$\gamma\delta$ T cells do not contribute to peripheral inflammatory pain

Jelena Petrović¹, Jaqueline Raymondi Silva^{1,2}, Julia P. Segal¹, Abigail S. Marshall¹, Cortney M. Haird¹, Ian Gilron^{1,2,3}, and Nader Ghasemlou^{1,2,3*}

Departments of ¹Biomedical & Molecular Sciences and ²Anesthesiology & Perioperative Medicine; and ³Centre for Neuroscience Studies, Queen's University, Kingston, Ontario, Canada

***Corresponding author:** Dr. Nader Ghasemlou

Address: Botterell Hall, Queen's University

18 Stuart St., room 754

Kingston, Ontario, K7L 3N6, Canada

Phone: 613.533.6854

Fax: 613.533.2022

Email: nader.ghasemlou@queensu.ca

Conflict of interest statement: The authors have nothing to disclose.

Acknowledgements: This work was supported by grants from the Canadian Institutes of Health Research, the J. P. Bickell Foundation, and Queen's University to NG. The authors thank Dr. Michael D. Kawaja for technical support.

Words: total = 4294; abstract = 178; discussion = 1297

Figures: 5; **Tables:** 0

Abbreviated title: $\gamma\delta$ T cells and inflammatory pain

Abstract

Circulating immune cells, which are recruited to the site of injury/disease, secrete various inflammatory mediators that are critical to nociception and pain. The role of tissue-resident immune cells, however, remains poorly characterized. One of the first cells to be activated in peripheral tissues following injury are $\gamma\delta$ T cells, which serve important roles in infection and disease. Using a transgenic mouse line lacking these cells, we sought to identify their contribution to inflammatory pain. Three distinct models of inflammatory pain were used: intraplantar injection of formalin and incisional wound (as models of acute inflammatory pain) and intraplantar injection of complete Freund's adjuvant (as a model of chronic inflammatory pain). Our results show that absence of these cells does not alter baseline sensitivity, nor does it result in changes to mechanical or thermal hypersensitivity after tissue injury. These results were consistent in both male and female mice, suggesting that there are no sex differences in these outcomes. This comprehensive characterization suggests that $\gamma\delta$ T cells do not contribute to basal sensitivity or the development and maintenance of inflammatory pain.

Introduction

Celsus assigned four cardinal signs of inflammation in the first century BC: *rubor* (redness), *calor* (heat), *tumor* (swelling), and *dolor* (pain); the Greek physician Galen added *functio laesa* (loss of function) in the 2nd century AD, a critical feature of inflammatory pain that may allow for healing and recovery by promoting post-injury immobility. Pathological inflammatory pain, however, serves no protective purpose; thus, identifying underlying mechanisms of disease will be critical to the development of new therapeutic options. The immune and nervous systems are intimately connected, particularly during inflammatory pain: immune cells and their secreted mediators act on nociceptors in the periphery while neurons, as a consequence, can modulate the inflammatory response [21; 35; 62]. Peripheral inflammation resulting from tissue injury, arthritis, and certain autoimmune diseases is brought on by the well-orchestrated recruitment and activation of circulatory and tissue-resident immune cells, including mast cells, neutrophils and macrophages [34; 66]. These cells and their secreted mediators can alter nociceptor function and activity to induce nociceptor activation and/or peripheral sensitization, triggering an increased responsiveness to noxious stimuli and pain hypersensitivity [21; 34; 35; 62; 66; 68; 85]. Under these conditions of peripheral sensitization, immune cell recruitment and activity can be altered by the neurogenic response from sensory neurons through the release of neuropeptides, neurotransmitters, and cytokines/chemokines [11; 68]. Thus, bi-directional interactions between immune cells and nociceptors are essential in the pathophysiology of inflammatory pain. However, these interactions, as well as the cells and mediators controlling these pain outcomes, remain poorly understood.

Many groups have begun to elucidate these complex neuro-immune interactions. Cytokines (interleukin-1 β , tumour necrosis factor), growth factors (nerve growth factor), lipids

(prostaglandins), neuropeptides (substance P, calcitonin gene-related peptide) and other inflammatory mediators have been shown to bring about peripheral sensitization and pain hypersensitivity [11; 21; 34; 35; 53; 69]. While the majority of this work has focused on circulating mediators, recent studies have identified specific contributions of immune cell subsets in mediating this pain sensitivity using animal models of injury and disease [5; 6; 19; 43; 44; 52; 70; 80]. These studies have found differing effects for various immune cells. Circulatory cells, including neutrophils and macrophages, have been shown by some groups to modulate inflammatory pain hypersensitivity [5; 6; 19; 33; 70; 80]. The role of tissue-resident cells on the other hand is less well known. Recent work using a transgenic mouse specifically lacking mast cells suggests that these cells have little to no effect on inflammatory pain hypersensitivity [44]. This was a surprising result given that mast cells are known producers of inflammatory mediators including cytokines, growth factors, and inflammatory mediators such as histamine that are known to alter hypersensitivity to stimuli [7; 77; 87]. This brings about the question of whether other tissue-resident immune cells may play a role in inflammatory pain and peripheral sensitization. To understand the role of T cells in inflammatory pain, there are two populations of these cells to consider: circulatory $\alpha\beta$ T cells and tissue-resident $\gamma\delta$ T cells, which are distinguished by their T cell receptors. In a previous study from our group, we evaluated the depletion of only $\alpha\beta$ T cells (using the TCR $\beta^{-/-}$ strain) and observed no role for this cell population in inflammatory pain. While $\alpha\beta$ T cells have not been shown to modulate inflammatory pain, they do play an important role in neuropathic pain with sexually dimorphic outcomes [47; 48; 74]. $\gamma\delta$ T cells are less abundant than $\alpha\beta$ T cells, are the primary T cell population found in the gut mucosa and skin, and are absent in TCR $\delta^{-/-}$ mice.

Tissue-resident $\gamma\delta$ T cells are considered to have qualities of both the innate and adaptive immune systems playing an important role in tissue surveillance, homeostasis and wound repair [27; 31; 60; 79; 83]. $\gamma\delta$ T cells respond within minutes to tissue injury, secreting inflammatory mediators such as growth factors and cytokines, are capable of serving as antigen presenting cells, and are critical for host immune defense against infections and autoimmune diseases (e.g., inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis). Recent work has shown that sensory neurons can suppress $\gamma\delta$ T cell numbers [1] and their production of specific cytokines [37], while others have shown $\gamma\delta$ T cells to modulate nerve regeneration [42]. Whether $\gamma\delta$ T cells can in turn alter nociceptor function/activity remains unknown. We therefore sought out to identify what role $\gamma\delta$ T cells might play in baseline sensitivity, due to their close proximity to sensory fibres in the skin, and the response to inflammatory pain using TCR $\delta^{-/-}$ mice, which lack these cells. Acute and persistent peripheral inflammation were used to mimic human clinical inflammation: the intraplantar injection of formalin (non-reflexive, spontaneous pain) or complete Freund's adjuvant (chronic inflammatory pain), and plantar incisional wound (acute post-surgical pain) [23; 59].

