Dermal resident versus recruited γδ T cell response to cutaneous Vaccinia virus infection

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Dedication

To all the science teachers, mentors, co-workers, classmates, friends and family who inspired, encouraged and supported me along the way – no one completes this journey on their own.

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University of Washington

Abstract

Dermal resident versus recruited γδ T cell response to cutaneous Vaccinia virus infection

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The study of T cell immunity at barrier surfaces has largely focused on T cells bearing the αβ T cell receptor. However, T cells that express the γδ T cell receptor are disproportionately represented in peripheral tissues of mice and humans, suggesting they too may play an important role responding to external stimuli. Here we report that in a murine model of cutaneous infection with Vaccinia Virus, dermal γδ T cell numbers increased ten-fold in the infected ear and resulted in a novel γδ T cell population not found in naïve skin. Circulating γδ T cells were specifically recruited to the site of inflammation and differentially contributed to dermal populations based on their CD27 expression. Recruited γδ T cells, the majority of which were $CD27^+$, were Granzyme B^+ and made up about half of the dermal population at the peak of the response. In contrast, recruited and resident γδ T cell populations that made IL-17 were $CD27^-$. Using a double chimera model that can discriminate between the resident dermal and recruited γδ T cell populations, we demonstrated their divergent functions and contributions to early stages of tissue inflammation. Specifically, the loss of the perinatal thymus-derived resident dermal population resulted in decreased cellularity and collateral damage in the tissue during viral infection. These findings have important implications for our understanding of immune coordination at barrier surfaces and the contribution of innate-like lymphocytes on the front lines of immune defense.

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Chapter 1:

Introduction

The immune system: T cells

The immune system is a network of cells with diverse functions dedicated to identifying and eliminating pathogens. Within this network are the innate and adaptive immune systems, which respond to broad classes of pathogens and mount specific, long-lasting responses, respectively. Communication between the innate and adaptive systems is critical for successful removal of and long-term protection against infections. One half of the adaptive immune response are classical T cells, which express an αβ T cell receptor (TCR) capable of recognizing foreign peptides in the context of self-MHC. The TCR dictates activation of T cells in the periphery and proper thymic selection ensures a TCR repertoire that is both diverse and non-reactive to host tissue.

More recently another lymphocyte subset was discovered that also develops in the thymus, but instead rearranges a TCR with γ and δ genes, termed γδ T cells. Data suggests these cells receive a TCR signal in the thymus, although their TCR-responsiveness may be altered to play a less prominent role in their activation in the periphery (1, 2). It has been proposed that these cells instead are poised to implement effector functions rapidly in response to cytokines or innate stimuli through TLRs (3). As a result, γδ T cells are considered part of the growing innate-like lymphocyte family, which also includes NK T cells and innate lymphoid cells. γδ T cells have a mostly unknown TCR specificity, although a few ligands have been identified, none are recognized in a MHC-dependent manner (4).

γδ T cell development and function

Although γδ T cells are a minor population of lymphocytes in the circulation of mice and humans, they are enriched in peripheral tissues such as the gut and skin (5). This is in part due to their unique developmental programming, which results in waves of γδ T cells being released from the thymus starting during fetal development of the mouse – a similar process also takes place during human fetal development (4). Each wave is characterized by transient and sequential expression of a single Vγ chain and the earliest waves express an invariant TCR. Early in development γδ T cells flood the circulation and seed peripheral tissues until soon after birth when αβ T cells dominate and a small variety of γδ T cells continue to be produced throughout life (4, 5).

Migration into peripheral tissues early in life is a hallmark characteristic of γδ T cells. Previous studies have assed the requirement for expression of various homing molecules, usually in the context of homeostasis and development (6). In the gut (CCR10 (7), CCR9 (8)) and skin (E/PSL, CCR4(9)), mice that lack expression have reduced γδ T cells in their respective tissues. The first γδ T cells to exit the thymus during embryonic development are dendritic epidermal T cells (DETC), which seed the murine epidermis and are not found elsewhere in the adult mouse (4). DETC express an invariant, germline encoded γδTCR (Vγ5/Vδ1). Although they are relatively immobile, their dendritic morphology allows them to sense keratinocyte health and plays a major role in the wound healing response (10-12). It has been proposed that their TCR recognizes a self ligand allowing them to rapidly respond to skin injury (13, 14). Similar clonal populations have also been found in the gut (Vγ7) and genital mucosa (Vγ6).

However, other tissues such as the lung and dermis are seeded in waves during development, but not with strictly clonal γδ T cell populations.

A distinguishing characteristic of γδ T cells is their ability to rapidly produce cytokines, which has also come to distinguish two major populations, Il-17- and IFNγ-producers. This functional commitment is thought to occur in the thymus and be controlled by TCR signal strength (1). On the whole, however, it is not clear what the TCR recognizes, either during development or in the periphery. Unlike αβ T cells, γδ T cells do not recognize antigen in a MHC-dependent manner and the structure of the TCR shares some similarities with the BCR. Some ligands for the γδTCR that have been identified in mice and/or humans are T10/T22, CD1c, MICA/B, phosphoantigens and the ATP synthase-1/apolipoprotien A-1 complex (15). The ability to recognize these structures does not appear to be constrained to a particular γδ TCR, further complicating our understanding of their antigen specificity.

The existence of large clonal populations of γδ T cells expressing identical or very similar TCRs calls into question their reliance on it for activation in the periphery. It is now appreciated that some γδ T cells received a strong TCR signal in the thymus, making TCR stimulation virtually dispensable in the periphery (1). As a result, γδ T cell functions may instead be controlled by innate sensors such as TLRs (16). Considering the DETC TCR is thought to recognize a self-ligand induced as a result of tissue damage, it is possible that many other γδ T cell subsets perform a similar function. This also makes sense given the fact that their TCR repertoire is more limited and would not perform the same role as the antigen-specific αβ T cell response.

The skin: resident T cell populations

The skin and its associated microbial flora are the body's first line of defense against infection. It is host to a network of immune cells that are in constant communication with commensal microbiota, keratinocytes and fibroblasts to maintain healthy tissue and respond quickly when the barrier is breached (17, 18). The initiation and requirements for residence of various immune subsets has been of great interest. T lymphocytes are one such cell type capable of permanent residence within the tissue, although some are more short lived (17, 18). Following antigen-specific activation in central lymphoid organs, TCRαβ effector cells traffic to peripheral sites, including the skin, and some become resident memory T cells (T_{RM}). These cells are recruited into the tissue as a result of local infection or inflammatory signals and remain there to protect against future external insults (19-24).

Two populations of γδ T cells are found in the skin. DETC, which have been mentioned above, are a relatively immobile population in the epidermis. They function to maintain keratinocyte health, sense tissue damage and aid in the repair process. More recently discovered are dermal γδ T cells. In contrast to TCRαβ T_{RM} cells, TCRγδ T cells have been shown to home to the dermis and acquire specific functions as a result of developmental programming and their response via TCR recognition of foreign antigens in poorly defined (25-27). We and others have observed that dermal γδ T cells appear to be a more motile and diverse population and comprise up to half of the dermal T cells in mice (28) and 2-9% in humans (29).

This population has been shown to participate in host defense against bacterial infection of the skin (28, 30) as well as to contribute to psoriasis in the mouse (31, 32)

and humans (33). It is now appreciated that in mice, dermal γδ T cells exit the thymus and seed the dermis near the time of birth (27, 34). Characterization of dermal γδ T cells by several groups has revealed their memory phenotype and functions related to that of $CD4^+$ T_H17 cells, being capable of producing IL-17 upon stimulation *in vitro.* Dermal γδ T cells express CCR6, CXCR6, CD103, CD44, IL-23R and IL-7R (33-35). However, it is unclear whether IL-17-producing dermal γδ T cell populations observed during infection or inflammation are derived from the resident population seeded there at birth or if unique populations are recruited from the circulation.

Questions to address

Given the mysterious nature of γδ T cells and their unconventional attributes, it is not surprising that we still have a great deal to learn about how they function within the immune system. Here we use a system of localized infection to investigate migration, plasticity and function of γδ T cells and put these within the context of development. The importance of immune coordination at barrier surfaces is being increasingly recognized for its ability to offer protection (21) or in some cases cause harm to the organism (36). The primary characteristics that we understand about γδ T cells are their unique programming to reside in barrier tissues and respond rapidly to inflammatory stimuli, which lends them well to helping maintain immunity to pathogens. Therefore, elucidating how γδ T cells participate in the immune response at these sites is imperative for a complete understanding of barrier immunity.

Chapter 2:

Materials and methods

Mice

C57BL/6, B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ and TCRδ$^{-/-}$ mice were obtained from the Jackson Laboratory and TCRδ-H2BeGFP (TCRδ-GFP) mice were obtained from I. Prinz (Hannover Medical School) and have been described previously (37). Mice were housed in specific pathogen-free conditions in the animal facilities at the University of Washington and unless otherwise specified were used at 6-10 weeks of age. All experiments were done in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Washington.

Infections

2x10^{6} plaque forming units (PFU) of recombinant Vaccinia Virus expressing full-length chicken ovalbumin (VV-OVA) was used for epicutaneous infection by skin scarification, as described previously (37). Mice were anesthetized with isoflurane and 5 µl of diluted virus was applied to the dorsal side of the ear. The skin area was then gently scratched 20 times with a 28-gauge needle. For flow cytometry experiments, both ears of mice were infected and combined to comprise one sample and the numbers were divided by two to obtain the number of cells/ear. VV-GFP was used for immunofluorescence experiments.

Adoptive transfer of γδ T cells and sorting

C57BL/6 or CD45.2$^+$ TCRδ-GFP mice received 7x10^5 γδ T cells enriched from spleen and skin dLN of CD45.1$^+$ TCRδ-GFP mice by i.v. injection one day before infection. Enrichment of γδ T cells was performed by incubating single cell suspensions with biotin-labeled anti-TCRβ and anti-CD19 mAb for 30 minutes on ice, followed by passage through the Stem Cell Biotin negative selection kit. Mice were infected on one ear and the infected (or uninfected) ears from two mice were combined for each sample. In some experiments, the enriched population was further purified as follows: cells were stained with fluorochrome-conjugated anti-CD27 and sorted on a FACS Aria for the GFP$^+$TCRβ$^-$CD19$^-$CD27$^+$ or GFP$^+$TCRβ$^-$CD19$^-$CD27$^-$ populations, of which >97% were TCRγδ$^+$. 6x10^5 CD27$^+$ or 2.7x10^5 CD27$^-$ sorted γδ T cells from B6.SJL-Ptprc^a Pepc^b/BoyJ (CD45.1) mice were transferred into separate C57BL/6 (CD45.2) hosts. Mice that received sorted cells were infected on both ears and ears from a single mouse were combined for a sample.

Adoptive transfer of OT-I T cells

Spleen and LN from GFP OT-I mice (whose CD8 T cells recognize the SIINFEKL peptide in OVA) were harvested and single cell suspensions obtained. The BD Biosciences CD8 T cell enrichment kit (plus CD44-Biotin) was used to enrich for OT-I T cells. Purity was determined and 1x10^4 OT-Is were transferred into each mouse.

Flow cytometry

Single cell suspensions were prepared from spleens and LN by mechanical disruption. Dorsal and ventral halves of ears were separated, minced and then enzymatically digested for 2 hrs at 37 °C in Liberase TM (Roche). Both ears from each mouse were combined for processing to obtain adequate cell numbers. All tissues were passaged through a 100-μm nylon sieve (BD Bioscience). In some experiments, to avoid the Liberase digestion (which cleaved CD27), dorsal and ventral halves of the ears were separated and floated dermal side down in complete media overnight at 37°C. Supernatant, into which cells had migrated, was collected the following day and stained. Cells were stained for 30 min on ice with the appropriate mixture of monoclonal antibodies and washed with PBS containing 1% BSA. The following conjugated mAb were obtained from BD Pharmingen, eBioscience or BioLegend: anti- TCRβ (H57-597), CCR6 (140706), CD27 (LG.3A10), CD103 (2E7), Vγ5 (BioLegend Vγ3 clone 536), CD44 (IM7), CD45.1 (A20), CD45.2 (104). In the ear, γδ T cells from TCRδ-GFP were identified as $GFP^{+}V\gamma5^{-}$ (dermal) or $GFP^{+}V\gamma5^{+}$ (DETC), according to the nomenclature described by Heilig & Tonegawa (38). For the analysis of intracellular IFNγ, IL-17 and Granzyme B, single cell suspensions were incubated in the presence or absence of 50 ng/mL PMA and 500 ng/mL Ionomycin for 4 hours at 37°C in the presence of Brefeldin A. Cells were stained for surface markers and then processed using the BD PharMingen Cytofix/Cytoperm kit. For analysis of Ki67, cells were stained for surface markers and then processed using the eBioscience FoxP3 kit. Samples were analyzed on a FACSCanto II (BD) using Flowjo software (Tree Star).

Bone marrow chimeras

Chimeras were made as previously described (22). Briefly, perinatal thymocytes (pThy) were harvested 0–48 h after birth and $5x10^6$ cells were transferred i.v. to congenic recipients that had been lethally irradiated with a dose of 1000 rad in a cesium irradiator. The next day, $1\text{-}2x10^6$ congenic bone marrow (BM) cells were transferred. These mice are referred to as “BMpThy chimeras” while controls without pThy are referred to as “BM chimeras”. Chimeras were analyzed or infected at least 8 weeks after reconstitution.

In situ proliferation

Infected mice were injected i.p. with 2mg in 200µl 50-Bromo-2-deoxyuridine (Brdu) 1 hour before being euthanized. Single cell suspensions were obtained from each tissue and stained using the BD Biosciences APC Brdu Kit. Cells were fixed with PFA before Cytofix/Cytoperm to ensure retention of TCRδ-GFP signal.

Cell transfer and in vivo proliferation

Cells were harvested as described for cell transfer experiments and labeled for 20 minutes at room temperature with 5µM of CellTrace Violet (CTV, Life Technologies). Following incubation cells were washed in serum, resuspended in PBS and $6\text{-}7x10^6$ γδ T cells were transferred into each mouse. For analysis of CTV dilution cells only live cells were gated on.

Cytokine analysis in the skin

Ears from infected mice were harvested, split open and added to tubes with 1 mL of cytokine extraction buffer (1 mini protease inhibitor/10mL, 0.4M NaCl, 0.5% BSA, 10mM EDTA, 0.05% Tween, 0.1mM benzethonium chloride) and beads. Tubes were placed in a bead beater for two 90 sec intervals (placed on ice in between). After homogenization, tubes were incubated on ice for 10 minute before being centrifuged for 5 min at 1300rpm. 500µl of the supernatant was transferred to a spin-x filter tube and centrifuged for another 5 min. Flow through was frozen at -80°C and thawed only once before use. For analysis of cytokines, the protocol for the BD Biosciences Cytometric Bead Array (CBA) Inflammation Kit was followed. Amount of each cytokine in the tissue was determined with standards provided in the kit and calculated based on MFI.

Virus titration by plaque assay

The viral load in organs was determined by plaque assays on 143B cells with dilutions of a 40% tissue homogenate added to confluent wells. Organs were harvested and homogenized in PBS using a bead beater. Samples were kept at -80°C and were frozen and thawed 3 times before being used. Titers reported are log10 PFU per whole LN or ear.

Virus titration by qPCR

Tissue samples were harvested and DNA was purified with the DNeasy Mini Kit (Qiagen 69504). The primers used in the quantitative PCR assay are specific for the ribonucleotide reductase Vvl4L of VV. The sequences are: (Fwd)

TTGTAGAATACCGTTACTGGCG and (Rev) CTTGTGCTTCCAGGCTATCA. Control primers from GAPDH are: (Fwd) CAATGGCTTGTTTCACTGCC and (Rev) CCAACAGGACCTCAGCCC. Amplification reactions were performed in a 96-well PCR plate (Bio-Rad Laboratory) in a 25µl volume containing 2x SybrGreen Master Mix (Applied Biosystems), 0.5µM forward primer, 0.5µM reverse primer and the template DNA. Thermal cycling conditions were: 95° C for 10 min, 94°C for 15 sec and 63°C for 1 min (45X). To calculate the virus load, a standard curve was established from DNA of a VV stock with previously determined titer. Corresponding CT values obtained by the real-time PCR methods were plotted on the standard curve to estimate viral load in the skin samples.

