5-2016

Microbiota activated CD103+ DCS stemming from oral microbiota adaptation specifically drive γδT17 proliferation and activation.

Christopher Jay Fleming

MICROBIOTA ACTIVATED CD103^{+} DCS STEMMING FROM ORAL MICROBIOTA ADAPTATION SPECIFICALLY DRIVE γδT17 PROLIFERATION AND ACTIVATION

By

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B.S., Centre College, 2009
M.S., University of Louisville, 2013

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A Dissertation Approved on

April 13, 2016

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DEDICATION

To God for giving me the strength, grace and courage to walk this difficult path

To Dr. Jun Yan for being a wonderful friend and role model for the last 5 years as well as a great mentor by always providing the necessary support I needed in order to succeed

To those at the University of Louisville who have shown tremendous kindness and love during this process

To the members of Dr. Jun Yan's lab for always being there for me and supporting me throughout my graduate studies

To Hillary for making me smile every day and pushing me to be my best

To my friends Michael and Ron for providing the essential balance in my life to allow me to be as productive as possible in my work life

To my parents Jay and Tina Fleming for supporting me every step of the way and always helping me to stay grounded and to remember who I am and where I come from

ABSTRACT

MICROBIOTA ACTIVATED CD103^{+} DCS STEMMING FROM ORAL MICROBIOTA ADAPTATION SPECIFICALLY DRIVE γδT17 PROLIFERATION AND ACTIVATION

Christopher Jay Fleming

April 13th 2016

IL-17 producing γδ T cells (γδT17) promote numerous autoimmune diseases such as psoriasis and arthritis, as well as, cancers of the colon, lung and breasts. Yet γδT17 peripheral regulation has yet to be thoroughly explored. In mice deficient in IL-17 signaling, we observed expansion of γδT17 in all major tissues. However, γδT17 expansion was not uniformly distributed systemically and was most prominent in oral draining cervical lymph nodes (LNs) with monoclonal expansion of Vγ6 γδT17. *In vitro* proliferation assays of these cervical LNs showed endogenous proliferation by γδT17 dependent on cell-to-cell contact with CD103^{+} DCs. CD86^{+} and CD80^{+} activated CD103^{+} DCs are increased in the oral draining LNs suggesting that perhaps microbiota-activated CD103^{+} DC expand γδT17. 16s rRNA FISH hybridization

shows increase in 16s rRNA in oral draining LNs. Treatment of mice deficient in IL-17 signaling with α-LTBR-Ig (to remove LNs) or oral broad-spectrum antibiotics abrogates γδT17 expansion. Cohousing IL-17r$^{-/-}$ mice with wild type mice induces γδT17 expansion in the wild type mice also showing increase numbers of activated CD80^{+} and CD86^{+} CD103^{+} DCs in the oral draining LNs. In germ-free mice which lack microbiota, γδT17 expansion is significantly inhibited while γδT17 cells are expanded in a *Porphyromonas gingivalis* oral infection model. γδT17 are promoters of different devastating diseases and this study provides new evidence showing they can be regulated by CD103^{+} DCs providing a new and specific cell-to-cell interaction that could be targeted by future therapeutics.

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INTRODUCTION

"If two people stare at each other for more than a few seconds, it means they are about to either make love or fight. Something similar might be said about human societies. If two nearby societies are in contact for any length of time, they will either trade or fight. The first is non-zero-sum social integration, and the second ultimately brings it."

— Robert Wright

General Introduction

The world we live in consist of interactions between groups that are either advantageous at the beginning or is in the process of becoming advantageous. The same can be said on the microscopic level as groups of cells and microorganisms interact and seek benefits to bring about survival short-term and prosperity long-term. To an outsider this complex array of interactions might initially seem chaotic and random; however as knowledge builds about each individual interaction more can be said about the array of interactions on a whole like learning the steps of a beautiful dance. At the host-environment interface, the immune system is one community and the

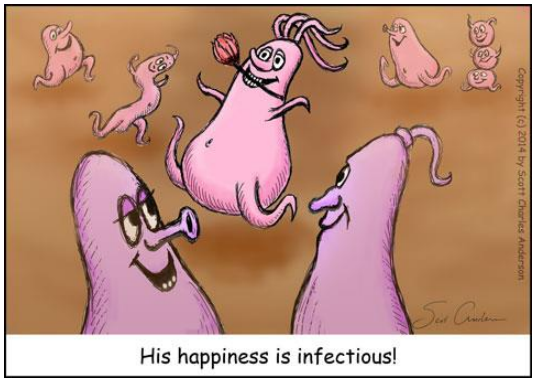


Figure 1 A Microbiota Dance Party

(Scott Charles Anderson ©2014)

microbiota is another. They learn from their interactions in order to bring about better and more efficient responses like two dance partners learning how the other moves and responds which is beneficial to the partnership and to the individual.

The immune system does not learn and dance for the sake of it but instead to bring about resilience and security to the host. The network of immune cells and the interactions within is immense and incredible but even within such beauty lays incredible control and order. Every cell is created for a specific process that has been selected due to millions of years of environmental pressures and cues. If a cell does not help in bringing forth prosperity to the host then it is removed. Cells of the healthy and thriving immune system do not exist due to mistake or accident. Each cell has a specific purpose and even if redundancies in the system exist there must be a specific niche or unique purpose for each cell otherwise that cell would no longer subsist.

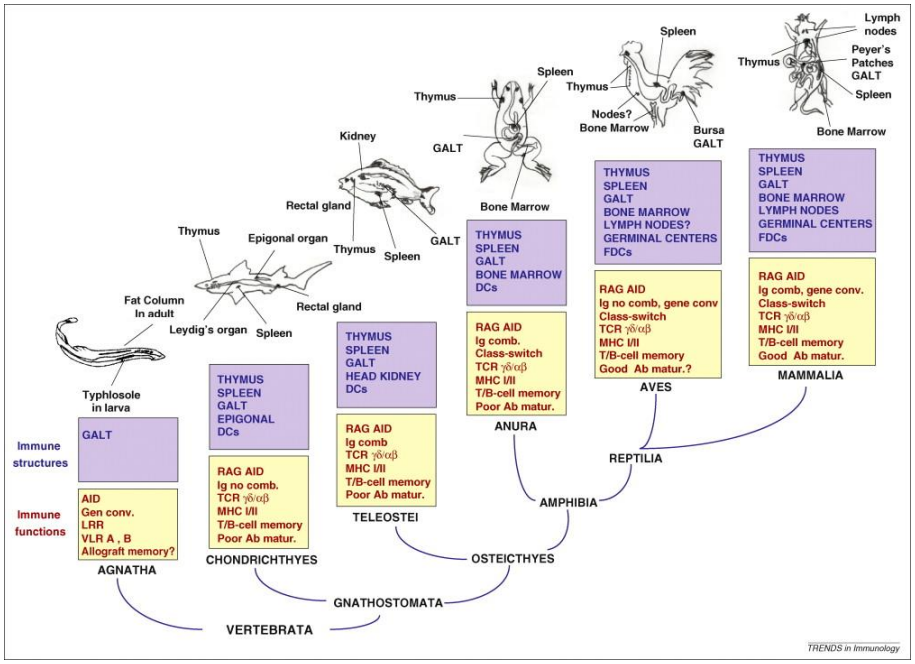


Figure 2. Conservation of ancient immune functions during the evolution of complex life forms. (Hofmann et al., 2010)

Rare cells of the immune system are of particular interest especially when you see them conserved throughout millions of years of evolution. T cells expressing the gamma and delta chain of their T cell receptor (γδT) evolved hundreds of

millions of years ago back to the origin of cartilaginous fish and have persevered throughout the evolution of man (Figure 2). They exist because they are important in some process(s) yet they remain rare and obscure. To be rare and obscure requires tremendous control thus energy. The host's need to use up energy to control such an obscure population is intriguing to say the least. Balance is a critical aspect of life and there are many examples of how when cells of few become cells of many suffering and tragedy shortly follows. Therefore the rare cells must be tightly controlled and regulated so the host continues to thrive.

