]. The ligand(s) of its TCR is/are unknown, as is the exact function of this invariant TCR. Upon activation, the cells round up [10] and retract their dendrites. Via phosphotyrosine-rich aggregates located on projections (PALP) at their tips, the DETC dendrites are stably attached to the inter-keratinocyte tight-junctions in steady state. The PALPs are concentration-points of the TCR and enriched in adhesion-molecules as CD103 (integrin α_E) [12]. $\gamma\delta$ T cells expressing other $V\gamma$ or $V\delta$ segments in $V\gamma 5^{-/-}$ [20] or $V\delta 1^{-/-}$ [21] mice appear in lower numbers than the original $V\gamma 5V\delta 1^+$ DETC, but seem to retain a certain degree of TCR-structure identity and also keratinocyte reactivity. Since Skint1, a butyrophilin homologue in mice, has been shown to be relevant for the development of the DETC-population [22]–[24], there are hints towards a TCR-dependent selection of DETCs. Moreover, mice lacking the $C\gamma 1$ cluster were devoid of DETC, which could be restored by introducing a $V\gamma 2$ - $J\gamma 1$ $C\gamma 1$ -construct. The emerging DETC population used different $V\delta$ -segments but preferentially $V\delta 7$. The authors deduced from this further evidence for a positive selection of $\gamma\delta$ DETC, since those transgenic DETC were activated by co-culture with a keratinocyte cell line and were selectively activated in the thymus for expression of certain skin-homing receptors [25]. Speaking for an initially more random generation of the DETC-TCR repertoire, early induction of terminal deoxynucleotidyl transferase (TdT) enzyme allows emergence of a DETC population with a more diverse TCR repertoire, that refocuses with time towards the original $V\gamma 5V\delta 1$ TCR [26].

Interestingly, Jameson et al. found that in *Tcrd*^{-/-} mice, the epidermal niche is populated by dendritic-shaped $\alpha\beta$ T cells, which seem to display a polyclonal TCR [13]. This somehow challenged the idea of a role of the original $V\gamma 5V\delta 1^+$ TCR or at least its specificity for the appearance of the DETC in their niche. However, these $\alpha\beta$ DETC appeared to be less non-functional in wound-healing as compared to the $\gamma\delta$ DETC [13] and they were unable to form stable dendrites immobilized at the inter-keratinocyte tight junctions [12].

Here, we revisited the similarities and differences of $\gamma\delta$ DETC and $\alpha\beta$ DETC regarding some prominent phenotypical properties and found a deranged cytokine-receptor expression on the $\alpha\beta$ DETC, despite largely similar expression levels of the TCR

complex and co-stimulatory molecules. Furthermore, we analysed the TCR repertoire of the mouse skin T cells and found that the $\alpha\beta$ DETC and a subpopulation of the dermal $\alpha\beta$ T cells in *Tcrd*^{-/-} mice showed properties characteristic for major histocompatibility complex (MHC)-independent T cell populations [27],[28]. This led us to conclude that the $\alpha\beta$ DETC are probably not just conventional $\alpha\beta$ T cells that manage to fill the empty DETC niche in *Tcrd*^{-/-} mice, but that there is rather a special embryonic mechanism that guides $\alpha\beta$ T cells into skin-homing and DETC phenotype independent of specific $\alpha\beta$ TCR – MHC interactions.

Results

***Tcrd*^{-/-} skin epidermis contains reduced numbers of TCR^{hi} DETC but elevated levels of dermal $\alpha\beta$ T cells**

To judge the comparability of $\gamma\delta$ DETC and $\alpha\beta$ DETC in WT and *Tcrd*^{-/-} mice, respectively, we first assessed all T cells in the entire skin of *Tcrd*^{-/-} mice. In the skin of WT mice it is well known that $\gamma\delta$ T cells show a special pattern of TCR complex expression with high TCR-complex levels in DETC and low TCR-complex levels in dermal $\gamma\delta$ T cells. Indeed, flow cytometric comparison revealed a population of skin T cells with lower TCR expression levels (TCR⁺) and a second one with very high TCR expression (TCR^{hi}) in both WT and *Tcrd*^{-/-} mice (**fig. 1A, full gating strategy in fig. S1A**). We next compared the numbers of TCR^{hi} DETC in WT and *Tcrd*^{-/-} mice. Since the presence of $\alpha\beta$ DETC in *Tcrd*^{-/-} mice has been reported to decrease with age [13], we analysed mice of approximately one to three months of age (**fig. 1B**). The mean age of the WT and the *Tcrd*^{-/-} mice did not differ significantly and the groups are sex-matched (WT: 5 males, 7 females; *Tcrd*^{-/-}: 10 males, 9 females). Here we could not see a similar situation in WT and *Tcrd*^{-/-} mice, since *Tcrd*^{-/-} skin displayed a significantly lower amount of DETC judged by cell number and frequency (**fig. 1C**). At the same time, the presence of TCR⁺ (dermal) $\alpha\beta$ T cells was enhanced in *Tcrd*^{-/-} mice, although only reaching statistical significance frequencywise. Additionally to TCR levels, also CD3 and the tyrosine-phosphatase CD45 involved in TCR-signalling regulation [29] showed higher expression levels on the TCR^{hi} DETC populations than in the TCR⁺ population (**fig 1D**). Since no DETC-population, neither $\gamma\delta$ DETC nor $\alpha\beta$ DETC, can be reconstituted in adult *Rag*-inducible mice, we hypothesized that the $\alpha\beta$ DETC develop in the same fetal time-window prior to onset of TdT, as $\gamma\delta$ DETC do. Therefore, we analyzed whole-body skin from one-day old newborn mice for presence

of $\alpha\beta$ T cells (fig. 7A, in preparation for the revision of the publication). Of note, TdT expression is known to start between postnatal day 3 and 5 in mice. Indeed, we could identify a CD3⁺ population (fig. 7B), that was expressing an $\alpha\beta$ TCR (fig. 7C). Different from adult mice, this population could not be subdivided into a TCR^{hi} and TCR⁺ population. However, this shows that in *Tcrd*^{-/-} mice already prior to onset of TdT $\alpha\beta$ T cells are present in the skin. Also a Thy1.2⁺CD3⁻ population, described earlier[30], was detected (fig. 7B). In adult WT mice, the TCR^{hi} expressing cells are known to be located in the epidermis, while the TCR⁺ population resides in the dermis. We asked, whether this distribution could also be observed in *Tcrd*^{-/-} mice and checked this by mechanical separation of dermal and epidermal layer of the skin prior to flow cytometric analysis. We found that also in *Tcrd*^{-/-} mice, the skin epidermis solely contained TCR^{hi} expressing cells (**fig. 1E, full gating strategy fig. S1B**). In sum, these data confirm, as previously reported [13], that despite the similar TCR-complex expression pattern in the skin of WT and *Tcrd*^{-/-} mice, the $\alpha\beta$ DETC are unable to maintain a comparable population size as $\gamma\delta$ DETC in WT mice do. Furthermore, the data suggest the existence of a hitherto unknown expanded dermal $\alpha\beta$ T cell population in *Tcrd*^{-/-} mice.

Disturbed cytokine-receptor and CD69 expression on $\alpha\beta$ DETC

To investigate further identical or divergent properties of DETC in WT versus *Tcrd*^{-/-}, we analysed the expression of several cell-surface proteins that have been reported to be expressed by $\gamma\delta$ DETC. We found that expression of surface markers as the epithelial-specific integrin α_E (CD103), shown to be important for the presence of DETC in their niche [31] and CD5, a TCR-signalling modulator raising the necessary signalling strength to yield a response that was shown to become down regulated with DETC maturation [32], were similarly expressed by both populations (**fig. 2A and B, full gating strategy fig. S1A**). Furthermore, the $\gamma\delta$ DETC-co-stimulatory molecules “junctional-adhesion-molecule-like” (JAML) [33] and NKG2D (CD314), whose ligand H60c was reported to be expressed specifically in the skin and to be necessary albeit not sufficient for DETC activation [34], were expressed at similar levels by both $\gamma\delta$ DETC and $\alpha\beta$ DETC (**fig. 2C and D**). Interestingly, some cytokine-receptors reported to be important for $\gamma\delta$ DETC development and function, including the IL7R α (CD127) [35] and the IL15R α (CD215) [18],[36], but not IL2R β (CD122), which is necessary for the emergence of DETC [37], showed lower expression by $\alpha\beta$ DETC (**fig. 2E to G**). Furthermore, a less intensive CD69 expression by $\alpha\beta$ DETC compared to $\gamma\delta$ DETC (**fig.**

2H) suggests a lower activation-state of this population, as suggested before [13]. Surprisingly, the dermal $\alpha\beta$ T cells from *Tcrd*^{-/-} mice seemed to display a significantly enhanced JAML expression compared to their WT counterpart, rather resembling the WT dermal $\gamma\delta$ T cells, albeit the absolute difference was low. Overall, in combination with the smaller size of the $\alpha\beta$ DETC population, the data suggest that the activation-state of the $\alpha\beta$ DETC does not allow the population to reach and maintain the size of the WT-population, potentially at least partially due to missing cytokine receptors, causing a lower self-renewal-rate.

$\alpha\beta$ DETC display a CD4/ CD8 double-negative phenotype

We next asked to which extent the $\alpha\beta$ DETC were conventional $\alpha\beta$ T cells that may recognize skin-specific MHC-presented peptides. First, we investigated their CD4 and CD8 expression. While $\gamma\delta$ DETC have been reported to be CD4⁻ and CD8⁻ [13],[38], previous reports suggested that $\alpha\beta$ DETC at least partially express CD8 α [13]. In our analysis we could not detect any CD8 α or CD4 expression by $\alpha\beta$ DETC or $\gamma\delta$ DETC (**fig. 3A and B, full gating strategy fig. S2A and B**). Of note, we used the WT dermal $\alpha\beta$ T cells as internal controls for the detection of, which we could both detect in this population. Also, we excluded the possible loss of CD8 due to the digestion during the isolation of the cells by parallel digestion of lymph node T cells under the same conditions (**fig. S2C**). Our data show that neither $\gamma\delta$ DETC nor $\gamma\delta$ dermal T cells from WT mice nor $\alpha\beta$ DETC express CD4 or CD8 α . The WT dermal $\alpha\beta$ T cells were mostly CD4⁺, a small fraction of CD8⁺ cells was also detectable. Interestingly, the *Tcrd*^{-/-} dermal population, was significantly shifted towards a CD4/ CD8 double-negative (DN) phenotype (**fig. 3B**). This, in combination with the expanded dermal $\alpha\beta$ T cell population in *Tcrd*^{-/-} mice (**fig. 1B**), led us to the idea, that potentially also in the dermis there might be a previously unknown replacement-population for the missing $\gamma\delta$ T cells. Furthermore, we realized that while the WT dermal $\gamma\delta$ T cells highly express integrin α_E (CD103), as $\gamma\delta$ DETC do, the *Tcrd*^{-/-} dermal $\alpha\beta$ T cells consisted of CD103^{hi} expressing cells and CD103⁺ cells, that resembled the expression height of WT dermal $\alpha\beta$ T cells (**fig. 3C and 3D**). Moreover, in the DN subpopulation of the *Tcrd*^{-/-} dermal $\alpha\beta$ T cells, the CD103^{hi} fraction was significantly enhanced compared to WT dermal $\alpha\beta$ T cells and resembled the WT dermal $\gamma\delta$ T cells, while within the single-positive (SP) subpopulation the fraction of CD103⁺ cells was significantly lower compared to the WT dermal $\gamma\delta$ T cells and resembled the WT dermal $\alpha\beta$ T cells (**fig. 3C**). To summarize,

these data suggest that epidermal $\alpha\beta$ DETC from *Tcrd*^{-/-} mice, unlike conventional $\alpha\beta$ T cells, do not seem to be MHC-restricted. Furthermore, in the dermis of *Tcrd*^{-/-} mice, there also seems to be a CD4 and CD8 DN MHC-independent CD103^{hi} replacement-population distinct from conventional $\alpha\beta$ T cells.

The TCR repertoire of the replacement-populations is composed of polyclonal but short and simple rearrangements

While it is widely accepted that the canonical V γ 5V δ 1 TCR of WT DETC is required for the maintenance and clonal expansion of the cells in the epidermal niche [15], it is unclear, which role it plays for their development and homing. Since also the developmental heritage of the $\alpha\beta$ DETC population is potentially not the same as for conventional T cells, because neither $\gamma\delta$ DETC, nor $\alpha\beta$ DETC could be restored after tamoxifen-induced conditional expression of “recombination-activating gene” (*Rag*) in *Rag*-Inducible mice, that otherwise lack RAG1 and RAG2 [30], or after conditional depletion of $\gamma\delta$ T cells in mice [39], we asked whether the $\alpha\beta$ DETC might resemble the structure or even the specificity of $\gamma\delta$ DETC. As a first rough estimator of the similarity between the $\alpha\beta$ DETC TCR and the WT canonical TCRs, we assessed whether the 17D1 antibody, which is known to bind both canonical V γ 5V δ 1 and V γ 6V δ 1 TCRs, does also bind to some extent to the $\alpha\beta$ DETCs. Absent binding of the 17D1 antibody (**fig. 4A**) suggested major structural differences between WT canonical TCRs and the $\alpha\beta$ DETC TCR. A second indicator of structural or specificity-related similarities might be a WT-level focussed TCR repertoire of the $\alpha\beta$ DETC. Therefore, we performed NGS TCR repertoire analysis of WT and *Tcrd*^{-/-} skin T cells. As a skin-internal not focused control TCR repertoire we used the dermal population of WT $\alpha\beta$ T cells and as a focused repertoire the one from epidermal WT $\gamma\delta$ DETC. In line with earlier reports[13], we could not find a highly focussed TCR repertoire regarding the beta sequences in the $\alpha\beta$ DETC population, albeit the diversity assessed by the inverse Simpson index is lower than in the dermal WT $\alpha\beta$ T cells (**fig. 4B**). Each sample represents sorted and pooled skin T cells from several mice. The pooling allowed us to reach sufficient cell numbers to yield an acceptable number of productive reads (**fig 4C**). When analysing the CDR3 lengths of the repertoires, we observed a one amino acid (AA) shortening of the peak CDR3 β -length (**fig. 4D**), which was also visible judging the data after pooling equivalent samples prior to analysis (**fig. S3A**) These data suggest, in line with earlier reports [30], a development of $\alpha\beta$ DETC prior to TdT expression. Interestingly, also the

dermal CD103^{hi} population displayed the shortening in its CDR3 β , whereas the dermal CD103⁺ population did not (**fig. 4D and S3A**). Together these data demonstrate the differences between the highly focussed $\gamma\delta$ DETC repertoire and the polyclonal $\alpha\beta$ DETC repertoire and suggest simple rearrangements in the $\alpha\beta$ DETC population, but also in a subset of dermal $\alpha\beta$ T cells from *Tcrd*^{-/-} mice. Since there was no CDR3-prolongation as reported for MHC-independent TCRs [27], but a one AA shortening of the CDR3 β , our data strongly suggest a simple TCR-gene rearrangement prior to onset of TdT, as known for $\gamma\delta$ DETC.

Finally, we wondered if the slightly diminished diversity observed for the replacement-populations compared to WT dermal $\alpha\beta$ T cells could be explained by either any kind of selection leading to certain, preferred specificities of the TCRs or by assumedly more simple rearrangements prior to onset of TdT expression in early T cell development. Therefore, we assessed the CDR3 β rearrangement probability using the program OLGA. We found that independent of the shortened CDR3 β of the two replacement-populations described above, the rearrangement probability of the $\alpha\beta$ DETC was significantly higher compared to the dermal WT $\alpha\beta$ T cell population over the full range of observed CDR3 β -lengths (**fig. 4E**). Re-evaluating the data after pooling equivalent samples corroborated this finding (**fig. S3B**). As suggested by the similarly shortened CDR3 β of the CD103^{hi} dermal population, these cells also showed high-probability TCR-rearrangements. Albeit this does not formally exclude the possibility of a structural selection of certain simple and short TCRs, this suggests that the process shaping the TCR repertoire diversity of the replacement-populations is rather regulated on the DNA segment recombination level than on a structural ligand-mediated TCR selection level.