Histology

Ears from infected mice were fixed in 10% buffered formalin for 3 days before being embedded vertically in paraffin. Sections were hematoxylin and eosin stained and the severity of inflammation and necrosis was determined on a scale of 0-4 (4 being the most severe). Scoring of all samples was blinded and uninfected mice were used as controls.

Immunofluorescence

Epidermal peels were obtained by a method given to us by the Havran Lab (11). Briefly, dorsal and ventral sides of the ears were separated and floated in a petri dish in a solution of 3.6% ammonium thiocyanate for about 15 min at 37°C. Epidermis could then be gently removed and transferred to a new dish to be stained. For skin cross-sections,

back skin was treated with Nair to remove hair and a small section cut and rolled before being frozen in Optimal Cutting Temperature (OCT) medium. Sections were cut, dried overnight and fixed in acetone before being stained. The following antibodies were used: γδTCR-PE, Vγ5-APC, CD3-PE.

Statistical analysis

Prism software (GraphPad) was used for all statistical analysis. The two-tailed, unpaired student *t*-test was used for comparisons of γδ T cell frequencies and histological scores.

Chapter 3:

γδ T cell functional subsets are determined during development, independent of TCR Vγ chain use

Introduction

γδ T cells were discovered over 30 years ago and originally noted for their prevalence during fetal development (38). Subsequent studies determined that γδ T cells exit the thymus in waves, which correlate with the use of specific TCR Vγ chain usage and homing to different tissues (5). Although few γδ T cells are found in the adult circulation, they are greatly enriched in peripheral tissues (5, 39). Considering γδ T cells were discovered because of their unique use of Vγ and Vδ genes, they have long been defined and subsetted based on their TCR expression. However, recent data suggests that the TCR may be dispensable for activation of some γδ T cells in the periphery (1, 40, 41) and other cell surface markers might be more accurate for describing lineages within the population.

It has been proposed for some time that γδ T cell function is determined in the thymus (2, 25, 42). Emerging data from studies of γδ T cells in central lymphoid organs suggest that CD27 delineates γδ T cell functional subsets (27, 41, 43). On activated αβ T cells, CD27 is thought to enhance survival and proliferation via interaction with CD70 (44). However, CD27 expression on γδ T cells appears to identify subsets determined during development and its expression by mouse γδ T cells is tightly correlated with their ability to produce IFNγ, whereas failure to express CD27 correlates with IL-17 production (43). Importantly, analogous γδ T cell populations have been found in human

PBMC (45-47), making this a relevant axis from which to orient experiments in model organisms.

Although these populations have been described in the circulation, their relevance to γδ T cell populations in peripheral tissues such as the skin has not been investigated. Several cell surface proteins have been used to characterize dermal γδ T cells, in particular CD103 and CCR6 are readily found on this population. A recent study identified a population of γδ T cells enriched in the skin dLN of mice that share similar properties with dermal γδ T cells (34). This discovery demonstrated a potential link between dermal γδ T cells and those found in the circulation. We used a combination of these markers in an attempt to relate known lineages to functional subsets associated with the tissue and determine how this might tie into developmental programming.

Results

CCR6 and CD27 define distinct γδ T cell subsets

The only unique marker to γδ T cells is their TCR. Staining for the γδTCR is not always clear or possible as some γδ T cells down-regulate the TCR upon activation. Further, for *in vitro* or transfer experiments it is not ideal to have an antibody attached to the TCR. In order to cleanly identify γδ T cells and use them untouched in some experiments, we obtained mice which express GFP under the TCRδ promoter (37). Mice are bred homozygous without impacting TCR expression, ensuring that all γδ T cells are GFP^+. GFP is bound to histones in the nucleus making intracellular staining possible without the loss of fluorescence. One caveat is that a small fraction of αβ T cells express GFP as a result of attempts to rearrange a productive TCR in the thymus;

this residual GFP is most likely on αβ T cells that have recently emigrated (48). In the circulation αβ T cells make up approximately half of the GFP^+ cells, but almost none are found in the skin. A GFP^{lo} population can also been found and these have been described as NK cells (49). Therefor we identify γδ T cells as $TCR\beta^-GFP^+$ in all our experiments (Figure 3.1A).

Staining of γδ T cells in the skin dLN revealed that CD27 and CCR6 were mutually exclusive in peripheral lymphoid organs and identified three γδ T cell populations (Figure 3.1A) with distinct surface and transcription factor staining patterns (Figure 3.1B). In line with previous reports that $CD27^-$ γδ T cells produce IL-17 upon stimulation, we found that baseline expression of the transcription factor RORγt was higher in $CD27^-$ than in $CD27^+$ γδ T cells. Conversely, the transcription factor Tbet was highest in the CD27+ γδ T cells. $CCR6^+CD27^-$ γδ T cells expressed the highest levels of CD44 and CD103, while the small number of $CD27^-CCR6^-$ cells and the $CD27^+$ cell subset had lower levels of CD44 and only half expressed CD103. Upon stimulation of naïve γδ T cells from the skin dLN with PMA/Ionomycin for 4 hours, IFNγ was produced by $CD27^+$ γδ Tcells and IL-17 was produced by $CD27^-CCR6^+$ γδ T cells (Figure 3.1C). Despite their phenotypic similarity, the $CD27^-CCR6^-$ γδ T cells produced little of either cytokine suggesting this population may have different functions and/or requirements for cytokine production.

Although there is significant evidence to suggest CD27 expression marks separate populations, it has not been definitively proven that they are independently maintained. Further, since there is lack of clarity on the subject, previous studies have interpreted changes in the CD27 ratio to be due to up- or down-regulation of this marker

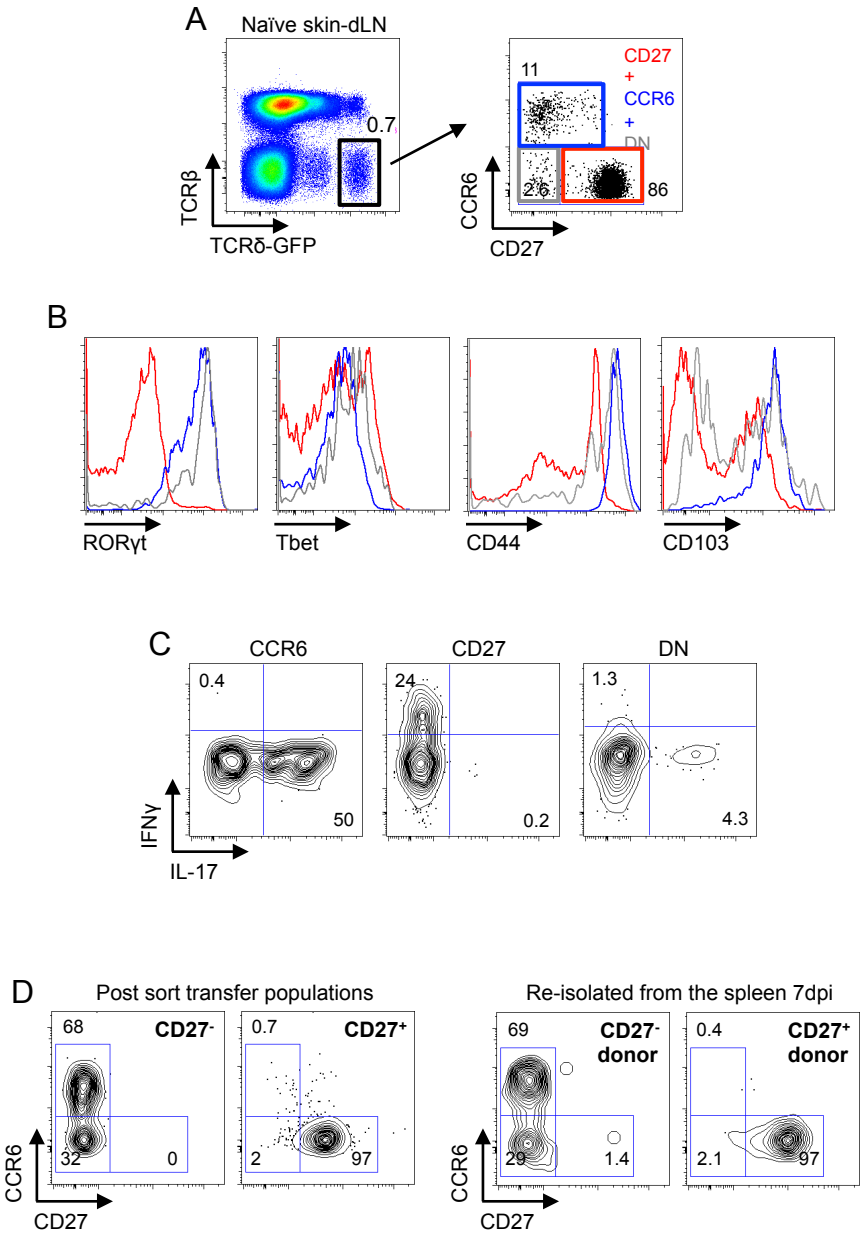

Figure 3.1. Phenotype of γδ T cells in the periphery of TCRδ-GFP mice. A) Representative flow plots show gating strategy for γδ T cells in the skin dLN and CCR6 by CD27 profile. Numbers denote percent of total dermal γδ T cell population within the indicated quadrant. B) Nuclear and surface staining for RORγt, Tbet, CD44 and CD103 on the three circulating γδ T cell populations. Colored boxes in A correspond with histograms. C) Single cell suspension from the skin dLN of naïve mice was re-stimulated with PMA/Ionomycin for 4 hours at 37°C followed by intracellular staining for cytokines. Representative flow plots are shown and gates are based on unstimulated naïve samples. Numbers indicate the percentage of that population in each quadrant. D) γδ T cells from spleen and skin dLN of TCRδ-GFP mice were sorted based on CD27 expression and $6x10^5$ $CD27^+$ or $2.7x10^5$ $CD27^-$ γδ T cells were transferred into separate C57BL/6 hosts. Recipients were infected with VV on the ear the following day. Flow plots show the phenotype of $CD27^-$ and $CD27^+$ at the time of transfer and 7dpi in the spleen. Numbers denote the percentage of cells within the indicated quadrant.

rather than expansion of one population. To ask whether CD27 is indeed a stable marker of γδ T cell lineages in the presence of inflammation, $CD27^+$ and $CD27^-$ γδ T cells were sorted from mice and transferred into separate congenically marked hosts. On day 7 post infection transferred cells were re-isolated from the spleen and stained for CD27 as well as CCR6. At this time point, no cells from either group changed their expression of CD27 (Figure 3.1D). It is also interesting to note that the ratio of $CCR6^+$ and $CCR6^-$ γδ T cells within the $CD27^-$ subset was relatively unchanged. These experiments demonstrate that in vivo in the presence of inflammatory signals, CD27 expression in the circulation remains constant as a marker of distinct γδ T cell lineages.

A wave of γδ T cells exit the perinatal thymus to form the circulating $CCR6^+CD27^-$ and dermal populations

Previous studies have demonstrated that near the time of birth, γδ T cells exiting the thymus contribute to the circulating $CD27^-$ population (27) and dermal γδ T cells (34). We analyzed the adult and perinatal (0-48 hours after birth) thymus to determine whether mature γδ T cell thymocytes ($GFP^+TCR\gamma\delta^+$) differed in CCR6 and CD27 expression. In the adult thymus γδ T cell subsets reflected closely the phenotype of the circulating population. In contrast, a much higher proportion of perinatal γδ T cells are $CD27^-$, and more specifically express CCR6 (Figure 3.2A).

A model was recently described to validate the existence of this wave and its unique ability to contribute to the dermal γδ T cell population (34). Following lethal irradiation, we confirmed that γδ T cells in the circulation and dermis are sensitive to whole body irradiation. Although donor bone marrow (BM)-derived γδ T cells were the

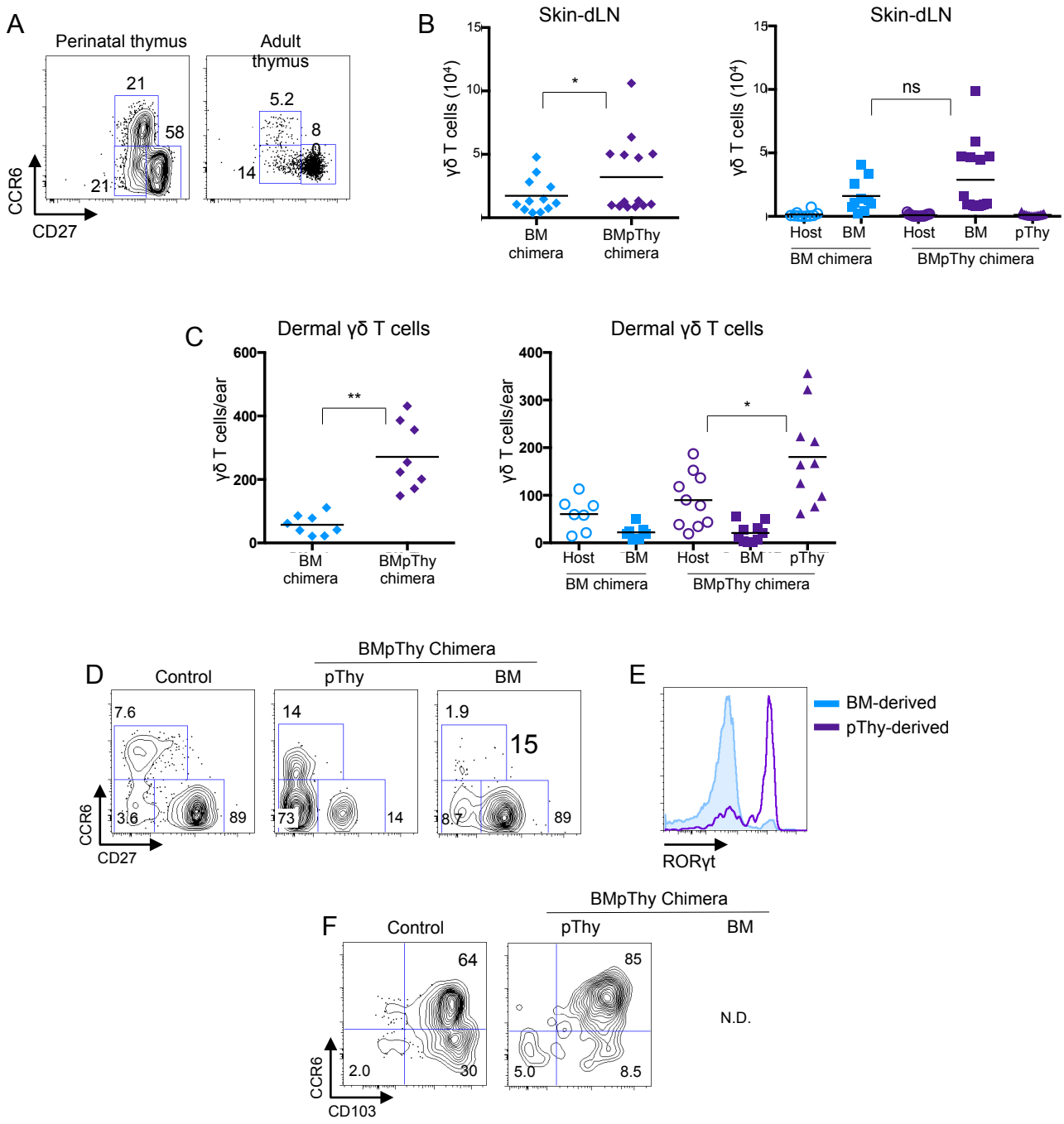

Figure 3.2. A wave of γδ T cells exit the perinatal thymus (pThy) to form the circulating CCR6⁺CD27⁻ subset and dermal γδ T cells. A) Perinatal (0-48hrs after birth) and adult thymus CCR6 by CD27 profiles. B-F) CD45.2⁺ TCRδ-GFP mice were irradiated and reconstituted with BM (BM only) or with BM plus pThy (BMpThy). B) Graphs show the total number of γδ T cells > 8 weeks following reconstitution in the dermis and skin dLN. Geometric mean is shown. C) Contribution of BM-derived and pThy-derived γδ T cells in the dermis and LN. Geometric mean is shown. D) CCR6 and CD27 profiles of γδ T cells in the skin dLN and E) RORγt profiles of pThy and BM γδ T cells in the skin dLN. F) CCR6 and CD103 profiles of γδ T cells in the dermis in WT mice and naïve chimeras (N.D. = not done). Numbers denote the percentage of that population within the indicated quadrant.

dominant population in the circulation (Figure 3.2B), they failed to reconstitute the dermal γδ T cell population (Figure 3.2C). In addition, the majority of γδ T cells in the spleen and LN were $CD27^+$ with a minority of $CD27^-CCR6^-$ and no $CD27^-CCR6^+$ γδ T cells (Figure 3.2D). Although small numbers of host γδ T cells remained in the dermis following irradiation, their phenotype was abnormal and their numbers were significantly lower than in unirradiated mice (data not shown).