Materials and Methods

All protocols were approved by the Queen's University Animal Care Committee and followed the ARRIVE guidelines [40] and those of the Canadian Council on Animal Care. All surgical and behavioural work was carried out between 9am to 5pm in a facility where lights are on between 7am to 7pm.

Animals. B6.129P2-Tcrd^{tm1Mom}/J mice (TCR $\delta^{-/-}$; Jackson Laboratory, Bar Harbor, ME), which lack $\gamma\delta$ T cells [30], were backcrossed to C57BL/6J mice (Jackson Laboratory) to generate TCR $\delta^{+/-}$ F1 progeny. Heterozygous mice from different parents were subsequently used to establish the colony including wildtype, heterozygous and knockout littermates. All experiments were carried out using adult mice between 6-12 weeks of age, housed at a maximum of four per cage on a 12-hour light/dark cycle in a temperature- (21°C ($\pm$ 1°C)) and humidity-controlled room, with food and water provided *ad libitum*. The colony was maintained and genotyped by an independent experimenter, ensuring that all work was carried out blinded to genotype. Wildtype, heterozygous, and knockout littermates were used in all experiments, with at least two independent replications.

Formalin. Male (n=6-9) and female (n=8-13) TCR δ littermates received intraplantar injections with 20 μ l of a 5% formalin solution [diluted from a 37% formaldehyde stock solution (Sigma-Aldrich, St. Louis, MO) re-suspended in 0.9% NaCl solution (Hospira, Saint-Laurent, QC)] using a Hamilton syringe with 27-gauge needle, as previously described [78].

Plantar incisional wound. Male (n=7-10) and female (n=8-12) TCR δ littermates were anesthetized with 2% (v/v) isoflurane USP (Fresenius Kabi Canada, Toronto, ON) and the left hindpaw sterilized three times sequentially with 10% (w/v) Proiodine[®] Povidone Iodine Solution (Rougier Pharma, Mirabel, QC) and 70% (v/v) ethanol. A 5-8mm incision was made using a #11 scalpel from the base of the heel to the first walking pad, cutting through the skin and underlying muscle along the midline of the plantar surface, as previously described [19]. The wound was closed at two sites with 6-O silk sutures (Ethicon, Cincinnati, OH). Pain hypersensitivity was measured post-incision at 3 and 6 hours, and 1, 2, 3, 4 and 7 days.

Complete Freund's adjuvant. Male (n=7-10) and female (n=8-12) TCR δ littermates received intraplantar injections with 20 μ l of complete Freund's adjuvant (Sigma-Aldrich) using a Hamilton syringe with 27-gauge needle, as previously described [19]. Mice were not anesthetized for the injections and were immediately returned to their home-cage until behavioural experiments were carried out. Pain hypersensitivity was measured at 3 and 6 hours, and 1, 2, 3, 4 and 7 days following plantar CFA injection.

Behavioural assays. Mice injected with formalin were immediately placed singly in clear glass chambers and nociceptive behaviour (characterized by licking and biting of the injected hindpaw) was observed over 60 minutes and recorded at 5-minute intervals. Behavioural analysis was further divided into two phases: an acute phase lasting 0-10 minutes and a tonic phase from 10-60 minutes. Mice undergoing intraplantar injection of CFA or plantar incisional wound were habituated to the equipment and experimenter over five consecutive days prior to all behavioural

testing with at least 30 minutes in each experimental apparatus. Baselines measurements were then taken on three separate days and the average response calculated. All behavioural assays were carried out using at least two independent cohorts of mice by a researcher blinded to animal genotype using methods previously described by our group [19] and briefly outlined below.

von Frey mechanical sensitivity assay. Mice were placed in polycarbonate boxes on a wire mesh with black dividers to reduce interactions between animals. The left hindpaw was stimulated with graded von Frey monofilaments (North Coast Medical, Gilroy, CA). Gentle stimulation with these monofilaments were used to measure the lowest force (in grams) at which the mouse responds at least 50% of the time (paw threshold response), as previously described [19].

Acetone cold sensitivity test. Similar to the von Frey assay, the acetone test is performed by placing mice on the same wire mesh apparatus. A small drop of acetone was gently applied to the left hindpaw using a 1cc syringe (Becton Dickinson, Franklin Lakes, NJ) and the amount of time (in seconds) spent licking and biting the affected paw was measured. At least two measurements were recorded and averaged per timepoint, with a minimum of five minutes between each stimulation.

Hargreaves radiant heat sensitivity test. Mice were assessed for thermal heat hypersensitivity by performing the Hargreaves radiant heat test [86]. Mice were placed in individual transparent polycarbonate compartments on a heated glass base set to a constant temperature of 30°C ($\pm 1^\circ\text{C}$) (IITC Life Science, Woodland Hills, CA). A radiant heat source was focused onto the plantar surface of the left hindpaw, creating a 4x6 mm radiant heat source for stimulation, and the

latency to withdrawal measured. A maximum cut-off of 30 seconds was used to prevent tissue damage. Three measurements were averaged from each timepoint.

Hot/cold plate test. Thermal sensitivity was assessed at specific temperatures using an air-cooled thermoelectric plate (TECA Corporation, Chicago, IL). The plate was set to 0, 50, 52, or 55°C, allowed to stabilize at the temperature for 15 minutes, and animals placed in the center of the platform. The time to first response (e.g., fast paw withdrawal, shaking, licking/biting) and jump (all four paws lifted off the plate) was recorded. A maximum cut-off of 30 seconds was used to prevent tissue damage. Only one measurement was taken at each temperature per day to prevent learning behaviours; mice that exhibited learning behaviours (e.g., scaling the enclosure) were excluded from analysis [44].