Interestingly some of the most rare and least known cells of the immune system exist at the host-environment interface where it would seem the least amount of control could be achieved. The most distal areas of the body, away from the developmental organs of the immune system which includes the thymus, liver, spleen and bone marrow, are the epithelial surfaces such as the skin, respiratory and GI tracts. These are the surfaces containing the highest numbers of microbiota. This is where self meets and interacts with non-self constantly. This is where the dance is most active and the most is being learned. This host-environment interface is the educational center of the innate immune system similar to the thymus for the adaptive side. This is where our immune system interacts and processes a mindboggling amount of data in order to determine what actions are needed in order to bring survival and prosperity to the host.

Interactions between two of the least frequent yet most potent populations of the immune system and their response to a changing microbiota is the subject of this dissertation. They are γδ T cells and $CD103^+$ dendritic cells (DC) which often only make up approximately 0.3-4% of the total hematopoietic cells present in the tissues discussed.

If the immune system is compared to a spear touching its sharp tip against the microbiota world to determine if its safe or dangerous, the γδT and $CD103^+$ DCs would be that tip (Figure 3). Even a rare and obscure population of cells can have a tremendous impact on the health and prosperity of the host.

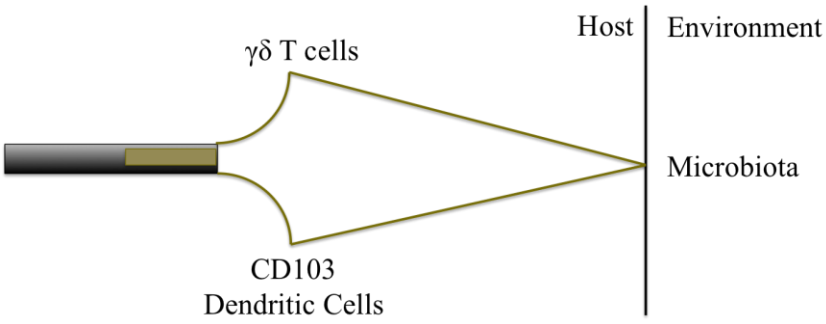


Figure 3. γδT and CD103 DCs are protectors of epithelial integrity

γδ T cells

γδ T cells are named for their variable Vgamma (γ) and Vdelta (δ) TCR chain usage, which is different than the alpha beta (αβ) chains of the more conventional $CD4^+$ and $CD8^+$ T cells. This alternative TCR expressing T cell was discovered in 1986 by three separate labs leading to three consecutive Nature publications in 1986 and 1987 (Bank I, 1986; Brenner M.B., 1986; Pardoll DM, 1987). The lymphocyte section of the immune system was thus divided into a tripartite system of αβ T cells, B cells and γδ T cells. A tripartite lymphocyte system has been observed and traced back to the most ancient of species some 450 million years ago (Hayday, 2000). With γδ T cells being such a rare population immunologist were instantly intrigued by their conservation for hundreds of millions of years. Obviously, an unknown importance was involved and has been a topic of investigation for the last thirty years.

By now, substantial progress in determining the functional roles and underpinning mechanisms of αβ T cells and B cells has been achieved. However, γδ T cells were more of less cast aside due to their small numbers and their TCR variable chains appeared for

the most part invariable compared to the mindboggling variability of the αβ TCR and B cell BCR (Li et al., 1998). Lack of MHC Class I or II restriction and the ability to recognize different antigens of biochemical makeup and size made γδ T cells appear as innate immune cells rather than cells of adaptive immunity (Asarnow DM, 1988).

Nevertheless, some immunologists were intrigued and began investigating the development, trafficking and effector function of γδ T cells in various mouse models even though it was not till later physicians became aware of the presence of γδ T cells in many human diseases in the clinic. Development has been one of the major areas of γδ T cell investigation because this particular subset has a very spatially and temporally defined maturation process. γδ T cell progenitor cells are formed in either the fetal liver or bone marrow during early embryogenesis and the vast majority are matured in the thymus in the last week of embryogenesis. There are also γδT cells which develop extrathymically that have been observed in athymic mice (Preffer FI, 1989). γδT cells are divided into two separate populations based on their effector cytokine production which is selected for intrathymically during this last week of development based on antigen exposure (Ribot et al., 2009). They are separated into IFN-γ producing and IL-17 producing γδ T cells. IFN-γ producing γδT cells are known to directly engage and kill virus infected or cancerous cells analogous to $CD8^+$ T cell responses except much quicker and less specific. γδT17 cells during health provide assistance to other cell types similar to that of T helper cells. As Vantourout and Hayday mentioned in their Nature Immunology Review of γδ functions that γδ T cells do not possess a unique function but as a heterogenous group with many different functions are unique in their ability to evoke these functions all in the same tissue (Figure 4)(2013). Due to this fact, use of $\delta TCR^{-/-}$

mice that are deficient in γδT cells must be used very carefully in order to determine specific roles of γδT cells.

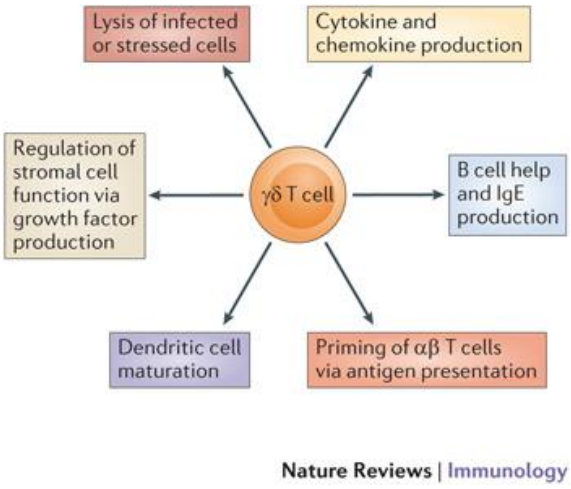


Figure 4. Vastly different Functions of γδT cells (Vantourout and Hayday, 2013)

IFN-γ producing γδ T cells are positively selected for through strong ligand interaction with molecules such as SKINT-1 and IL-17 producing cells are negatively selected for through weak ligand interaction (Barbee et al., 2011; Jensen et al., 2008; Ribot et al., 2009; Turchinovich and Hayday, 2011). IFN-γ producing γδ T cells reside primarily in the blood and lymphatic system including the spleen while IL-17 producing cells are primarily found at epithelial surfaces such as the skin, lungs, G.I. tract and vagina. γδ T cells develop earlier than αβ T cells and their program of development is synchronously connected to the development of other tissues (Carding and Egan, 2002; Shibata, 2012). Vγ usage is tied to functionality and plasticity with some subsets such as Vγ1 and Vγ4 being more plastic with a variety of inducible, adaptive-like effector functions while Vγ6 γδ T cells are more programmed, terminally differentiated innate-like effector cells (Bonneville et al., 2010).

γδT17 cells

γδT17 cells are major producers of IL-17 and are an important source of this cytokine at mucosal and epithelial barriers where IL-17 plays a critical role in maintaining epithelial integrity. They can be found in the skin, tongue, lungs, colon,

vagina and upper respiratory tract. Much investigation has been used in order to elucidate the signaling requirements needed for the differentiation of γδT17 cells in the thymus. Hayes and Laird described these

events in a 2012 review showing at different stages throughout the thymus the events required for mature effector γδT17 cells to develop (Hayes and Laird, 2012) . It has also been reported that Sox4 and Sox13 are important transcription factors in the early development of Vγ4 γδT17 cells but not Vγ6 (Malhotra et al., 2013). A transcription factor specific for Vγ6 γδT17 development is still unknown. Evidence suggests that antigen exposure in the thymus is important for development of IFN-γ γδT cells but not γδT17 cells which show little antigen exposure (Jensen et al., 2008). γδ T cells in humans however show a greater level of plasticity with *in vitro* treatment of Vγ9Vδ2, which makes up approximately 70% of all human γδ T cells, with various cytokine cocktails leading to differentiation into different effector subsets including IL-17 and IFN-γ producing γδT cells (Caccamo et al., 2013; Moens et al., 2011). *Ex vivo* staining of human samples however has shown that the majority of γδT17 producers in humans express the TCR Vδ1 chain (Wu et al., 2014). However in mice the predominant γδT17 cells consist of $V\gamma6^+$ and $V\gamma4^+$ γδT cells with the majority being committed during embryonic development in the thymus.These two different