Importantly, γδ T cell populations lost due to irradiation could be replaced by transferring perinatal thymocytes (pThy) into irradiated hosts along with congenically marked BM cells, referred to as BMpThy chimeras. This resulted in the restoration of both $CD103^+CCR6^+$ and $CD103^+CCR6^-$ dermal γδ T cell populations and the circulating $CCR6^+$ population (Figure 3.2C & D). pThy also expressed high levels of RORγt while this was mostly absent from the BM-derived γδ T cells (Figure 3.2E). Total γδ T cells numbers in the dermis returned to normal levels and the phenotype of the pThy-derived dermal γδ T cells resembled that of unirradiated mice (Figure 3.2F). Although pThy-derived γδ T cells contributed to all three circulating populations defined by CD27 and CCR6 staining, $CD27^+$ cells were a relatively minor population.

Functional γδ T cell subsets are determined independently of TCR Vγ chain expression

In order to look at the distribution of Vγ chain usage we used commercially available flow antibodies (Vγ1, 2, 4 and 5) to stain spleen, lymph node and skin. Of the seven Vγ chain genes, γδ T cells are known to utilize six (Vγ3 is excluded). Using immunofluorescence we confirmed previous reports and found high expression of Vγ5 used exclusively by DETC in the skin epidermis (Figure 3.3A & B). As a result, whole

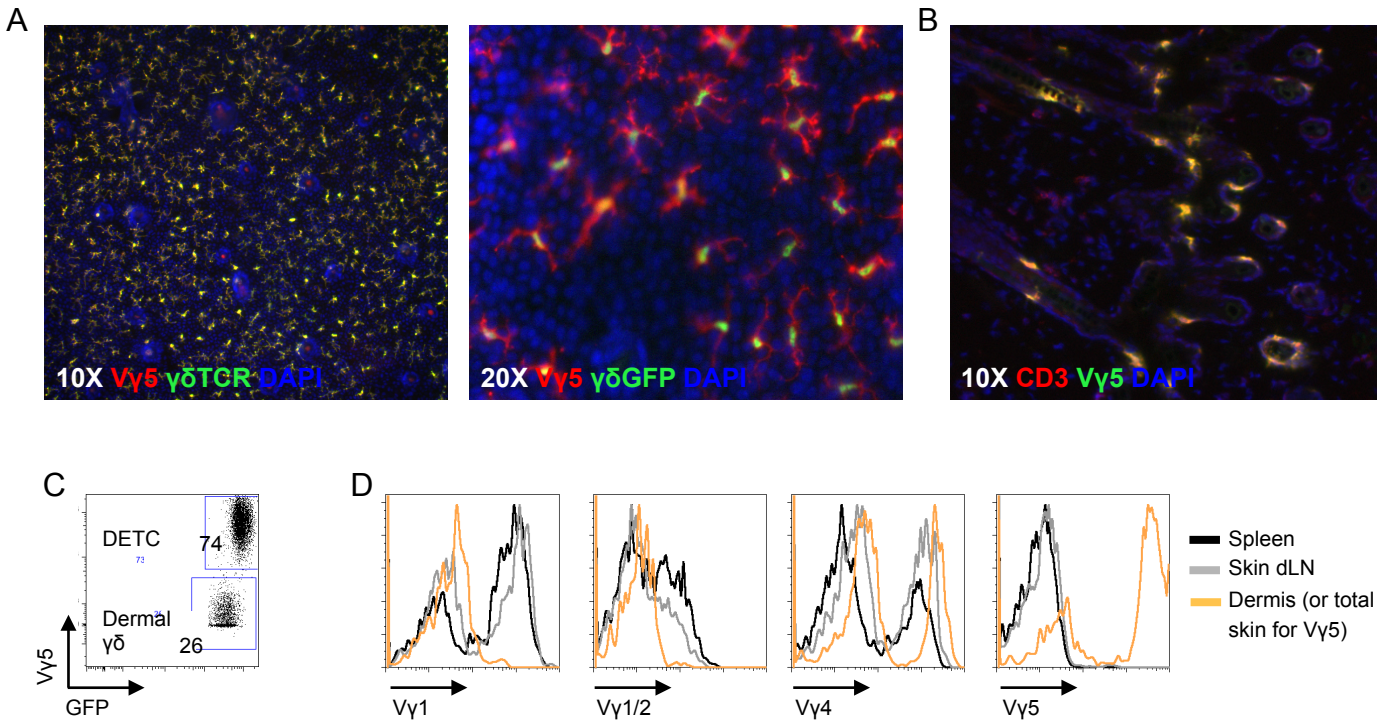

Figure 3.3. γδ T cell Vγ expression in the circulation and skin. A) Immunofluorescence of an epidermal peel from C57BL/6 (left) and TCRδ-GFP (right) mice and B) frozen skin cross sections from C57BL/6 mice. Samples were stained with Vγ5 (DETC-specific) and/or γδTCR as well as dapi (nucleus). C) Representative flow plot of skin demonstrating gating strategy for skin experiments. D) Representative histograms show levels of Vγ1, 1&2 and 4 expression in spleen, skin dLN and dermis. Vγ5 expression is shown for total skin.

skin was digested for flow experiments and dermal γδ T cells were identified as Vγ5$^-$ (Figure 3.3C). In the dermis, Vγ4 is expressed by up to half of dermal γδ T cells and a minority expressed Vγ1 (Figure 3.3D). In the circulation the majority were Vγ1$^+$ and Vγ4$^+$ also made up a large portion. Vγ2 is identified with an antibody that stains both Vγ1 and Vγ2, however we did not find this antibody to stain a higher proportion than Vγ1 alone, suggesting this TCR is not readily used in the circulation or dermis. Results are summarized in Table 3.1.

Considering that CD27 aligns tightly with γδ T cell function, we asked whether γδ T cells with a particular TCR Vγ chain favored its expression. Although it does not cover the breadth of Vγ usage, we used Vγ4 as a proxy to determine the likelihood that γδ T cells with a specific function would all use the same TCR chains. We found that neither the CD27$^+$ nor CD27$^-$ populations were homogeneous populations (Figure 3.4A). Approximately 20-30% of CD27$^+$ γδ T cells and 25-60% of CD27$^-$ γδ T cells used Vγ4. Based on this it seems that during development, expression of CD27 is not directly tied to TCR rearrangement although perhaps CD27$^-$ γδ T cells favor Vγ4.

To better address this question we also analyzed γδ T cells from the thymus of adult mice and within 48 hours after birth. In the adult thymus, almost 50% of GFP$^+$γδTCR$^+$ cells expressed a Vγ4 TCR and similarly, approximately 40% of total pThy were Vγ4$^+$ (Figure 3.4B). In the perinatal thymus, half of the CD27$^+$ population expressed Vγ4 while only a fraction of the CD27$^-$ γδ thymocytes did (Figure 3.4C). In the adult thymus, all populations similarly used Vγ4. In fully reconstituted BMpThy chimeras where pThy make up a minority of γδ T cells, which are mostly CD27$^-$ and

Table 3.1. γδ T cell Vγ genes, available antibodies and tissue localization.

Heilig & Tonegawa nomenclature	Vγ1	Vγ2	Vγ3	Vγ4	Vγ5	Vγ6	Vγ7
Commercially available Antibody	Yes	Yes*	NA	Yes	Yes	No	No
LN/Spleen	✓	✗/?	NA	✓	✗	?	?
Dermis	✓/?	✗	NA	✓	✗	?	?
Epidermis	✗	✗	NA	✗	✓	✗	✗

*Stains both Vγ1 & Vγ2

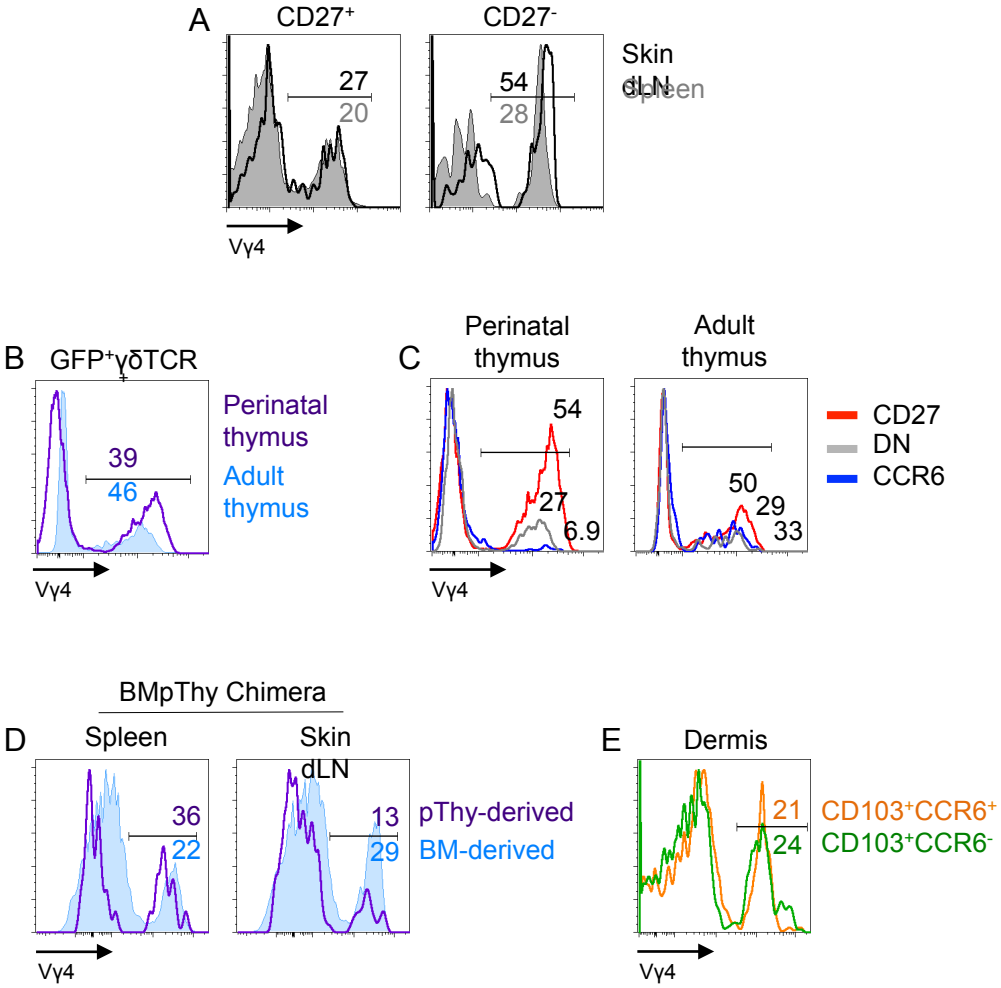

Figure 3.4. Functional γδ T cell subsets are determined independently of TCR Vγ chain expression. Vγ4 expression was analyzed to determine whether functional or developmental subsets were uniform in TCR usage. A) Representative histograms for CD27$^+$ and CD27$^-$ γδ T cells in spleen and skin dLN of naïve mice. B) Total adult and perinatal thymocytes were compared for Vγ4 use. C) Vγ4 on thymocytes from perinatal (left) and adult (right) that were subsetted based on CCR6 and CD27 expression. D) Vγ4 expression on pThy-derived and BM-derived in the spleen and skin dLN from reconstituted BMpThy chimeras. Numbers denote the percentage of that population within the indicated quadrant.

BM-derived γδ T cells are almost all CD27$^+$, the BM- and pThy-derived γδ T cells reflected similar Vγ proportions (Figure 3.4D).

Although DETC within the epidermis all express an identical germline encoded TCR, dermal γδ T cells do not appear to be a homogenous population. Using CD103 and CCR6 expression to identify dermal subsets, we found that 20-25% of both populations were Vγ4$^+$ (Figure 3.4E). Overall this challenges the notion that γδ T cells exit the thymus in strict Vγ chain waves, each with a specific purpose, and instead that functional waves of γδ T cells may exit the thymus that are not restricted to particular TCRs.

Discussion

One of the most challenging aspects of understanding γδ T cells is a lack of context from which to study them. The γδTCR has long been the only way of identifying them, as no other marker is currently known to be unique to γδ T cells. As such, TCR chain usage has been a primary method of categorizing them, but more recently it has become clear that this is possibly too simplistic and inaccurate in some cases. The most well characterized γδ T cells are DETC, which follow the classical line of thought, having exited the thymus in a distinct wave with expression of a discrete TCR and entered the epidermis where they reside throughout life (50). However, it seems that this type of γδ T cell population may be the exception rather than the rule, especially considering this population is not conserved across species.

The most effective way to investigate the γδ T cell role in the immune system is to understand how individual cells fit within a network of fixed lineages and plastic

subsets. Recent work done by our group as well as others attempts to elucidate this system by evaluating the relationship between cell surface proteins, cell function and γδ T cell development, which may still play an important role in determining their fate. CD27 is now recognized as a significant cell surface marker for γδ T cells with an ability to distinguish populations fated to produce either IFNγ or IL-17 (43). Although there has been emphasis on CD27 in the circulation and its importance for determining function, less has been done to look at its expression on γδ T cells in peripheral tissues such as the skin. In contrast, CCR6 is known to be expressed on γδ T cells in the dermis and the associated lymphatics (34), but has not been studied well in the context of γδ T cell lineages.

Here we characterize distinct subsets within the dermal and circulating γδ T cell populations (Figure 3.5). CD27 delineates two independently maintained lineages of γδ T cells, which remain stable in the presence of inflammation. These populations have been shown during the steady state to have distinct epigenomes (41) confirming our understanding that these are indeed separate subsets of γδ T cells. Further, within the $CD27^-$ population we identified two subsets based on CCR6 expression, each with unique properties. $CD27^-$ γδ T cells are found readily in the thymus near the time of birth and seed the circulation and dermis at this early point in development, which persist as long-lived populations. $CD27^+$ γδ T cells exit the thymus throughout life and contribute primarily to the circulating population where they are the majority. Although these populations are derived at different times during development, this does not necessarily correlate with TCR Vγ chain expression. The dermal γδ T cell population is exclusively

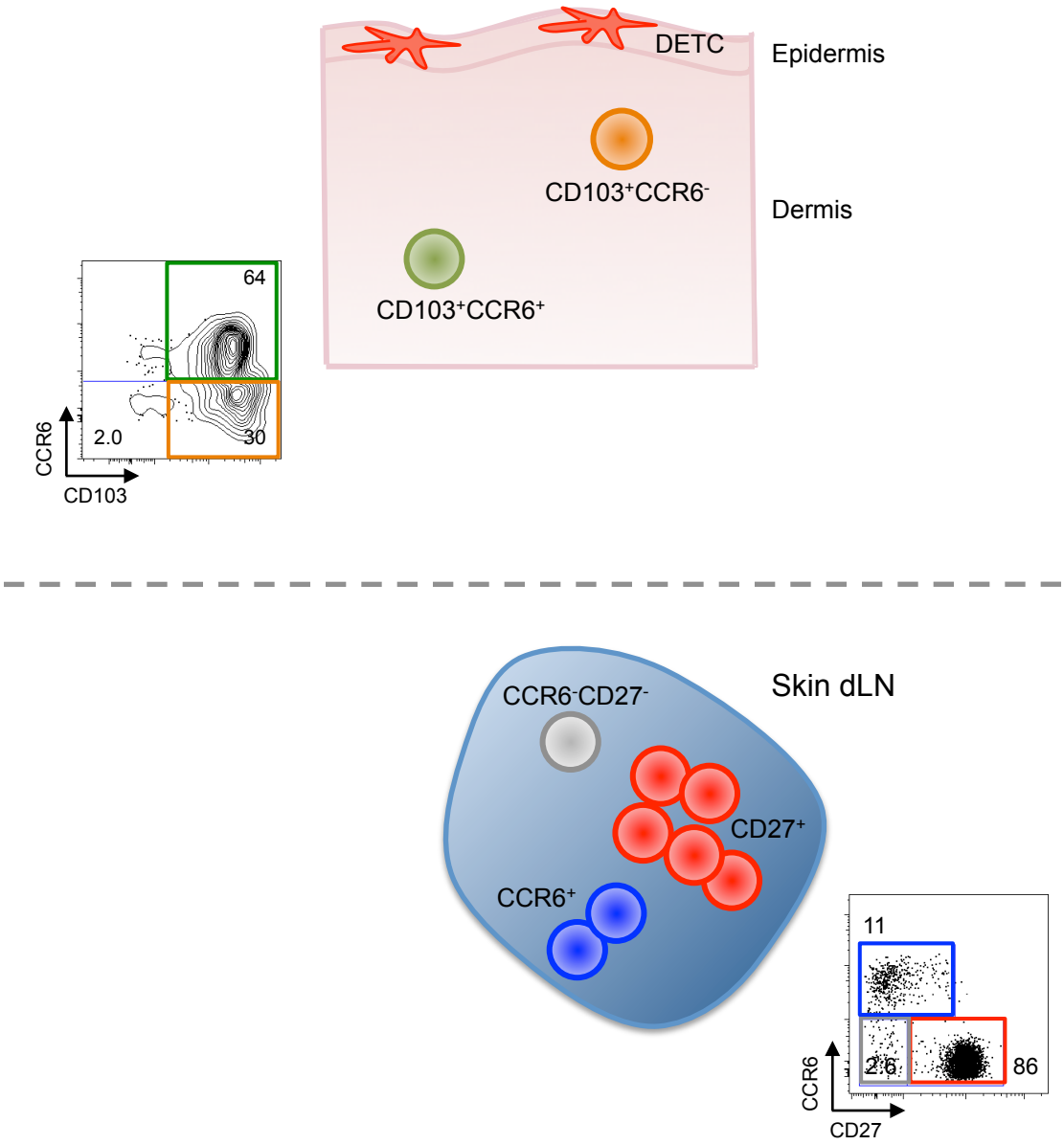

Figure 3.5. Model of γδ T cell populations in the skin and skin dLN under steady state conditions.