Immunohistochemistry. Immunostaining for $\gamma\delta$ T cells was carried out as described elsewhere (Marshall, Silva, Gilron, and Ghasemlou, in preparation). Briefly, mice were deeply anesthetized and sacrificed by transcardial perfusion with 2% paraformaldehyde in 0.1M phosphate buffer. Ears were removed, post-fixed for 1 hour, and cryoprotected in 30% sucrose. Serial cryostat sections (15 μ m thickness) were obtained for histological analysis. Samples were incubated with hamster anti-mouse TCR δ (1:100; Invitrogen, Waltham, MA), followed by goat anti-hamster IgG conjugated to FITC (1:200; BioLegend, San Diego, CA). Slides were coverslipped using Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA) and visualized using an AxioSkop2 fluorescent microscope and AxioVision software (Carl Zeiss AG, Jena, Germany).

Statistical analysis. All statistical analyses were carried out using SigmaPlot version 11.0 software package (Systat Software, San Jose, CA). Data are expressed as mean $\pm$ standard error of the mean (SEM) throughout the text and in figures. One-way analysis of variance (ANOVA) was used for a direct comparison between two or more groups, while a two-way repeated-measures ANOVA was used to assess the effect of time between two or more groups. *Post-hoc* Tukey tests were used where appropriate with significance set at $P < 0.05$.

Results

$\gamma\delta$ T cells do not contribute to baseline thermal or mechanical sensitivity

Immunohistochemical analysis was used to visualize presence/absence of these cells in $\text{TCR}\delta^{+/+}$ and $\text{TCR}\delta^{-/-}$ mice to ensure complete absence of $\gamma\delta$ T cells in null mice. As expected, $\gamma\delta$ T cells were present in the epidermal layer of $\text{TCR}\delta^{+/+}$ mice, yet absent in $\text{TCR}\delta^{-/-}$ littermates (Figure 1A). We began our work by assessing whether loss of $\gamma\delta$ T cells causes a change to either baseline mechanical or thermal sensitivity in male and female mice, as these cells are resident in the skin and other barrier organs and could interact with sensory fibres during development. Mechanical sensitivity, measured as the 50% threshold using graded von Frey monofilaments, did not show any difference between $\text{TCR}\delta^{+/+}$, $\text{TCR}\delta^{+/-}$, and $\text{TCR}\delta^{-/-}$ mice in either males (Figure 1B; $n=17-22$ per genotype; one-way ANOVA, $P=0.402$) or females (Figure 1B; $n=15-18$ per genotype; one-way ANOVA, $P=0.276$). Mechanical threshold was slightly, but not significantly, increased in $\text{TCR}\delta^{-/-}$ males relative to wild-type ($1.39\pm 0.12\text{g}$ vs. $1.21\pm 0.09\text{g}$, respectively), though female mice did not exhibit such an effect.

We next measured responses to thermal stimuli at baseline. Cold sensitivity was assessed using the acetone test and heat sensitivity was measured using the Hargreaves radiant heat test. The response time to application of acetone to the hindpaw did not show a significant effect in either males (Figure 1C; $n=14-16$ per genotype; one-way ANOVA, $P=0.669$) or females (Figure 1C; $n=10-15$ per genotype; one-way ANOVA, $P=0.758$). The response to the radiant heat source was not different between the three genotypes in either male (Figure 1D; $n=17-19$ per genotype; one-way ANOVA, $P=0.086$) or female mice (Figure 1D; $n=15-18$ per genotype; one-way ANOVA, $P=0.679$). Although the response time was slightly faster in male $\text{TCR}\delta^{-/-}$ mice relative to wild-type littermates ($13.55\pm 1.00\text{s}$ vs. $16.55\pm 0.71\text{s}$, respectively), such an effect was not

evident in female mice. The cold and hot plate test was used to identify whether there were any differences in noxious thermal response between the three genotypes that could not be measured using the Hargreaves and acetone tests, using fixed temperatures between 0 to 55°C. As before, male $\text{TCR}\delta^{+/+}$ (n=7-11), $\text{TCR}\delta^{+/-}$ (n=8-15), and $\text{TCR}\delta^{-/-}$ (n=6-12) mice did not show any significant differences at 0, 50, 52, or 55°C in either time to first response (e.g. flinching of the hindpaw; Figure 2A; one-way ANOVA, $P \geq 0.193$) or first jump (Supplemental Figure 1A; one-way ANOVA, $P \geq 0.444$). Female $\text{TCR}\delta^{+/+}$ (n=12-17), $\text{TCR}\delta^{+/-}$ (n=6-13), and $\text{TCR}\delta^{-/-}$ (n=6-13) mice also did not show any differences in response time to flinch at 0, 50, or 52°C (Figures 2B; one-way ANOVA, $P \geq 0.099$). While there was a group effect between the three strains at 55°C for latency to first response (one-way ANOVA, $P=0.039$), *post-hoc* analysis did not show significant differences between the female strains at 55°C (e.g., $\text{TCR}\delta^{+/+}$ vs $\text{TCR}\delta^{-/-}$, $\text{TCR}\delta^{+/-}$ vs $\text{TCR}\delta^{-/-}$, etc.; one-way ANOVA, with *post-hoc* Tukey test $P=0.063$). Time to first jump was not significant at any temperature in female mice (Supplemental Figure 1B; one-way ANOVA, $P \geq 0.129$). Thus, loss of $\gamma\delta$ T cells does not appear to alter basal mechanical or thermal sensitivity.

$\gamma\delta$ T cells do not contribute to inflammatory pain

Specific circulating and skin-resident immune cells have been found to control inflammatory pain responses. The contribution of $\gamma\delta$ T cells to the inflammatory pain response was assessed using standard assays, where all experiments were conducted using at least 2 cohorts of littermates.

Formalin. We first assessed the contribution of $\gamma\delta$ T cells to acute inflammatory pain outcomes

using the formalin test [72; 78], where nociceptive pain, measured as time spent licking/biting the affected hindpaw, often lasts for approximately 60 minutes. Male TCR δ mice (n=6-9 per genotype) did not show an effect across the three genotypes studied over the course of their response (Figure 3A; two-way repeated measures ANOVA, P=0.403). Differences were also not observed when the response times were divided into acute (0-10min) and tonic (10-60min) phases (Figure 3B; one-way ANOVA, p $\geq$ 0.400). Female mice (n=8-13 per genotype) similarly do not show an effect over the duration of their response (Figure 3C; two-way RM-ANOVA, P=0.353), or over the acute or tonic phases (Figure 3D; one-way ANOVA, p $\geq$ 0.338). This suggests that $\gamma\delta$ T cells do not contribute to formalin-induced inflammatory pain.