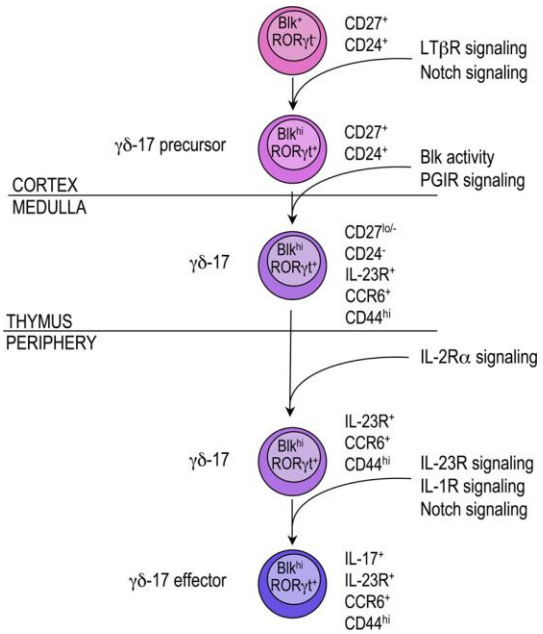


Figure 5. γδT17 Develop ment in Thymus and Peripheral Regulation

(Hayes and Laird, 2012)

γδT17 subsets in mice appear to develop differently and leave the thymus at different times in what has been called "Functional embryonic waves" (Figure 5) (Cai et al., 2014; Haas et al., 2012). As shown in Haas et al.'s diagram, Vγ6 γδT17 cells are developed in the thymus before birth meaning that they must undergo self-renewal for long-time persistence throughout a host's life. Vγ4 and Vγ1 continue to be developed in the thymus and these have the added advantage of being inducible γδT17 cells (Chien et al., 2013).

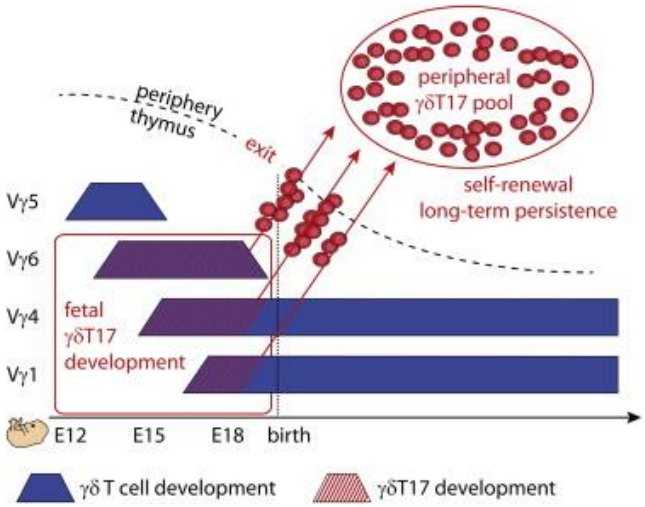


Figure 6. Functional Embryonic Waves of γδT17 cell egress from the thymus.

(Haas et al., 2012)

Disproportionally located in the mucosal linings of different organs in mice and humans, γδT17 cells are able to quickly respond to inflammatory events and foreign pathogen insults. Most studies have shown that γδT17 get activated through archaic signals of injury and infection such as inflammatory cytokines, pathogen pattern recognition receptors (PPARs) and complement receptors (Martin et al., 2009; Sutton et al., 2009; Xu et al., 2010). Vγ1 and Vγ4 γδT17 cells can also be induced to produce IL-17 with minimal clonal expansion through TCR-dependent antigen induction with phycoeurythrin (PE) (Zeng et al., 2012). Chien et al.'s study provided a paradigm shift in γδT17 biology since we now have two different pathways of γδT17 responses much like regulatory Tregs with natural and inducible Tregs. γδT17 have since been looked at as natural and inducible or programmed and programmable.

γδT17 in Health

IL-17 plays a major role at epithelial barriers as it induces epithelial cells to secrete anti-microbial products such as defensins in order to maintain microbiota populations (Pappu et al., 2012). γδT17 cells are one of the major contributors of IL-17 and due to their innate abilities are the predominant earlier responders to epithelial insults. This IL-17 response is important in the release and maturation of neutrophils from the bone marrow as well as trafficking of neutrophils into the site of inflammation. Neutrophils then play a role in decreasing the pathogen load and helping to restore the tissue to health (Chien et al., 2013).

γδT17 cells play a critical role in restoring tissue to homeostasis after injury or disease. Li et al. found that γδT17 cell are important in corneal nerve regeneration due to the downstream effects of IL-17 and VEGF necessary for neutrophil involvement (2011b). This is only one of a few reports citing the importance of γδT17 in neuropathology however this field is growing tremendously. Braun et al. using models of lung inflammation including bleomycin inhalation was able to show that γδT17 are important in controlling the inflammation necessary to restore the lungs to health and prevent fibrosis development (2008).

γδT17 in Disease

γδT17 cytokine effector function is the primary means in which they have been studied in the context of disease. Due to their ability to induce a powerful immune response under the necessary circumstances, if they become deregulated it will lead to the initiation and progression of various inflammation driven diseases. Inflammation is a tightly regulated

process directing the immune response to initiate and follow through with injury or infection resolution. Their role therefore in the pathophysiology of autoimmune diseases and cancer reflects their ability to be potent IL-17 producers thus acting as attractors of other immune cells such as MDSCs, macrophages and neutrophils (Rei et al., 2014; Shibata, 2012; Wu et al., 2014).

The role of γδT17 in human colon cancer shows that a vicious γδT17 cycle could play a major role in the theory of cancer as a "never healing wound" (Wu et al., 2014). The cycle begins with the initial inflammatory events that activate γδT17 cells either directly from injured epithelial cells, local antigen presenting cells such as DCs and/or γδT17 recognition of foreign material using their own PAMP or DAMP receptors. γδT17 cells then attract myeloid-derived suppressor cells (MDSCs) or neutrophils to the sight of inflammation. MDSCs are well known for their ability to regulate inflammation so that a wound or injury can be resolved; however, they also produce IL-1β and IL-23 inducing proliferation of γδT17 cell. The increased IL-17 production will lead to more MDSC attraction and accumulation thus propelling the cycle (Figure 6) (Wu et al., 2014; Yan and Huang, 2014).

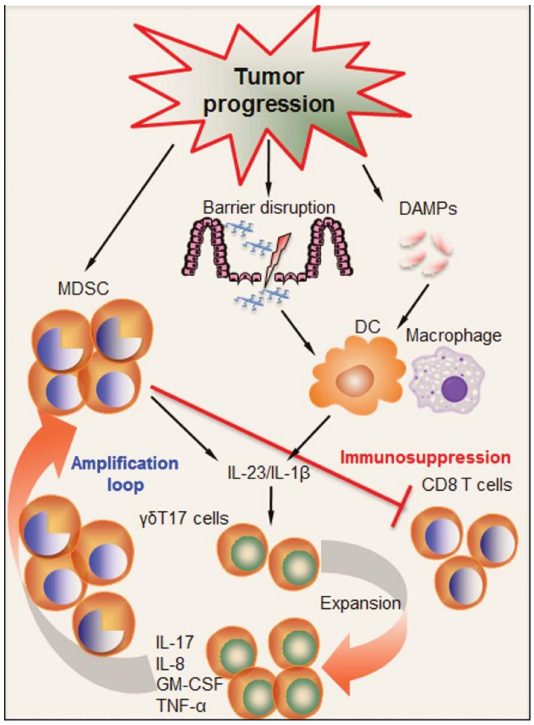


Figure 7. γδT17-MDSC Inflammation Cycle. (Yan and Huang, 2014)

IL-17 production not only leads to the accumulation of inflammatory cells known to promote various diseases but it is also an upstream signaling pathway for markers known to be important in cancer metastasis. IL-17 is a direct upstream and potent inducer of VEGF leading to angiogenesis, a hallmark of cancer progression and metastasis. In fact, VEGF blockade led to increase levels of IL-17 which induced additional angiogenesis factors leading to cancer progression (Chung et al., 2013). IL-17 also induces the production of metalloproteinases such as MMP-1, 2, 9 and 13 (Feng et al., 2014; Li et al., 2011a). These proteins are essential for cell migration through tissue and barriers resulting in lymph node involvement and metastasis to other organs.