MHC-independent characteristics of replacement TCR repertoires

Next, we analysed the AA usage in the CDR3-region of different populations, since it has been reported that the CDR3 AA usage is altered in MHC-independent T cell populations [27]. We observed similar alterations in the $\alpha\beta$ DETC repertoire. While the dermal WT $\alpha\beta$ T cells displayed an AA usage of CDR3 β largely similar to conventional lymph-node T cells (**fig. 5A**), the $\alpha\beta$ DETC showed an enhanced tryptophan-usage (**fig. 5A to C**), an enhanced cysteine-usage (**fig. 5A and D**) and a diminished usage of lysine, proline, phenylalanine, serine, and threonine in their CDR3 β . While the tryptophane-usage data are at the limit of significance (**fig. 5C**). Effect size analysis of

the data after pooling of all samples prior to analysis shows a sustained tryptophane-elevation in $\alpha\beta$ DETC ($d > 0.8$) (**fig. S3C and D**). Furthermore, the highly hydrophobic AA tryptophane was most frequently present at the position 6 and 7 of the CDR3 β of both $\alpha\beta$ DETC and CD103^{hi} dermal $\alpha\beta$ T cells (**fig. 5B**), the notorious P6-7 doublet shown to be directly interacting with pMHC-complexes [28]. Hydrophobic AA at this position are associated with stronger TCR-pMHC interactions and are thus normally depleted during thymic selection [27],[28]. Another strongly interacting AA is cysteine, which was also reported to induce depletion during thymic negative selection. Notably, such cysteine usage and the usage of hydrophobic and strongly interacting AA was also enhanced in the CDR3 of MHC-independent repertoires [27]. Importantly, while there was not a single sequence in the WT dermal $\alpha\beta$ T cells that contained a cysteine, we found elevated cysteine usage in the CDR3 β (**fig. 5D**) of the $\alpha\beta$ DETC population as well as in the dermal population. Looking at the CD103⁺ and the CD103^{hi} dermal populations, cysteine was almost absent in the CD103⁺ one but elevated in the CD103^{hi} dermal cells, corroborating the existence of a CD103^{hi} replacement-population in the dermis. Again, effect size analysis after pooling of all data prior to analysis (**fig. S3E**) showed a “very large” elevation of cysteine levels in all *Tcrd*^{-/-} populations ($d > 1.2$), including pLN, while there were “very small” differences between the skin T cells from *Tcrd*^{-/-} mice ($d < 0.04$). The CD103⁺ dermal cells and the pLN cells in contrast showed “large” difference to the CD103^{hi} and the total dermal *Tcrd*^{-/-} population and the $\alpha\beta$ DETC ($d > 1.0$). In line with these AA-usage data, an amino acid profile analysis comparing the skin $\alpha\beta$ T cell populations to NGS-samples of WT conventional T cell populations and MHC-independent T cell populations from *Quad*^{-/-} mice, previously published by Lu et al. [27], showed similar properties of the CDR3 β from MHC-independent T cell subsets and $\alpha\beta$ DETC.

Analysis of the physicochemical properties of the respective CDR3 regions revealed an enhanced usage of rather large, negatively charged or hydrophobic than polar AA (**fig. 6A to D**) for CDR3 β . Accordingly, the CDR3 are more strongly interacting with other proteins (**fig. 6E**). As a result, the CDR3 of the $\alpha\beta$ DETC displayed a higher mj-energy (**fig. 6F**), a lower degree of disorder (**fig. 6G**) and prefer locations inside the protein (**fig. 6H**), meaning that the CDR3 likely is less flexible and less exposed. Although, these differences did not in all cases reach statistically significant levels, a clear pattern is visible. The CD103^{hi} dermal replacement-population displayed comparable properties, albeit here, a shift towards hydrophobic AA was not detected.

In sum, these data suggest that the TCR repertoire of replacement $\alpha\beta$ DETC and of the dermal CD103^{hi} $\alpha\beta$ T cells is not shaped by the same processes as the one from conventional T cells. Both $\alpha\beta$ T cell populations clearly show characteristics of MHC-independent TCR repertoires.

Discussion

In this study, we aimed to bring more insight into a genetically modified mouse line which is often only used as a $\gamma\delta$ T cell-deficient model to investigate the functional consequences of absent $\gamma\delta$ T cells. The relatively mild immune phenotype of *Tcrd*^{-/-} mice makes these mice a cumbersome subject of investigation. Since it has been also repeatedly reported that in *Tcrd*^{-/-} mice, conventional T cells at least partially overtake the functions of missing $\gamma\delta$ T cells, one might speculate that these “wannabe $\gamma\delta$ T cells”, i.e. $\alpha\beta$ T cells replacing $\gamma\delta$ T cells, may teach us about the selection requirements of innate T cells. Actually, the results of this work taught us that things are not as they seem at the first look. First, we could confirm earlier reports of $\alpha\beta$ DETC phenotypes in the epidermis of *Tcrd*^{-/-} mice. Along with their lower numbers, we could find more differences, but also similarities in comparison to $\gamma\delta$ DETC. On the one hand, the very high TCR-complex expression is maintained in $\alpha\beta$ DETC as well as the expression of the co-stimulatory molecules NKG2D and JAML, suggesting that $\alpha\beta$ DETC adopt important basic phenotypic properties of WT $\gamma\delta$ DETC. But still, they differ from $\gamma\delta$ DETC in their expression of cytokine-receptors IL-7R α and IL15R α , which are important for $\gamma\delta$ DETC development and function, and obviously they do not express any $\gamma\delta$ TCR but instead an $\alpha\beta$ TCR. On the other hand, $\alpha\beta$ DETC are very different from conventional $\alpha\beta$ T cells, since they display an MHC-independent TCR repertoire, are consequently DN for CD4 and CD8, and develop prior to onset of TdT. Furthermore, we unexpectedly found a population of DN dermal $\alpha\beta$ T cells displaying a similar MHC-independent TCR repertoire, which at least regarding their CD103 expression, resembled the missing dermal $\gamma\delta$ T cells. The existence of both populations underlines a robust and genetically deep-anchored mechanism that drives the quick development of MHC-unrestricted T cells, normally $\gamma\delta$ T cells, for populating defined sites of the organism as we usually see it for $\gamma\delta$ T cells expressing certain canonical TCRs.

Regarding the question, which role the canonical V γ 5V δ 1 TCR plays for the presence and persistence of the $\gamma\delta$ DETC population in the epidermis, our data suggested that neither the original V γ 5V δ 1 TCR nor any other $\gamma\delta$ TCR is necessary for the appearance of DETCs. To which extent any TCR is necessary for the appearance of an epidermal “T cell population” is also debatable, since the existence of dendritic-shaped epidermal cells with or without a TCR at all or with a certain specificity has been shown in several species [11], but not really in mice. As reported before, our results show that the specificity of the epidermal TCR does not necessarily need to be highly focussed, hinting towards a more important role of the TCR for the maintenance of the DETC in their niche after having populated it.

The shortened CDR3 β length of $\alpha\beta$ DETC and the identification of $\alpha\beta$ TCR expressing cells in newborn skin revealed in our study suggests a TCR-rearrangement prior to postnatal onset of TdT [40], as known for $\gamma\delta$ DETC, constituting the first wave of $\gamma\delta$ T cells leaving the thymus around embryonic day E15 [1],[6]. In line with that, induction of RAG in *Rag*-inducible mice after birth does not allow a restoration of any DETC population [30]. Therefore, the $\alpha\beta$ DETC and $\gamma\delta$ DETC very likely underlie the same time-restricted and highly orchestrated mechanism of development and skin-homing, for which the TCR does not seem to play a pivotal role.

Since TCR-specificity seems to have a rather limited influence on the initial epidermal seeding by any DETC population, there are two possible influences shaping the TCR repertoire of those populations. The first and simple one is being at the right place in the right time, meaning that due to the impossible $\gamma\delta$ TCR rearrangement, more $\alpha\beta$ TCR rearrangement takes place, resulting in an exclusive presence of $\alpha\beta$ DETC-precursors, which are then driven to the skin by the same mechanisms as otherwise $\gamma\delta$ DETC. This would mean that no thymic selection, as proposed for Skint1 [22], takes place and the canonical V γ 5V δ 1 TCR dominates the DETC TCR repertoire just by its predetermined simple rearrangements, which were somewhat copied by the replacement-populations. The second option is that any kind of MHC-unrelated molecular interaction of the TCR could sustain this positive selection process. From an evolutionary or simplistic perspective this would be rather unlikely, since there is no reason to develop two mechanisms yielding the same result, which is in this case a focussed TCR repertoire. Apart from that, the alteration of the $\gamma\delta$ DETC repertoire upon V γ 5 [20] or V δ 1 [21] deficiency, its diversification upon early TdT-induction, focussing

with time [26], its diversification upon Skint1 deficiency [24] and the possibility to express a TCR α transgenic TCR alongside as well as in place of the original DETC[41] advocate for the hypothesis that rearrangement on DNA-level is the decisive mechanism shaping the DETC-TCR repertoire and not any following selection. Still, Skint1 clearly promotes survival of the original $\gamma\delta$ DETC. But since in absence of Skint1, as the assumed decisive positive selecting molecule, other $\gamma\delta$ DETC appear in the skin and the initial repertoire generated in the thymus around E15/E16 with or without Skint1, does not differ in its constitution, the function of Skint1 cannot be selecting anything, neither homing, nor generation of any DETC, but only to maintain the desired type of DETC.

But still, Xiong et al. [25] interpreted the preferential usage of a certain V δ -segment by Vy2-transgenic DETC and the predominant activation of those T cell precursors and not others for expression of certain skin-homing receptors as strong evidence for “positive selection” of DETC in the thymus. However, selection defined in a wider biological sense is a process taking advantage of a certain degree of variance within a population, which enhances the probability that one or more of the different individuals in the population are able to survive under given circumstances. Selection of T cells additionally requires that out of the diverse population only those with TCRs displaying certain properties are actively filtered out. Following this rational, DETC that cannot be sufficiently activated by keratinocytes or during thymic development, may die and the original DETC may have advantages in surviving in the epidermal niche or during development due to their TCR-specificity. But first of all, there is no real diversity in the natural WT $\gamma\delta$ DETC-precursors that can be selected afterwards [40],[42]. Inducing a higher diversity by early onset of TdT indeed leads to appearance of a diverse repertoire in the skin. So, either those more diverse cells, that afterwards cannot survive in the skin, can recognize the positively selecting ligand, assuming there is one, so that they are “selectively driven” to the skin, but lose their ability to be activated by their selecting ligand only after homing to the skin, or the “selective skin-homing” is rather independent from the TCR-specificity. The preferential but not exclusive usage of V δ 7-segment usage in Vy2-transgenic mice [25], the preferential but not exclusive Vy1-segment usage in Vy5^{-/-} mice [20] as well as the slightly, but not to WT-level reduced diversity of $\alpha\beta$ DETC that we observed here, are unlikely to be the result of positive selection, but should rather result from simple rearrangements and from clonal

expansion. Furthermore, the progress in $\gamma\delta$ TCR-ligand search in the past years rather hints towards a multifaceted ligand-recognition by $\gamma\delta$ T cells [43].

Assuming that the $\gamma\delta$ DETC TCR repertoire and thus, as we described, also the $\alpha\beta$ DETC TCR-repertoire, and potentially also the repertoires of other canonical TCR expressing $\gamma\delta$ T cells are not shaped at all by a selection comparable to $\alpha\beta$ T cells, what can we propose as an alternative? What shapes the repertoire of a T cell population? Classically the well-known selective pressure for recognizing MHC, that has been shown to be also TCR-inherent [44], and not recognizing self-antigens has a huge influence onto the CDR3 AA usage, length [27] and exact sequence. Prior to those influences, other processes such as rearrangement biases shape TCR repertoires. While the DNA-level rearrangement can be absolutely random, the resulting AA sequence needs to be in frame and allow at least acceptable folding of the TCR chains to be successfully expressed on the surface. Therefore, the TCR-structure itself gains much influence on the TCR repertoire, in case the classical thymic selection does not apply. Reminiscent of that is our finding that the $\alpha\beta$ DETC seem to prefer tryptophane, which is one of two AA for which only a single codon encodes, in its CDR3 β . It is unlikely that this is a hint towards any kind of selection that prefers tryptophane in the CDR3 β , since also the dermal MHC-independent population we described displays this enhanced tryptophane usage and tryptophane in the P6-7 doublet is associated with enhanced self-reactivity. Apart from that, our data revealed that over the full range of observed CDR3 β lengths, the replacement-populations display TCR β sequences with an enhanced generation probability, which demonstrates that the random rearrangement on DNA-level has much more influence in those populations than selection of very specific clones. From a deterministic point of view, it would make sense to first provide a certain TCR-repertoire bias on DNA-level than selecting it from a vast range of recombinations on the protein-level, only to yield an almost monoclonal population homing to a specific site of the organism.

It is known that the rearrangement of canonical TCRs, both V γ 5V δ 1 and also V γ 6V δ 1, is predetermined and simple [40],[42]. In the right time and place, the population then seems to be driven to the epidermis, irrespective of the specificity of the TCR or even the type or presence of any TCR. Assuming that there is an evolutionary reason and a utility for the original canonical $\gamma\delta$ DETC population in the epidermis, which is suggested by wound-healing functions, the logical consequence is that the presence

and specificity of the canonical TCR plays a role in place in the epidermis, as proposed before [11]. The strongest argument for this is the refocusing of a TdT-induced more diverse $\gamma\delta$ T cell repertoire towards the V γ 5V δ 1 TCR with time [26] in the epidermis and is sustained by the lower number of $\alpha\beta$ DETC that we and others [13] showed. The role the $\gamma\delta$ TCR might play is to ensure the reported persistence of the population in the epidermis by self-renewal [19], potentially by interacting with one or more $\gamma\delta$ TCR-ligands on keratinocytes [14]. The phenotypic characteristics of lower activation levels and of deranged cytokine-receptor expression of $\alpha\beta$ DETC sustain this hypothesis. The previously suggested decrease of $\alpha\beta$ DETC in aged mice is likely to be a consequence of the insufficient activation for self-renewal [13]. Interestingly, this mechanism seems to work not only for the isolated epidermal niche, but also for the higher frequented dermis, since we could find a DN MHC-independent population of $\alpha\beta$ T cells coexisting alongside with conventional $\alpha\beta$ T cells. This also implies a third important part of the mechanism, which is to make the cells stay where they are supposed to be. Otherwise, the dermal replacement-population would very likely not be able to persist in the relatively easy-to-escape dermal niche. Probably they would just be washed out with time. A part of that mechanism might be the integrin α_E (CD103), that we found to be highly expressed by the DN replacement-populations in *Tcrd*^{-/-} mice, since CD103 deficiency leads to a diminishment of the DETC population [31] and a deficiency in Runx3, a transcription factor controlling CD103, leads to complete lack in DETC [45].

In humans, Dimova et al. reported that fetal V γ 9V δ 2 $\gamma\delta$ T cells display a semi-invariant TCR repertoire. This was surprising, since in adults there is a considerable diversity. Intuitively, this made the authors draw similarities to mouse V γ 5V δ 1 DETC and intuitively, a “selection” of human fetal V γ 9V δ 2 $\gamma\delta$ TCR for phosphoantigen-reactivity, was proposed [46]. However, pragmatically judged, it shows another fetal T cell population with a semi-invariant TCR repertoire that changes after birth in place in the periphery.

Finally, it needs to be determined in future studies whether only the $\gamma\delta$ T cell populations expressing confined canonical TCRs developing prior to TdT onset, i.e. V γ 5V δ 1-expressing DETC and V γ 6V δ 1-expressing dermal $\gamma\delta$ T cells, have specific corresponding replacement-populations in *Tcrd*^{-/-} mice, and whether other $\gamma\delta$ T cell populations might become functionally replaced by other mechanisms. In case of wide

functionality of the replacement-populations, this would by far decouple the $\gamma\delta$ TCR from the functions of $\gamma\delta$ T cells and degrade the $\gamma\delta$ TCR itself to an evolutionary rudiment. Considering the high conservation of $\gamma\delta$ T cells among jawed vertebrates [47], this seems rather unlikely. It is more likely that $\gamma\delta$ T cells overtake certain basic rough-and-ready functions, which could also be fulfilled by conventional $\alpha\beta$ T without much specialisations. An answer to this question might be provided by in-depth investigating the specificity of the $\alpha\beta$ TCR of the replacement-populations and comparing them to the respective $\gamma\delta$ TCR and of course by functional analyses. Also, in the light of recently reported $\alpha\beta\gamma\delta$ T cells existing in the human dermis during the fetal period [48], answering these questions will allow a better understanding of the development of a “fetal immune system” and its functions. Since we could not find a predominant clone or even few highly similar clones shared among individual mice, our data do so far not suggest that the TCR of $\alpha\beta$ DETC are reactive towards one specific molecule.

Methods

Animals

C57BL/6-N/Crl mice and B6.129P2-Tcrd^{tm1Mom}/J (*Tcrd*^{-/-}) mice were bred in the animal facility of the central animal facility at Hannover Medical School (Hannover, Germany). All animal experiments were conducted in compliance with the German animal protection law (TierSchG BGBl. I S. 1105; 25.05.1998).