Incisional wound. We next considered the contribution of $\gamma\delta$ T cells to the development and maintenance of acute inflammatory pain (lasting 2-4 days after injury) following plantar incisional wound, a model of post-surgical pain. Mechanical hypersensitivity in the injured hindpaw showed no difference between male TCR $\delta^{+/+}$, TCR $\delta^{+/-}$ and TCR $\delta^{-/-}$ mice (n=7-10 per genotype, P=0.064, two-way RM-ANOVA; Figure 4A). Similarly, hypersensitivity to heat stimuli also showed no significant differences (P=0.215, two-way RM-ANOVA, Figure 4B). Female TCR δ mice (n=8-12 per genotype) also did not show any significant effects after incisional wound for either mechanical hypersensitivity (P=0.942, two-way RM-ANOVA, Figure 4C) or thermal hypersensitivity (P=0.675, two-way RM-ANOVA, Figure 4D), suggesting $\gamma\delta$ T cells do not affect post-surgical inflammatory pain.

Complete Freund's adjuvant. To determine whether $\gamma\delta$ T cells contribute to chronic inflammatory pain, we carried out intraplantar injection of CFA. Mechanical and thermal

hypersensitivity do not return to baseline levels following intraplantar CFA injection as occurs following incisional wound, providing an opportunity to assess the contribution of these cells to a prolonged inflammatory response. Similar to the formalin and incisional wound models, no significant effects were observed in male (n=9-12 per genotype) or female (n=6-9 per genotype) TCR δ littermates when assessed for mechanical (P=0.226 [male], P=0.530 [female], two-way RM-ANOVA; Figures 5A, C) and thermal hypersensitivity (P=0.943 [male], P=0.857 [female], two-way RM-ANOVA; Figures 5B, D). Thus, $\gamma\delta$ T cells likely do not contribute to chronic inflammatory pain outcomes.

Discussion

Inflammatory pain is associated with tissue damage, infection, or autoimmune disease, as well as the presence of immune cells that can contribute to pain outcomes and wound healing. This pain often serves an important role in allowing healing and recovery to occur by promoting temporary immobility, such as after injury or infection. However, it can also be pathological in nature as it transitions into persistent pain where it serves no purpose, as is often the case in autoimmune diseases and in many patients following surgery once their wounds have healed [28; 38; 39]. Understanding how the immune cells and their mediators contribute to the development and maintenance of pain will be crucial to the development of safe and efficacious therapeutics for the treatment of inflammatory pain.

We therefore set out to determine the contribution of $\gamma\delta$ T cells to the development and maintenance of acute and chronic inflammatory pain, beginning by first backcrossing the B6.129P2-TCR $\delta^{tm1Mom/J}$ mice to the C57BL/6J background, in an effort to account for genetic background-dependent variations in behavioural phenotypes [41; 57; 58]. We began by backcrossing B6.129P2-*Tcrd* $^{tm1Mom/J}$ mice with wildtype C57BL/6J mice to generate TCR $\delta^{+/+}$, TCR $\delta^{+/-}$, and TCR $\delta^{-/-}$ littermates that were used in all studies, resulting in a total of 14 backcrosses for this strain. All experiments were carried out using both male and female mice in an effort to identify any effects controlled by sex. We first found that loss of these cells did not have an effect on basal sensitivity, as these cells may interact with sensory fibres in the skin during development and thus affect their function. Using three models of peripheral inflammatory pain, including intraplantar injection of formalin or CFA, and incisional wound, our work revealed that $\gamma\delta$ T cells do not alter mechanical or thermal sensitivity. It is important to note that the pain outcomes observed in TCR δ wildtype and heterozygous animals in the

formalin, incisional wound, and CFA models matches that observed in C57BL/6 mice in previous studies by our group and others [14; 19; 58]. We previously identified the contribution of myeloid and lymphoid cells to inflammatory pain, using cell-specific strategies to deplete neutrophils, non-neutrophil myeloid, and $\alpha\beta$ T cells [19]. While only non-neutrophil myeloid cells were found to alter behavioural outcomes, the role of most tissue-resident cells to inflammatory pain remained unknown.

$\gamma\delta$ T cells are a population of skin-resident cells that lie at the intersection of the innate and adaptive immune response and are among the first cells to be activated in the skin following tissue injury or viral/bacterial infection [12; 17; 31; 63; 65; 71]. These cells are known for their dendritic-like projections that allow them to intimately interact with their local environment and neighbouring cells, such as keratinocytes, Langerhans cells, and melanocytes [24; 45]. $\gamma\delta$ T cells are similar in appearance to microglia: stationary under homeostatic conditions, with dendritic projections that allow them survey their immediate milieu for signs of injury or infection [13; 22]. While our results suggest that $\gamma\delta$ T cells do not contribute to inflammatory pain hypersensitivity, only three models of disease were used. Assessing the role of these cells may show an important effect in pain outcomes following bacterial infection, where they are known to have an effect in both skin [15; 46] and lung [1; 8; 55], or in models of inflammatory bowel disease [16; 29; 36]. We hypothesize this to be possible due to the high number of these cells in the lungs and lining the gut mucosa. The site of injury/infection may be critical, given that $\gamma\delta$ T cells are more prevalent in the ear and back skin than in the hindpaw, where their number and morphology are more similar to that observed in human skin [unpublished observations, 4].

$\gamma\delta$ T cells participate in the early stages of an immune response by recognizing lipid-ligand antigens, unprocessed proteins, and phosphor-antigens without MHC-restricted

presentation [3; 10; 25; 26]. Several studies showed that $\gamma\delta$ T cells contributes to the development of an inflammatory response in various tissues (e.g., gut, lungs, spinal cord), through expression of inflammatory cytokines and regulatory factors including interferon (IFN)- γ , IL-17, TNF- α , granzymes, and insulin-like growth factor 1 [10] This can result in the recruitment of circulating immune cells to the site of inflammation [18] and can modulate $\gamma\delta$ T cell interaction with other immune cells, including B and $\alpha\beta$ T cells [50; 64; 67; 75; 76]. Two major families of mediators secreted by $\gamma\delta$ T cells include keratinocyte and fibroblast growth factors (KGFs and FGFs). While several FGF family members have been found to directly activate sensory neurons [49; 62; 73], KGFs are not known to affect sensory neuron activity, though keratinocytes themselves have recently been implicated in modulating nociception [2; 61], as well as itch [51; 81] and mechanosensitivity [9; 56]. Although our results do not show an effect for $\gamma\delta$ T cells in the nociceptive response following peripheral inflammation, these cells may still play an important role in itch and other skin pathologies and could prove useful in identifying novel underlying cellular and molecular mechanisms. When evaluating the influence of these cells in the skin, there are some conflicting results in literature. Some studies suggest that $\gamma\delta$ T cells are important for the development of the inflammatory response, with mechanisms similar as described above. Other studies have shown that these cells are not required or negatively regulate skin inflammation [20; 84]. These studies have almost exclusively studied $\gamma\delta$ T cells in the ear and back, and no studies have been carried out in the footpad.