γδT17 in Cancer

γδT17 cells have been shown in five top-tier journal reports to play an important role in the progression of various cancers including colon, lung, breast, liver, and ovarian. In colon cancer, γδT17 cells are stimulated by microbiota activated inflammatory DCs causing them to produce vast amounts of IL-17 which attracts immune suppressive MDSCs into the tumor microenvironment (Wu et al., 2014). In a similar mechanism in breast cancer, γδT17 cells attracted neutrophils into the breast cancer microenvironment causing them to become suppressive and play a role in the metastasis of the disease (Coffelt et al., 2015). Ma et al. found using a hepatocellular

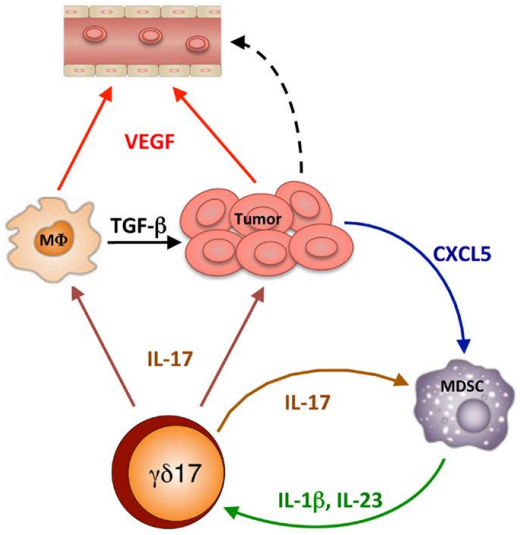


Figure 8. γδT17 IL-17 Effector Function in Cancer.

(Lo Presti et al., 2014)

cancer model that γδT17, mainly Vγ4, recruited suppressive MDSCs in a CXCL5/CXCR2 dependent manner and γδT17 could also induce additional suppressive activity from the recruited MDSCs (2014). Using ovarian cancer as their model, Rei et al. showed tumor challenge induced the proliferation of γδT17 cells and this caused recruitment and increase pro-tumorigenic activity from small peritoneal macrophages (SPMs) (2014). However, in lung cancer challenge, using IV injection, γδT17 have been shown to be protective in mice. By decreasing levels of IL-6 and IL-23, Cheng et al. found that γδT17 cells are defective resulting in increased susceptibility to tumor foci development (2014). By supplementing and reconstituting γδT17 cells they were able to decrease the number of tumor foci. These studies show that γδT17 cells are in general indicators of increased cancer progression and worst prognosis because of their ability to attract immunosuppressive populations into the tumor microenvironment; however, as with everything there are exceptions.

γδT17 in Autoimmune disease

γδT17 have also been shown by our group as well as others to play a major role in the promotion of certain autoimmune diseases including psoriasis, EAE, collagen induced arthritis and EA uveitis. In *Immunity* 2011, we showed that a new subset of T cells called dermal γδT cells were pathogenic in the progression of psoriasis through TLR7-dependent recruitment of neutrophils to the psoriatic plaques (Cai et al., 2011). Becher et al. showed the role of γδT17 in psoriasis nicely in their Nature Medicine review (Figure 8) (2012).

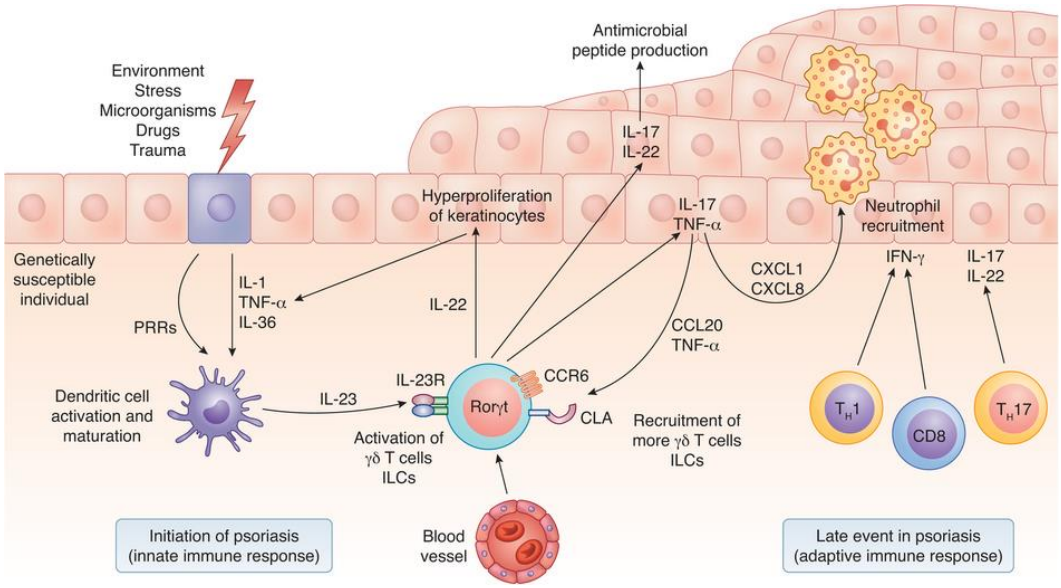


Figure 9. Role of γδT17 in psoriasis development. (Becher and Pantelyushin, 2012)

In 2014, we showed that Vγ6 as well as Vγ4 can contribute to the pathogenic role of γδT17 cells in psoriasis and that the two subsets have differential development programs between egress of thymus and establishment in epithelial tissue (Cai et al., 2014). Petermann et al. found using an EAE model that γδT17 cells influence other T cell subsets by making them refractory to suppressive activity and preventing the induction of Tregs in the periphery (2010). It was also found that γδT17 cells in EAE can promote IL-17 production from Th17 cells thus amplifying their response (Sutton et al., 2009). Intradermal injection of CFA with collagen was also found to induce γδT17 responses and promote the development of collagen-induced arthritis again through support of Th17 responses at the joints (Roark et al., 2013). Liang et al. found that γδT17 that are slightly activated express higher levels of IL-23r and are the cells responsible for promoting autoimmune driving Th17 cells. Highly activated γδT17 cells don't express IL-23r therefore there is a fine balance in activation and functionality in γδT17 (2013).

γδT17 and Microbiota

Having both γδT17 and Th17 cells seems redundant however they are capable of producing IL-17 at different phases of the immune response; therefore, they each fill their own niche. γδT17 can produce IL-17 much quicker than Th17 cells and will be directors of the immune response long before antigen-specific Th17 cells realize a pathogen invasion or injury has occurred (Figure 10) (Roark et al., 2008).

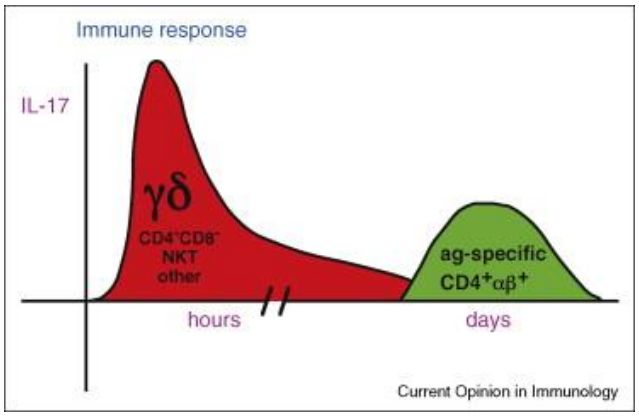


Figure 10. γδT17 response time and potency compared to Th17 response.
(Roark et al., 2008)

γδT cells that are positive for surface marker chemokine receptor $CCR6^+$, which are IL-17 capable γδT cells, have different PPAR receptors on their surface compared to $CD27^+$ γδT cells which are IFN-γ capable (Martin et al., 2009). $CCR6^+$ γδT cells express TLR1, TLR2 and Dectin-1 exclusively while $CD27^+$ γδT cells express TLR4. Martin et al. show that different γδT cells respond differently to different types of microbes. This is consistent with what we know about the location and function of these T cells. γδT17 cells are located near the periphery where the environment-host interface occurs. TLR1 recognizes lipoproteins and unconventional LPS expressed on bacteria. TLR2 interacts with peptidoglycan and zymosan therefore they specialize in bacterial and fungal interaction. Dectin-1 identifies β-glucan and carbohydrates specific for fungal cell walls therefore fungal recognition is priority. Duan et al. found that $IL\text{-}1r^+$ γδT17 in the gut responded to bacteroides differences through not TLR signaling but guanine nucleotide

exchange factor VAV1 (2010). These studies are advancing our knowledge of how complex γδT17 cells are with varied capabilities in responding to microbiota populations.