Isolation of skin lymphocytes

Directly before the experiment, the mice were sacrificed by CO₂ inhalation and cervical dislocation and the ears were removed. Hairs were removed from the ears by scraping with a scalpel. The dorsal and the ventral layer of the ears were separated by forceps, placed into 2 mL of digestion-medium (RPMI 1640, DNaseI 187,5 µg/mL, Col IV 50 µg/mL) and minced by scissors. After 75 min incubation in a thermo-shaker (37°C, 1400 rpm), 121 µL 0.5 M EDTA was added and the incubation was continued for another 15 min. Then, the suspension was filtered through a 100 µm cell strainer into a 15-mL falcon. The falcon was filled up to 14 mL, centrifuged at 1250 rpm for 5 min and the supernatant was removed. The pellet was resuspended in 3 mL 40% Percoll (1.596 mL RPMI 1640, 84 µL FCS, 120 µL 10X PBS, 1.2 mL Percoll). The suspension was underlaid with 3 mL 70% Percoll (0.6555 mL RPMI 1640, 34.5 µL FCS, 210 µL 10X PBS, 2.1 mL Percoll) and a density-gradient centrifugation was performed at 2000 rpm for 20 min at RT. The interphase containing the lymphocytes was transferred into a fresh 15-mL falcon that was filled up with MACS buffer to 14 mL and centrifuged at 1250 rpm for 5 min at 4°C. The pellet was resuspended in MACS buffer for flow cytometric analysis or sorting.

Separation of dermis and epidermis for flow cytometric analysis

The separation of dermis and epidermis is an additional step in the protocol for Isolation of the lymphocytes from ear skin and was performed immediately before the digestion of the tissue. The separated dorsal and ventral layer of the ears were incubated overnight at 4°C the dermal side facing the liquid on 2.4 U/mL Dispase in PBS. Then, the dermal layer was carefully removed from the epidermal layer by forceps. The isolation of lymphocytes from the separated dermal and epidermal tissue was then continued with the digestion.

Flow cytometric analysis and cell-sorting

After washing in MACS, the cells were blocked 5 min on ice with 5 % F_c-block (clone 2.4G2) in MACS buffer and stained with the respective antibodies (diluted in MACS buffer, table S1) for 30 min on ice. Flow cytometric analysis was performed employing a Becton Dickinson LSRII, FACSDiva software version 8.0.1, FlowJo version 10 and GraphPad Prism version 7.05. The cell-sorting was performed in the sorting facility at Hannover Medical School (Hannover, Germany).

NGS TCR sequencing

For the TCR repertoire analysis, WT dermal $\alpha\beta$ T cells (CD3⁺TCR β ⁺), WT dermal $\gamma\delta$ T cells (CD3⁺TCR $\gamma\delta$ ⁺), WT $\gamma\delta$ DETC (CD3^{hi}TCR $\gamma\delta$ ^{hi}), *Tcrd*^{-/-} dermal $\alpha\beta$ T cells (CD3⁺TCR β ⁺), *Tcrd*^{-/-} $\alpha\beta$ DETC (CD3^{hi}TCR β ^{hi}), CD103⁺ *Tcrd*^{-/-} dermal $\alpha\beta$ T cells (CD3⁺TCR β ⁺CD103⁺) and CD103^{hi} *Tcrd*^{-/-} dermal $\gamma\delta$ T cells (CD3⁺TCR β ⁺CD103^{hi}) were sorted from ear skin of mice. *Tcrd*^{-/-} pLN T cells (CD3⁺TCR β ⁺) were sorted from inguinal, axillar and brachial lymph-nodes.

A 5' RACE cDNA synthesis, followed by a PCR employing a constant region reverse primer and a UPM (primer sequences in table S2), was performed. In brief, RNA extraction from sorted cells was performed using RNeasy Plus Micro kit (Qiagen). The cDNA was generated by employing ingredients from the TAKARA cDNA Synthesis Kit (TAKARA Bio Europe), based on the manufacturer's protocol. A mixture from 1 μ l 12 μ M OligodT and 4.5 μ l RNA was incubated for 3 min at 72°C, followed by 2 min at 42°C and placed on ice immediately. Directly, 4.5 μ l RACE-Mastermix (1X First-Strand-buffer, 2.5 mM DTT, 1 mM dNTP mix, 0.6 μ M SMARTer IIA Oligonucleotide, 10 U RNase Inhibitor and 50 U SMARTScribe Reverse Transcriptase) was added, and the PCR program was continued for 90 min at 42°C and 10 min at 72°C. Subsequently, the DNA-amplicons for Illumina sequencing were generated by using Advantage2 PCR-Kit (TAKARA Bio Europe), employing a mTCR constant region reverse primer and a Universal primer-mix (UPM). The fully generated cDNA (10 μ l) was processed. After agarose-electrophoresis, the DNA was purified using the Qiaquick Gel Extraction Kit (Qiagen). The purified product of each sample was indexed by an indexing-PCR with Nextera-Primers. Finally, a pool of the samples was sequenced, employing Illumina MiSeq v2 Reagent Kit (Illumina).

TCR repertoire analysis

For the analysis of TCR sequencing data, samples were aligned to the international immunogenetics information system (IMGT) database from 22.05.2018, using MiXCR software [49]. The TCR diversity analysis, CDR3 length analysis, CDR3 AA profile analysis (analysis of physicochemical properties of CDR3) and sample-pooling was performed employing VDJtools [50]. The AA distribution analysis was calculated employing Microsoft excel 2010. The CDR3 generation probability was calculated employing OLGA v.1.2.3 [51]. The CDR3-pictograms were generated using the WebLogo generator [52] from University of California, Berkeley. The final presentation of the data was prepared employing GraphPad Prism version 7.05.

Data availability

Sequencing data were deposited at NCBI GEO with accession number GSE168123, freely available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168123>.

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 7.05 or Microsoft excel. Effect size analysis was performed and judged as described previously [53]–[55]. A p-value below 0.05 was considered as significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns (not significant).

TCR γ and TCR δ nomenclature

Throughout this publication, we use the TCR γ and TCR δ nomenclature as defined by Heilig and Tonegawa [56].

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Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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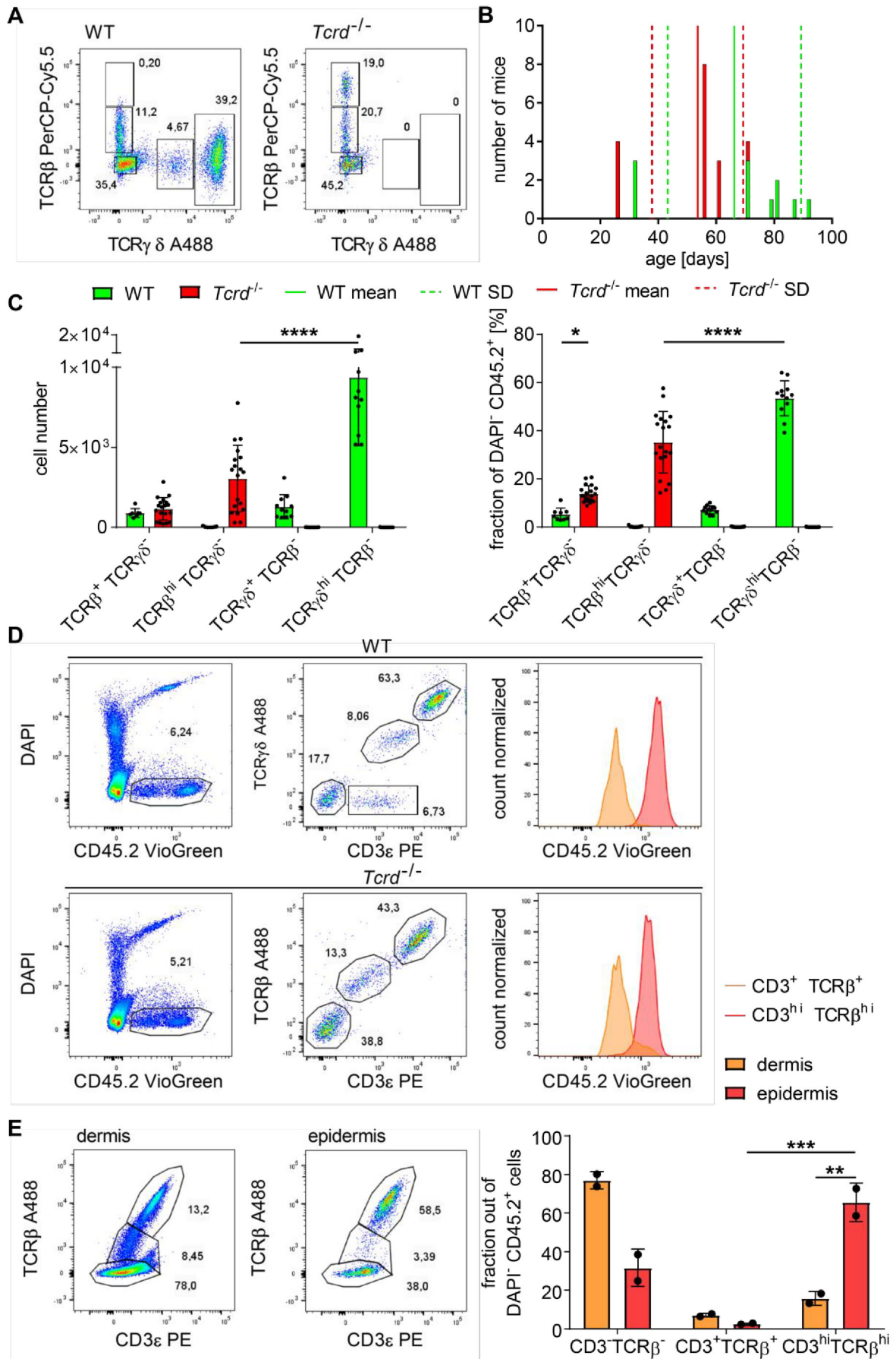


Figure 1: TCR-expression patterns in WT and *Tcrd*^{-/-} mice

(A) Representative FACS-plots of TCR-expression pattern in WT and *Tcrd*^{-/-} mouse ear skin, gated as DAPI-CD45.2⁺ cells or DAPI-CD45.2⁺CD3⁺ cells. (B) Histogram of the age of the mice analyzed. Mean and SD of WT or *Tcrd*^{-/-} mice age is depicted as line (mean) and dotted line (SD). (C) Absolute and relative abundance of skin T cell populations in WT and *Tcrd*^{-/-} mice. Absolute and relative WT (n=11) and *Tcrd*^{-/-} (n=19) data were obtained from 4 independent experiments. (D) FACS-plots demonstrating enhanced CD45.2 expression by high TCR-complex expressing cells in WT and *Tcrd*^{-/-} mice. Data are representative of the data from (C). (E) Fraction of TCR^{hi} and TCR⁺ cells in the skin of WT and *Tcrd*^{-/-} mice measured after mechanical separation of dermis and epidermis. In all diagrams, each point represents one mouse, mean and SD are shown. Statistical analysis was performed by Two-way ANOVA and Holm-Sidak multiple-comparisons.

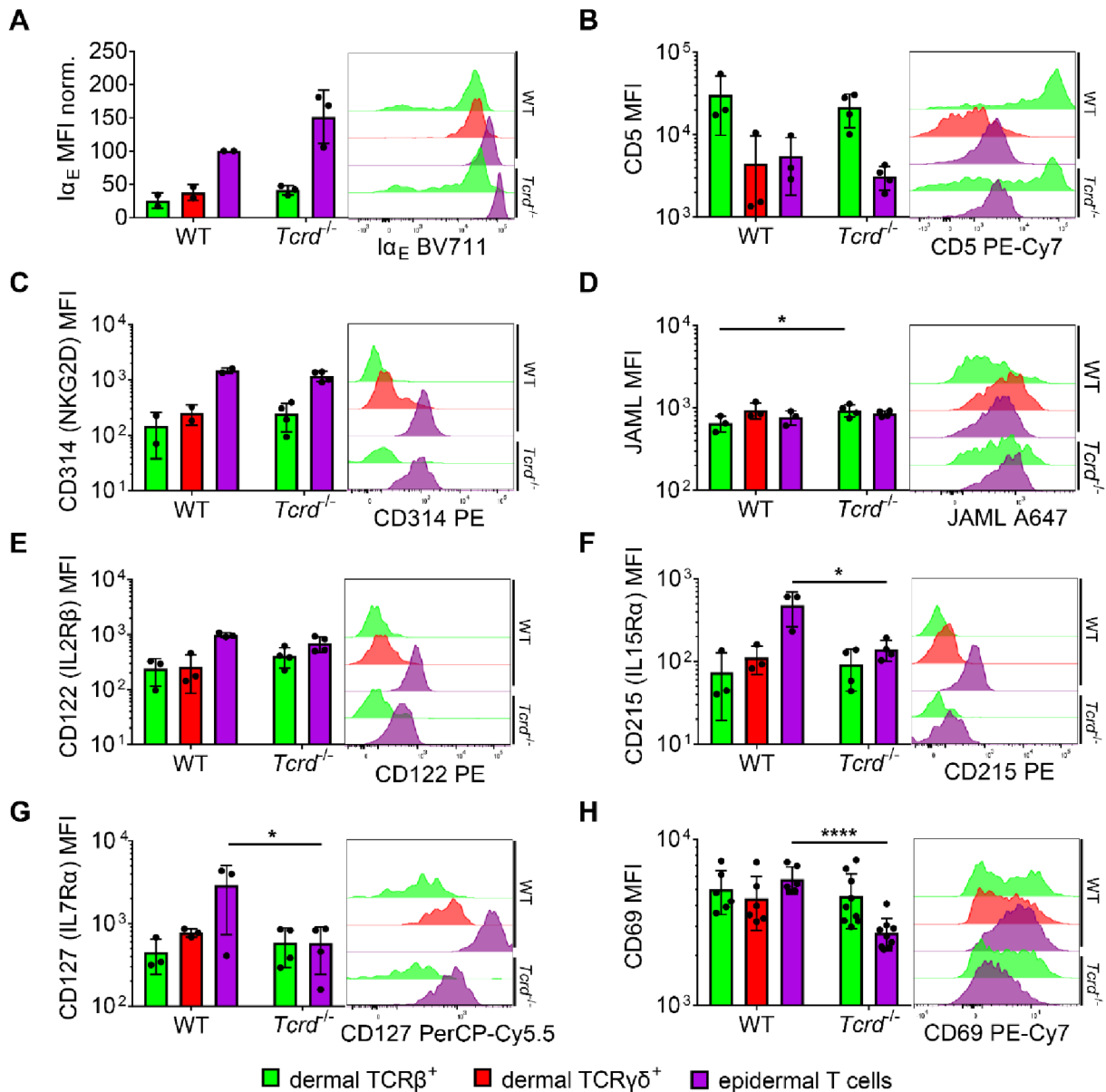


Figure 2: Flow-cytometric analysis of WT and *Tcrd*^{-/-} skin T cells for expression of prominent surface-proteins

(A to H) Expression of CD103 (Integrin α_E), CD5, CD314 (NKG2D), JAML, CD122 (IL2R β), CD215 (IL15R α), CD127 (IL7R α) and CD69 by WT and *Tcrd*^{-/-} skin T cell populations, assessed by measuring the mean fluorescence intensity (MFI) of fluorophore conjugated antibody bound cells by flow cytometry, and representative histograms. For (A) CD103, the MFI was normalized to the MFI of WT DETC due to a high difference between the absolute values measured in two independent experiments. The data for each surface-protein were obtained from two independent experiments, except CD69 (three independent experiments). In all diagrams, each

point represents one mouse, mean and SD are shown. Statistical analysis was performed by Two-way ANOVA and Holm-Sidak multiple-comparisons.