CD27$^-$ under steady state conditions and all of them are CD103$^+$ with about half expressing CCR6.

Linking cell surface phenotype with TCR specificity and ultimately γδ T cell function is an important avenue of research. Although the specificity of the γδ TCR has remained mostly elusive, a few antigens have been identified. The most well characterized population Vγ9Vδ2-expressing γδ T cells, which seem to recognize phosphoantigens and are found only in primates (51, 52). Their reliance on TCR stimulation for activation or other signals to perform effector functions is not well known, however they have potent effects on infected (53, 54) and cancerous cells (55, 56). Some Vγ9Vδ2$^+$ γδ T cells do express CD27 and it has been proposed that this signal integrates with TCR stimulation and contributes to their survival (45). NKG2D is another cell surface protein that has been proposed to work in concert with TCR stimulation for γδ T cell activation (57, 58). It is an activating receptor, which recognizes MICA/MICB as well as ULBP molecules expressed on infected or cancerous cells (59).

Considering the limited nature of TCR expression and the variety of activating molecules on γδ T cells, it is possible that even cells expressing the same TCR, Vγ9Vδ2 for example, have unique sets of co-receptors that work to fine tune alternative responses and activation requirements. This allows for a wide variety of activating requirements in the periphery and the possibility that in some cases the primary function of the TCR was during thymic development rather than recognition of foreign antigen like classical αβ T cells.

Chapter 4:

Vaccinia Virus infection results in accumulation of γδ T cells in the tissue and alters the dermal γδ T cell population

Introduction

Although γδ T cells make up a small portion of T cells in the circulation, they are found at higher proportions in peripheral tissues such as the gut, vaginal mucosa and skin (5). While migration into tissues for αβ T cells is most often in response to infection, injury or normal flora, γδ T cells seem to undergo programming during development that directs them to these locations following their exit from the thymus. This hardwiring, which drives them particularly to barrier tissues such as the intestine, reproductive tract and skin, seems ideally suited to ensure that γδ T cells participate in innate host defense.

While several models of systemic viral infection (60, 61) have elicited a γδ T cell response in the spleen and LN, these studies did not address questions concerning tissue trafficking or provide a relevant context for natural modes of infection. Importantly, studies with human PBMC have found that γδ T cells in the circulation are impacted in patients with inflammatory bowel disease (62), melanoma (63) and psoriasis (64), suggesting that local inflammation can impact global γδ T cell homeostasis. These findings point to the need to understand the trafficking patterns of γδ T cells during steady state conditions and in the context of disease.

In the current study, we analyzed the γδ T cell response in mouse skin to local infection with Vaccinia Virus (VV). We sought to dissect the contribution of resident

versus immigrant γδ T cells following infection and to determine whether γδ T cells recruited from the circulation give rise to long-term residents in the skin after the cutaneous infection has cleared. Many previous reports have studied the γδ T cell response in TCRα$^{-/-}$ or TCRβ$^{-/-}$ mice which lack TCRαβ T cells (60, 61) or performed transfers of γδ T cells isolated from TCRβ$^{-/-}$ mice (65) to investigate the anti-viral response. In such scenarios the γδ T cell makeup and response is likely abnormal (66). Analyzing γδ T cells in a wild-type setting allowed us to better appreciate their role in a skin infection.

Our experiments revealed that CD27^{+} and CD27^{-} γδ T cells are specifically recruited from the circulation to the site of infection and contribute to different populations in the dermis. Surprisingly neither population becomes resident in the tissue, even when encountering a relatively empty niche.

Results

γδ T cells accumulate at the site of infection with the emergence of a novel dermal population

Although it is known that γδ T cells reside at barrier surfaces in mice and humans (28, 29), few studies have addressed the role they play during infection of the skin, particularly in response to viral infection. To address this, we used a model of Vaccinia VV scarification, in which virus localizes primarily to the site of inoculation (67). Following infection of the ear pinna, we observed the number of dermal γδ T cells increased approximately ten-fold in the infected ear and dLN (Figure 4.1A & B). The response kinetics were slightly unusual, as the numbers in the skin and dLN remained

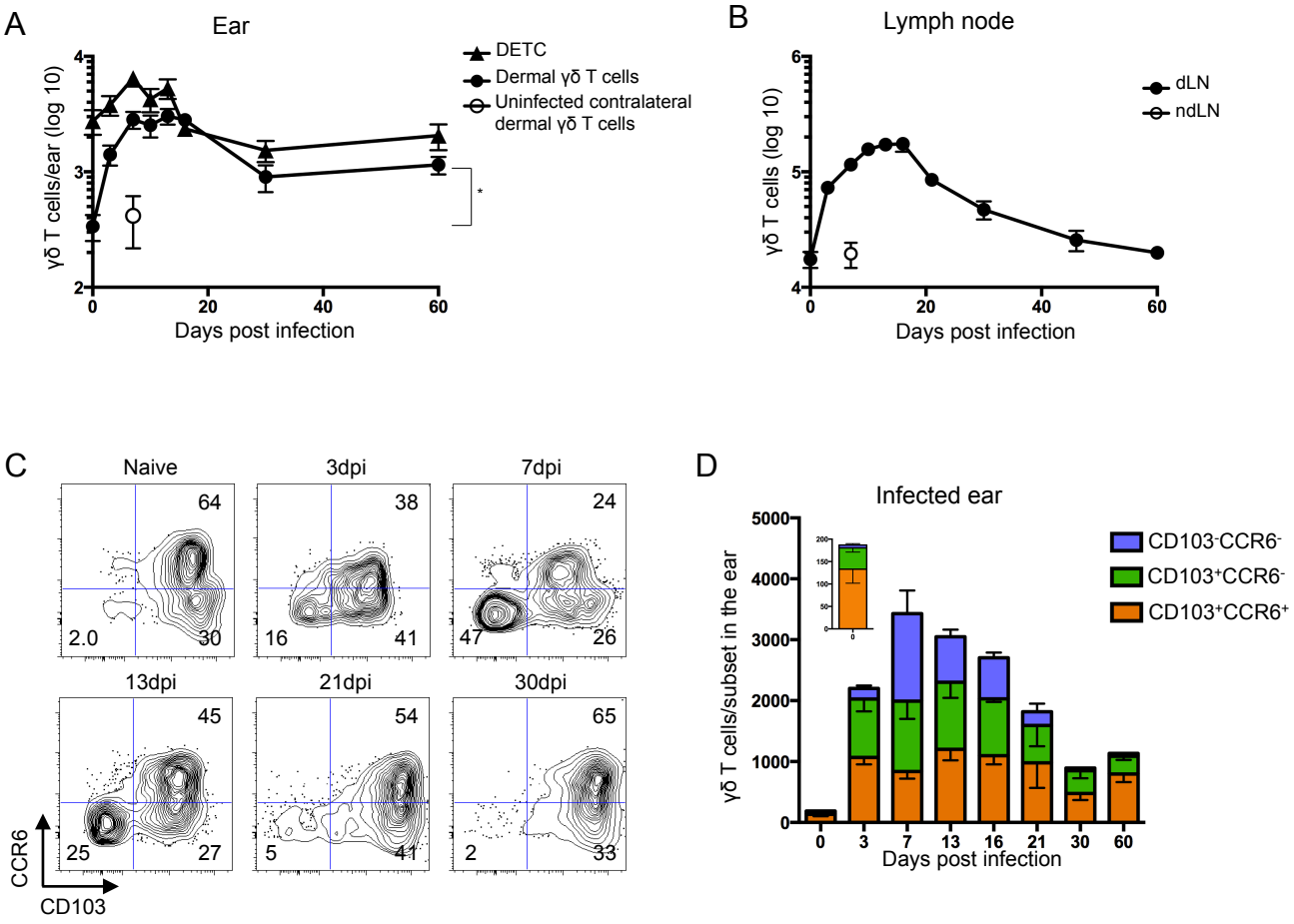

Figure 4.1. Following skin scarification with Vaccinia virus (VV), γδ T cells accumulate in draining lymph node and dermis. TCRδ-GFP mice were infected with VV on the ear by scarification. γδ T cells were gated as TCRβ^{-}GFP^{+} in the LN and as TCRβ^{-}GFP^{+}Vγ5^{+} (Dendritic Epidermal T Cells, DETC) or TCRβ^{-}GFP^{+}Vγ5^{-} (dermal γδ T cells) in the ear. A) Number of DETC and dermal γδ T cells in the ear following infection. Open symbol represents the number of dermal γδ T cells in the contralateral uninfected ear at 7dpi. B) Number of γδ T cells in the dLN following infection. Open symbol represents the number of γδ T cells in the contralateral non-draining LN (ndLN) at 7dpi. C) Representative flow plots show CCR6 and CD103 profile of dermal γδ T cells following infection. Numbers denote percent of total dermal γδ T cell population within the indicated quadrant. D) Cell numbers within the indicated dermal γδ T cell subsets over the course of infection per ear (insert, day 0). Error bars signify SEM.

elevated for a protracted period of time. Furthermore, the number of dermal γδ T cells at late time points was significantly higher than in naive ears, suggesting that infection could result in prolonged alterations in the dynamics of the dermal γδ T cell population. The number of DETC also increased about three-fold following infection and returned to the number found in control ears at late time points.

In resting skin, all dermal γδ T cells are $CD103^+$ and over half are $CCR6^+$; however, skin scarification with VV resulted in dramatic changes in the dermal γδ T cell population. By three days post infection, a novel subset appeared in the dermis that was CCR6 and CD103 double negative (DN) (Figure 4.1C & D). This novel dermal γδ T cell subset accounted for almost half of the γδ T cell population at the peak of the response, then disappeared entirely from the dermis by 30 days post infection.

Circulating γδ T cells are rapidly recruited to the VV infected ear

We performed an adoptive transfer to investigate the origin of the dermal γδ T cell populations following infection and to determine the contribution of circulating γδ T cells to the expanded dermal population. Accordingly, $7x10^5$ γδ T cells isolated from the spleen and skin dLN of naïve TCRδ-GFP mice were transferred intravenously into normal C57BL/6 recipients before VV scarification. Infection of the recipients showed that donor γδ T cells were specifically recruited to the skin of the infected ear and their numbers peaked 7 days post infection (Figure 4.2A). While these adoptively transferred, circulating cells were capable of entering the dermis during active infection, very few remained permanently integrated into the dermis at a late time point.

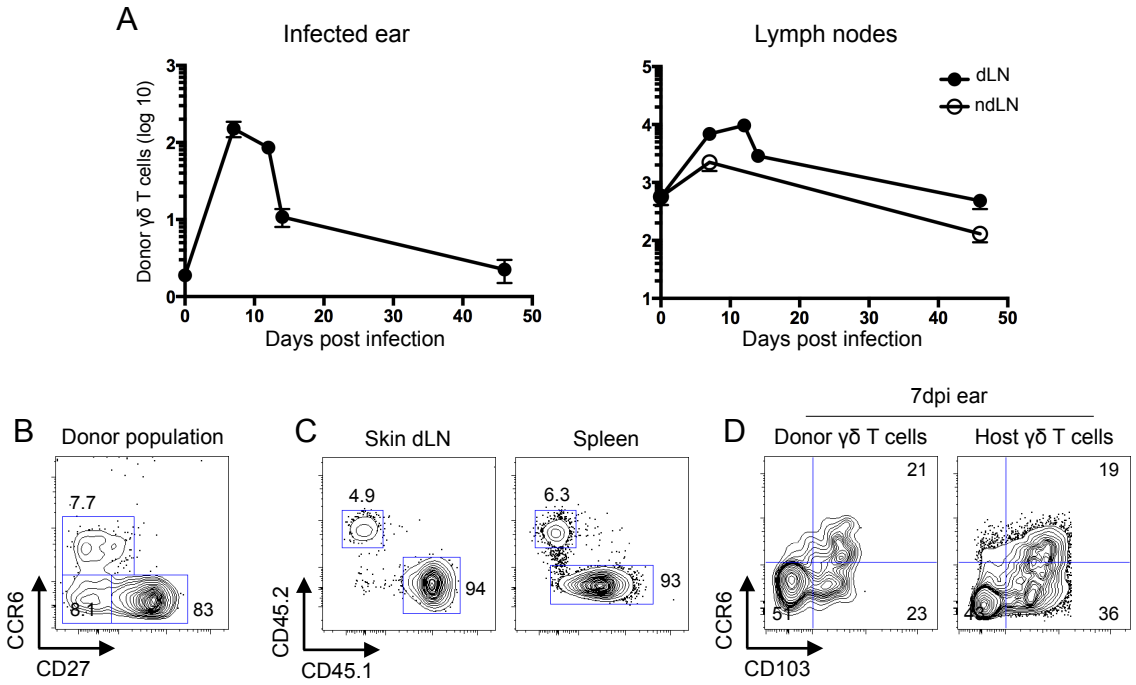

Figure 4.2. Circulating γδ T cells migrate to VV infected skin and contribute to all three dermal subsets. C57BL/6 mice (CD45.2) or CD45.2$^+$ TCRδ-GFP mice received 7x10^5 γδ T cells isolated from spleen and skin dLN of CD45.1$^+$ TCRδ-GFP mice. The following day, mice were infected with VV on the ear. A) Graphs show numbers of transferred TCRδ-GFP$^+$ cells recovered from the LN or ear, shown as +/- SEM. B) Representative flow plot shows the CCR6 and CD27 profile of the donor γδ T cell population prior to transfer. C) Representative flow plots showing the proportion of donor (CD45.2) to host (CD45.1) in the skin dLN and spleen 1 day post transfer. D) Representative flow plots show CCR6 and CD103 profile of donor and recipient dermal cells at 7dpi.

The donor population accurately reflected the typical makeup of circulating γδ T cell proportions (Figure 4.2B) and made up 5% of the total γδ T cell population in the spleen and LNs before infection (Figure 4.2C). Transfer experiments revealed that the donor γδ T cells contributed to all three dermal γδ T cell populations defined by CD103 and CCR6 staining (Figure 4.2D), but it was not clear how these populations related to those in the circulation.