Our knowledge of γδT17 interaction with microbiota or microbial pathogens remains for the most part uncertain except to say that they are innate immune cells responding to microbes using the conventional methods of pattern recognition and cytokine induction. How γδT17 cells interact with microbiota under homeostatic conditions and the role they play in shaping the "microbiota" has yet to be investigated. It is still known whether microbiota play a role in regulating γδT17 cells as well. More investigation is necessary into the regulatory mechanisms at the epithelial interface between microbiota and the immune system especially looking at γδT17 cells.

Dendritic Cells and Microbiota

Dendritic cell (DC) interaction with commensal microbiota has been thoroughly investigated in the GI tract showing that DCs play a critical role for maintaining the microbiota populations through induction of the adaptive immune response. DCs sample bacterial products at the epithelial surface using their dendrites

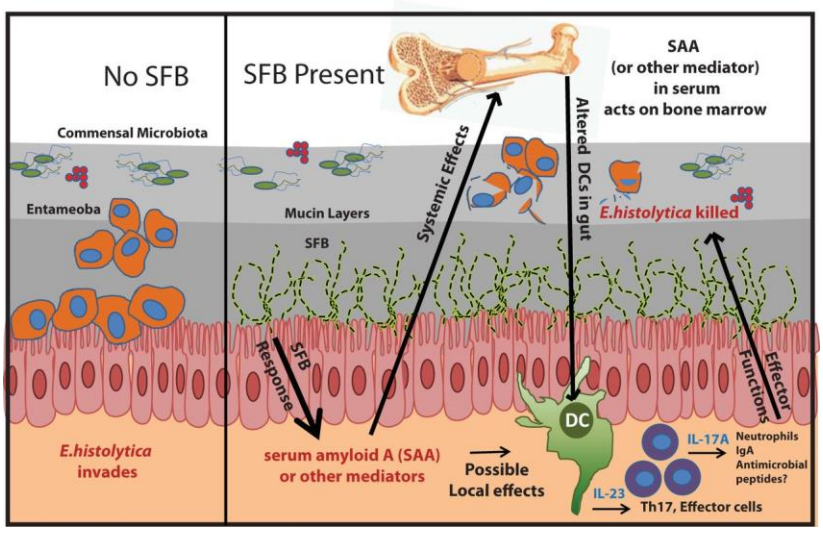


Figure 11. SFB Induced Protection against Entamoeba histolytica Initiated Colitis through DC polarization of Th17 cells. (Burgess et al., 2014)

and in the peyer patches activate B cells to produce an appropriate amount of IgA necessary to maintain microbiota populations at equilibrium (Macpherson and Uhr, 2004). Goto et al. investigating the regulation of Th17 cells in murine gut showed that segmented filamentous bacteria (SFB) engulfed and presented by intestinal dendritic cells requires MHC Class II expression and is essential for Th17 maintenance (2014). Burgess et al. found that SFB activation of DC can lead to protection against colitis initiated through Entamoeba histolytica infection (Figure 11) (Burgess et al., 2014). Microbiota or pathogens that escape the initial DC barrier at the epithelial surface has been shown to stimulate and induce the differentiation of monocytes into DCs capable of classical DC antigen presentation (Cheong et al., 2010).

Dendritic cells and γδT17 cells

Dendritic cells are known to induce IL-17 responses from γδT17 cells through the production of IL-1β and IL-23 (Cai et al., 2011; Sutton et al., 2009; Wu et al., 2014). Besides these two conventional cytokines, dendritic cells activated through anaphylatoxin C5a produces

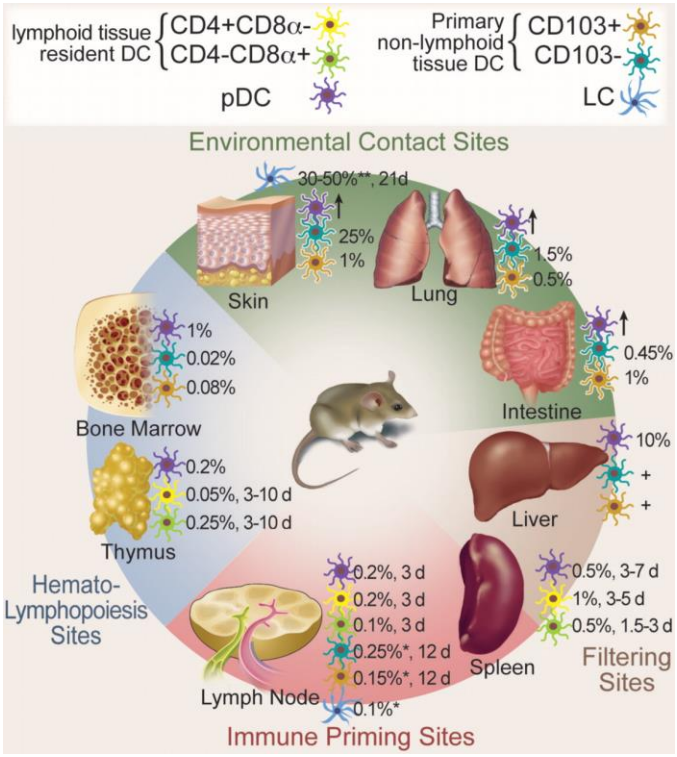


Figure 12. DC Heterogeneity, Location and Frequency in Mice (Manz, 2009)

IL-6 and TGF-β that were capable of inducing IL-17 responses from peritoneum γδT17 cells (Xu et al., 2010). In humans, it was shown that DCs can use antigen presentation using MHC like molecules CD1b and CD1c to induce γδT17 responses through the presentation and recognition of lipid A (Cui et al., 2009). Studies investigating the activation of γδT17 cells from DCs to this point have focused primarily on the origin of the cytokines stimulating γδT17 cells for mechanistic purposes therefore less focus has been placed on what particular subsets of DCs are capable of inducing γδT17 responses. This is important because DCs are heterogeneous in terms of their location and effector function (Figure 12); therefore, it is probable that only certain types of DC types and responses can in fact induce a specific γδT17 response under certain conditions (Manz, 2009).

$CD103^+$ DCs at the host-environment interface

$CD103^+$ DCs is a specific DC subset known to be particularly capable at migration and antigen presentation once activated at the epithelial surface (del Rio et al., 2010). Rio et al. showed that $CD103^+$ DCs in the lungs are potent at activating CD8 T cell responses while $CD103^-$ DCs were better at activating CD4 T cell response (2007). Bedoui et al. showed that langerin-positive $CD103^+$ DCs and no other DCs in the skin are specifically capable of processing viral and self-antigens and presenting through cross presentation to CD8 T cells (2009). With close interactions to commensal microbiota populations, investigators have shown that $CD103^+$ DCs are particularly susceptible to microbiota-induced regulation of the immune system. $CD103^+$ DCs have been shown to be capable of inducing Treg induction in the absence of TGF-β unlike other $CD103^-$ DC populations placing particular importance on this subset in immune tolerance (Coombes et al., 2007).

Interestingly, it was found that epithelial cells which are in close contact with $CD103^+$ DCs condition intestinal DCs in the gut to express $CD103^+$ converting them to a more suppressive role in inducing Tregs (Iliev et al., 2009). However showing their adaptability, $CD103^+CD11b^+$ DCs in the gut through TLR5 recognition of flagellin produce high levels of IL-23 which leads to the activation of Th22 cells and IL-22 production (Kinnebrew et al., 2012). IL-22 is a close relative to the IL-17 cytokine family normally both being secreted simultaneously by Th17 cells (Sutton et al., 2009). In the murine small intestine a couple of studies have shown that $CD103^+$ DCs producing IL-6 and TGF-β are capable of regulating Th17 cells. Located at the same epithelial surfaces as γδT17 cells $CD103^+$ DCs could play an important role in the activation and expansion of γδT17 cells especially under homeostatic conditions where microbiota signals play such a critical role in the maintenance of innate immune cells.