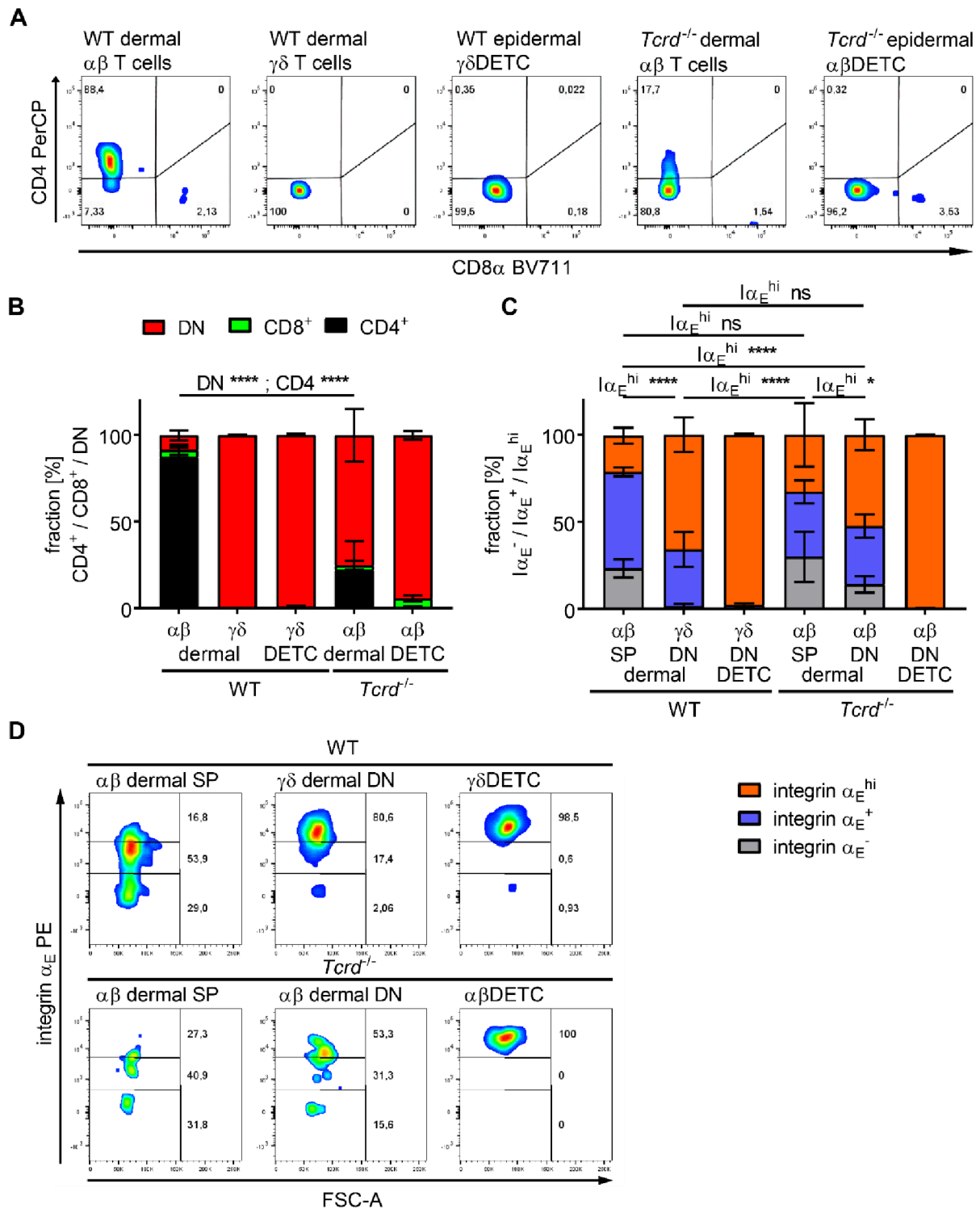


Figure 3: Flow-cytometric analysis of WT and *Tcrd*^{-/-} skin T cells for expression of CD4/CD8 and integrin α_E

(A) FACS-plots and (B) relative analysis of CD4/CD8 expression by skin T cells of WT (n=5) and *Tcrd*^{-/-} (n=6) mice. Data were obtained from two independent experiments. (C) Relative analysis and (D) representative FACS-plots of CD103 (Integrin α_E) expression by skin T cells of WT (n=4) and *Tcrd*^{-/-} (n=4) mice. In all diagrams mean

and SD are shown. Statistical analysis was performed by Two-way ANOVA and Holm-Sidak multiple-comparisons.

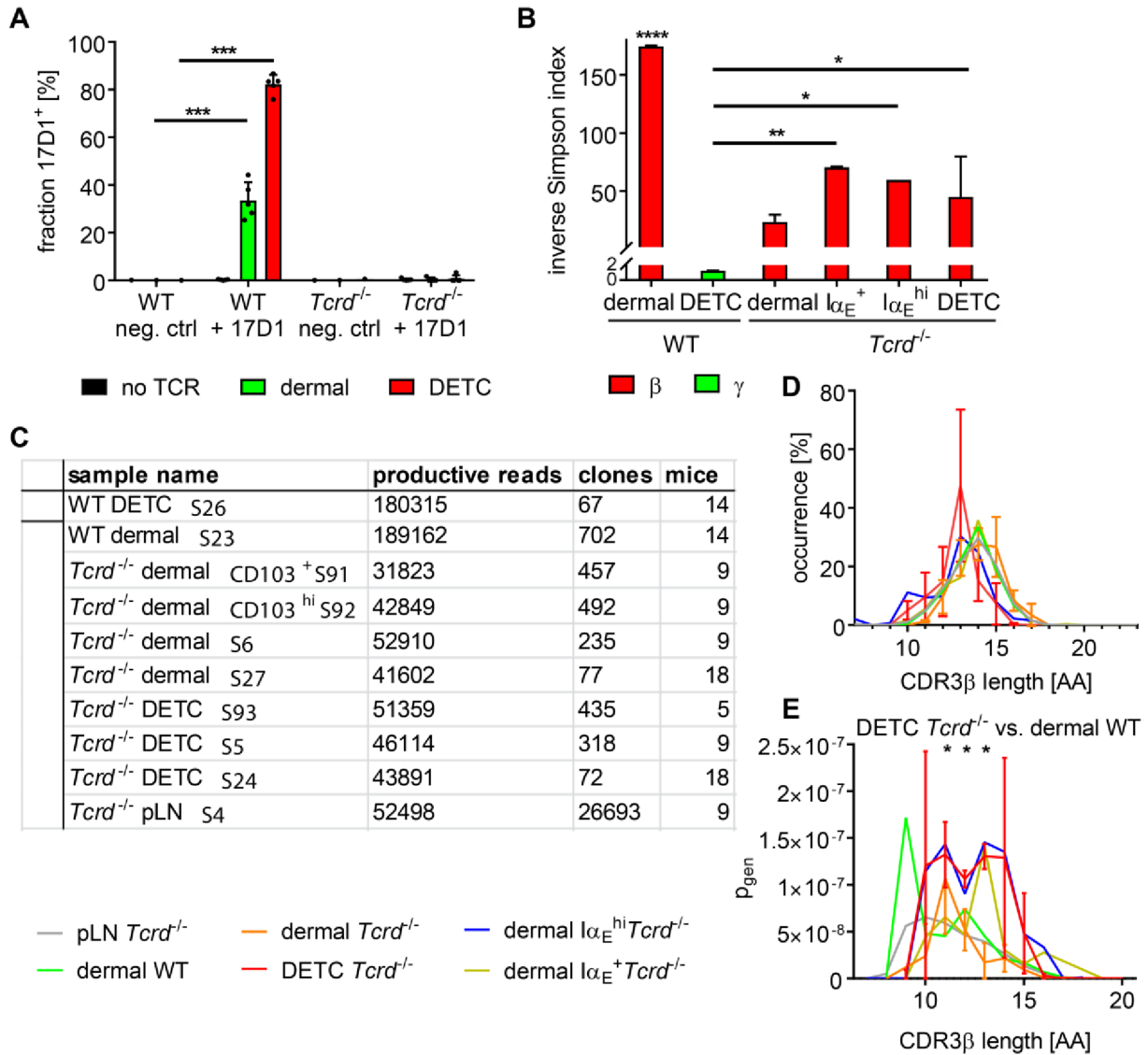


Figure 4: Analysis of 17D1-reactivity, TCR-repertoire diversity, CDR3 β length and rearrangement-probability of WT and *Tcrd*^{-/-} skin T cells

(A) Fraction of 17D1-reactive skin T cells in WT or *Tcrd*^{-/-} mice, gated as DAPI⁻ CD45.2⁺ TCR^{hi} (for epidermal) or DAPI⁻ CD45.2⁺ TCR⁺ (for dermal) cells. Data were obtained from 2 independent experiments, each point in the diagram represents one mouse. Mean and SD are shown. Statistical analysis was performed by Two-way ANOVA and Holm-Sidak multiple-comparisons. (B) The diversity of the NGS-samples was assessed by the inverse Simpson index. Mean and SD of three resampling-processes from each sample are shown. Statistical analysis was performed by One-way ANOVA and Holm-Sidak multiple-comparisons. (C) Productive read and identified clone numbers of the NGS-samples, generated from the indicated number of mice. (E) CDR3 β length distribution and (F) TCR β -rearrangement generation probability of WT dermal (n=1), *Tcrd*^{-/-} pLN (n=1), *Tcrd*^{-/-} dermal T cells (n=2), *Tcrd*^{-/-} DETC (n=3), *Tcrd*^{-/-}

$^{-/-}$ dermal $\text{I}\alpha\text{E}^+$ T cells ($n=1$) and $\text{Tcrd}^{+/-}$ dermal $\text{I}\alpha\text{E}^{\text{hi}}$ T cells ($n=1$). Mean and SD are shown. Statistical analysis for (F) was performed by one-sample t-test comparing groups with $n > 1$ to dermal WT as control.

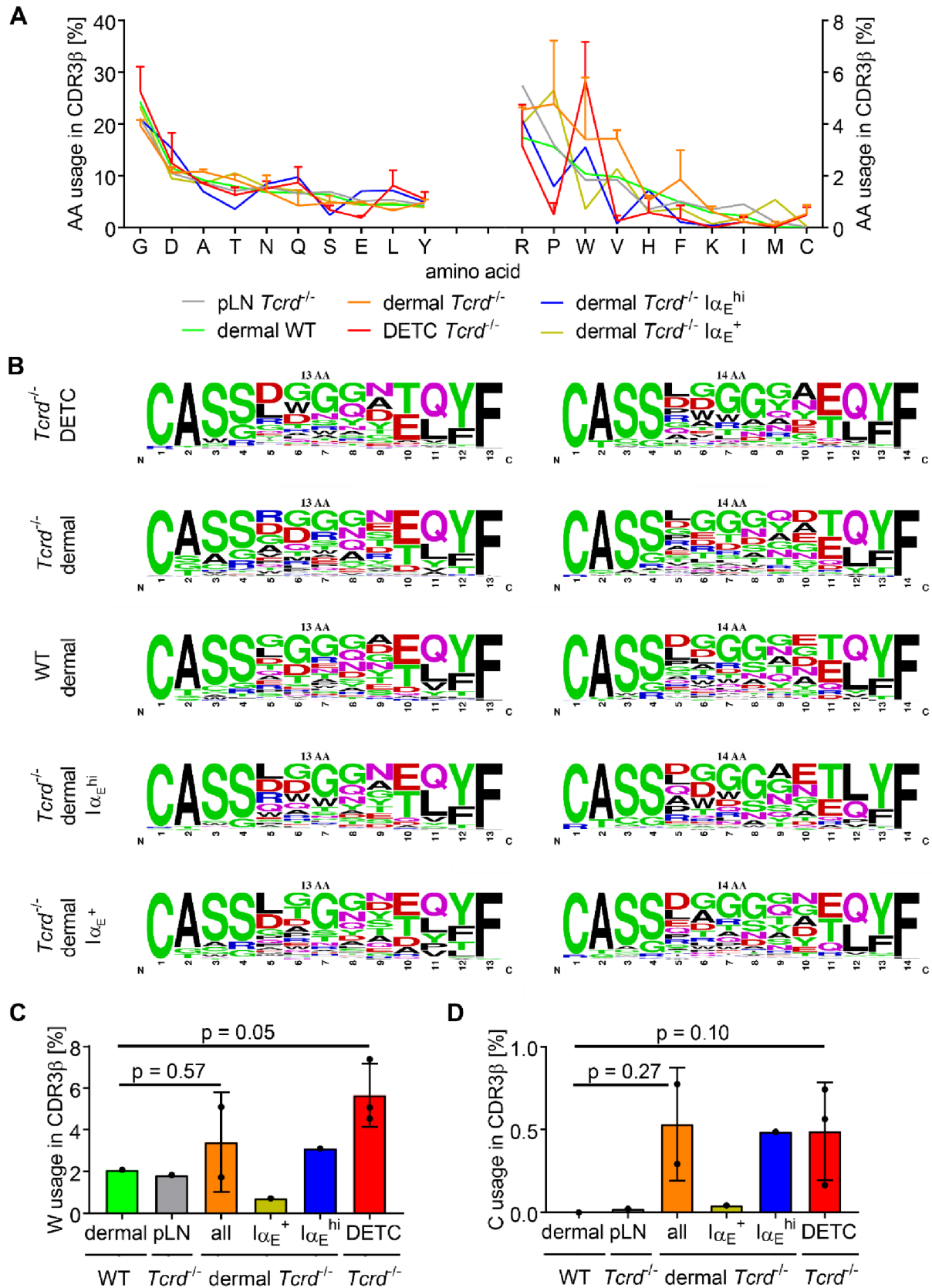


Figure 5: AA usage in CDR3β of WT and *Tcrd*^{-/-} skin T cells

(A) CDR3β AA usage of WT dermal αβ T cells (n=1), *Tcrd*^{-/-} pLN (n=1), *Tcrd*^{-/-} dermal T cells (n=2), *Tcrd*^{-/-} DETC (n=3), *Tcrd*^{-/-} dermal Iα_E⁺ T cells (n=1) and *Tcrd*^{-/-} dermal Iα_E^{hi} T cells (n=1). The conserved cysteine plus three following and the conserved

phenylalanine plus three preceding AA were excluded from the analysis. Mean and SD are shown. (B) Pictograms of the CDR3 β of WT dermal $\alpha\beta$ T cells (n=1), *Tcrd*^{-/-} pLN (n=1), *Tcrd*^{-/-} dermal T cells (n=2, pooled prior to analysis), *Tcrd*^{-/-} DETC (n=3, pooled prior to analysis), *Tcrd*^{-/-} dermal I α _E⁺ T cells (n=1) and *Tcrd*^{-/-} dermal I α _E^{hi} T cells (n=1) from sequences with a CDR3 β length of 13 AA (left) and 14 AA (right). CDR3 β tryptophane (C) and cysteine (D) usage of samples indicated for (A). (C) and (D) show cysteine and tryptophane usage in the CDR3 β in higher resolution than presented in (A). Each point in the diagrams represents one sequencing-sample. Statistical analysis was performed by one-sample t-test comparing all samples with n > 1 to dermal WT as control.

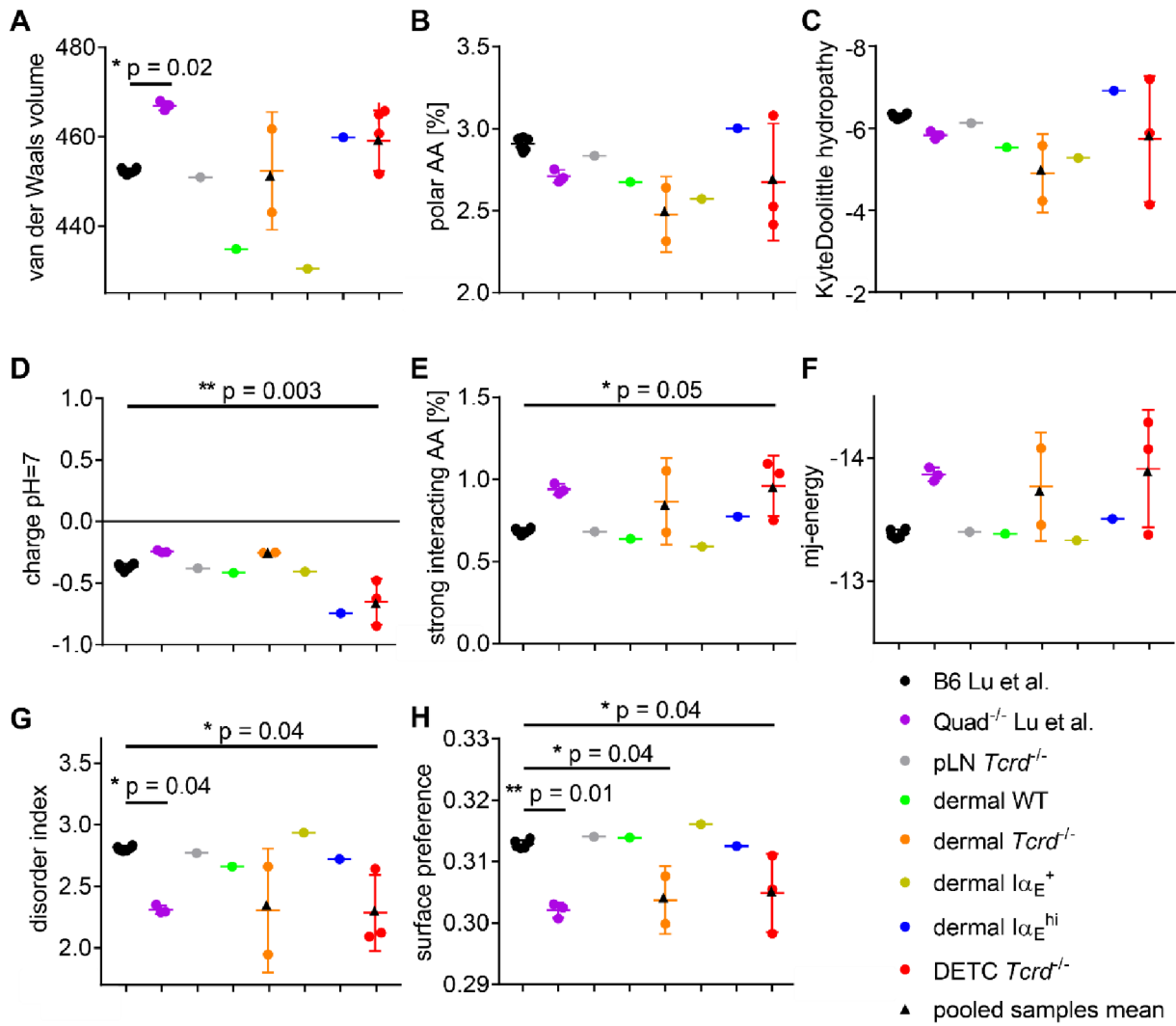


Figure 6: Comparison of physicochemical properties of CDR3 β AA of WT and *Tcrd*^{-/-} skin T cells

(A to H) CDR3 β AA physicochemical properties. The data consider the center-5 AA of the CDR3 β , are clonotype-frequency-weighted and not normalized to the number of AA that were considered (in all samples 5 AA). In all diagrams, each point represents one sequencing-sample. Mean and SD are shown. The black triangles indicate the respective value obtained after pooling equivalent samples prior to analysis. The first two data-sets presented (B6 and Quad^{-/-}) are derived from previously published work from Lu et al. [27] as control-samples. Statistical analysis was performed by One-way-ANOVA and Holm-Sidak multiple comparisons considering all samples with $n > 1$.