Besides their potential role in skin inflammation, it is well known that $\gamma\delta$ T cells play an important role in wound healing. Skin-resident $\gamma\delta$ T cells are called dendritic epidermal T cells (DETCs) when strictly limited in their distribution to the epidermis. Their distribution in the skin epithelium allows the intimate contact with other resident cell types, as well as with the

extracellular matrix. They recognize an unidentified antigen expressed by damaged, stressed, or transformed keratinocytes in the epidermis, which results in the production of inflammatory mediators that have specific effects on other epithelial cells [82]. Among these regulatory factors, it has been demonstrated that the insulin-like growth factor-1, KGFs, and FGFs, are involved in tissue repair after injury. In fact, rapamycin treatment of mice, which disables $\gamma\delta$ T cell proliferation, migration, and expression of normal levels of growth factors, compromises the wound repair in the injured skin. However, treatment with insulin-like growth factor-1, produced by DETCs, results in restoration of normal wound closure in those animals [54]. Similarly, the addition of DETCs or recombinant KGF in skin organ culture from $\gamma\delta$ T cell- deficient mice restored normal wound healing in this tissue [31]. Finally, upon TCR stimulation in vitro, DETCs produce FGF, which has been suggested to promote keratinocyte proliferation and accelerates wound repair [32]. Altogether, these findings suggest that DETCs and $\gamma\delta$ T cells might play an important role in the biological function of wound repair. It has also been suggested that $\gamma\delta$ T cells might have regulatory roles in the inflammatory response initiated after tissue injury. For instance, these cells are responsible for the modulation of myeloid cell infiltration after burn-induced wound by suppressing the inflammatory response, leading to the initiation of the proliferative phase of wound healing [65].

While our results demonstrate that $\gamma\delta$ T cells do not contribute to inflammatory nociception, this is limited by our use of three specific models – the intraplantar injection of formalin or complete Freund’s adjuvant and incisional wound. While this is the first study of the function of these cells in mediating pain outcomes, our work is limited to inflammation in the hindpaw. We speculate that future work examining the function of $\gamma\delta$ T cells in other models of pain/nociception may yet identify a role for these cells.

References

- [1] Baral P, Umans BD, Li L, Wallrapp A, Bist M, Kirschbaum T, Wei Y, Zhou Y, Kuchroo VK, Burkett PR, Yipp BG, Liberles SD, Chiu IM. Nociceptor sensory neurons suppress neutrophil and gammadelta T cell responses in bacterial lung infections and lethal pneumonia. *Nat Med* 2018;24(4):417-426.
- [2] Baumbauer KM, DeBerry JJ, Adelman PC, Miller RH, Hachisuka J, Lee KH, Ross SE, Koerber HR, Davis BM, Albers KM. Keratinocytes can modulate and directly initiate nociceptive responses. *Elife* 2015;4.
- [3] Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* 2010;10(7):467-478.
- [4] Bos JD, Teunissen MB, Cairo I, Krieg SR, Kapsenberg ML, Das PK, Borst J. T-cell receptor gamma delta bearing cells in normal human skin. *J Invest Dermatol* 1990;94(1):37-42.
- [5] Brack A, Rittner HL, Machelska H, Leder K, Mousa SA, Schafer M, Stein C. Control of inflammatory pain by chemokine-mediated recruitment of opioid-containing polymorphonuclear cells. *Pain* 2004;112(3):229-238.
- [6] Carreira EU, Carregaro V, Teixeira MM, Moriconi A, Aramini A, Verri WA, Jr., Ferreira SH, Cunha FQ, Cunha TM. Neutrophils recruited by CXCR1/2 signalling mediate post-incisional pain. *Eur J Pain* 2013;17(5):654-663.
- [7] Chatterjea D, Martinov T. Mast cells: versatile gatekeepers of pain. *Mol Immunol* 2015;63(1):38-44.
- [8] Cheng P, Liu T, Zhou WY, Zhuang Y, Peng LS, Zhang JY, Yin ZN, Mao XH, Guo G, Shi Y, Zou QM. Role of gamma-delta T cells in host response against *Staphylococcus aureus*-induced pneumonia. *BMC Immunol* 2012;13:38.
- [9] Chiang LY, Poole K, Oliveira BE, Duarte N, Sierra YA, Bruckner-Tuderman L, Koch M, Hu J, Lewin GR. Laminin-332 coordinates mechanotransduction and growth cone bifurcation in sensory neurons. *Nat Neurosci* 2011;14(8):993-1000.
- [10] Chien YH, Meyer C, Bonneville M. gammadelta T cells: first line of defense and beyond. *Annu Rev Immunol* 2014;32:121-155.
- [11] Chiu IM, von Hehn CA, Woolf CJ. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. *Nat Neurosci* 2012;15(8):1063-1067.
- [12] Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, Magorien JE, Blauvelt A, Kolls JK, Cheung AL, Cheng G, Modlin RL, Miller LS. IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest* 2010;120(5):1762-1773.
- [13] Chodaczek G, Papanna V, Zal MA, Zal T. Body-barrier surveillance by epidermal gammadelta TCRs. *Nat Immunol* 2012;13(3):272-282.
- [14] Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ. Inflammation-induced decrease in voluntary wheel running in mice: a nonreflexive test for evaluating inflammatory pain and analgesia. *Pain* 2012;153(4):876-884.
- [15] Dillen CA, Pinsker BL, Marusina AI, Merleev AA, Farber ON, Liu H, Archer NK, Lee DB, Wang Y, Ortines RV, Lee SK, Marchitto MC, Cai SS, Ashbaugh AG, May LS, Holland SM, Freeman AF, Miller LG, Yeaman MR, Simon SI, Milner JD, Maverakis E, Miller LS. Clonally expanded gammadelta T cells protect against *Staphylococcus aureus* skin reinfection. *J Clin Invest* 2018;128(3):1026-1042.