Our initial attempts to stain for CD27 on γδ T cells isolated from the ear were negative. Further analysis revealed that CD27 was cleaved during the digestion process used to isolate cells from the ear (Figure 4.3A). Floating ears (dorsal and ventral sides separated) overnight in complete media without enzymatic digestion released some γδ T cells from the tissue and permitted analysis of CD27 expression (Figure 4.3B). Although $CD27^+$ γδ T cells predominate in the circulation, none were found in naïve dermal tissue.

Using this technique with infected samples revealed that although CD27 was not found on dermal γδ T cells in naïve animals, it was expressed on some dermal γδ T cells following infection, all of which belonged to the $CD103^-CCR6^-$ DN population (Figure 4.3C). This suggested that the $CD103^-CCR6^-$ DN subset appearing in infected ears was an immigrant population of circulating $CD27^+$ γδ T cells. Because yields and subset composition from the floating technique were variable, we opted to use CD103 and CCR6 for further analyses of skin samples.

To ask whether $CD27^+$ or $CD27^-$ γδ T cells from the circulating pool differentially contributed to the three dermal γδ T cell subsets, we sorted and transferred them into separate hosts before ear scarification with VV. The $CD27^-$ donor γδ T cells contributed

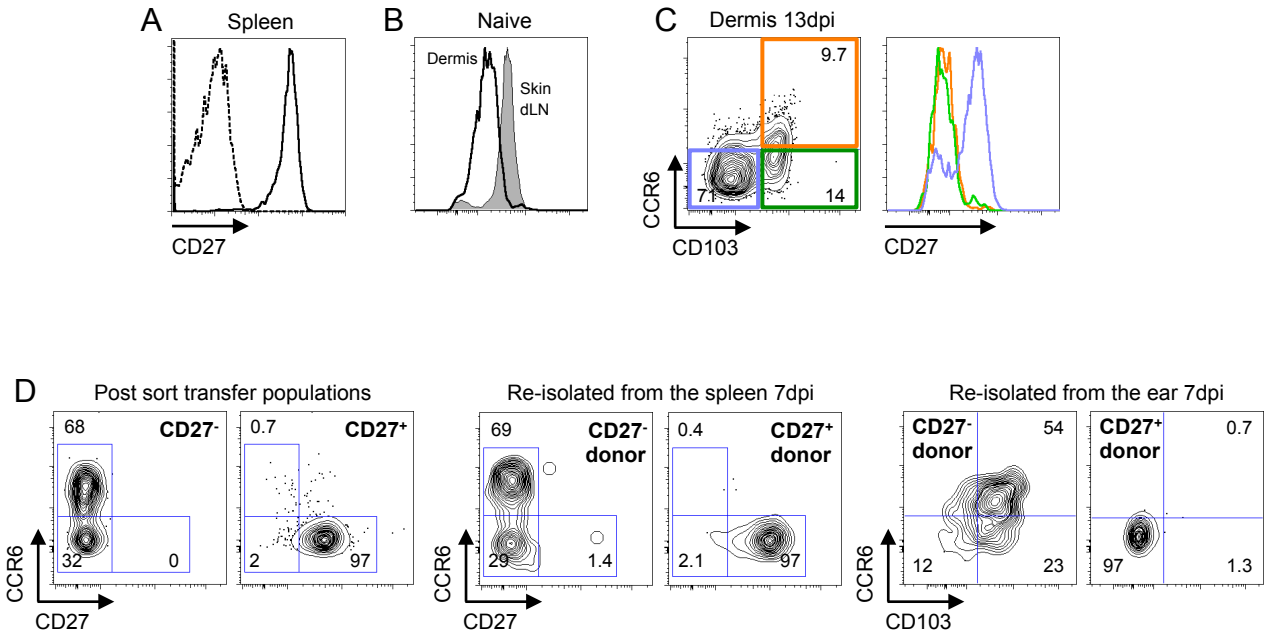

Figure 4.3. CD27^{+} γδ T cells are only found in inflamed skin and contribute specifically to the CD103^{-} dermal population. A) Representative flow plot of CD27 expression on γδ T cells from spleen samples treated with liberase (dashed line) or left untreated (solid line). B) To avoid liberase digestion of skin, naïve ears were floated in media overnight. Representative flow plot of CD27 expression on dermal γδ T cells (black line) released from the tissue by floating and skin dLN (grey filled). C) CD27 staining on dermal γδ T cells floated from 13dpi infected ears without enzymatic treatment. D) γδ T cells from spleen and skin dLN of TCRδ-GFP mice were sorted based on CD27 expression and 6x10^{5} CD27^{+} or 2.7x10^{5} CD27^{-} γδ T cells were transferred into separate C57BL/6 mice. Recipients were infected with VV on the ear the following day. Flow plots show the phenotype of CD27^{-} and CD27^{+} at the time of transfer and 7dpi in the spleen and ear. Numbers denote the percentage of cells within the indicated quadrant. Data are representative of at least two independent experiments, n=3-7 mice per group.

to all three dermal populations defined by CD103 and CCR6, although few were DN and most expressed CD103 (Figure 4.3D). In contrast, immigrant γδ T cells from the $CD27^+$ donor population were exclusively $CD103^-CCR6^-$ DN, even though 50% of cells in the donor population expressed CD103. On a per cell basis, the $CD27^-$ γδ T cells were recruited more efficiently to the infected skin.

γδ T cells show limited proliferation following infection

In addition to recruitment, we wanted to know whether the changes we saw in the number of γδ T cells could be do to proliferation. To do this we took several approaches commonly used to assess cell proliferation. Ki67 is a protein strictly expressed in all cells undergoing division (68), therefore we stained the dLN and skin at day 3 post infection when the cell numbers start to increase. Although staining was above background (determined by an isotope control antibody), there was no difference between naïve and infected samples in any of the populations by percent in the skin dLN or dermis (Figure 4.4A & B). The total number of γδ T cells is higher at day 3 and so there might be slightly more total $Ki67^+$ cells in the infected samples, but the vast majority of cells are not undergoing active proliferation by this method of detection at this time point when we see γδ T cell accumulation in these tissues.

We next assessed proliferation with Bromodeoxyuridine (Brdu), which is incorporated into the DNA of replicating cells. Depending on the length of time, delivery method and amount of Brdu in the animal, a window of proliferation is captured. We chose to inject mice on day 3 or day 5 post infection with Brdu 1 hour before tissues were harvested to determine how much proliferation is occurring over a short period of

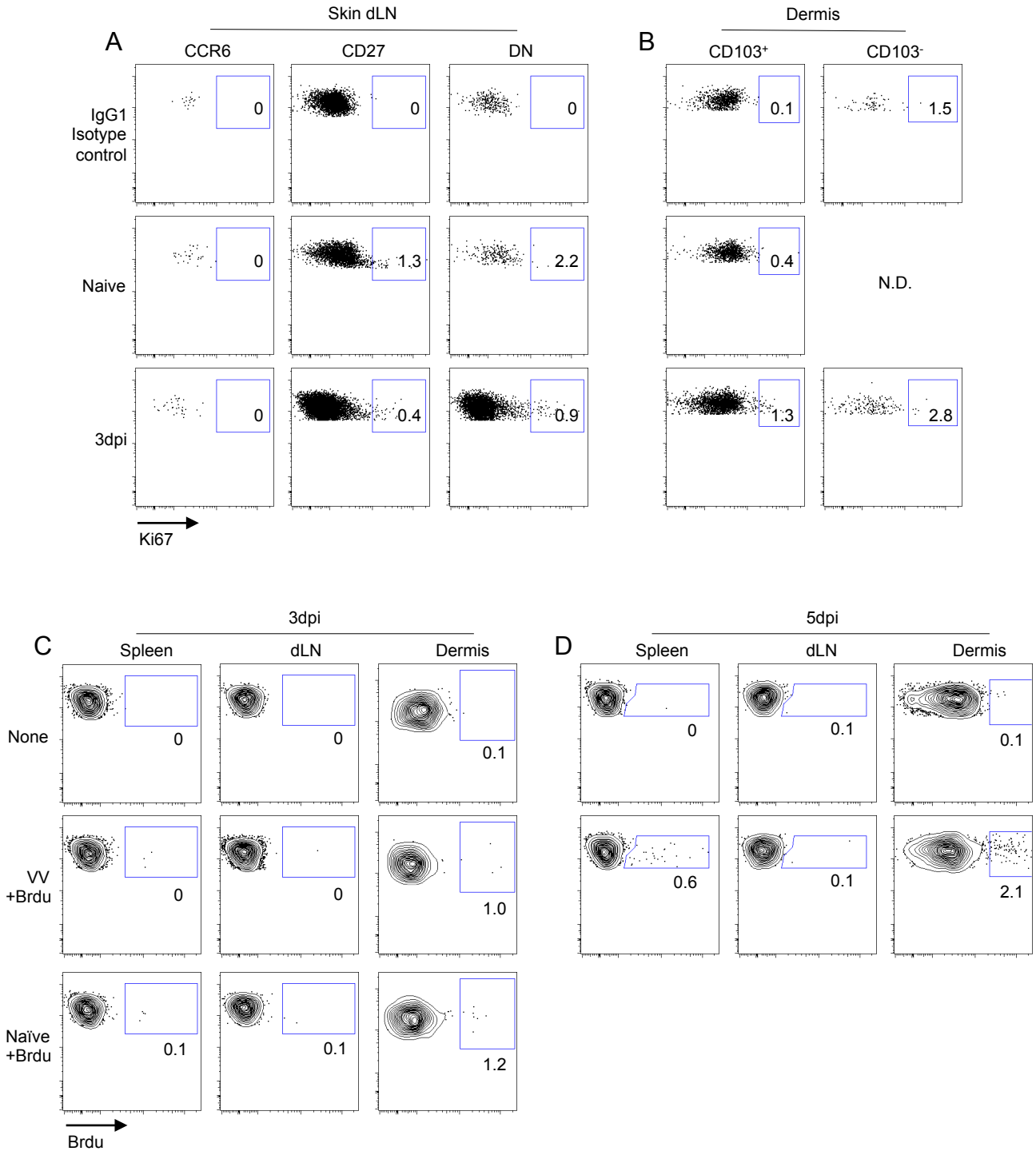

Figure 4.4. Low level of γδ T cell proliferation in situ following VV infection. Representative flow plots of Ki67 expression in γδ T cells in the A) dLN (or skin dLN for naïve) and B) dermis of naïve and 3 day infected mice (N.D. = no data). γδ T cell Brdu incorporation after 1 hour on C) day 3 and D) day 5 post infection in the spleen, skin dLN and dermis. Numbers indicate percent of total population.

time and exactly in what location. These results confirmed what we saw with Ki67 that very little proliferation is happening at day 3 (Figure 4.4C), however by day 5 Brdu incorporation could be seen in the dermis and to a lesser extent the spleen (Figure 4.4D). Proliferation in the dermis over a short period of time suggests that dermal γδ T cells are expanding within the tissue.

The last method we used is one commonly applied to antigen-specific αβ T cells whereby cells are isolated, stained with a dye (CTV used here) that enters the cytoplasm of cells and transferred into a new host. The most brightly stained cells are undivided and at each cell division the dye is halved, resulting in a series of fluorescent peaks. By day 7 post infection, very few cells had diluted the CTV (Figure 4.5). This further indicates that very little proliferation among circulating γδ T cells has occurred in response to infection by the peak of the response. Together this data supports the notion that a fraction of dermal γδ T cells proliferate and the majority of γδ T cells in circulation migrate to the site of infection and dLN.

Adult BM- and pThy-derived γδ T cells occupy discrete niches

We have established that BMpThy chimeras allow us to identify circulating (BM-derived) and resident dermal (pThy-derived) γδ T cells by taking advantage of when each population exists the thymus. Using BMpThy chimeras, we confirmed that, following VV infection, adult BM-derived γδ T cells (largely $CD27^+$ in the circulation) were transiently recruited from the circulation and contributed primarily to the $CCR6^-$ $CD103^-$ DN dermal subset (Figure 4.6A & B).

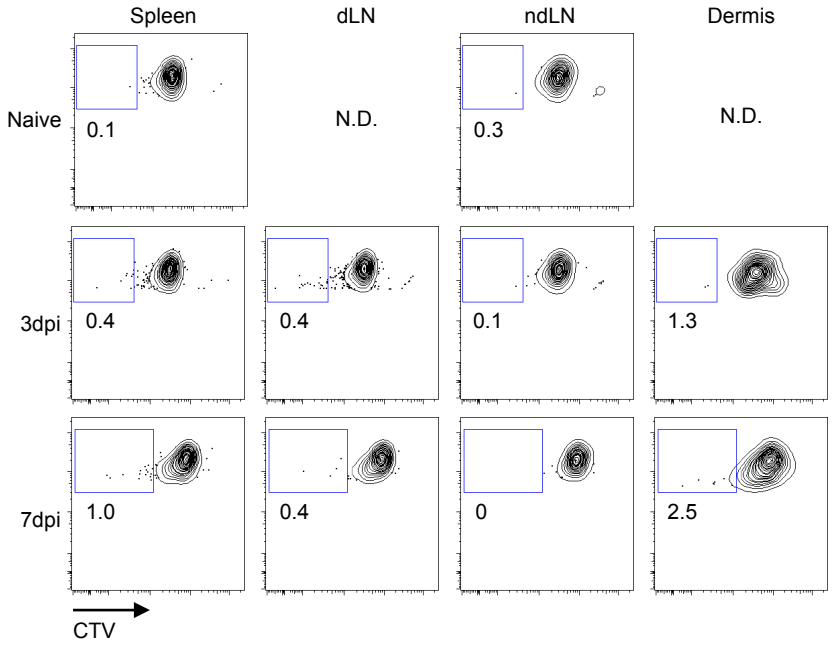

Figure 4.5. Low level of transferred γδ T cell proliferation and accumulation in vivo following VV infection. Representative flow plots of CTV dilution in γδ T cells in the spleen, dLN, ndLN and dermis. Cells were harvested from skin dLN and spleen of TCRδ-GFP mice, labeled with CTV and transferred one day before infection. Gated on live cells. Numbers indicate percent of total population.

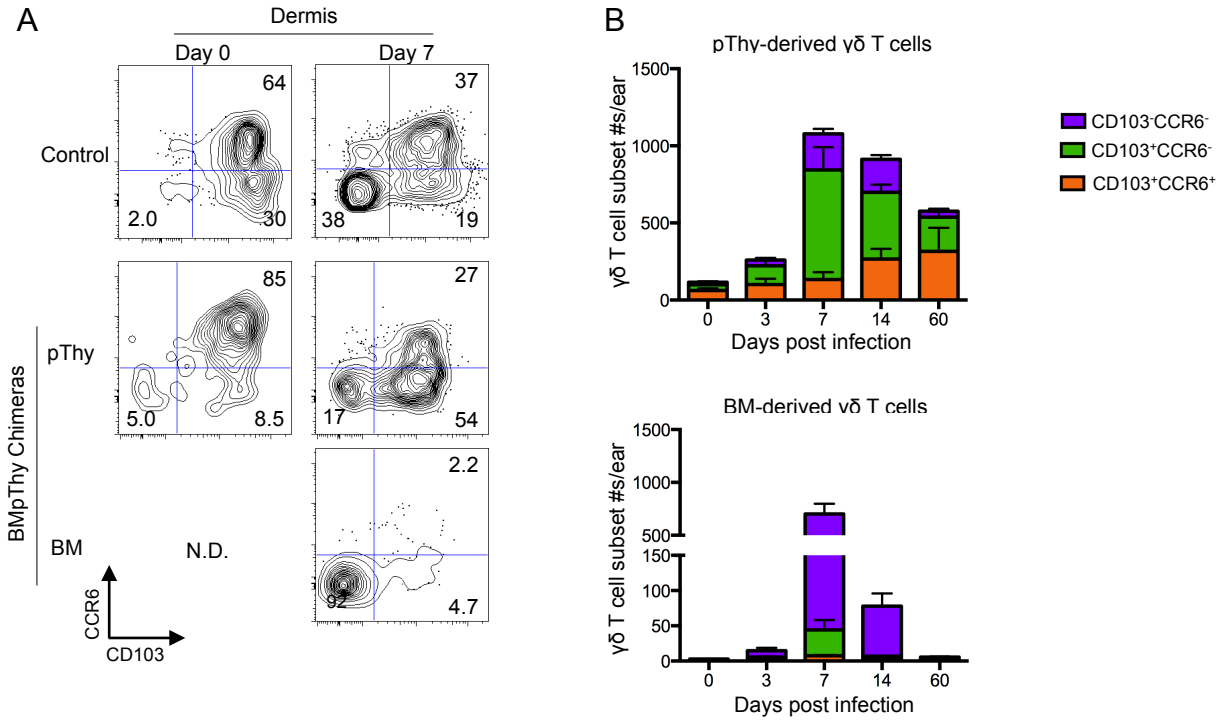

Figure 4.6. Contribution of adult BM-derived and perinatal thymus-derived γδ T cells to the dermal response. CD45.2$^+$ TCRδ-GFP mice were irradiated and reconstituted with BM (BM only) or with BM plus pThy (BMpThy). BMpThy chimeras were infected with VV on the ear > 8 weeks following reconstitution. A) CCR6 and CD103 profiles of γδ T cells in the dermis at day 0 and 7dpi (N.D. = not done). Numbers denote the percentage of that population within the indicated quadrant. B) The number of BM- and pThy-derived γδ T cells contributing to subsets in the dermis following infection. Error bars signify SEM. Statistics performed with the two-tailed, unpaired student *t*-test, *p<0.02, **p<0.0001, NS = not significant. Data show results >2 independent experiments, n=3-15 mice per group.