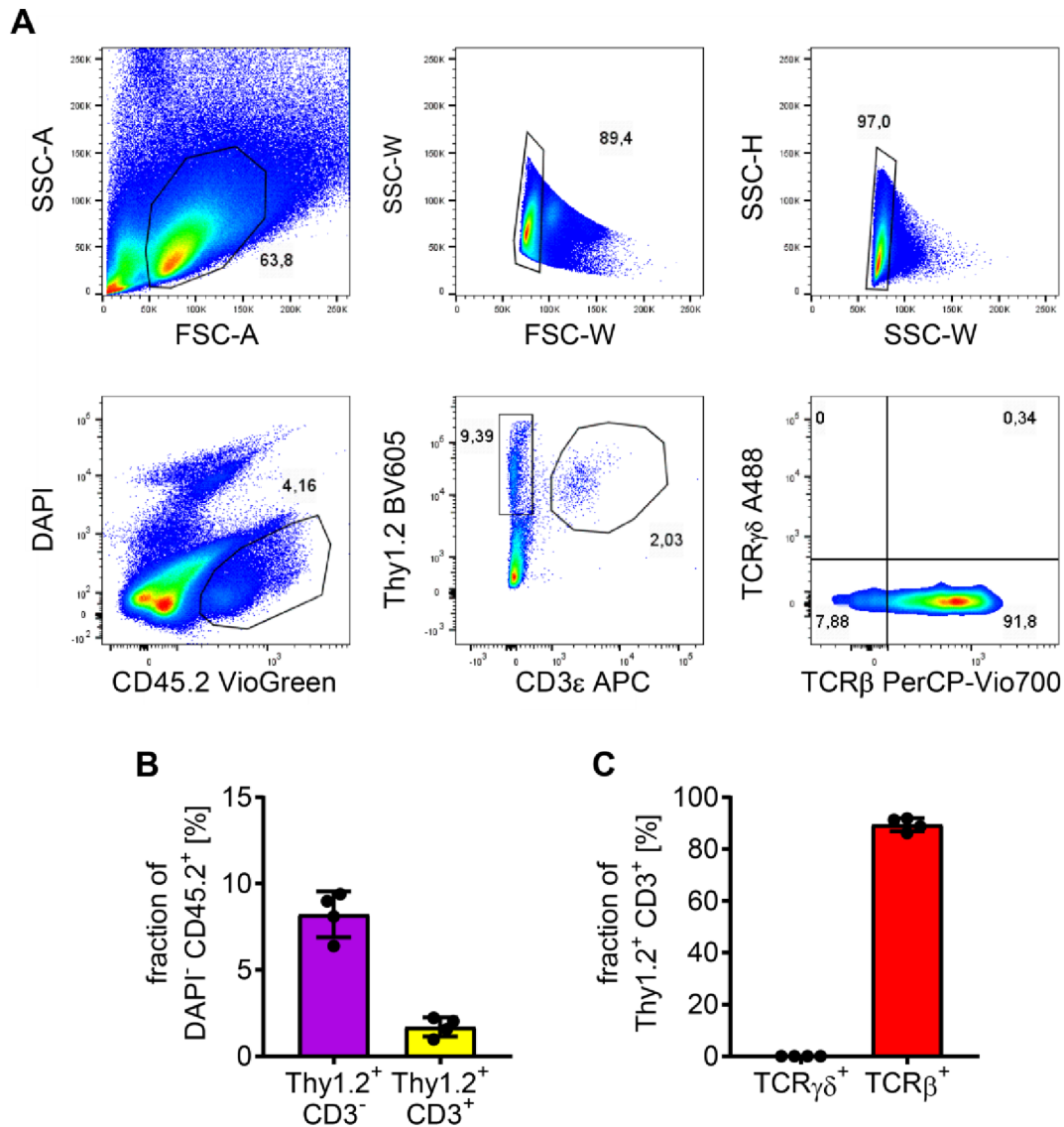


Figure 7: Flow-cytometric analysis of newborn *Tcrd*^{-/-} skin

(A) representative gating strategy for the flow-cytometric analysis of the skin from newbrn *Tcrd*^{-/-} mice. (B) percentage of Thy1.2⁺CD3⁻ cells and Thy1.2⁺CD3⁺ cells of all DAPI⁻CD45.2⁺ cells. (C) TCRγδ and TCRβ expression of CD3⁺ cells in newborn *Tcrd*^{-/-} skin. Each point in the diagrams represents a mouse, mean and SD are shown. (This figure is in preparation for the revision of the manuscript.)

Supplementary information for

MHC-independent $\alpha\beta$ T cells replace epidermal and dermal $\gamma\delta$ T cells in *Tcrd*^{-/-} mice

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Supplemental informations:

Table S1: antibody-list

antigen	fluorophore	clone	company
TCR Vg5/Vg6:Vd1		17D1 (IgM)	homemade
anti-rat-IgM	PE	RM7B4	eBioscience
CD103	BV711	2E7	BioLegend
CD103	PE	2E7	Miltenyi
CD122	PE	TM- β 1	BioLegend
CD127	PerCP-Cy5.5	A7R34	BioLegend
CD215	PE	6B4C88	BioLegend
CD3	APC	145-2C11	BioLegend
CD3	PE	145-2C11	BD Pharmingen
CD3	PE	REA641	Miltenyi
CD3	PE-Cy7	145-2C11	Invitrogen
CD4	Cy5	GK1.5	homemade
CD4	PerCP	RM4-5	BioLegend
CD45.2	VioGreen	104-2	Miltenyi
CD5	PE-Cy7	53-7.3	BioLegend
CD69	PE-Cy7	H1.2F3	BioLegend
CD8 α	APC-Vio770	REA601	Miltenyi
CD8 α	BV711	53-6.7	BioLegend
JAML	A647	4E10	BioLegend
CD314	PE	C7	BioLegend

antigen	fluorophore	clone	company
Tcr β	A488	H57-597	BioLegend
Tcr β	APC-Vio770	REA318	Miltenyi
Tcr β	PE-Cy7	H57-597	BioLegend
Tcr β	PerCP-Vio700	REA318	Miltenyi
TCR $\gamma\delta$	A488	GL3	homemade
TCR $\gamma\delta$	APC	GL3	BioLegend

Table S2: primer sequences for the NGS

primer	sequence
mTCR β	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGCTCAAA CAAGGAG
mTCR γ	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTATGGAG ATTTGTTTCAGCA
long primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAG AGT
short primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAATACG ACTCACTATAGGGC

Supplemental figures:

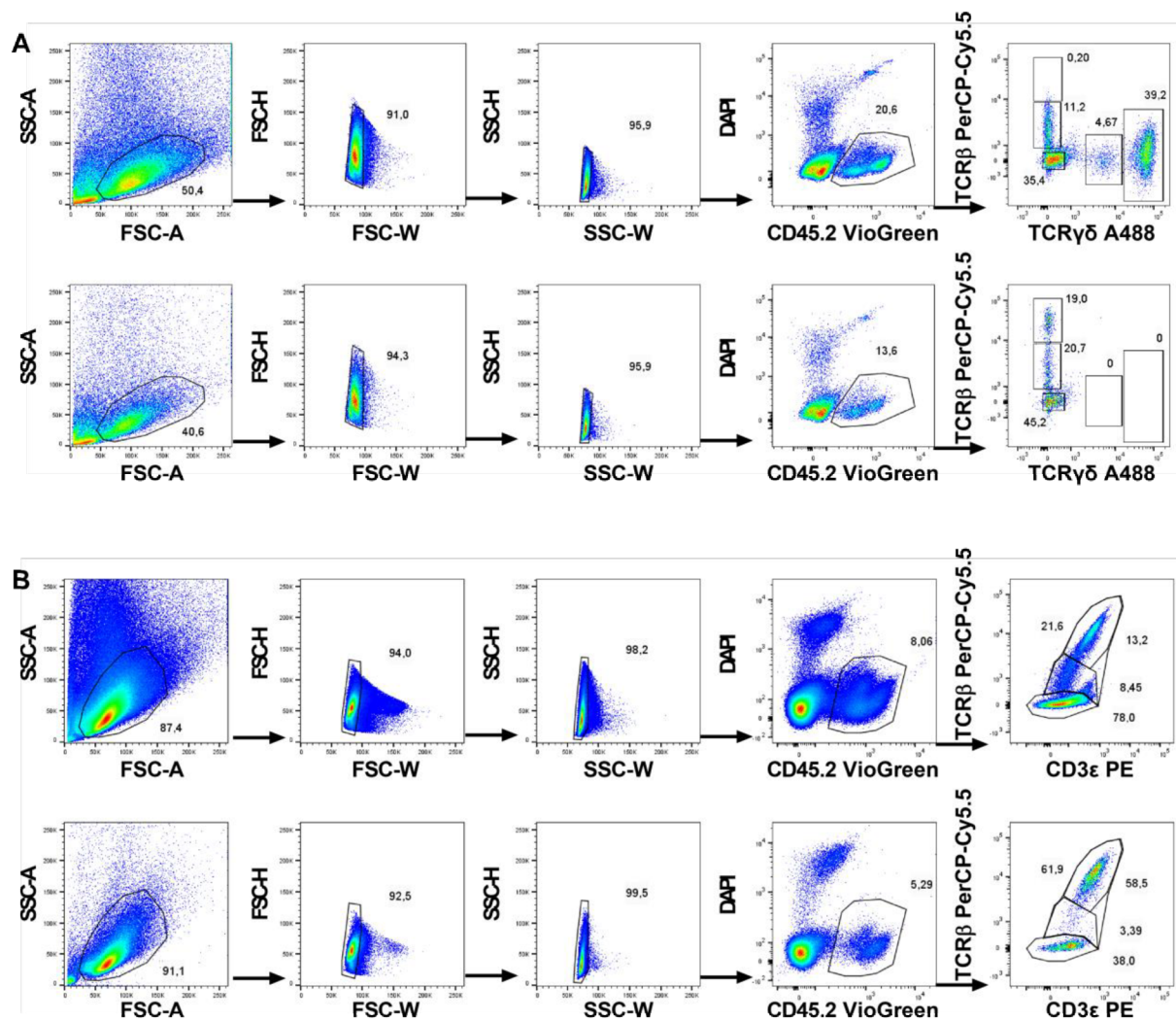


Figure S1: CD8α is not quantitatively digested during the lymphocyte-isolation from the tissue

Representative gating strategy for ear skin cells (A) isolated from a *Tcrd*^{-/-} mouse (upper panel) and WT mouse (bottom panel) for analysis of cellular abundance and surface-protein expression. Representative gating strategy for analysis of ear skin T cells after mechanical separation of dermis (upper panel) and epidermis (bottom panel).

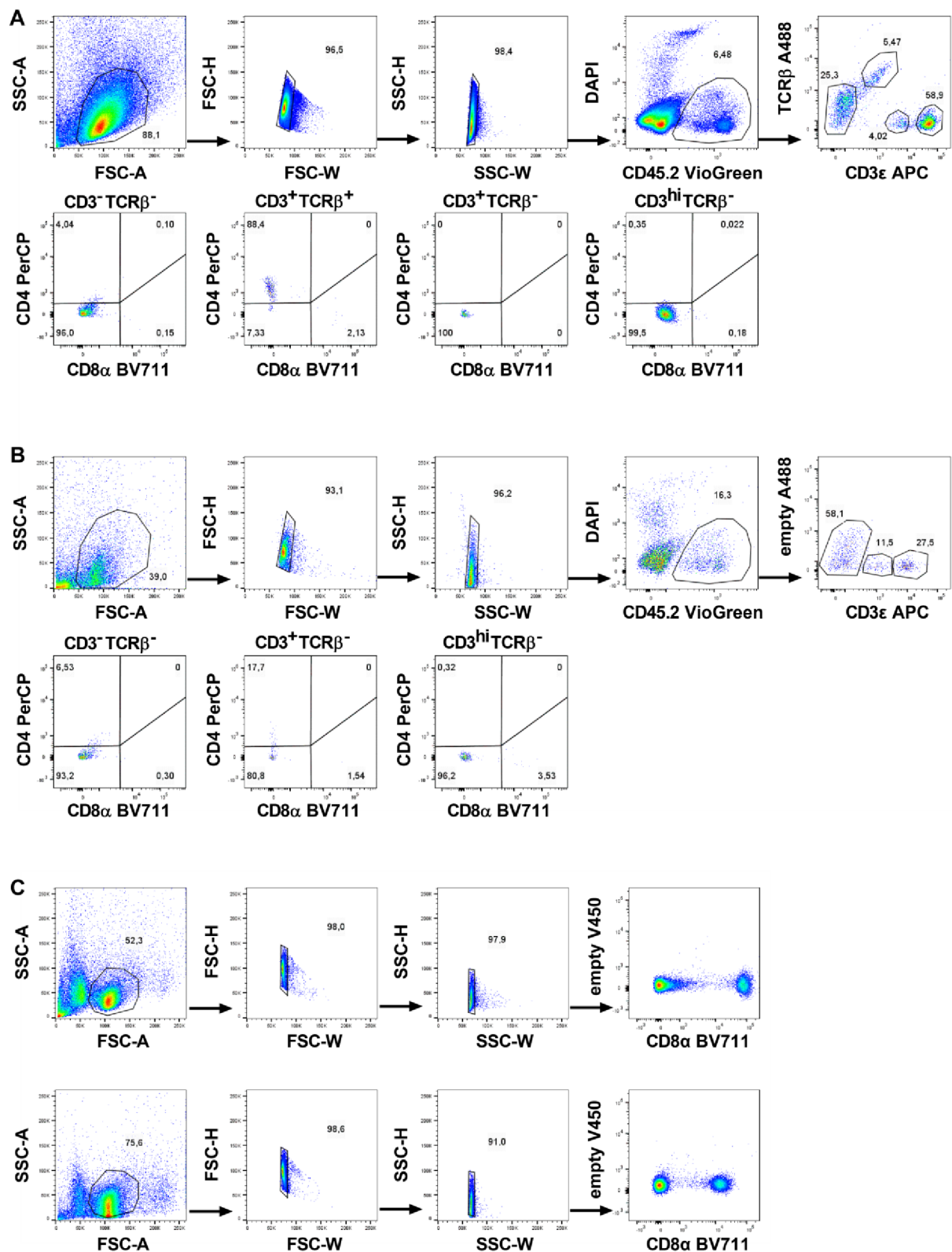


Figure S2: Gating strategy for CD4/ CD8 expression analysis

Representative gating strategy for the identification of CD4/ CD8 expressing skin T cells in WT (A) and *Tcrd*^{-/-} mice (B). Lymph-node cells (C) not digested (upper panel)

and digested (bottom panel) under the same conditions as ear skin cells during the isolation of lymphocytes from the skin-tissue.