RATIONALE, SIGNIFICANCE AND MAIN FINDINGS

γδT17 cells in various disease settings have been shown to either be a subset necessary for healthy recovery from wounds and infections or an inflammatory subset driving the immune response out-of-control leading to disease initiation and progression. Our elementary understanding of this subset in the disease setting is based on our conventional thinking that these professional IL-17 producers are sentinels of the innate immune response. Sentinels however require much more subtle regulation in times of peace in order to not cause undue damage and catastrophe. During homeostasis they must learn and grow so they'll be even more prepared for future incidences such as infection or injury. New therapies have been introduced such as neutralizing IL-17 or IL-

17r blocking antibodies which is the primary effector cytokine of this important subset. However, little is known about how this will effect the γδT17 cell population and our ability to recover from injury or fight off pathogen infection. How γδT17 cells at the epithelial border are regulated during homeostasis and whether they require signals from the environment including 1) autocrine IL-17 signaling, 2) cellular interaction with other immune cells and 3) signals from the steady-state host microbiota or "microbiota" is not known. Knowing how γδT17 cells are regulated should be a priority considering how important they are becoming in many inflammation-driven diseases and also the introduction of new immunotherapies specifically targeting the effector functions of γδT17 cells. We demonstrate in this study that IL-17 signaling is important in the intrinsic and extrinsic regulation of γδT17 cells proliferation and activation. We first show that γδT17 cells are drastically expanded in the absence of IL-17 signaling. Second we show that the BTLA-RORγt pathway contributes the intrinsic regulation of γδT17 cells. Third, we demonstrate that oral microbiota activated $CD103^+$ DCs drive γδT17 expansion via cell-to-cell contact. Fourth, we show that microbiota plays a central role in γδT17 peripheral regulation by inducing microbiota exchange through co-housing which induces γδT17 expansion. Lastly, we show that absence of microbiota in germ free mice leads to the collapse of the γδT17 population and by inducing a dysbiotic environment such as *P. gingivalis* infection of the oral cavity we can induce the expansion of γδT17 cells in draining cervical lymph nodes. These findings are the first to demonstrate that γδT17 cells are directly regulated through microbiota activated $CD103^+$ DCs and that γδT17 cells are regulated through cell-to-cell contact. These findings are the first to show

that the oral microbiota plays an important role in the proliferation and activation of γδT17 population.

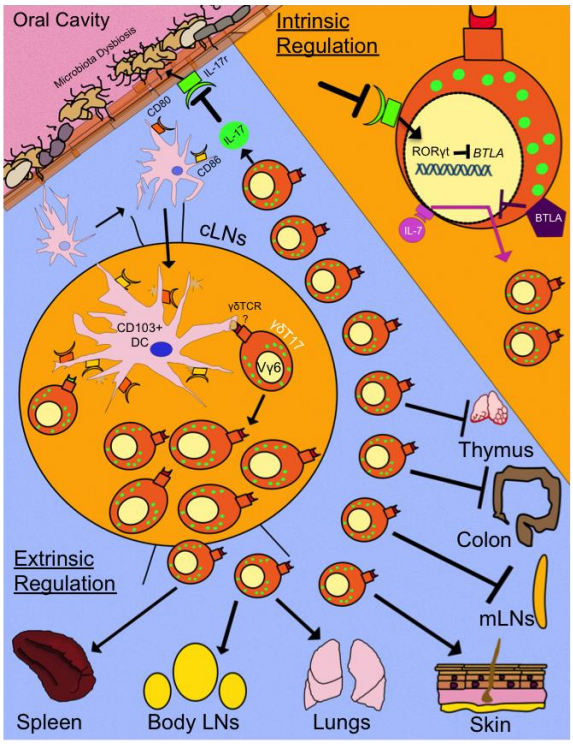


Figure 13. Schematic showing how γδT17 cells are intrinsically and extrinsically regulated in the periphery. Deficiency in IL-17 leads to oral microbiota dysbiosis which is detected through antigen sampling from $CD103^+$ DCs. Activated $CD103^+$ DCs migrate to cLNs where they induce γδT17 proliferation through cell-to-cell contact leading to systemic expansion of γδT17s except in the mLNs, colon and thymus. Intrinsically, IL-17r signaling deficiency leads to increase expression of RORgt which inhibits BTLA transcription. BTLA is a suppressor of IL-7 signaling thus γδT17 respond more to IL-7 signaling leading to increase expansion.

MATERIALS AND METHODS

Mice

WT and IL-1r$^{-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratory and homebred in our SPF murine facility. *Il17ra*$^{-/-}$ mice were purchased from Taconic (Cambridge City, IN) and have been previously described (Ye P, 2001). WT and IL-1r$^{-/-}$ mice were homebred and housed in University of Louisville's SPF Clinical and Translational Research Mouse Facilities. Germ-Free and Specific-pathogen-free mice were housed and bred at University of Chicago and were kindly provided by Dr. Yang-Xin Fu (UTMB, Galveston, TX). *P. gingivalis* and sham treated WT mice were housed and bred in Baxter II Vivarium (University of Louisville) and were kindly provided by Dr. Huizhi Wang (University of Louisville). All animals were housed and treated in accordance with institutional guidelines and approved by the IACUC at the University of Louisville.

Preparation of single cell suspensions

Samples from different tissues were processed for single cell suspensions. Mouse lungs were flushed with PBS, minced, digested with collagenase/hyaluronidase/DNAse digestion buffer for 45 min at 37 °C, and passed through a cell strainer to obtain a single cell suspension. Red blood cells (RBC) were lysed by ACK lysis buffer. Cells were then washed with complete RPMI. LNs and spleens were processed by meshing against a cell strainer using the plunger end of 5 ml syringe. RBCs were then lysed same as the lungs.

Colon cecum and anal verge were harvested for cell preparations. Colon sections were thoroughly washed using repetitively PBS inversions in 50 mL tubes to remove all fecal material. After washes colon samples were processed similar to skin tissues as described in (Cai et al., 2011).

***Ex vivo* immunostaining and flow cytometry analysis**

Mouse CD3, CD45, CD4, γδTCR, TCRVγ1, TCRVγ4, CD27, CD11c, CD11b, CD19, CD80, CD86, I-A/I-E, CD103, RORγT, BTLA, IFN-γ and IL-17A mAbs were obtained from Biolegend. Mouse CCR6 mAbs were obtained from R&Dsystem. Mouse Vγ6 TCR antibodies were kindly provided by Dr. Tigelaar (Yale University, New Haven, CT). For intracellular staining of cytokines, cells were stimulated with PMA and ionomycin in the presence of Golgiplug (BD-Bioscience) for 5 h and stained with different cell surface Abs and then fixed, permeabilized using Biolegend fixation/permeabilization buffers followed by staining intracellularly for IL-17 and IFN-γ. For RORγt intracellular staining Ebioscience FoxP3 fixation/permeabilization kit was utilized. The appropriate isotype control mAbs were also used.

Carboxyfluorescein succinimidyl ester (CFSE) labeling and co-culture assay

Single cell homogenate from LNs, spleen, lungs and bone marrow were resuspended at a concentration of $1x10^7$ cells per ml and incubated with CFSE (1μM) at 37 °C for 10 min. The reaction was then quenched by addition of ice-cold FBS. After CFSE labeling, $1x10^5$ γδ T cells were plated per well of a 96-well plate. $1x10^4$ DCs were added. After 5 days the cells were harvested.

LTBR-Ig Partial LN Depletion

Two pregnant IL-17r$^{-/-}$ females ~ E13-14 were I.P. injected either with the systemic antagonist, mLTBR-mIgG1 (LTBR-Ig) or control monoclonal IgG1 antibody with a single dose of 100 μg. LTBR-Ig was kindly provided by Dr. Fu (University of Chicago, Chicago, IL). 6 weeks after birth the adult mice were euthanized and successful partial LN removal was indicated when iLNs were absent, cLNs were reduced in size and mLN we observed no difference.

Neonatal Vγ6 γδT WT:IL-17r$^{-/-}$ Reconstituted Chimerism

D1-2 newly born IL-17r$^{-/-}$ (CD45.2) and SJL (CD45.1) pups were euthanized by induced hypothermia using ice. Thymuses were harvested and processed by meshing with 40 μm cell strainer. Thymocytes were stained with CD3, γδTCR, Vγ6 and 7AAD. Vγ6 γδT cells were sorted using Beckman Coulter MoFlo. After sorted Vγ6 γδT, WT and SJL were mixed in 1:1 ratio which was confirmed using BD FacsCanto and FlowJo analysis. Adult δTCR$^{-/-}$ mouse was euthanized using CO_2 gas and bone marrow (BM) was harvested and processed using 40 μm cell strainer and treated with ACK lysis buffer. 3 δTCR$^{-/-}$ mice were irradiated using a single dose of 950 Rads for 10 minutes. Irradiated mice, after resting for 5 hours, were each injected with a thymocyte/bone marrow mixture containing $10x10^7$ δTCR$^{-/-}$ BM cells and $6x10^4$ SJL:$4x10^4$ IL-17r$^{-/-}$ sorted Vγ6 γδT cells. After 8 weeks of reconstitution, mice were euthanized using CO_2 and tissues were harvested, stained and analyzed. Degree of chimerism was calculated using the before and after reconstitution ratio of WT:IL-17r$^{-/-}$ Vγ6 γδ T based on CD45.1 and CD45.2 expression.