In contrast, resident dermal pThy-derived γδ T cells contributed mainly to the two $CD103^+$ subsets in the dermis and their expansion during infection. As early as day 3 following infection, pThy-derived γδ T cells had expanded in the dermis and their numbers remained elevated for a protracted period of time (Figure 4.6B). In contrast, the number of the BM-derived γδ T cells peaked at 7 days post infection and then declined dramatically. Importantly, at a late time point the BM-derived γδ T cells were absent from the ear while the pThy-derived γδ T cells plateaued at a level similar to wild type (WT) mice following infection. This suggests that while immigrant γδ T cells are not able to join the resident dermal population, the existing dermal population may expand and be permanently altered following infection.

Discussion

A challenge to studying γδ T cells is that their numbers are very small in the circulation of mice. To get around this, several groups have used $TCR\beta^{-/-}$ mice so that the only T cells in the mouse express the γδTCR (60, 61, 65). However, this only forces the thymus to create a full T cell repertoire with different genes and does not simply remove one cell type. It has been shown that doing so alters the γδ T cell makeup and their response to infection is likely abnormal (66). We chose to perform all experiments analyzing γδ T cells from WT mice, including all transfers experiments. In this way γδ T cells developed under normal constraints in a full compartment.

The fact that γδ T cells are found more readily in tissues, however, led us to use a model that was perhaps more relevant to their function. VV is used as a vaccine for smallpox as it is a less severe form of the disease. Administration of the vaccine is done

by skin scarification with a bifurcated needle, which is the virus' natural mode of transmission (69, 70). Although one study looked at the γδ T cells response to VV (61), it was following a very high done via the intraperitoneal route of infection, which does not accurately portray viral spread or cell trafficking.

Following scarification with VV, we found that γδ T cells accumulate in the dermis of the infected ear and the corresponding dLN. DETC also expanded to some extent and this is the first time, to our knowledge, of documentation of their response to viral infection of the skin. In addition to the expansion of the resident $CD103^+$ populations, we noticed the presence of a novel $CD103^-$ population that appeared within a few days of infection and peaked around day 7. Although it constituted half of the γδ T cells in the dermis at the peak, this population was not found at a memory time-point. Interestingly, the total number of dermal γδ T cells remained elevated for a prolonged period of time before declining. At a memory time-point there were significantly more dermal γδ T cells, suggesting that infection permanently altered the population. Our limited understanding of the adaptive properties of γδ T cells makes it difficult assess whether this is a form of 'memory', but the change in total number suggests some capacity for modification in response to a pathogen.

Considering the appearance of a new population we asked whether any of the three populations in the inflamed ear were derived from the circulation. Transferring γδ T cells first revealed that the dermal population is independently maintained from those in the circulation as they did not enter the dermis of naïve mice, which has been demonstrated previously in the gut (8). Importantly, following infection the transferred population specifically entered the infected ear. It was interesting to find that the

transferred γδ T cells that entered the dermis had a similar phenotype to the total host population, supporting the notion that a large number of the γδ T cells in the dermis during infection have been recruited. Further supporting this, we tested several methods of assessing proliferation and found that overall γδ T cells did not readily undergo cell division. The most we were able to observe was by Brdu, which revealed local proliferation of dermal γδ T cells within the tissue.

Finally, we used BMpThy to demonstrate different roles for the circulating vs. resident dermal γδ T cells during infection. pThy-derived γδ T cells, which dominate in the dermis, expand and contribute largely to the $CD103^+$ population. BM-derived γδ T cells expand in the circulation and enter the dermis, peaking at day 7 post infection. Confirming results obtained with the transfer experiments, largely $CD27^+$ BM-derived γδ T cells made up the DN dermal population.

Using an infectious model to investigate γδ T cell population dynamics uncovered previously unappreciated ways of looking at γδ T cells in the dermis and circulation. We originally thought that CD103 clearly distinguished between resident and immigrant γδ T cells in the dermis during an inflammatory state. Further experiments revealed that it was not so straightforward because circulating γδ T cells were able to contribute to all three populations – while all $CD103^-$ γδ T cells are immigrants, not all $CD103^+$ are resident. Another way of looking at these populations is by CD27 expression. $CD27^+$ γδ T cells exclusively contribute to the $CD103^-$ population, however while the majority of $CD27^-$ γδ T cells were $CD103^-$ in the dermis, a few were found in the $CD103^-$ compartment. Considering these cells are all CCR6 we conclude that these few $CD27^-$ $CD103^-$ γδ T cells, which are capable of producing IL-17, came from the circulating

CCR6$^-$CD27$^-$ population. Lastly, a wave of γδ T cells exists the thymus near the time of birth that become CCR6$^+$CD27$^-$ or CCR6$^-$CD27$^-$ in the circulation and fill the dermal compartment. It seems that although the circulating population does not enter the skin during the steady state, these cells are rapidly recruited to the skin following infection and for the most part resemble resident dermal γδ T cells – a subset they likely emigrated into the periphery with.

Considering how inflammation impacts the γδ T cell network in the dermis, we have created new terminology that temporarily applies to dermal γδ T cells during this state. Since CD103 does delineate the appearance of a novel population, CD103$^-$ designates “inflammatory recruited γδ T cells” and CD103$^+$ designates “inflammatory resident γδ T cells”. The inflammatory recruited population is entirely made up of γδ T cells that have come from the circulation and will not remain long term. All of them are CCR6$^-$ and the majority is CD27$^+$. This population can be derived from the BM although some may be restricted to a pThy wave. Within the inflammatory resident population are a mixture of permanent resident dermal γδ T cells and some that have been recruited from the circulation. All are CD27$^-$, are derived from the pThy wave and approximately half express CCR6. Transfer studies support the notion that even these recruited cells will not stay long term, but the original dermal γδ T cell population may expand and leave behind some of those generated during infection.

Chapter 5:

Function and role of inflammatory resident and recruited γδ T cells

Introduction

The most well defined function of γδ T cells is their ability to rapidly produce cytokines during the early stages of infection, before the initiation of the αβ T cell response (3). A portion of them, now known to be $CD27^-$, produce IL-17 (28, 30, 33, 71, 72) while the majority, which express CD27, are IFNγ-producers (73). Because of their ambiguous nature, many approaches have been taken to determine the role of γδ T cells. As a result they appear to be a jack-of-all-trades, capable of performing a wide variety of functions. Previous studies attribute them with the ability to perform antigen presentation (74-79), enhance $CD8^+$ T cell priming via dendritic cell maturation (80), boost $CD8^+$ T cell memory formation (81), recruit neutrophils (28, 32, 59, 71, 82) and many other roles that are critical to the anti-viral response. We therefore sought to determine whether γδ T cells were performing any of these or other functions during VV infection.

Considering the close relationship between γδ T cell development, localization and phenotype, we also wanted to determine whether populations derived at different periods throughout life had distinct functions during infection. BMpThy chimeras previously revealed that BM- and pThy-derived γδ T cells occupy distinct niches in the circulation and dermis. Further, they uniquely contribute to the different inflammatory dermal populations, making it an ideal system to assess the relationship between development and function in an infectious setting. This system also allows us to further

probe whether the inflammatory resident or recruited populations serve unique functions in the anti-viral response.

Results

Loss of γδ T cells does not impact inflammatory cytokine production in the tissue

In order to assess whether the presence of γδ T cells in the tissue affects the cytokine milieu, we used a CBA kit to measure the levels of six cytokines associated with inflammation: IL-6, IL-10, MCP-1, IFNγ, TNF and IL-12p70. C57BL/6 or TCRδ$^{-/-}$ mice were infected with VV scarification on the ear and tissues were harvested at 24 hour intervals through day 4. At various time points following infection, tissue from the TCRδ$^{-/-}$ mice had higher levels of several cytokines, namely MCP-I, TNF and IL-12p70; however these differences were not significant (Figure 5.1).

γδ T cells do not significantly impact the antigen specific CD8^{+} T cell response or resolution of infection

Since it has been suggested that γδ T cells may directly or indirectly impact CD8^{+} T cells during infection (80, 81), we assessed the total number of antigen specific CD8^{+} T cells at the peak of the response. To do this we transferred OT-I cells, which are specific for OVA (SIINFEKL peptide), into WT and TCR$^{-/-}$ mice and infected them with VV expressing OVA. We found no difference in the number of OT-I in the infected ear 7 days post infection (Figure 5.2A).

We took two approaches to determine how the loss of γδ T cells would impact viral load and we also wanted to confirm that scarification did indeed produce a

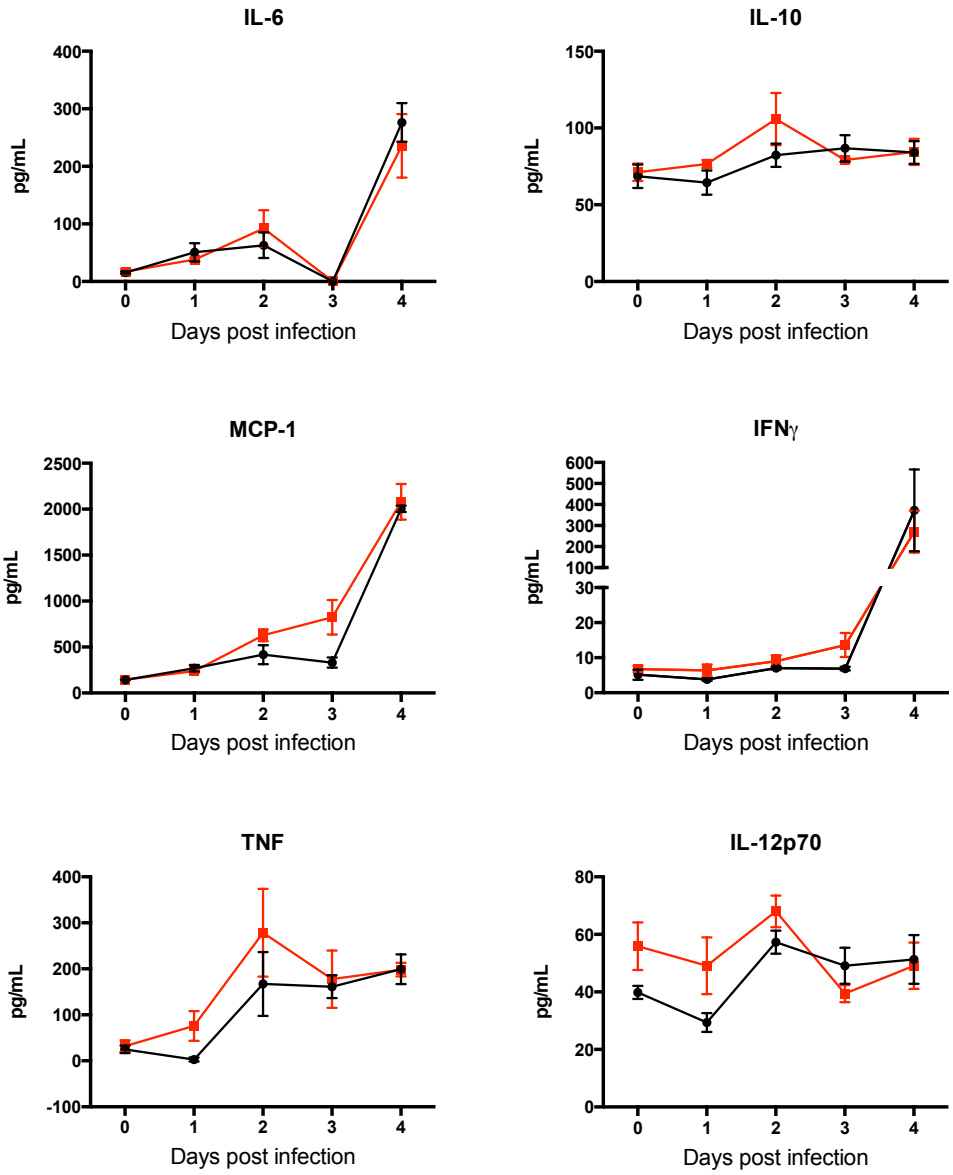

Figure 5.1. Cytokine production in the ear following VV scarification. WT (black) and TCRδ$^{-/-}$ (red) mice were sacrificed at the indicated time points following infection and ears were processed then stained with a CBA kit to determine levels of each cytokine. SEM is shown. N=3 mice per group.

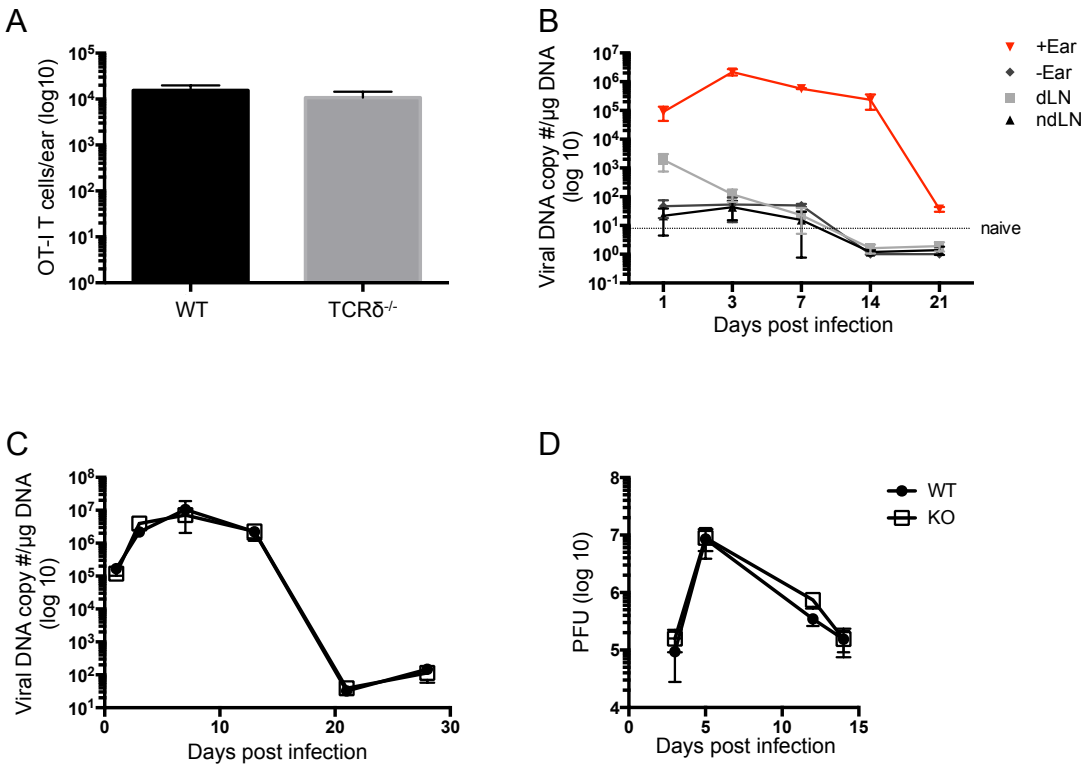

Figure 5.2. Loss of γδ T cells does not impact antigen specific CD8 T cell response or viral load. A) Number of OT-I T cells in the ear of WT (black) and TCRδ$^{-/-}$ (grey) mice at 7 days post infection. B) VV infected ear (+Ear), corresponding dLN, contralateral control ear (-Ear) and corresponding ndLN were harvested at the indicated time points following infection and analyzed by qPCR for viral DNA. C and D) VV infected ears from WT (solid circle) and TCRδ$^{-/-}$ (open square) mice were analyzed by C) qPCR and D) plaque assay to determine viral titer at the site of infection. N=3-6 mice per group.

localized infection with limited dissemination. WT mice were scarified with VV and the infected ear, contralateral control ear and their corresponding dLNs were harvested and analyzed by qPCR. Confirming previous reports, the majority of viral DNA was found in the infected ear with perhaps a small amount in the dLN and almost none in the contralateral tissues (Figure 5.2B).