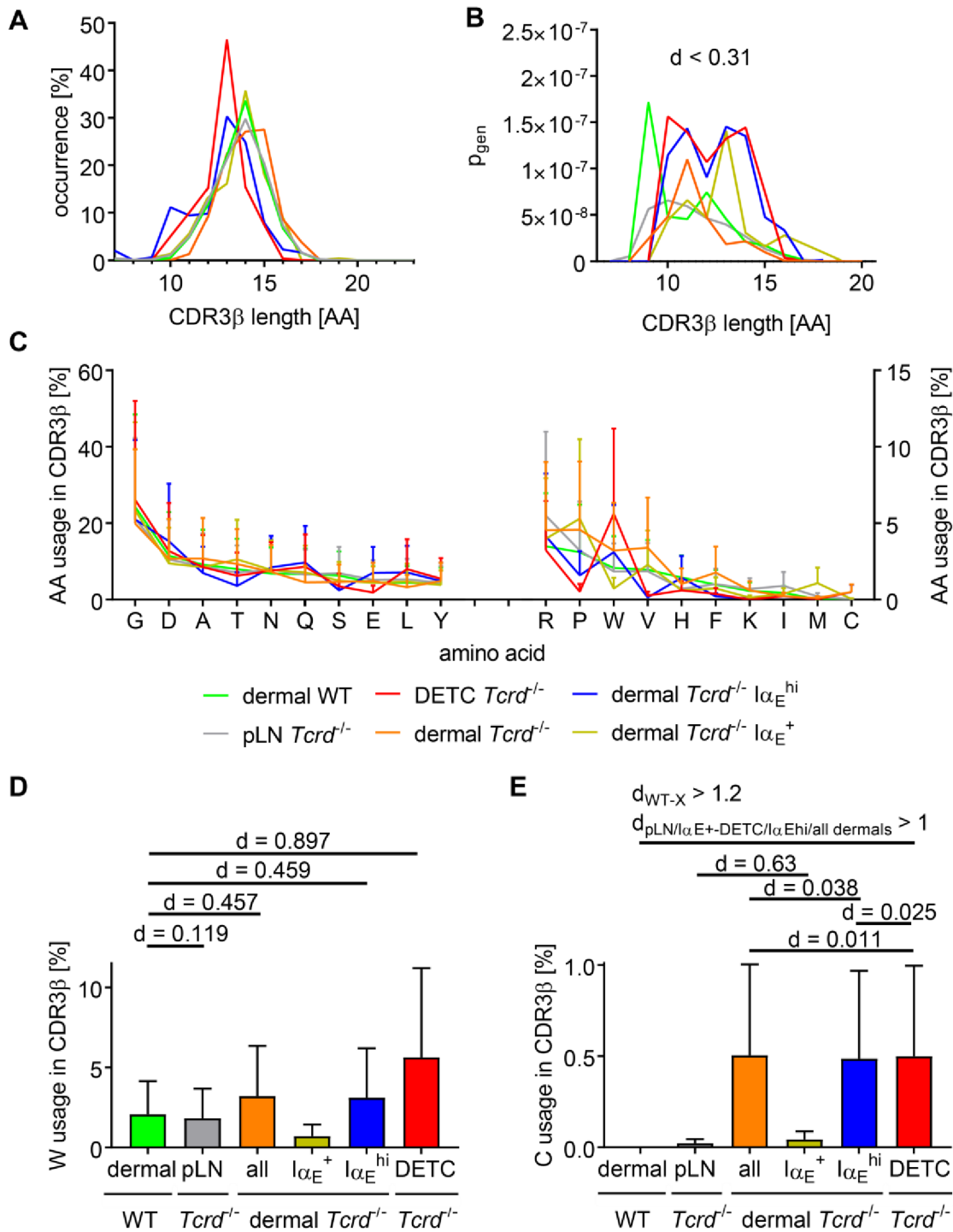


Figure S3: Assessment of the NGS-data after pooling equivalent samples prior to analysis

(A) CDR3 β length, (B) CDR3 β rearrangement probability, (C) CDR3 β AA usage, (D) CDR3 β tryptophane and (E) cysteine usage of samples indicated in fig 4C. During AA usage analysis (C, D, E), the conserved cysteine plus three following and the conserved phenylalanine plus three preceding AA were excluded from the analysis. Mean and SD are shown. (D) and (E) show tryptophane and cysteine usage in the CDR3 β in higher resolution than presented in (C). The data were analysed after

pooling all equivalent samples of a respective population. Mean and SD are shown. Statistical analysis was performed by Cohen's d effect size analysis (in " $d_{\text{index-index}}$ " the index states which groups are compared or "X" for any group).

7.3.2 Manuscript II

Interleukin-17 is disease promoting in early stages and protective in late stages of experimental periodontitis

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Abstract

Periodontitis is one of the most common infectious diseases in humans. It is characterized by a chronic inflammation of the tooth-supporting tissue that results in bone loss. However, the role of the pro-inflammatory cytokine interleukin-17 (IL-17) and of the cells producing it locally in the gingiva is still controversial. First, there are divergent results regarding the question whether in periodontitis IL-17 plays a tissue-damaging role or whether it is rather limiting tissue-damage. Moreover, it is not clear, which cell type is the main producer of IL-17 in periodontitis and decisive for the outcome of the severity of periodontal inflammation. Th17 $\alpha\beta$ T cells, CD4⁺ exFoxP3⁺ $\alpha\beta$ T cells, or IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ 17 cells) seem to be prime candidates. To address these issues in an experimental model for periodontitis, we employed several genetic mouse models deficient for either $\gamma\delta$ T cells or IL-17 cytokines. We found that $\gamma\delta$ T cells are not the decisive IL-17 producers during the course of periodontitis. Apart from that, our data support earlier studies that suggested bone-damaging effects of IL-17 in early periodontitis and rather bone-protective effects in late stages of experimental periodontitis.

Introduction

$\gamma\delta$ T cells are innate T cells developing in embryonic and postnatal phase¹, conserved among almost all jawed vertebrates² including mice and humans. In mice, $\gamma\delta$ T cells are known to constitute the majority of epithelial-resident T cells³. After their development in the thymus, they egress in several waves of cells expressing distinct $\gamma\delta$ TCRs to home to these epithelial tissues^{1,4}, as for example the skin and the gingiva^{3,5}. The dermal layer of the skin is populated by $V\gamma 4^+$ and $V\gamma 6^+$ $\gamma\delta$ T cells that rapidly produce IL-17 to induce inflammation^{3,6,7}. Recently we and others found that also the gingiva is populated by mainly $V\gamma 6^+$ $\gamma\delta$ T cells⁸.

The gingiva is an extraordinary site of the body-barrier, with the junctional epithelium (JE) directly lining the teeth, where also the $\gamma\delta$ T cells concentrate⁸. The JE represents a transmucosal passage in the external body-barrier connecting the bacteria inflicted oral cavity with the subjacent tissue layers including bone. While the epithelium of the oral cavity as well as other epithelial sites is protected by tight junctions, the JE is very thin and only weakly attached to the teeth via hemidesmosomes⁹. Apart from that, the JE is much closer to the underlying alveolar bone than any other external body barrier epithelia. On the other hand, the JE is exposed to constant mechanical forces by mastication and to the oral bacteria, which both influence the oral immune homeostasis^{10–12}.

With a prevalence of almost 50% in adults, periodontitis is a very common^{13,14} inflammatory disease of the tooth-supporting tissue. It is induced by oral bacteria as well as by the subsequent reaction of the host immune system¹⁵. Untreated it may lead to tooth-loss, since the chronic inflammation is accompanied by the loss of periodontal attachment and retraction of the alveolar bone¹⁶. *Porphyromonas gingivalis*, together with *Tennerella forsythia* and *Treponema denticola*, belongs to the “red complex” of bacteria, which are strongly associated with periodontal disease, originally described by Soransky *et al.*¹⁷. The invading oral bacteria can be disseminated to other sites of the organism¹⁸ and drive inflammation there^{19,20}, which was already proposed in 1891 in the aptly named article “The human mouth as a focus of infection”²¹. Furthermore, periodontitis is linked to diseases like cardiovascular diseases²² and rheumatoid arthritis^{23,24}.

During the course of periodontitis, osteoclasts in the tooth-supporting tissue are activated by “receptor activator of NF κ B ligand” (RANKL)²⁵ and “secreted osteoclastogenic factor f activated T cells” (SOFAT)²⁶ resulting in the destruction of

alveolar bone. RANKL expression has been reported to be induced on osteoblastic and ligament cells by IL-17 produced by T cells^{27,28} as well as being expressed by those T cells themselves, both also true for periodontitis¹⁸. SOFAT, a RANKL-independent activator of osteoclastogenesis, is directly secreted from T cells and increased in periodontitis patients.

It is unclear whether exFoxp3 Th17 $\alpha\beta$ T cells¹⁸ or amphiregulin producing $\gamma\delta$ T cells²⁹, that were shown to produce IL-17 in the gingiva⁸, play the most decisive role for the outcome of the inflammation. A study from Krishnan *et al.* suggested an enhanced bone-loss in *Tcrd*^{-/-} mice compared to WT after ten days of inflammation, while Tsukasaki *et al.* could not see any difference between both genotypes at that time point. However, after ten days of inflammation, bone loss was reduced in *IL17af*^{-/-} mice¹⁸. The IL-17 produced during the inflammation has been shown to be indispensable for the recruitment of neutrophils to the gingiva³⁰, whose efferocytosis by macrophages in turn limits the production of IL-17³¹⁻³³. In IL-17RA knockout mice, this recruitment is not possible and the alveolar bone loss is enhanced after six weeks of inflammation³⁰. Furthermore, at other barrier sites, $\gamma\delta$ T cells have been reported to be beneficial with respect to body-barrier integrity^{34,35}, host-defense⁵ or host-commensal homeostasis³⁶. Therefore, it is unclear whether IL-17 and its producers have a bone-protective or a bone-damaging role. It has been proposed that IL-17 is a “double-edged sword”¹⁸ in this context, fighting pathogens but simultaneously damaging the bone-supporting tissue.

To shed more light onto these partially contradicting results, we used an experimental model of ligature-induced periodontitis and analyzed the ligature-induced bone loss in *Tcrd*-H2BeGFP (as WT-control expressing eGFP in all $\gamma\delta$ T cells), *Tcrd*^{-/-} and *IL17af*^{-/-} mice after four weeks of inflammation was analyzed, representing a time point between the two assessed ones in the publications mentioned above^{18,29,30}. Surprisingly, we could not detect any differences between those three genotypes. Therefore, we conclude that the IL-17 production by the present $\alpha\beta$ T cells must be more relevant during the course of periodontitis, than the one by $\gamma\delta$ T cells, as previously shown by Tsukasaki *et al.* and that IL-17 indeed acts as a double-edged sword. Moreover, our data add to the idea that the bone-damaging effects of IL-17 in periodontitis might be an evolutionarily beneficial emergency-stop switch for the inflammation, that otherwise persists for a longer period and threatens the whole organism.

Results

***P. gingivalis* is dispensable for bone loss in ligature-induced periodontitis in mice**

Since there are different methods in use to assess the bone erosion in periodontitis experimentally^{37–39}, we aimed to investigate the bone erosion in *Tcrd*-H2BeGFP mice, *Tcrd*^{−/−} mice and *IL17a*^{−/−} mice performing two slightly distinct models and applying two different ways of analysis afterwards. The first approach was based on an initial reduction of oral microbiota by application of ampicillin and kanamycin for five days. After two days without antibiotics, ligatures were placed, tying a piece of suture material around both second molars of the maxilla and fixing it with two nuds on the lingual side of the teeth. *P. gingivalis* was subsequently administered orally five times per week for four consecutive weeks (fig. 1A). On the other hand, we only applied a ligature around the second molars of the mice, sacrificed and analyzed the mice 28 days later. The analysis of bone erosion was performed after imaging the jaws by measuring the cemento-enamel-junction to alveolar-bone-crest (CEJ-ABC) distance at defined tooth-structures³⁷ at the ligated second molar or by measuring the area of the teeth laying open between CEJ and ABC (fig. 1B). Both models induced significant and comparable bone-loss (fig. 1C and 1D). There seemed to be a more severe bone erosion in the *Tcrd*-H2BeGFP mice compared to the *Tcrd*^{−/−} and *IL17a*^{−/−} mice, which showed very similar amounts of bone loss, but no statistically significant differences were detectable between the three genotypes. Furthermore, we analyzed the immune cells in the gingiva of the respective mice by flow cytometry. The number of CD45⁺ cells or of T cells did not increase in response to either treatment (fig. 2A). Looking into the T cell compartment we could not detect any significant changes induced by the treatment regarding αβ T cells, γδ T cells or the predominant Vγ6⁺ γδ T cells in percentagewise or absolute presence in the gingiva, despite there seemed to be a slight tendency towards an increase in both subsets for the ligature and ligature plus *P. gingivalis* treatment group (fig. 2B and 2C), confirming earlier reports¹⁸. While neutrophils in ligature treated animals, in line with the literature^{18,30}, seemed to increase percentagewise and showed a statistically significant increase in absolute numbers, the macrophages showed a significant percentagewise decrease and no absolute decrease, suggesting an enhanced neutrophil presence in the gingiva (fig. 2D). Due to the high similarity of the outcomes of both models, we decided to restrict the following experiments to the ligature-model.

RT-PCR reveals protective role of IL-17 in periodontitis independent of $\gamma\delta$ T cells

The bone loss observed in the model used here, is a result of the inflammation-associated T cell activation in the gingiva, leading to RANKL secretion by T cells, that in turn binds to its receptor RANK on osteoclasts inducing their final differentiation and thereby intensifying the bone loss²⁵. A RANKL-inhibitory protein is osteoprotegerin (OPG), that is secreted by osteoblasts and blocks the RANKL-RANK binding by binding to RANKL²⁵. Besides a qPCR-measurement of their induction during the experimental periodontitis, we assessed the osteoclast-activity based on the induction of the tartrate-resistant acid-phosphatase (TRAP), an enzyme responsible for bone resorption by osteoclasts⁴⁰ and growth-arrest-specific-6 (GAS6), that has been reported to be a key factor for establishment of oral homeostasis¹¹ and that activates osteoclasts via the Tyrosine-protein-kinase-receptor Tyro-3⁴¹.

Notably, induction of IL-17 expression was only detectable in *Tcrd*-H2BeGFP and *Tcrd*^{-/-} mice to a comparable level and not at all in *IL17af*^{-/-} mice (fig. 3A). Apart from that, induction of IL-23 expression is diminished in *IL17af*^{-/-} mice in response to the treatment (fig. 3B), which suggests in line with the missing IL-17 in these mice a weaker inflammation. Neither GAS6 nor TRAP expression was differently induced by the treatment in any group (fig. 3C-D), supporting our data not showing a difference in bone loss.

Furthermore, the RT-PCR showed no difference in RANKL or OPG induction by the treatment in *Tcrd*-H2BeGFP or *Tcrd*^{-/-} mice (fig. 3E-F). In *IL17af*^{-/-} mice, RANKL expression seems to be slightly more induced and OPG expression slightly less induced compared to *Tcrd*-H2BeGFP and *Tcrd*^{-/-} mice (fig. 3E-F). Accordingly, a significantly increased RANKL/OPG ratio induced by the treatment occurs in *IL17af*^{-/-} mice (fig. 3G). Together those data suggest, there is no difference in real osteoclast activity, whereas the milieu might become in *IL17af*^{-/-} mice more and more osteoclastogenic, taking into account the elevated RANKL/OPG ratio. Apart from that, the data suggest, that the protective role of IL-17 is in the context of periodontitis largely independent of $\gamma\delta$ T cells, as proposed before¹⁸.

Equal alveolar bone loss in *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice after four weeks of inflammation

After no difference in bone loss in response to periodontitis in *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice, there was a need to corroborate the data regarding the bone-loss-measurements, since they suggested, although statistically not significant, a tendency of diminished bone erosion not only in the *IL17af*^{-/-} mice, but also in the *Tcrd*^{-/-} mice. Therefore, we measured the CEJ-ABC distance on HE-stained cryo-sections. This analysis did not show any significant differences between the genotypes, whereas again the *IL17af*^{-/-} mice seem to develop a less severe bone loss (fig. 4A and 4B).

Furthermore, we assessed the bone erosion in *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice in response to ligature-application by μ CT-imaging of the respective jaws and subsequent measurement of the CEJ-ABC distance. The 3-dimensionality of the data allowed us to assess the CEJ-ABC distance from both the palatal (fig. 4C-D) and the buccal side (fig. 4E-F) of the jaws. Applying this analysis, we could not detect any differences in bone erosion between the three genotypes (fig. 4D and F).