- [16] Do JS, Kim S, Keslar K, Jang E, Huang E, Fairchild RL, Pizarro TT, Min B. gammadelta T Cells Coexpressing Gut Homing alpha4beta7 and alphaE Integrins Define a Novel Subset Promoting Intestinal Inflammation. *J Immunol* 2017;198(2):908-915.
- [17] Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, Craft J, Yin Z. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med* 2003;198(3):433-442.
- [18] Gelderblom M, Arunachalam P, Magnus T. gammadelta T cells as early sensors of tissue damage and mediators of secondary neurodegeneration. *Front Cell Neurosci* 2014;8:368.
- [19] Ghasemlou N, Chiu IM, Julien JP, Woolf CJ. CD11b+Ly6G- myeloid cells mediate mechanical inflammatory pain hypersensitivity. *Proc Natl Acad Sci U S A* 2015;112(49):E6808-6817.
- [20] Girardi M, Lewis J, Glusac E, Filler RB, Geng L, Hayday AC, Tigelaar RE. Resident skin-specific gammadelta T cells provide local, nonredundant regulation of cutaneous inflammation. *J Exp Med* 2002;195(7):855-867.
- [21] Grace PM, Hutchinson MR, Maier SF, Watkins LR. Pathological pain and the neuroimmune interface. *Nat Rev Immunol* 2014;14(4):217-231.
- [22] Gray EE, Suzuki K, Cyster JG. Cutting edge: Identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J Immunol* 2011;186(11):6091-6095.
- [23] Gregory NS, Harris AL, Robinson CR, Dougherty PM, Fuchs PN, Sluka KA. An overview of animal models of pain: disease models and outcome measures. *J Pain* 2013;14(11):1255-1269.
- [24] Havran WL, Jameson JM. Epidermal T cells and wound healing. *J Immunol* 2010;184(10):5423-5428.
- [25] Hayday A, Tigelaar R. Immunoregulation in the tissues by gammadelta T cells. *Nat Rev Immunol* 2003;3(3):233-242.
- [26] Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 2009;31(2):184-196.
- [27] Holtmeier W, Kabelitz D. gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy* 2005;86:151-183.
- [28] Hughes JP, Chessell I, Malamut R, Perkins M, Backonja M, Baron R, Farrar JT, Field MJ, Gereau RW, Gilron I, McMahon SB, Porreca F, Rappaport BA, Rice F, Richman LK, Segerdahl M, Seminowicz DA, Watkins LR, Waxman SG, Wiech K, Woolf C. Understanding chronic inflammatory and neuropathic pain. *Ann N Y Acad Sci* 2012;1255:30-44.
- [29] Inagaki-Ohara K, Chinen T, Matsuzaki G, Sasaki A, Sakamoto Y, Hiromatsu K, Nakamura-Uchiyama F, Nawa Y, Yoshimura A. Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *J Immunol* 2004;173(2):1390-1398.
- [30] Itohara S, Mombaerts P, Lafaille J, Iacomini J, Nelson A, Clarke AR, Hooper ML, Farr A, Tonegawa S. T cell receptor delta gene mutant mice: independent generation of alpha beta T cells and programmed rearrangements of gamma delta TCR genes. *Cell* 1993;72(3):337-348.
- [31] Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, Havran WL. A role for skin gammadelta T cells in wound repair. *Science* 2002;296(5568):747-749.
- [32] Jameson JM, Sharp LL, Witherden DA, Havran WL. Regulation of skin cell homeostasis by gamma delta T cells. *Front Biosci* 2004;9:2640-2651.

- [33] Jha MK, Jeon S, Jin M, Ock J, Kim JH, Lee WH, Suk K. The pivotal role played by lipocalin-2 in chronic inflammatory pain. *Exp Neurol* 2014;254:41-53.
- [34] Ji RR, Chameissian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. *Science* 2016;354(6312):572-577.
- [35] Ji RR, Xu ZZ, Gao YJ. Emerging targets in neuroinflammation-driven chronic pain. *Nat Rev Drug Discov* 2014;13(7):533-548.
- [36] Kadivar M, Petersson J, Svensson L, Marsal J. CD8alphabeta+ gammadelta T Cells: A Novel T Cell Subset with a Potential Role in Inflammatory Bowel Disease. *J Immunol* 2016;197(12):4584-4592.
- [37] Kashem SW, Riedl MS, Yao C, Honda CN, Vulchanova L, Kaplan DH. Nociceptive Sensory Fibers Drive Interleukin-23 Production from CD301b+ Dermal Dendritic Cells and Drive Protective Cutaneous Immunity. *Immunity* 2015;43(3):515-526.
- [38] Kehlet H, Jensen TS, Woolf CJ. Persistent postsurgical pain: risk factors and prevention. *Lancet* 2006;367(9522):1618-1625.
- [39] Kidd BL, Photiou A, Inglis JJ. The role of inflammatory mediators on nociception and pain in arthritis. *Novartis Found Symp* 2004;260:122-133; discussion 133-128, 277-129.
- [40] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010;8(6):e1000412.
- [41] Leo S, Straetmans R, D'Hooze R, Meert T. Differences in nociceptive behavioral performance between C57BL/6J, 129S6/SvEv, B6 129 F1 and NMRI mice. *Behav Brain Res* 2008;190(2):233-242.
- [42] Li Z, Burns AR, Han L, Rumbaut RE, Smith CW. IL-17 and VEGF are necessary for efficient corneal nerve regeneration. *Am J Pathol* 2011;178(3):1106-1116.
- [43] Liu T, van Rooijen N, Tracey DJ. Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain* 2000;86(1-2):25-32.
- [44] Lopes DM, Denk F, Chisholm KI, Suddason T, Durrieux C, Thakur M, Gentry C, McMahon SB. Peripheral inflammatory pain sensitisation is independent of mast cell activation in male mice. *Pain* 2017;158(7):1314-1322.
- [45] Macleod AS, Havran WL. Functions of skin-resident gammadelta T cells. *Cell Mol Life Sci* 2011;68(14):2399-2408.
- [46] Malhotra N, Yoon J, Leyva-Castillo JM, Galand C, Archer N, Miller LS, Geha RS. IL-22 derived from gammadelta T cells restricts *Staphylococcus aureus* infection of mechanically injured skin. *J Allergy Clin Immunol* 2016;138(4):1098-1107 e1093.
- [47] Mapplebeck JC, Beggs S, Salter MW. Sex differences in pain: a tale of two immune cells. *Pain* 2016;157 Suppl 1:S2-6.
- [48] Mapplebeck JC, Beggs S, Salter MW. Molecules in pain and sex: a developing story. *Mol Brain* 2017;10(1):9.
- [49] McMahon SB, La Russa F, Bennett DL. Crosstalk between the nociceptive and immune systems in host defence and disease. *Nat Rev Neurosci* 2015;16(7):389-402.
- [50] Mehta P, Nuotio-Antar AM, Smith CW. gammadelta T cells promote inflammation and insulin resistance during high fat diet-induced obesity in mice. *J Leukoc Biol* 2015;97(1):121-134.
- [51] Meng J, Moriyama M, Feld M, Buddenkotte J, Buhl T, Szollosi A, Zhang J, Miller P, Ghetti A, Fischer M, Reeh PW, Shan C, Wang J, Steinhoff M. New mechanism underlying IL-31-induced atopic dermatitis. *J Allergy Clin Immunol* 2018;141(5):1677-1689 e1678.