We next compared VV DNA levels in the infected ears of WT and $TCR^{-/-}$ mice and found no difference at any time point (Figure 5.2C). Since viral DNA remained for a long period of time after live virus is presumably cleared from the mouse, we turned to plaque assay for more sensitive analysis. Using this method we again found no viral spread to the dLN or other tissues (data not shown) in WT or $TCR\delta^{-/-}$ mice and no difference in the viral load over the course of infection (Figure 5.2D). Therefore we concluded that γδ T cells are not necessary for VV containment or clearance.

Inflammatory resident and recruited γδ T cells are functionally distinct

Several groups have established that dermal γδ T cells are fated to produce IL-17 (28, 33, 72). We found that a fraction of dermal γδ T cells in uninfected ears produced this cytokine following *in vitro* stimulation with PMA/Ionomycin (Figure 5.3A). On day 3 after VV scarification, $CD103^{+}$ γδ T cells in the dermis were capable of producing IL-17 at a level similar to that of naïve tissue. Although they represented a minority at day 3, it was surprising that $CD103^{-}$ γδ T cells also produce IL-17 in the dermis. Using the float method in order to preserve CD27 expression, we found that all the IL-17 production was by $CD27^{-}$ γδ T cells (Figure 5.3B), which we predict are derived from the circulating $CCR6^{-}CD27^{-}$ population.

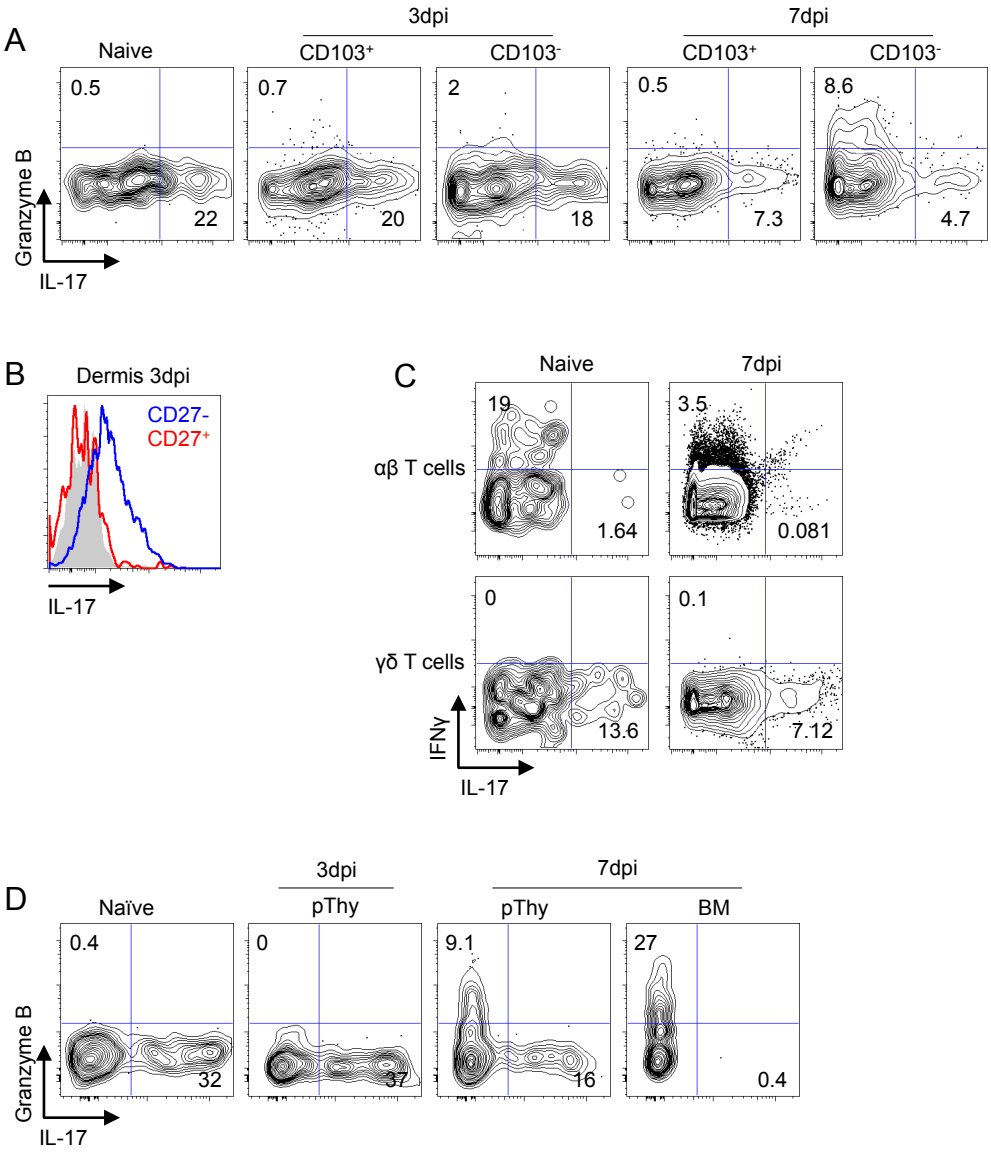

Figure 5.3. Adult BM-derived and pThy-derived γδ T cells in the dermis are functionally distinct. A) TCRδ-GFP mice were infected with VV on the ear and sacrificed on days 3 and 7. Ears were digested in Brefeldin A and cells were re-stimulated with PMA/Ionomycin for 4 hours at 37°C followed by intracellular staining for cytokines and Granzyme B. Representative flow plots are shown and gates are based on unstimulated naïve samples. Numbers indicate the percentage of that population in each quadrant. Data are representative of at >2 independent experiments. B) Cytokine staining of dermal γδ T cells isolated from day 3 infected ears by flotation in complete medium. IL-17 production by $CD27^+$ (blue) and $CD27^-$ (red) γδ T cells. Gated on $CD103^-$ dermal γδ T cells. Gray histogram is unstimulated naïve control. C) Cytokine staining for IL-17 and IFNγ in αβ versus γδ T cells in the dermis of TCRδ-GFP mice 7dpi. D) BMpThy were infected and analyzed as in A).

Notably, there were no Granzyme B^+ γδ T cells in the naïve dermis at day 3 post infection, but by day 7 (which is the peak for the immigrant population), the $CD103^-$ γδ T cells produced Granzyme B (Figure 5.3A). Although $CD27^+$ γδ T cells from the naïve circulation, which make up a majority of the $CD103^-$ population in the dermis, are readily capable of producing IFNγ, none of the γδ T cells in the dermis stained positive for IFNγ at any time following VV scarification (Figure 5.3C).

We repeated these intracellular staining experiments with BMpThy chimeras to determine whether the BM- and pThy-derived populations had distinct functions. At day 3 post infection, there were too few BM-derived cells to be analyzed, but the pThy-derived γδ T cells readily produced IL-17 (Figure 5.3D). By day 7 post infection, a clear distinction between the two populations could be seen, demonstrating that in this infection model, the function of adult BM-derived γδ T cells from the circulation was to make Granzyme B while the dermal pThy-derived γδ T cells gave rise to a population capable of making either IL-17 or, to a lesser extent, Granzyme B.

Resident dermal γδ T cells enhance the early immune response to infection

To determine whether γδ T cells contributed to inflammation, we assessed tissue pathology in wild type (WT) and $TCR\delta^{-/-}$ mice, which lack all γδ T cells. By 3 days post infection, WT ears had evidence of both epidermal and cartilage necrosis, often with entire sections of the epidermis replaced by serocellular crusts (Figure 5.4A). The dermis of these mice predominantly contained accumulation of intact and degenerate neutrophils along with some macrophages and fewer lymphocytes. In contrast, there was less infiltration of neutrophils and macrophages in the ears of $TCR\delta^{-/-}$ mice and the

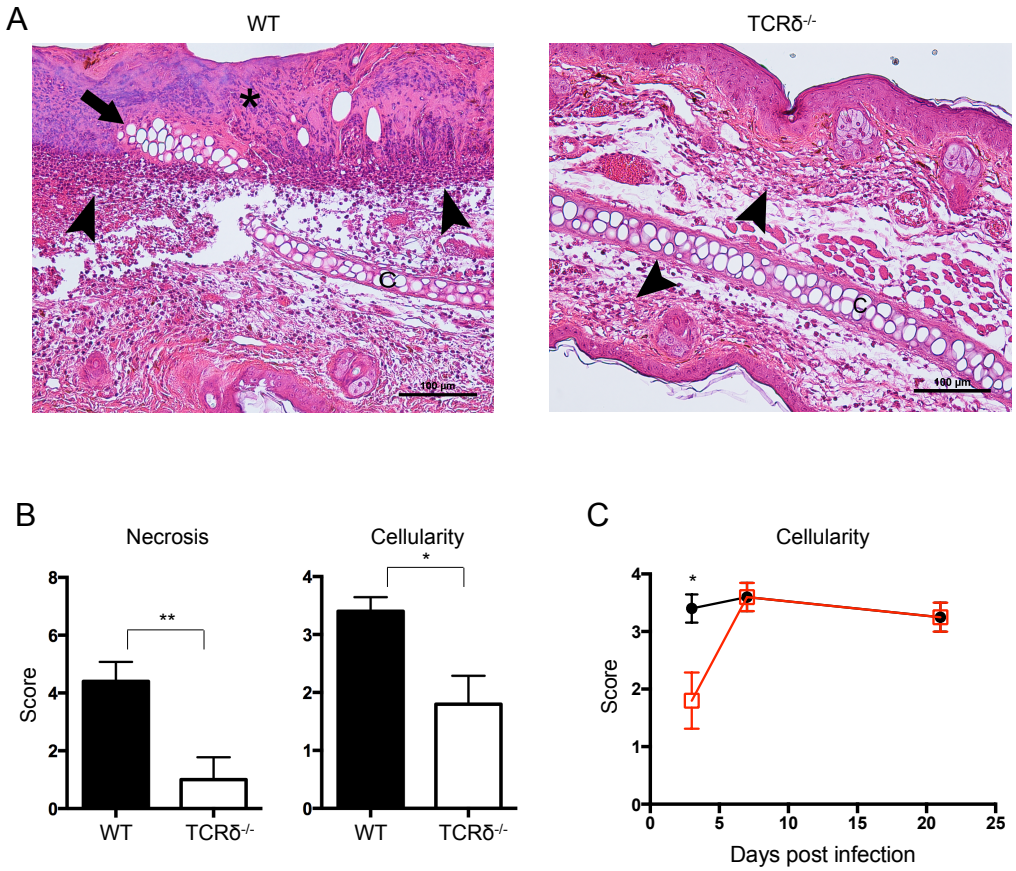

Figure 5.4. Absence of γδ T cells reduces tissue pathology during VV infection. Mice were infected with VV on the ear. A) Representative histological sections of ears from C57BL/6 WT and TCRδ$^{-/-}$ mice 3dpi at 40x magnification. Arrows indicate necrotic cartilage, arrowheads indicate neutrophilic infiltrates and asterisks indicate serocellular crusts. B) Graphs show scores for necrosis (epidermis and cartilage scored separately and then combined) and cellularity. Mean +/- SEM is shown. C) Graph of cellularity score over time for WT (black) and TCRδ$^{-/-}$ (red). Statistics performed with the two-tailed, unpaired student *t*-test, n=5-6 mice per group. *p<0.05, **p<0.01, ***p<0.005 Data are representative of >1 independent experiments, n=3-6 mice per group (C) or >2 (A, B) independent experiments, n=5-6 mice per group.

cartilage and epidermis remained largely intact. Comparing the histological analysis for necrosis (combined for epidermis and cartilage) and cellularity, WT mice scored significantly higher than TCRδ$^{-/-}$ mice (Figure 5.4B). By day 7 post infection and for later time points both groups had similar scores for cellularity (Figure 5.4C) and the same pattern was seen for necrosis (data not shown).

We next compared the histologic changes of BMpThy chimeras and BM chimeras to determine whether the differences between WT and TCRδ$^{-/-}$ mice could be assigned to the presence or absence of resident dermal γδ T cells. By and large, the BMpThy chimeras recreated the picture seen in WT mice with high scores for necrosis and cellularity (Figure 5.5A & B). However, the BM chimeras had mild neutrophil infiltration, little to no cartilage necrosis and rare epidermal necrosis. It was striking to see that replacement of dermal γδ T cells in BMpThy mice recapitulated results obtained with the WT mice and similarly, that BM mice mirrored γδ T cell null mice (Figure 5.5C). Thus, the loss of the dermal CD103^{+} γδ T cell population appears sufficient to reduce ear inflammation and damage.

Discussion

γδ T cells have been proposed to play a variety of roles in the immune response to infection in mice and primates. Studies have largely relied on systemic models of infection and generally observed an increase in the number of γδ T cells in the circulation. A few studies have analyzed γδ T cells in the lung (73, 83), intestine (84) and genital mucosa (85) where local accumulation of γδ T cells occurs. However, many

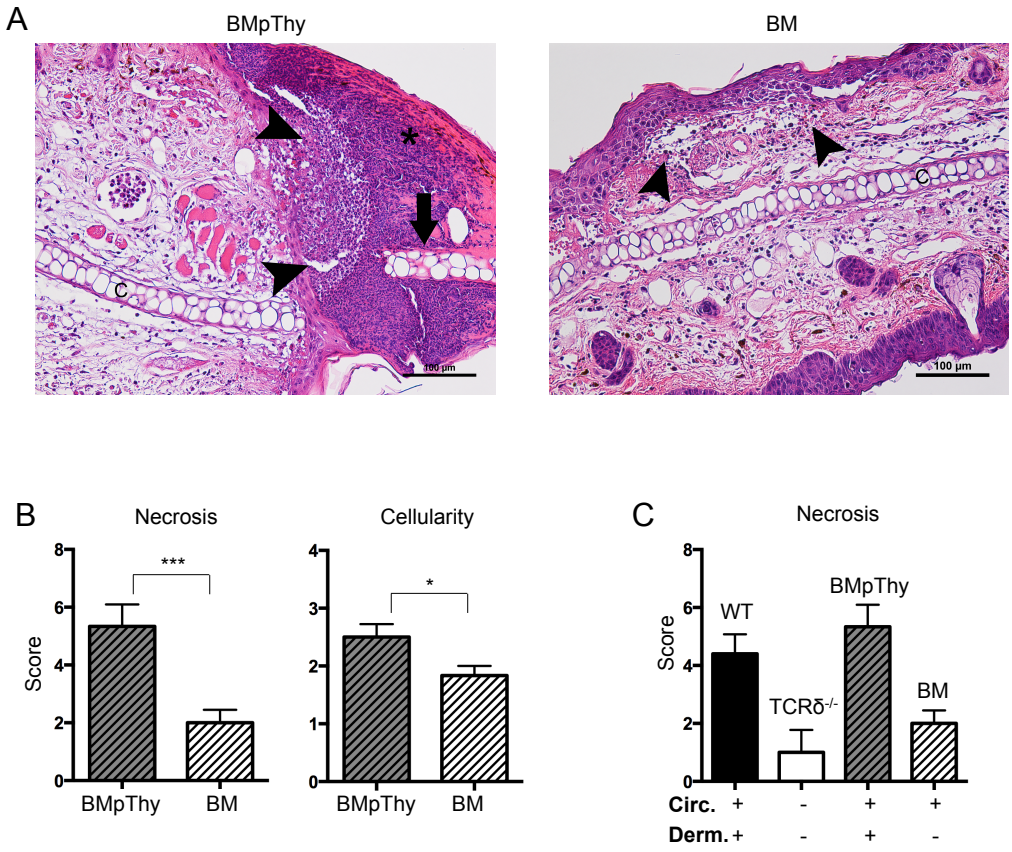

Figure 5.5. Dermal γδ T cells accelerate early collateral damage and contribute to increased tissue cellularity during VV infection. Mice were infected with VV on the ear. A) Representative histological sections and B) scores for BM chimeras and BMpThy chimeras 3dpi. Mean +/- SEM is shown. C) Comparison of necrosis scores for WT, TCRδ$^{-/-}$, BMpThy chimeras and BM chimeras in relation to the presence (+) or absence (-) of γδ T cell populations (Circ = circulating γδ T cells, Derm = dermal γδ T cells). Arrows indicate necrotic cartilage, arrowheads indicate neutrophilic infiltrates and asterisks indicate serocellular crusts. Statistics performed with the two-tailed, unpaired student *t*-test, n=5-6 mice per group. *p<0.05, **p<0.01, ***p<0.005 Data are representative of >2 independent experiments, n=5-6 mice per group.

of these studies use systems that favor γδ T cells to some extent, which inaccurately portrays their role in the immune system.