Tissue sectioning and microscopy

cLNs were frozen in OCT and stored at -80°C. Sections of approximately 7 µm were fixed with cold acetone for 15 min followed by 30 min of air dry. Slides were then blocked using 20% FBS in PBS for 1 h. Ab staining occurred overnight at 4°C in humidifier staining container. After 16 h staining, secondary Ab was added for 5 h at 4°C. For 16s rRNA fish hybridization, the slides were air dried and incubated with oligonucleotide probe Cy3-EUB338 (5 ng/µl). This probe (5′-GCT GCC TCC CGT AGG AGT-3′) is complementary to a region of the 16S rRNA, a highly conserved domain in Bacteria. The probe is labeled with Cy3 fluorophore at 5' end (Integrated DNA Technologies, CA). The hybridization was carried out in the presence of 50 µl of 1x *in situ* hybridization buffer (Enzo) containing EUB338 probe (5 ng/µl). A large cover slip was placed on the slides and carefully pressed until the hybridization solution was evenly distributed over the respective section and incubated for 1 hr in humidified chamber at 46 ºC. The cover slip was carefully removed and the slides were rinsed with distilled water and incubated with DAPI containing anti-fade mounting media (BioMedia, CA). The cover slips were fixed to the slides overnight and the slides were analyzed the following day. The fluorescence images were captured using Nikon A1R confocal microscope with appropriate lasers.

Co-housing and antibiotics *in vivo* studies

WT and IL-17r$^{-/-}$ mice once reaching 4 weeks old were separated into either cohoused groups or WT alone and IL-17r$^{-/-}$ alone control groups. The mice were cohoused for 4-5 weeks and then were euthanized. In the antibiotics protocol, a pregnant IL-17r$^{-/-}$ female 1-2 days prior to delivering pups was given fresh antibiotics water described previously (Rakoff-Nahoum et al., 2004) consisting of Ampicillin (1 g/L), Vancomycin (500 mg/L),

Neomycin Sulfate (1 g/L) [GoldBio] and Metronidazole (1 g/L) [Sigma]. The female was given antibiotics water till delivery and pups were maintained on antibiotics water till 6 weeks of age when the experiment reached the terminal timepoint.

P. gingivalis Growth and Infection

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee, University of Louisville (IACUC # 10045). *P. gingivalis* ATCC 33277 cells were grown in TSB (BD, Franklin Lakes, NJ, USA) under anaerobic conditions (85% N2, 10% H2, 5% CO2) at 37°C. Porphyromonas gingivalis ATCC 33277 was obtained from the American Type Culture Collection (Manassas, VA, USA). The oral microflora was suppressed in 10- to 12-week old C57BL/6 mice by sulfamethoxazole (800 μg/ml) and trimethoprim (400 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) provided ad libitum in water for 10 days. The mice then received pure drinking water for 3 days followed by oral infection with 1x109 CFU of live *P. gingivalis* suspended in 100 μL of phosphate-buffered saline with 2% carboxymethylcellulose directly by gavage. Infections were performed four times at 2 day intervals. Vehicle control was also established. To enumerate *P. gingivalis* colonization, oral samples were collected along the gingiva of the upper molars using a 15-cm sterile polyester-tipped swabs (VWR, Radnor, PA, USA) at 1, 3 and 5 weeks after the first bacterial infection. Total genomic DNA was purified and amplified by qPCR. Numbers of *P. gingivalis* were calculated by comparison with a standard curve derived from known amounts of *P. gingivalis*. Wizard Genomics DNA Purification Kit was from Promega (Madison, WI). SYBRTM Green Master Mix qPCR Kit was from ThermoFisher (Waltham, MA), and the

primer sequences of *P. gingivalis* 16S rDNA are: 5'-AGGAACTCCGATTGCGAAGG-3' (Forward) and 5'-TCGTTTACTGCGTGGACTACC-3' (Reverse).

A single cell gene sequencing

Lymphocytes from adult cLNs of wildtype and IL-17r$^{-/-}$ mice were immunostained for Vγ6 γδTCR then single cell sorted into 96-well plate containing 10 µl Qiagen One Step PCR 1x Buffer using Beckman Coulter MoFlo. Plates were spun down at 3000 rpm for 2 min then frozen at -20 °C immediately. RT-PCR for Vγ6 TCR gene sequence was performed at Stanford University using Barcode enabled high throughput single cell TCR determination described previously (Wei et al., 2015).

Fecal Transfer *in vivo* studies

Adult male WT mice were give broad-spectrum antibiotics 1 week prior to fecal transplant. 1 hr prior to fecal transplant mice were injected IP with 3 mg of cimetidine HCl and 0.02 mg of sincalide to reduce gastric acid levels and enhance reconstitution. After 1h, mice were given 12 mg fecal transplant using oral gavage injection. 5 WT each were transplanted with either IL-17r$^{-/-}$ or WT fecal samples. Mice were treated with 1 injection per week for 3 weeks followed by 4 weeks of rest for gut microbiota reconstitution.

Oral and fecal microbiota sequencing

The v1-v3 regions of 16s rRNA gene was amplified using 27f (AGAGTTTGATCCTGGCTCAG) and 534r (ATTACCGCGGCTGCTGG) primers (1 µM). The primers were anchored adaptor (adopter A: 5'

CCATCTCATCCCTGCGTGTCTCCGACTCAG 3' and adopter B: 5' CCTATCCCCTGTGTGCCTTGGCAGTCTCAG 3') and Multiplex Identifiers (MIDs; 10 bp long). The multiplexed amplicons were gel purified and sequenced using the 454 Jr. Sequencing platform. The 16S rRNA sequences were analyzed using QIIME (1.9.0) platform scripts (www.qiime.org) (Caporaso et al., 2010). The sequences were rarified at randomly selected 2000 sequences/sample and downstream analysis was performed. The microbial classification was performed using Green Genes reference database (gg_13_8_otus) using QIIME tools (Caporaso et al., 2010). The sequences reference picked into Operational Taxonomic Units (OTUs) by clustering 97% sequence similarity (uclust) and classified at various taxonomic ranks (phylum, order, class, family, genus, and species). The beta diversity principle co-ordinate plots were generated using phylogenetic metrics of UniFrac distances. The Unifrac metric distances were used to calculate the significance between two groups using ANOSIM with 999 permutations.

Statistical Analysis.

Results were exhibited as means ± SEM. Statistical analysis was performed using GraphPad Prism software. The statistical significance of differences between groups was determined by the Student's t test. All data were analyzed using two-tailed tests unless otherwise specified, and a P value < 0.05 was considered statistically significant.

RESULTS

γδT17 cell proliferation is peripherally regulated by the presence of IL-17 signaling

In the absence of pro-inflammatory cytokine IL-17 signaling, we observed a drastic systemic expansion of total γδ T cells particularly γδT17 cells. To extensively dissect this phenotype, we performed *ex vivo* immunostaining on cell homogenate from skin, lungs, body LNs, spleen, colon, and thymus. We found expansion of the total γδ T cell population in all tissues except the thymus and colon (Figure 14A and data not shown). In addition, $CCR6^{+}CD27^{-}$ γδT cells, known for being capable of producing IL-17 (Haas et al., 2009; Ribot et al., 2009), were specifically increased (Figure 14B). In contrast, $CCR6^{-}CD27^{+}$ γδT cells, which are capable of producing IFN-γ remained unchanged or significantly decreased (Figure 14B). Consistent with CCR6 expression, *in vitro* γδT17 cells were increased in frequency (Figure 15A) as well as IL-17 intensity on a per cell basis (MFI, Figure 15B). Lack of total γδT cell expansion in the thymus suggests the expansion was regulated peripherally not centrally. Further support for peripheral expansion was the observation that γδ T cell expansion did not occur in the spleens and lungs till two days after birth (data not shown). CD4 T cells also showed more polarization towards a Th17 phenotype (Figure 17A and 17B). However, the total percentage of CD4 T cells was not changed (Figure 16). This data shows that γδT17 cells are regulated by the presence of IL-17 signaling not only in the *de novo* synthesis of IL-17 (Smith et al., 2008) but also in the expansion of γδT17 cells in the periphery during homeostasis.