- [52] Mert T, Gunay I, Ocal I, Guzel AI, Inal TC, Sencar L, Polat S. Macrophage depletion delays progression of neuropathic pain in diabetic animals. *Naunyn Schmiedeberg's Arch Pharmacol* 2009;379(5):445-452.
- [53] Miller RJ, Jung H, Bhangoo SK, White FA. Cytokine and chemokine regulation of sensory neuron function. *Handb Exp Pharmacol* 2009(194):417-449.
- [54] Mills RE, Taylor KR, Podshivalova K, McKay DB, Jameson JM. Defects in skin gamma delta T cell function contribute to delayed wound repair in rapamycin-treated mice. *J Immunol* 2008;181(6):3974-3983.
- [55] Misiak A, Wilk MM, Raverdeau M, Mills KH. IL-17-Producing Innate and Pathogen-Specific Tissue Resident Memory gammadelta T Cells Expand in the Lungs of Bordetella pertussis-Infected Mice. *J Immunol* 2017;198(1):363-374.
- [56] Moehring F, Cowie AM, Menzel AD, Weyer AD, Grzybowski M, Arzua T, Geurts AM, Palygin O, Stucky CL. Keratinocytes mediate innocuous and noxious touch via ATP-P2X4 signaling. *Elife* 2018;7.
- [57] Mogil JS, Ritchie J, Sotocinal SG, Smith SB, Croteau S, Levitin DJ, Naumova AK. Screening for pain phenotypes: analysis of three congenic mouse strains on a battery of nine nociceptive assays. *Pain* 2006;126(1-3):24-34.
- [58] Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* 1999;80(1-2):67-82.
- [59] Muley MM, Krustev E, McDougall JJ. Preclinical Assessment of Inflammatory Pain. *CNS Neurosci Ther* 2016;22(2):88-101.
- [60] Nielsen MM, Witherden DA, Havran WL. gammadelta T cells in homeostasis and host defence of epithelial barrier tissues. *Nat Rev Immunol* 2017;17(12):733-745.
- [61] Pang Z, Sakamoto T, Tiwari V, Kim YS, Yang F, Dong X, Guler AD, Guan Y, Caterina MJ. Selective keratinocyte stimulation is sufficient to evoke nociception in mice. *Pain* 2015;156(4):656-665.
- [62] Pinho-Ribeiro FA, Verri WA, Jr., Chiu IM. Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation. *Trends Immunol* 2017;38(1):5-19.
- [63] Rani M, Schwacha MG. The composition of T-cell subsets are altered in the burn wound early after injury. *PLoS One* 2017;12(6):e0179015.
- [64] Rani M, Zhang Q, Scherer MR, Cap AP, Schwacha MG. Activated skin gammadelta T-cells regulate T-cell infiltration of the wound site after burn. *Innate Immun* 2015;21(2):140-150.
- [65] Rani M, Zhang Q, Schwacha MG. Gamma delta T cells regulate wound myeloid cell activity after burn. *Shock* 2014;42(2):133-141.
- [66] Raoof R, Willemen H, Eijkelkamp N. Divergent roles of immune cells and their mediators in pain. *Rheumatology (Oxford)* 2018;57(3):429-440.
- [67] Rei M, Goncalves-Sousa N, Lanca T, Thompson RG, Mensurado S, Balkwill FR, Kulbe H, Pennington DJ, Silva-Santos B. Murine CD27(-) Vgamma6(+) gammadelta T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. *Proc Natl Acad Sci U S A* 2014;111(34):E3562-3570.
- [68] Ren K, Dubner R. Interactions between the immune and nervous systems in pain. *Nat Med* 2010;16(11):1267-1276.
- [69] Rittner HL, Brack A, Stein C. The other side of the medal: how chemokines promote analgesia. *Neurosci Lett* 2008;437(3):203-208.

- [70] Sahbaie P, Li X, Shi X, Clark JD. Roles of Gr-1+ leukocytes in postincisional nociceptive sensitization and inflammation. *Anesthesiology* 2012;117(3):602-612.
- [71] Schwacha MG, Rani M, Nicholson SE, Lewis AM, Holloway TL, Sordo S, Cap AP. Dermal gammadelta T-Cells Can Be Activated by Mitochondrial Damage-Associated Molecular Patterns. *PLoS One* 2016;11(7):e0158993.
- [72] Shibata M, Ohkubo T, Takahashi H, Inoki R. Modified formalin test: characteristic biphasic pain response. *Pain* 1989;38(3):347-352.
- [73] Si W, Zhang Y, Chen K, Hu D, Qian Z, Gong S, Li H, Hao Y, Tao J. Fibroblast growth factor type 1 receptor stimulation of T-type Ca(2+) channels in sensory neurons requires the phosphatidylinositol 3-kinase and protein kinase A pathways, independently of Akt. *Cell Signal* 2018;45:93-101.
- [74] Sorge RE, Mapplebeck JC, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin JS, Sotocinal SG, Chen D, Yang M, Shi XQ, Huang H, Pillion NJ, Bilan PJ, Tu Y, Klip A, Ji RR, Zhang J, Salter MW, Mogil JS. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci* 2015;18(8):1081-1083.
- [75] Sun G, Yang S, Cao G, Wang Q, Hao J, Wen Q, Li Z, So KF, Liu Z, Zhou S, Zhao Y, Yang H, Zhou L, Yin Z. gammadelta T cells provide the early source of IFN-gamma to aggravate lesions in spinal cord injury. *J Exp Med* 2018;215(2):521-535.
- [76] Tedesco D, Thapa M, Chin CY, Ge Y, Gong M, Li J, Gumber S, Speck P, Elrod EJ, Burd EM, Kitchens WH, Magliocca JF, Adams AB, Weiss DS, Mohamadzadeh M, Grakoui A. Alterations in Intestinal Microbiota Lead to Production of Interleukin 17 by Intrahepatic gammadelta T-Cell Receptor-Positive Cells and Pathogenesis of Cholestatic Liver Disease. *Gastroenterology* 2018;154(8):2178-2193.
- [77] Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, Vasiadi M, Weng Z, Miniati A, Kalogeromitros D. Mast cells and inflammation. *Biochim Biophys Acta* 2012;1822(1):21-33.
- [78] Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. *Pain* 1992;51(1):5-17.
- [79] Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol* 2013;13(2):88-100.
- [80] Willemen HL, Eijkelkamp N, Garza Carbajal A, Wang H, Mack M, Zijlstra J, Heijnen CJ, Kavelaars A. Monocytes/Macrophages control resolution of transient inflammatory pain. *J Pain* 2014;15(5):496-506.
- [81] Wilson SR, The L, Batia LM, Beattie K, Katibah GE, McClain SP, Pellegrino M, Estandian DM, Bautista DM. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. *Cell* 2013;155(2):285-295.
- [82] Witherden DA, Havran WL. Cross-talk between intraepithelial gammadelta T cells and epithelial cells. *J Leukoc Biol* 2013;94(1):69-76.
- [83] Witherden DA, Watanabe M, Garijo O, Rieder SE, Sarkisyan G, Cronin SJ, Verdino P, Wilson IA, Kumanogoh A, Kikutani H, Teyton L, Fischer WH, Havran WL. The CD100 receptor interacts with its plexin B2 ligand to regulate epidermal gammadelta T cell function. *Immunity* 2012;37(2):314-325.
- [84] Woodward AL, Spergel JM, Alenius H, Mizoguchi E, Bhan AK, Castigli E, Brodeur SR, Oettgen HC, Geha RS. An obligate role for T-cell receptor alphabeta+ T cells but not T-cell receptor gammadelta+ T cells, B cells, or CD40/CD40L interactions in a mouse model of atopic dermatitis. *J Allergy Clin Immunol* 2001;107(2):359-366.