The three different imaging methods, two models and four variants of analysis we performed did not reveal any significant differences in bone loss between *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice suggesting that, in line with previous reports¹⁸, in the gingiva, the IL17-response by $\alpha\beta$ T cells is in contrast to other epithelial sites decisive for the outcome of the inflammation. However, in contrast to earlier reports showing an amelioration of bone loss in *IL17af*^{-/-} mice ten days after inflammation¹⁸, four weeks after inflammation the alveolar bone loss of *Tcrd*-H2BeGFP and *IL17af*^{-/-} mice seems to equalize.

$\gamma\delta$ T cells are not functionally replaced by $\alpha\beta$ T cells in *Tcrd*^{-/-} gingiva

Other experimental models suggest that $\gamma\delta$ T cells can be functionally replaced by $\alpha\beta$ T cells⁴². Therefore, we hypothesized that potentially Th17 $\alpha\beta$ T cells functionally replaced the missing $\gamma\delta$ T cells in the gingiva of the *Tcrd*^{-/-} mice, hiding the expected phenotype of reduced bone loss. To test this, we applied the ligature-model to *Tcrd*-GDL mice that can be conditionally depleted of all $\gamma\delta$ T cells by diphtheria-toxin treatment. The comparison of $\gamma\delta$ T cell depleted and not depleted *Tcrd*-GDL mice did not reveal an amelioration of bone loss after ligature-treatment (fig. 5). This suggests that different from other sites, in periodontitis $\gamma\delta$ T cells are not functionally replaced

by $\alpha\beta$ T cells and the equal bone-loss in *Tcrd*-H2BeGFP and *Tcrd*^{-/-} mice is due to an anyway more decisive function of the $\alpha\beta$ T cells.

Discussion

In this study, we aimed to clarify the contradicting effects of IL-17 and its producers in the course of periodontitis. While IL-17 had a bone-damaging effect around day ten of periodontitis^{18,29}, it seemed to display an overall protective role in case the inflammation persisted for a period as long as six weeks³⁰. In other epithelial sites of the organism than the gingiva, IL-17 is associated with inflammation-exacerbation and in case of deregulation with the establishment of chronic inflammation, as for example psoriasis in the skin⁷, enthesial inflammation⁴³ or inflammations in the lung⁴⁴. In those sites, IL-17 production by $\gamma\delta$ T cells plays a prominent role for induction of the inflammation and associated tissue-damaging side effects. The situation in early periodontitis reflected this effect of IL-17, although it has been shown that in the gingiva the IL-17 production by CD4⁺ $\alpha\beta$ T cells plays an important role^{45,46} that seems to be more decisive¹⁸ than the one by $\gamma\delta$ T cells. Our data, measured at a medium-duration of periodontitis (four weeks) showed no differences between *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17a*^{-/-} mice. The conditional depletion of the $\gamma\delta$ T cells, also not leading to a difference in bone loss, sustains these data. Despite that, the RANKL/OPG ratio seems to be slightly enhanced in *IL17a*^{-/-} mice while osteoclast-markers also do not suggest differences in osteoclast activity. We hypothesize that the seemingly contradictory conclusions drawn in past are caused by the different timespan between induction of periodontitis and assessment of bone-loss. From an evolutionary point of view, the teeth are very important. On the one hand you cannot eat and survive without them. On the other hand they are a relatively fragile site in the outer body-barrier⁹. Their consistency out of mineral materials is necessary to ensure the sufficient hardness of the teeth, but also makes the sealing of the connective site between tooth and gingiva difficult and weak. The reported dissemination of oral bacteria¹⁸ to other sites of the body is reminiscent of this paradox. In other words: In case of a periodontal inflammation, which is common in humans, the organism needs to resolve the problem to prohibit potential extension of the inflammation to the fragile neighboring gingiva, to minimize dissemination of bacteria in the body and to avoid tooth-loss. Our data now suggest that in absence of IL-17 after four weeks of periodontitis, the bone-damage reaches the level of *Tcrd*-H2BeGFP (i.e. wild type) mice. Therefore, we hypothesize

that the function of IL-17 in early periodontitis is primarily the same as in other epithelial inflammations. It is pro-inflammatory and by this, in the unique situation in the tooth-supporting tissue, it is bone-damaging. This damage may be an evolutionary useful collateral damage, since it limits the timespan of chronic periodontitis by ultimately leading to tooth-loss and sealing of the body-barrier. Since our data suggest that in case of ongoing inflammation, bone loss in *IL17a^{f/-}* becomes as intensive as in wild type mice and others showed a worsening after six weeks³⁰, the bone-damaging effects of IL-17 limit the timespan of bacterial dissemination to other sites, of potential spreading of the periodontitis to other tooth and potentially hampered mastication due to pain. Based on our data here, and integrating multiple prior publications using similar models, we suggest that the organism finally prefers to effectively fight the bacterial infection, at the price of tooth loss, which is the lesser evil, so that in the end IL-17 has a protective function for the organism, but a damaging for the bone.

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Methods

Animals

C57BL/6-Trdc^{tm1Mal}/J (*Tcrd*-H2BeGFP) mice⁴⁷, B6.129P2-Trdc^{tm1Mom}/J (*Tcrd*^{-/-}) mice⁴⁸, B6.Cg-Il17a/Il17f^{tm1.1Impr} (*IL17af*^{-/-}) mice⁶ and C57BL/6-Trdc^{tm1}(eGFP-2A-hDTR-2A-CBGr99 luciferase)Impr (*Tcrd*-GDL)⁴² mice were bred in the animal facility of the central animal facility at Hannover Medical School (Hannover, Germany). All animal experiments were conducted in compliance with the German animal protection law (TierSchG BGBI. I S. 1105; 25.05.1998). All animal experiments were approved by the Lower Saxony Committee on the Ethics of Animal Experiments as well as the Lower Saxony State Office of Consumer Protection and Food Safety under the permit number 33.12-42502-04-16/2167.

P. gingivalis culture and preparation for infection

Culture and handling of *Porphyromonas gingivalis* (ATCC 33277) was performed in an anaerobic incubator under an atmosphere containing 80% nitrogen, 10% carbon dioxide and 10% hydrogen. Every other day Schaedler medium (Oxoid Limited), supplemented with vitamin K (10 µg/ml, Carl Roth GmbH & CO. KG, Karlsruhe, Germany) was inoculated with *P. gingivalis* to prepare liquid cultures. 6x10¹² cells per animal per day were resuspended in 50 µl carboxymethyl cellulose (CMC) 2% dissolved in PBS to prepare the inoculum for oral infection.

Ligature application and *P.gingivalis* administration for 28 days

Prior to ligature placement all animals received a combination of ampicillin and kanamycin (2 mg each per day and animal dissolved in ultrapure water) over 5 days. The antibiotics were administered directly into the oral cavity. After two days of recovery, ligatures were applied. In brief, the mice were anesthetized (Xylacin 5 mg/kg body weight and Ketamin 100 mg/kg body weight) and a piece of suture material (PremiCron 6/0, B.Braun Surgical, S.A. Rubi. Spain) was tied around each maxillar second molar and fixed with two nods on the lingual side of the teeth. The ligature was not applied in control mice. The following 28 days, the mouth of the “ligature + *P. gingivalis*” group was flushed 5 days per week with 50 µL of bacterial inoculum, prepared as described above. The “ligature” group of mice and control mice only got CMC. At day 28, the mice were sacrificed and further processed.

Defleshing, staining and microscopy of extracted jaws

For the quantification of alveolar bone loss around ligature treated teeth, the maxillae of the mice were partly dissected, including the complete molar area and fixed in paraformaldehyde (4%, dissolved in PBS) for 72h at 4°C. After fixation the samples were rinsed several times in PBS and stored afterwards in PBS at 4°C until further processing. To remove all the soft tissue from the jawbone and teeth, the dissected samples were incubated in 25% NaOH in a 15ml clear plastic tube (Sarstedt, Sarstedt AG & Co. KG, Nümbrecht, Germany) at room temperature and shaken vigorously several times within the tubes. When the jawbone and teeth of the maxilla were completely defleshed, they were rinsed several times in PBS, until a stable neutral pH was detectable. Afterwards they were stored in PBS until further processing. The jaws were stained in a solution of 0.5% methylene blue, dissolved in PBS, at a pH of 7.5 for 2-3 minutes. The staining facilitated the detection of the cemento-enamel junction (CEJ). Both rows of maxillary molars were photo documented from the lingual and the buccal side using a stereomicroscope (Type M3Z, Wild, Heerbrougg, Switzerland). The resulting images were analyzed using the IMS Client V16Q2 software. For the detection of alveolar bone loss the exposed area between CEJ and the alveolar bone crest was measured on the buccal and lingual side of the second molar, which had been fitted with the ligature in the experimental group.

Histological analysis

After sacrifice of the mice and removal of the jaws, the tissue was fixated over-night in 4% paraformaldehyde (PFA). The fixated jaws were then decalcified by incubating them in 0.5 M EDTA in PBS for one week, with a daily renewal of the EDTA solution. For another night the jaws were kept in 30% (mass percentage) sucrose. The fixed and decalcified tissue was embedded in Tissue Tek® O.C.T™ Compound and frozen at -20°C. The frozen blocks were cut into 10 µm sections by using a Leica cryotome CM3050, mounted on glass slides and dried for 30 min at 37°C. The sections were stained with Eosin and Hematoxylin. The imaging was performed employing a Zeiss Axio Scan.Z1 slide scanner and the resulting images were analyzed using the Zen (Blue edition) software version 3.2 from Zeiss and GraphPad Prism version 7.05.

μCT analysis

After sacrifice of the mice and removal of the maxilla, the right and left maxillary teeth-rows were excised and the gingival tissue was removed by forceps. The bone and teeth were subjected to μCT analysis, performed by the small-animal-imaging center of the central animal facility of Hannover medical school (Hannover, Germany) in a Siemens Inveon μCT (80kV, 500μA, 720 projections, binning 1; effective pixel size 8.17μm). For the analysis, the 3D-datasets were analyzed within the Inveon research workspace version 4.2 by positioning the jaws horizontally to allow a view onto the palatal or buccal side of the jaw and subsequent measurement of the CEJ-ABC distance in the software. Further data processing was performed in GraphPad Prism version 7.05.

qPCR

After sacrifice of the mice and removal of the maxilla, the right and left maxillary teeth-rows were excised and the gingival tissue was removed by forceps. The remaining bone and teeth were subjected to μCT analysis. The gingival tissue was stored in PBS and subjected to RNA-isolation by an Ultra-turrax T18 basic from IKA and Qiagen RNeasy micro-kit. 4 μg RNA were subjected to cDNA synthesis (4μg RNA in 12 μl dH₂O, 1 μl oligodT (12 μM), 1 μl dNTP (2.5 mM)) and heated in a PCR-cycler for 5 min to 65°C. Immediately the mixture was placed on ice and a PCR-mastermix (4 μl superscript III 5X FS buffer buffer, 1 μl DTT (100 μM), 1 μl superscript III (200 U/μL), 0.1 μl RNase out (40 U/μL)) was added. The mixture was then placed into the PCR-cycler and subjected to cDNA synthesis (60 min 50°C followed by 15 min 70°C). The following real-time qPCR was performed employing the SYBR® Premix Ex Taq™ II Kit from Takara Bio and HPLC-purified customized primer supplied from Sigma Aldrich (*GAS6* forward: AGGTCTGCCACAACAAACCA; *GAS6* reverse: GCGTAGTCTAATCACGGGGG; *IL-17* forward: GCCCTCAGACTACCTCAACC; *IL-17* reverse: GTCCTAGTAGGGAGGTGTGAAGTTG; *IL-23* forward: AGCGGGACATATGAATCTACTAAGAGA; *IL-23* reverse: GTCCTAGTAGGGAGGTGTGAAGTTG; *OPG* forward: TACCTGGAGATCGAATTCTGCTT; *OPG* reverse: CCATCTGGACATTTTTTGCAA; *RANKL* forward: TGTACTTTCGAGGGCAGATG; *RANKL* reverse: AGGCTTGTTTCATCCTCCTG; *TRAP* forward: GCTGGAAACCATGATCACCT; *TRAP* reverse: GAGTTGCCACACAGCATCAC). During the data analysis according to

the $\Delta\Delta C_t$ method employing microsoft excel 2010 and GraphPad Prism version 7.05, only samples displaying C_t values lower than 35 were included. Data were normalized to HPRT-induction as housekeeping-gene.

Isolation of gingival lymphocytes for flow cytometry

Isolation of gingival lymphocytes was performed as described previously⁸. In brief, after sacrifice of the mice, the maxilla was carefully removed. The right and the left molar teeth-rows and the adjacent gingiva were excised with a scalpel. The tissue was subjected to a digestion by collagenase IV (2 mg/mL) and DNaseI (0.025 mg/mL) in RPMI 1640. After incubation in a shaker (1400 rpm) at 37°C for 1h, the digestion was stopped by adding 150 μ L 0.5 M EDTA and further incubation under the same conditions for 15 min. After removal of the gingival tissue from the bone and its mincing, the lymphocytes were then isolated by filtering the medium and the minced gingival tissue through a cell-strainer (100 μ m), washing of the cell-strainer by 6 mL MACS buffer (3% FCS (volume percentage) and 0,8% 0.5 M EDTA (volume percentage) in PBS) and centrifugation at 1250 rpm and 4°C for 5 min. The supernatant was removed and the cells were resuspended in 100 μ L of PBS for live/dead staining (Zombie Aqua dye, BioLegend) for 5 min on ice. After washing in MACS, the cells were blocked 5 min on ice with 5 % F_c-block (clone 2.4G2) in MACS buffer and stained with the respective antibodies (CD45.2-APC-eF780, eBioscience, clone 104; CD3e-PB, BioLegend, clone 17A2; Tcr β -PerCP-Cy5.5, eBioscience, clone H57-597; TCR $\gamma\delta$, homemade, clone GL3; IgM-PE, eBioscience, clone RM-7B4; Vg5/Vg6, homemade, clone 17D1; Ly6G-PE, BioLegend, clone 1A8; Ly6C-PE-Cy7, BioLegend, clone HK1.4) diluted in MACS buffer for 30 min on ice. Flow cytometric analysis was performed employing a Becton Dickinson LSRII, FACSDiva software version 8.0.1, FlowJo version 10 and GraphPad Prism version 7.05.

Conditional ablation of $\gamma\delta$ T cells in vivo

The $\gamma\delta$ T cell depletion was performed as previously described⁴². In brief, *Tcrd*-GDL mice were treated two times with 15 ng diphtheria-toxine (DTx) per gram body weight by intraperitoneal injection. The first DTx injection was performed 7 days before the ligature-application, the second one 5 days before the ligature-application. *Tcrd*-GDL mice in control groups were injected with PBS. Finally, the mice were subjected to the ligature-induced periodontitis model.

Statistical analysis

Statistical analysis was performed in GraphPad Prism version 7.05. A p-value below 0.05 was considered as significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns (not significant).