For infectious models it is often the primary goal to show increased morbidity or mortality in the mice, which lack a certain cell type or effector molecule. This has unfortunately led to studies using extremely high doses of the pathogen in order to see differences in TCRδ$^{-/-}$ mice compared to WT (61, 65, 86). In addition to the fact that we did not see a difference in viral load with VV, our attempts to repeat previously published data in other models was not successful. Indeed we did not observe significant differences in infectious load or mortality in models of West Nile Virus or *Yersinia pseudotuberculosis* (data not shown). In addition, we also found no significant difference in the antigen specific CD8^{+} T cell response between WT and TCRδ$^{-/-}$ mice following VV scarification or in other models of *Listeria monocytogenes* and Vesicular Stomatitis Virus infection (data not shown). Further, other experiments not included here determined that antigen presentation was not hindered in mice lacking γδ T cells.

Characterization of the different γδ T cell populations in the inflamed dermis confirmed the IL-17-producing capacity of CD103^{+} dermal γδ T cells, even in naïve tissue. A small population of the CD103^{-} γδ T cells also appeared to be capable of producing IL-17 and this was determined to be from CD27^{-} immigrants, which we predict originated from the circulating CD27^{-}CCR6^{-} population. Interestingly Granzyme B was only produced by the inflammatory recruited γδ T cells and not until the peak of the response.

It was surprising however that IFNγ was not expressed at any point by γδ T cells in the dermis. This was not due to technical difficulties in staining for IFNγ as we were

able to see a positive population among the αβ T cells. This suggests that perhaps a subset within the $CD27^+$ γδ T cell population that does not possess IFNγ-producing capabilities is specifically recruited to the tissue. Alternatively they lose that ability in response to inflammatory signals upon entering the tissue. In either case, by the parameters assessed here, a large portion of the inflammatory recruited γδ T cells have an unknown function the remains to be determined.

Since γδ T cells appeared to be a dynamic population capable of acquiring functions in response to infection, but their loss did not impact the cytotoxic T cell response or viral titers, we took a different approach to assess what purpose they could have. Histologic analysis offered qualitative and quantitative data at the site of infection that revealed an overall more quiet state in mice that lacked all γδ T cells. More specifically, it turned out to be the dermal γδ T cell population that was primarily responsible for the increased cellularity and necrosis. Other studies have shown that γδ T cell production of IL-17 early on contributes to neutrophil-mediated tissue damage (87) as well as enhanced inflammation (88), indicating they are active participants in the inflammatory response even if they do not play a central role. These experiments reveal that the inflammatory immigrant γδ T cells have functional attributes distinct from the inflammatory resident dermal γδ T cells, the latter being primarily responsible for enhancing the inflammatory response in the tissue.

Chapter 6:

Concluding remarks and future directions

Scarification of the skin with VV was used as a vaccine to eradicate small pox infection and the virus is now appreciated as an important vector for vaccine development and potentially cancer treatment (70). We have employed VV scarification of mouse ears to interrogate the distinct roles of recruited and resident dermal γδ T cells and to better understand their diversity. In this study, we describe for the first time the expansion, recruitment and contraction of γδ T cells in the dermis following localized VV infection. About half of the expanded dermal γδ T cells at day 7 were comprised of a unique population of $CD103^{-}CCR6^{-}$ γδ T cells that rapidly appeared in the dermis in response to infection (Figure 6.1). This novel population was derived primarily from circulating $CD27^{+}$ γδ T cells. A portion of the immigrant γδ T cells were phenotypically similar to the $CD103^{+}$ IL-17 producing dermal-resident γδ T cell population and derived from circulating $CD27^{-}$ γδ T cells, although some were also found in the $CD103^{-}$ fraction. These immigrant populations were functionally distinct and played a discrete role from the resident dermal population, which was ultimately responsible for driving increased cellularity and tissue damage at an early time-point.

The number of dermal γδ T cells increased approximately 10-fold following VV infection and our data suggests that most of this increase can be ascribed to recruitment and immigration rather than expansion of the resident population. Thus $7x10^{5}$ adoptively transferred γδ T cells made up 5% of the total γδ T cell population in the spleen and LNs before infection and this donor:host γδ T cells ratio was maintained

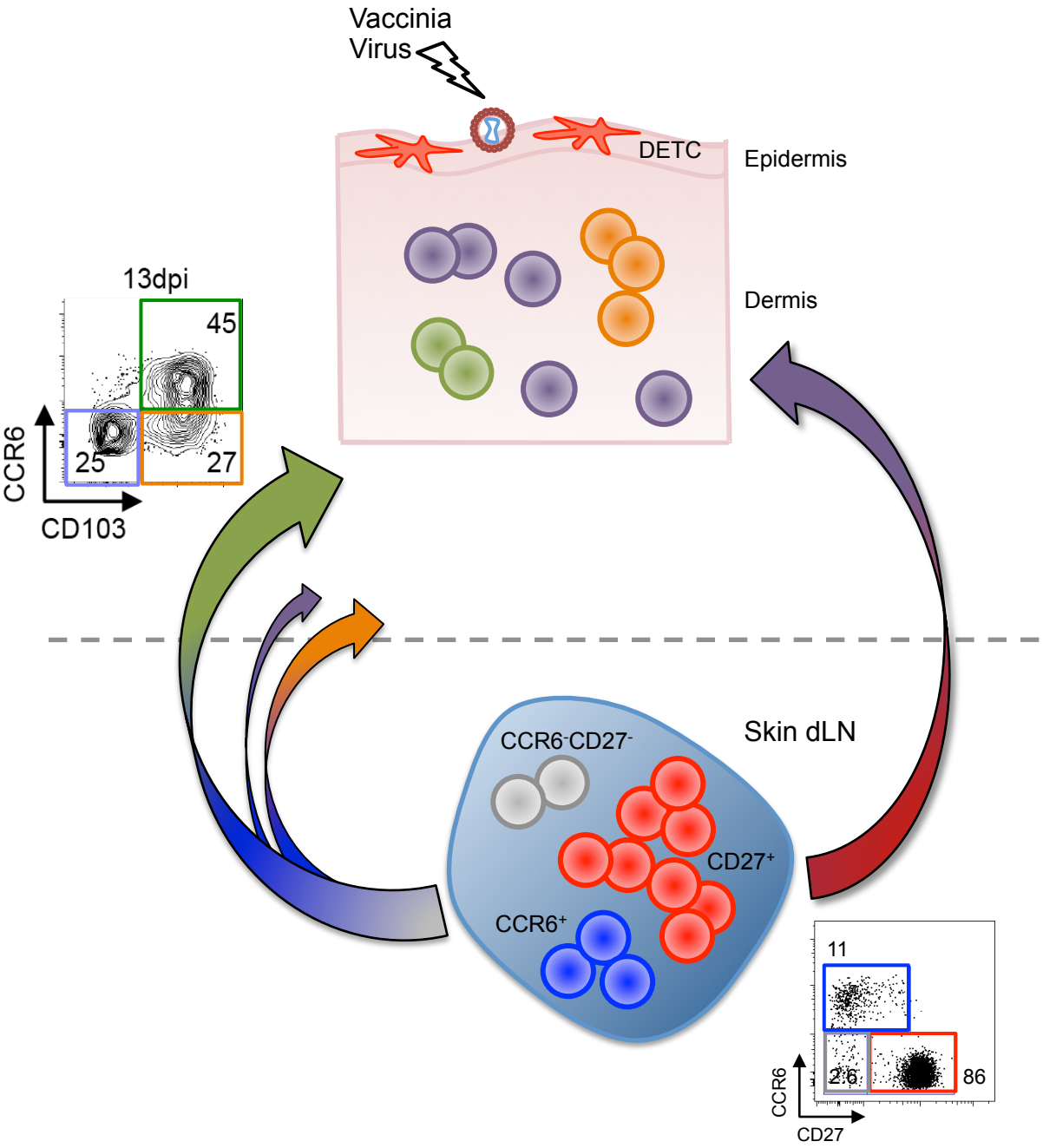

Figure 6.1. Model of γδ T cell response following scarification with VV.

in the dermis at day 7 following infection. Furthermore, the CD103/CCR6 profile of the adoptively transferred immigrants resembled that of the host γδ T cells in the dermis day 7 post-infection. The ability of immigrant cells to diversify into all the dermal subsets lead us to ask what the contribution was of the individual populations in the circulation. Emerging evidence confirms that CD27 delineates functional γδ T cell subsets in both mice (27, 41, 43) and humans (45-47). Transfer of $CD27^+$ and $CD27^-$ circulating γδ T cells into separate hosts followed by VV scarification demonstrated their unique contribution to $CD103^-$ or $CD103^+$ dermal populations, respectively. These experiments also confirmed the independent maintenance of these two populations even in the presence of inflammation. The stability of these two subsets and the constancy of the $CCR6^+$ and $CCR6^-$ ratio within the $CD27^-$ population offer an important tool for studying γδ T cells during inflammation. Although other groups have compared $CD27^+$ and $CCR6^+$ γδ T cells (27, 41), the circulating $CCR6^-CD27^-$ subset has received less attention. This latter population is uniformly $ROR\gamma t^+$ and distinguished from the $CCR6^+$ population by lower levels of CD103 and CD44. Future studies will be needed to explore the nuances between these populations.

Although there is some disagreement concerning the radiosensitivity of γδ T cells and their reconstitution in an irradiated adult mouse (27, 28, 34), our chimera experiments revealed that, unlike the DETC population, γδ T cells in the circulation and dermis are radiosensitive. Our results confirm that transfer of perinatal thymocytes into an irradiated host generates bonafide $CCR6^+CD27^-$ γδ T cells in the circulation and residents in the dermis. Differential kinetics and functional profiles of the BM (immigrant) and pThy (resident) γδ T cell populations in the BMpThy chimeras allowed us to ask

whether they had distinct roles following infection. Determining the role of dermal γδ T cells by comparing WT and TCRδ$^{-/-}$ mice is complicated by the absence of normal DETC in the null mice (11), cells that are known to play a role in keratinocyte growth and survival, perhaps impacting tissue damage or recovery. Whole body irradiation allowed us to specifically deplete the resident dermal γδ T cells, creating mice that lack this population while retaining healthy BM-derived circulating γδ T cell populations. Comparing mice specifically with or without dermal γδ T cells revealed that the dermal resident population was responsible for the increased inflammation observed in infected WT animals.

The ability of pThy-derived dermal γδ T cells to recruit inflammatory cells, such as neutrophils, is likely due to their production of IL-17 (28, 32, 89) and recruitment of neutrophils by γδ T cells directly results in increased tissue damage (87). We demonstrated increased necrosis and cellularity of infected ears 3 days after VV infection, indicating that the resident IL-17^{+} γδ T cells are early responders to viral infection. In contrast, the immigrating population of CD103^{-}CCR6^{-} DN γδ T cells, which appeared concurrently, only upregulated Granzyme B expression at a later time-point. Although classically associated with cytotoxicity, which has been shown for γδ T cells (54), Granzyme B has direct antiviral effects within cells and can cleave structural proteins when released into the extracellular matrix (90). Importantly for our study, extracellular Granzyme B release has been shown to have remodeling activity (91), potentially facilitating migration of cells into the tissue and impacting wound repair. Considering the absence of γδ T cells had no effect on viral load and the number of γδ T cells within the dermis remained elevated for an extended period of time, these

Granzyme-producing cells may play a non-traditional role during inflammation and in the recovery process after viral clearance. There were also large populations within both subsets that were negative for the parameters we tested. Future studies should take an unbiased approach to determine what other functions they may be performing.

Little is known about whether γδ T cells can form 'memory'. The majority of studies have examined at total γδ T cell population re-expansion (53, 92) or relied on CD44 expression (47), which is now appreciated to be regulated differently in γδ T cells than classical αβ T cells (1). When we transferred congenically marked γδ T cells, none of the immigrant γδ T cells remained in the dermis as residents after infection was cleared (Figure 4.1). This was true for the $CD27^+$ circulating γδ T cells characterized as $CD103^-CCR6^-$ DN cells in the dermis and for the $CD27^-$ circulating cells that give rise to all three populations in the dermis at day 7 post infection. Similarly in the BMpThy chimeras, BM-derived circulating γδ T cells that entered during the infection did not remain there long term. This indicates that inflammation is not sufficient to drive the long-term skin residence of γδ T cells that enter the skin from the circulation. In contrast, it has been observed after oral *Listeria monocytogenes* infection that a population of γδ T cells with memory-like function develop and persist in intestinal tissues (84). However, generating resident dermal γδ T cells requires a neonatal population (or their precursors), derived in our case from the transfer of perinatal thymocytes into irradiated hosts.

The presence of pThy-derived γδ T cells in the dermis was required to increase cellularity near the site of infection and to amplify the overall potency of the immune response as demonstrated by increased tissue damage in WT and BMpThy mice. While

the complete absence of γδ T cells did not translate to increased viral load in this or related systems (85, 93), other models have shown that mice lacking γδ T cells are more susceptible to bacterial infection (71) and have a decreased capacity for wound healing (10).

Together, the data indicate that γδ T cells are a dynamic population with a range of functions, but that they might not respond equally to all inflammatory cues. Importantly, since there is no DETC counterpart in human skin (94), it has become increasingly important to isolate the relevance of γδ T cells in the dermis in models relevant to human disease. Our study employing the adoptive transfer of circulating γδ T cells into normal hosts and the creation of chimeras specifically lacking a dermal resident population will be useful for interrogating γδ T cell subsets and furthering our understanding of the dermal immune network.

This study also challenges the notion that terminology used to describe classical αβ T cells can be transferred to also characterize γδ T cells. While labels such as “naïve”, “central memory” and “effector memory” have distinct meaning and are tightly correlated with cell surface proteins that are well defined for αβ T cells, these same surface proteins may not carry the same meaning on γδ T cells. Here we have demonstrated that CD27 stably marks separate γδ T cell lineages where expression of this same protein is constitutively expressed on all naive αβ T cells and can be modified by external cues (95).

Therefore, the function of a cell surface protein should be detached from its classical terminology when describing γδ T cells as the purpose of their expression continues to be investigated. The expression of a marker such as CD44 on the cell

surface serves a specific function and the fact that has long been used to designate memory T cells should not influence discussion of its expression on other cell types. On γδ T cells it is now appreciated that even the TCR itself may even serve a different function on the $CD27^-$ population, which express high levels of CD44 – after receiving a strong TCR signal in the thymus these γδ T cells are licensed to respond quickly in the periphery. Therefore CD44 expression indicates their ability to rapidly perform effector functions, perhaps similar to αβ T cells, but otherwise shares no resemblance to a traditional memory response.

The fact that γδ T cells have persisted over time and across species (6, 96), dominating the T cell compartment in some cases, suggests their ability to perform important functions. It is likely that many of these are redundant in organisms that have developed a dominant αβ T cell population. However, this does not necessarily make γδ T cell existence inconsequential in these systems. Although in many situations γδ T cells may be dispensable for clearance of a pathogen, their tendency towards enhancement of immune pathology offers a clue into their usefulness in immune therapy. In settings of autoimmunity (97) or cancer (98), targeted approaches against γδ T cells may prove important for modulating the immune response.

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