- [85] Woolf CJ, Ma Q. Nociceptors--noxious stimulus detectors. *Neuron* 2007;55(3):353-364.
- [86] Yalcin I, Charlet A, Freund-Mercier MJ, Barrot M, Poisbeau P. Differentiating thermal allodynia and hyperalgesia using dynamic hot and cold plate in rodents. *J Pain* 2009;10(7):767-773.
- [87] Zuo Y, Perkins NM, Tracey DJ, Geczy CL. Inflammation and hyperalgesia induced by nerve injury in the rat: a key role of mast cells. *Pain* 2003;105(3):467-479.

Figure Legends

Figure 1. Absence of $\gamma\delta$ T cells does not affect basal mechanical or thermal sensitivity. (A)

Sections of the ear from wildtype, heterozygous, and knockout TCR δ mice immunostained for $\gamma\delta$ T cells using an antibody recognizing the δ T cell receptor subunit. Representative micrographs show $\gamma\delta$ T cells are present in TCR $\delta^{+/+}$ and TCR $\delta^{+/-}$ mice, but not in TCR $\delta^{-/-}$ littermates. (B)

Mechanical thresholds, measured as the von Frey monofilament corresponding to a 50% response, is not affected by loss of $\gamma\delta$ T cells in male ($P=0.402$, one-way ANOVA; $n=17-22$ per genotype) or female ($P=0.276$, one-way ANOVA; $n=15-18$ per genotype) mice. (C) Cold

thermal responses were assessed using the acetone test, measured as total response time (e.g., licking and biting of the affected hindpaw), was not different between male ($P=0.669$, one-way ANOVA; $n=14-16$ per genotype) or female ($P=0.758$, one-way ANOVA; $n=10-15$ per genotype) littermates. (D) Thermal heat hypersensitivity was measured as the latency to response following stimulation of the hindpaw by a radiant heat source. No differences were observed in either male ($P=0.086$, one-way ANOVA; $n=17-19$ per genotype) or female ($P=0.679$, one-way ANOVA; $n=15-18$ per genotype) mice. Graphs show mean $\pm$ SEM, scale bar=50 μ m.

Figure 2. Response to noxious heat and cold are unaffected by loss of $\gamma\delta$ T cells. (A) No

differences were observed in latency to paw withdrawal (e.g., flinch) using the hot and cold plate test in male TCR δ littermates ($P \geq 0.193$, one-way ANOVA; $n=6-15$ per genotype) at any of the temperatures examined. (B) Female mice assessed for latency to first response did not exhibit differences at 0, 50, or 52°C ($P \geq 0.099$, one-way ANOVA; $n=6-17$ per genotype). While there was a significant group effect for genotype at 55°C ($*P=0.039$, one-way ANOVA), *post-hoc* Tukey analysis was not significant between the three groups ($P \geq 0.063$).

Figure 3. Response to formalin is unaffected by absence of $\gamma\delta$ T cells. TCR δ littermates were injected with formalin and the response time measured over 60 minutes. Male mice (n=6-9 per genotype) did not show an effect over the duration of response (**A**; $P=0.403$, two-way RM-ANOVA) or during acute and tonic phases (**B**; $P\geq 0.400$, one-way ANOVA). Female mice (n=8-13 per genotype) also did not show a significant effect over the duration of response (**C**; $P=0.353$, two-way RM-ANOVA) or in acute/tonic phases (**D**; $P\geq 0.338$, one-way ANOVA).

Figure 4. $\gamma\delta$ T cells do not contribute to mechanical and thermal hypersensitivity after incisional wound. Male TCR δ littermates (n=7-10 per genotype) did not exhibit differences in mechanical thresholds (**A**; $P=0.064$, two-way RM-ANOVA), measured with von Frey monofilaments, or heat hypersensitivity (**B**; $P=0.215$, two-way RM-ANOVA), measured as the latency of response to a radiant heat stimulus. A similar effect was observed in female TCR δ littermates (n=8-12 per genotype) for both mechanical (**C**; $P=0.942$, two-way RM-ANOVA) and thermal (**D**; $P=0.675$, two-way RM-ANOVA) hypersensitivity.

Figure 5. CFA-induced hypersensitivity is unaffected by loss of $\gamma\delta$ T cells. TCR δ littermates received intraplantar injections of complete Freund's adjuvant and pain outcomes were measured over 7 days. Differences in mechanical (**A**; $P=0.226$, two-way RM-ANOVA) or thermal (**B**; $P=0.943$, two-way RM-ANOVA) in male mice (n=9-12 per genotype). Female littermates (n=6-9 per genotype) also did not show any differences in mechanical (**C**; $P=0.530$, two-way RM-ANOVA) or thermal (**D**; $P=0.857$, two-way RM-ANOVA) responses.