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Authors contributions

Investigation, A.W., C.B., E.B., S.L., I.S., F.R., A.D.; writing – original draft, C.B.; writing – review & editing, C.B., I.P., M.S., S.L., E.B., A.W.; visualization, C.B.; supervision A.H., I.P., M.S.; project administration I.P.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

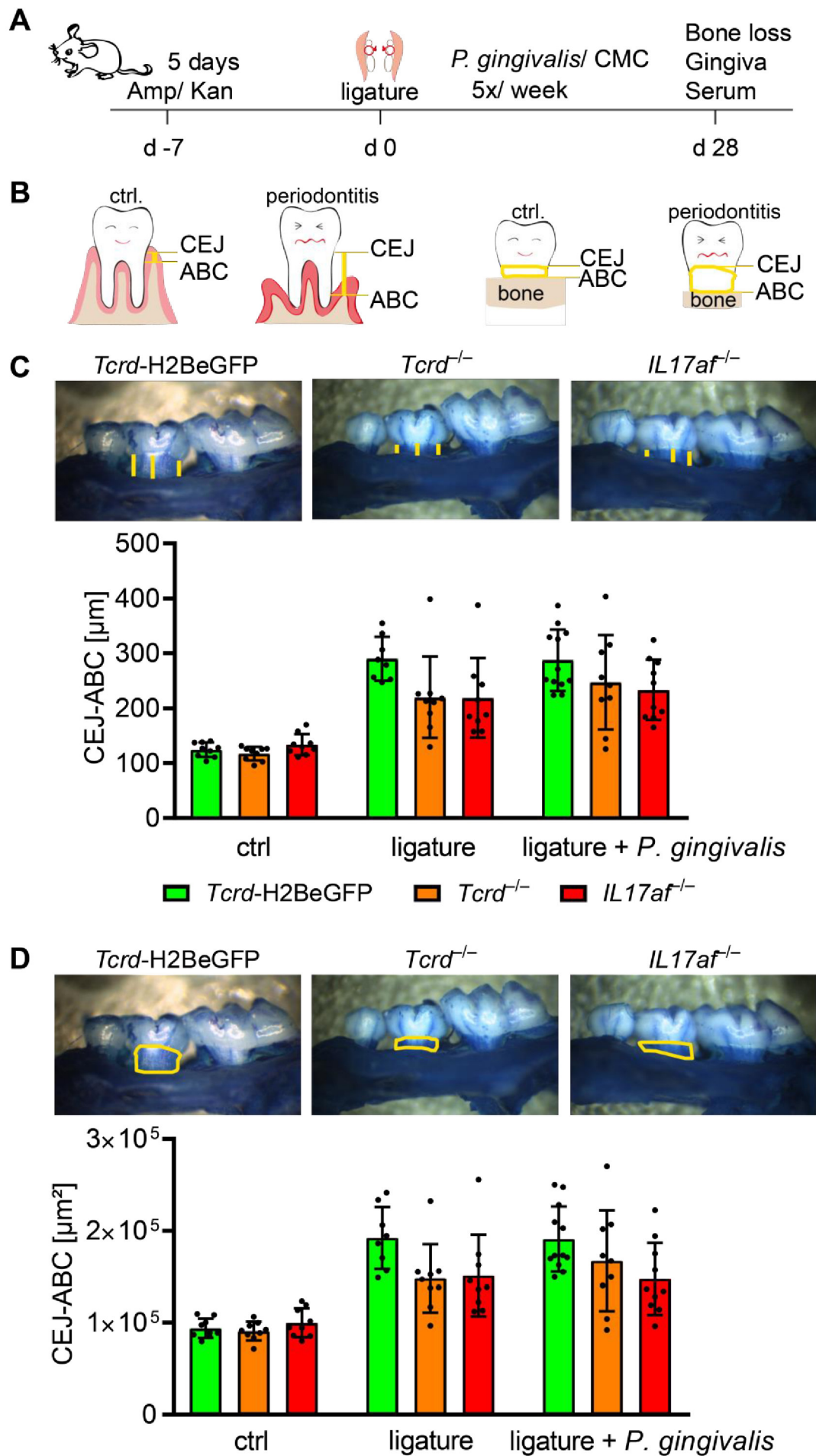


Figure 1: No differences in ligature-induced bone-loss between *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17a*^{-/-} mice with or without presence of *P. gingivalis*

(A) scheme of the procedure applied to induce periodontitis. (B) Scheme of the bone-loss-analysis by measuring the CEJ-ABC distance (left) or the area laying open between CEJ and ABC (right). (C) CEJ-ABC distance and representative raw-data of defleshed jaws after methylene blue staining. Each point represents a mouse. Data were statistically analyzed by Kruskal-Wallis test, mean and SD are shown. (D) CEJ-ABC area and representative raw-data of defleshed jaws after methylene blue staining. Each point represents a mouse. Data were obtained from three independent experiments and statistically analyzed by Kruskal-Wallis test and Dunn's multiple comparisons, mean and SD are shown. A p-value below 0.05 was considered as significant. All genotypes show statistically significant bone loss compared to control, which is not depicted in the figure.

Figure 2: Cellular immune reaction to ligature-induced periodontitis

(A) Number of CD45⁺ lymphocytes and CD3⁺ T cells isolated from gingival tissue of ligature-treated and control mice. (B) Fraction and number of $\gamma\delta$ T cells isolated from gingival tissue of ligature-treated and control mice in CD45⁺ CD3⁺ or CD45⁺ CD3⁺ TCR $\gamma\delta$ ⁺ gate, respectively, and representative gating. (C) Fraction and number of $\alpha\beta$ T cells isolated from gingival tissue of ligature-treated and control mice in CD45⁺ CD3⁺ gate. (D) Fraction and number of neutrophils and macrophages in CD45⁺ CD3⁻ gate. In all diagrams each point represents a mouse. Data were obtained from three independent experiments and statistically analyzed by Kruskal-Wallis test and Dunn's multiple comparisons, mean and SD are shown. A p-value below 0.05 was considered as significant.

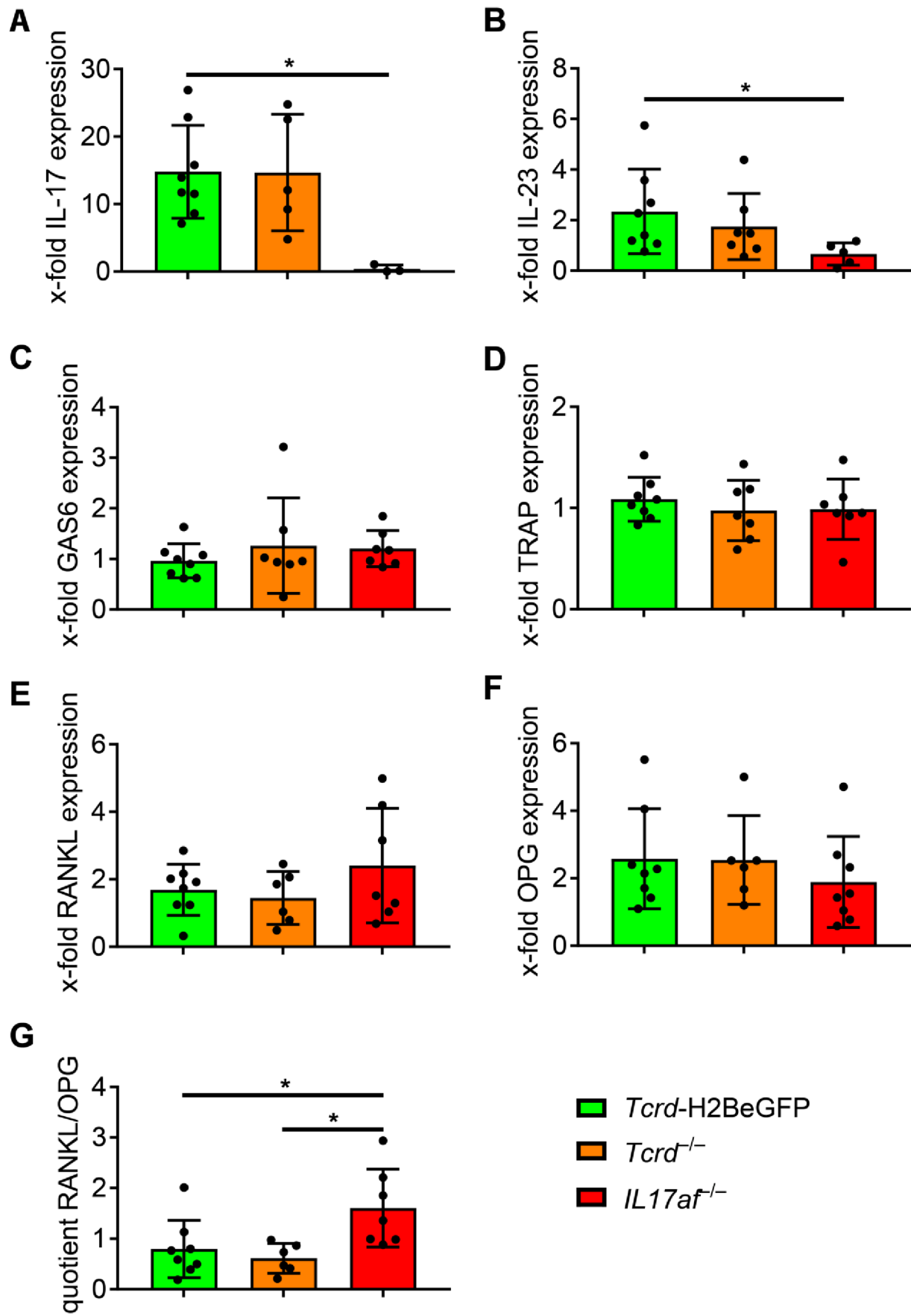


Figure 3: qPCR shows no difference in acute osteoclast-activity between *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17a*^{-/-} mice, while RANKL/OPG ratio suggests an osteoclastogenic milieu in *IL17a*^{-/-} mice

The ligature-induced induction of IL-17 (A), IL-23 (B), GAS6 (C), TRAP (D), RANKL (E) and OPG (F) expression in *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17a*^{-/-} mice measured by qPCR-analysis employing the $\Delta\Delta C_t$ method and RANKL/OPG ratio (G) as a measure of osteoclastogenic milieu. In all diagrams each point represents a mouse. The data are normalized to HPRT-induction by ligature-induced periodontitis. Data were obtained from two independent experiments and statistically analyzed by Kruskal-Wallis test and Dunn's multiple comparisons, two outliers were removed by Grubb's test, mean and SD are shown. A p-value below 0.05 was considered as significant.

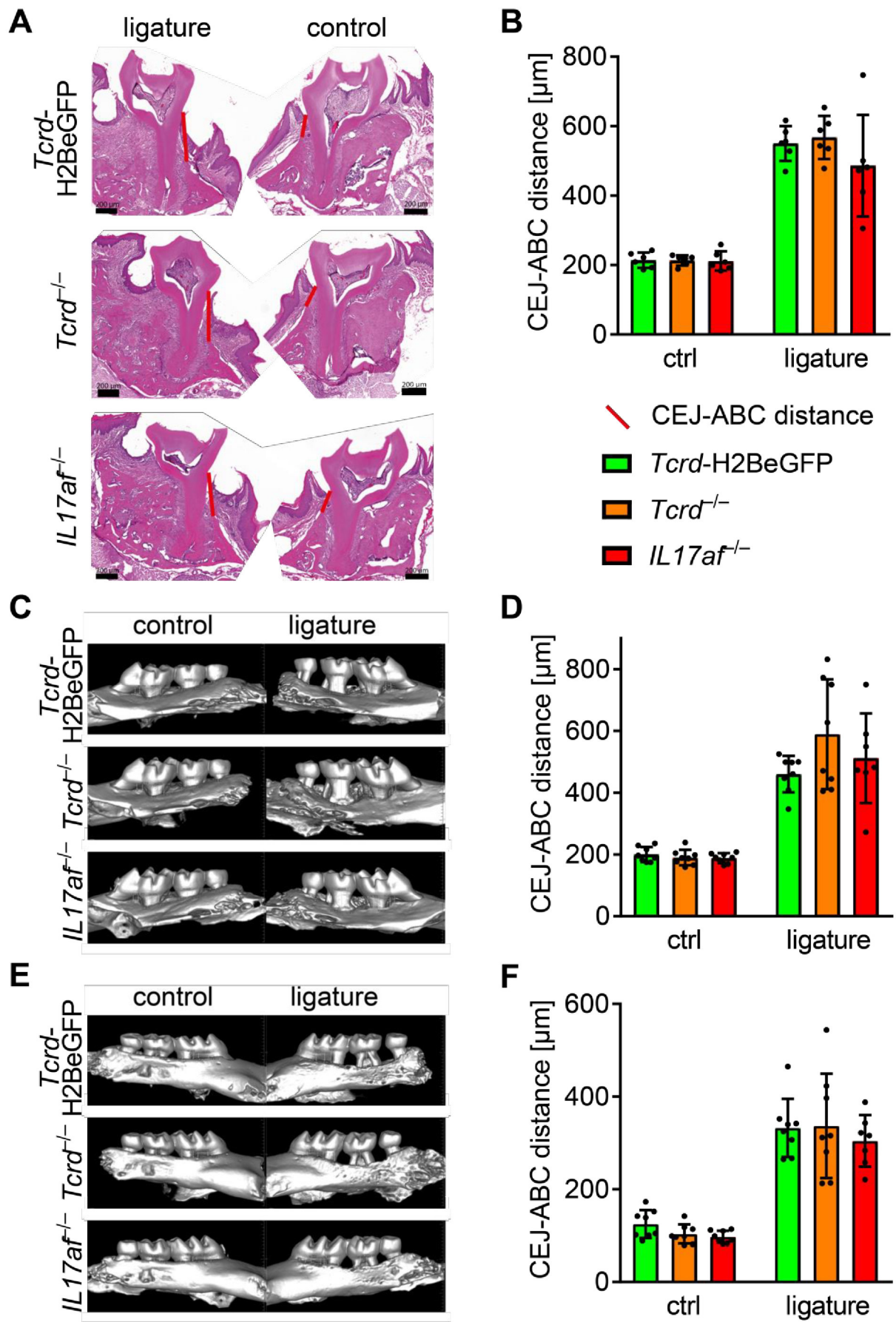


Figure 4: No difference in ligature-induced bone loss between *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice

Representative HE-stained cryo-sections (A) of jaws from ligature-treated and control *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice and (B) measured CEJ-ABC distance. Representative μ CT images of jaws from ligature-treated and control *Tcrd*-H2BeGFP, *Tcrd*^{-/-} and *IL17af*^{-/-} mice and measured CEJ-ABC distance from palatal (C, D) and buccal (E, F) side. In all diagrams each point represents a mouse. Both the HE and μ CT data sets were obtained from two independent experiments and statistically analyzed by Kruskal-Wallis test and Dunn's multiple comparisons, mean and SD are shown. A p-value below 0.05 was considered as significant. All genotypes show statistically significant bone loss compared to control, which is not depicted in the figure.

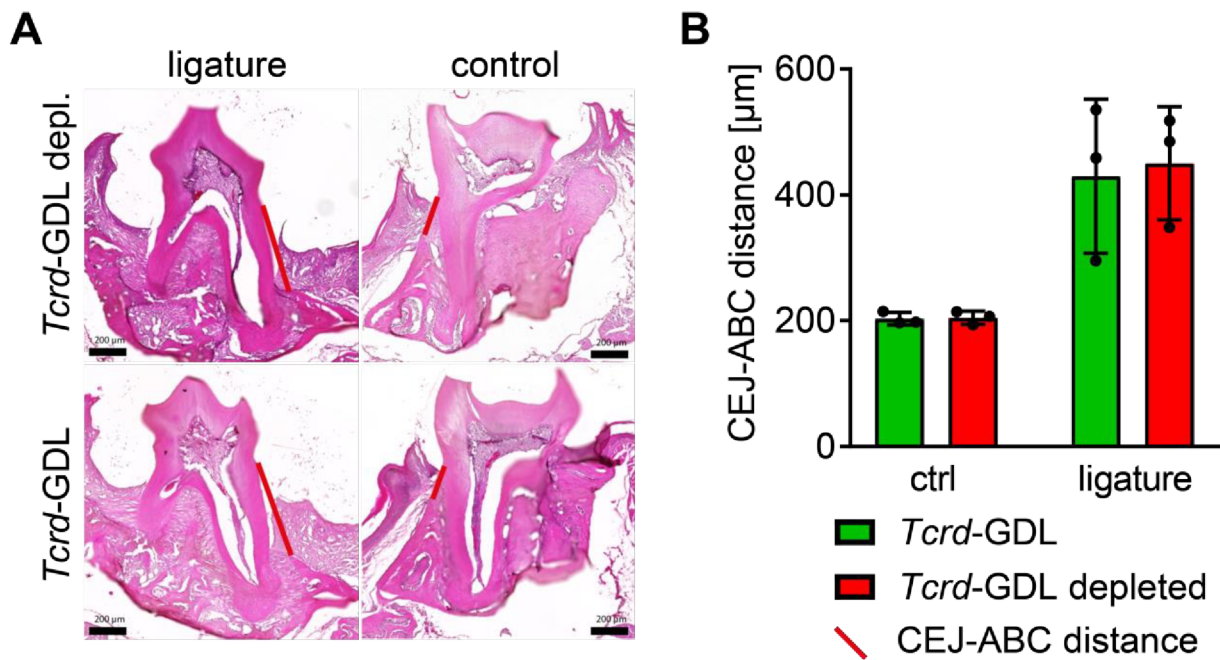


Figure 5: Conditional depletion of $\gamma\delta$ T cells suggests no functional replacement of missing $\gamma\delta$ T cells by $\alpha\beta$ T cells in the gingiva

Representative HE-stained cryo-sections (A) of jaws from ligature-treated and control *Tcrd*-GDL and $\gamma\delta$ T cell depleted *Tcrd*-GDL mice and (B) measured CEJ-ABC distance. Each point represents a mouse. Data were obtained from one experiment and statistically analyzed by Kruskal-Wallis test and Dunn's multiple comparison, mean and SD are shown. A p-value below 0.05 was considered as significant. All genotypes show statistically significant bone loss compared to control, which is not depicted in the figure.

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Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently, in compliance with “the policy of Hannover Medical School on the safeguarding of good scientific practice and procedural rules for dealing with scientific misconduct” and that I have not submitted it at any other university worldwide.

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Hannover, 04th of August 2021

Christoph Binz