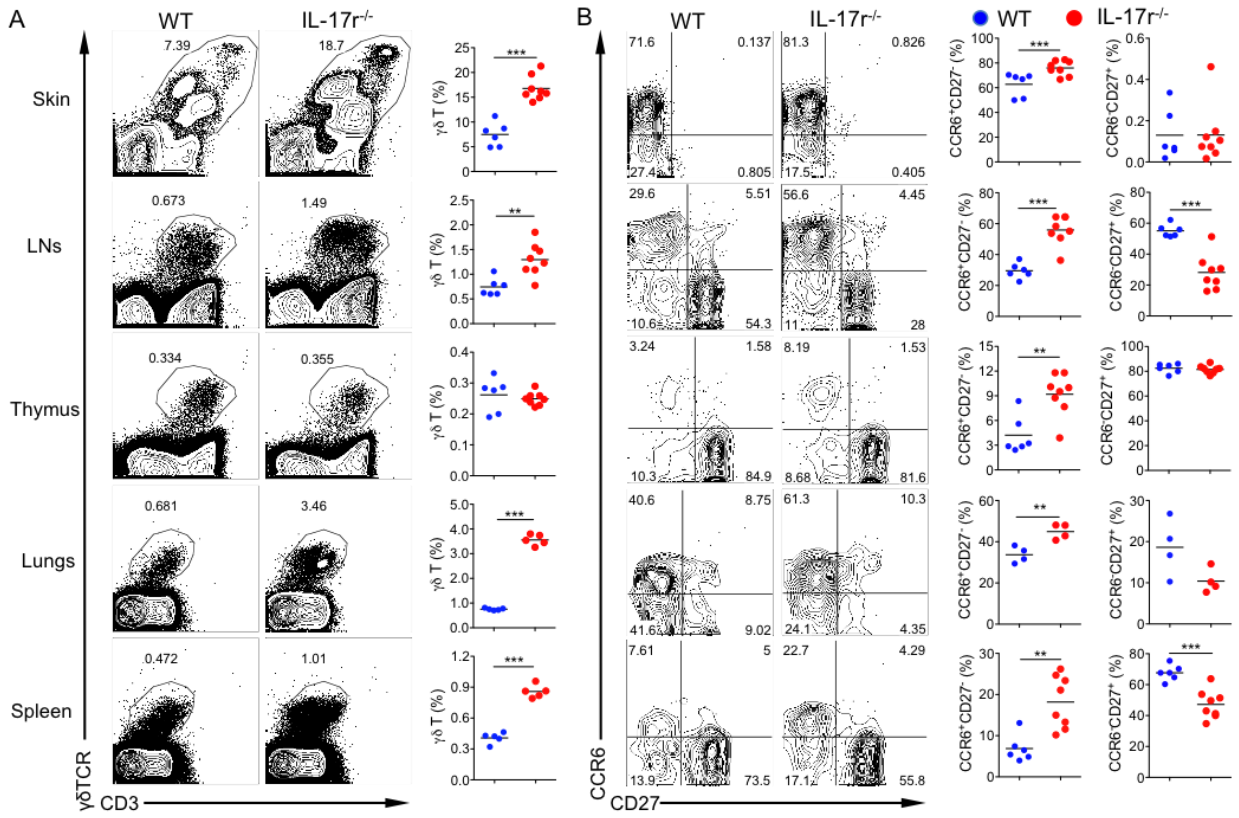


Figure 14. IL-17-capable γδT cells expanded in mice deficient in IL-17 signaling

Flow cytometry studies staining homogenous tissue samples from different organs from IL-17r$^{-/-}$ mice and WT mice of similar age and sex from the same facility.

(A) Gating from the total live lymphocyte population then gated on CD3 versus γδTCR for total γδ T cell percentage in each tissue. Plots representative of 7-8 experiments. **p<0.01, ***p<0.001

(B) Gating from total γδ T cells then looking at CCR6 versus CD27 in each tissue to calculate CCR6^{+}CD27^{-} (IL-17 capable γδ T cells) and CCR6^{-}CD27^{+} (IFN-γ capable γδ T cells). Plots representative of 3 experiments. **p<0.01, ***p<0.001

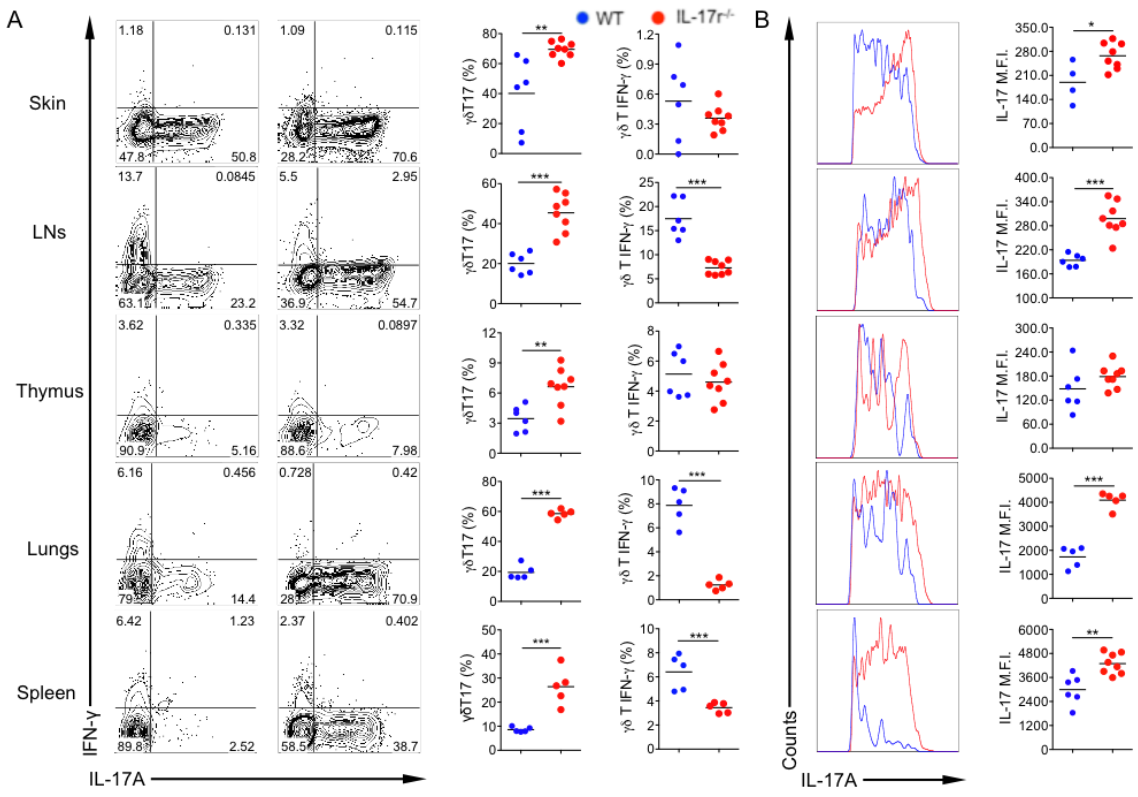


Figure 15. γδT17 cells expand and IL-17 producing capability increases in mice deficient in IL-17 signaling

Flow cytometry studies staining homogenous tissue samples from different organs from IL-17r$^{-/-}$ mice and WT mice of similar age and sex from the same facility.

(A) Gated on total γδ T cells stimulated with PMA/Ionomycin for 5 h to calculate IL-17 or IFN-γ-producing γδ T cells. Figures representative of 7-8 experiments. **p<0.01, ***p<0.001

(B) Gated on IL-17-producing γδ T cells from IL-17r$^{-/-}$ (red) and WT mice (blue) to compare mean fluorescence intensity (MFI) of IL-17 production level on a per cell basis. Histograms representative of 7-8 experiments. *p<0.05, **p<0.01, ***p<0.001

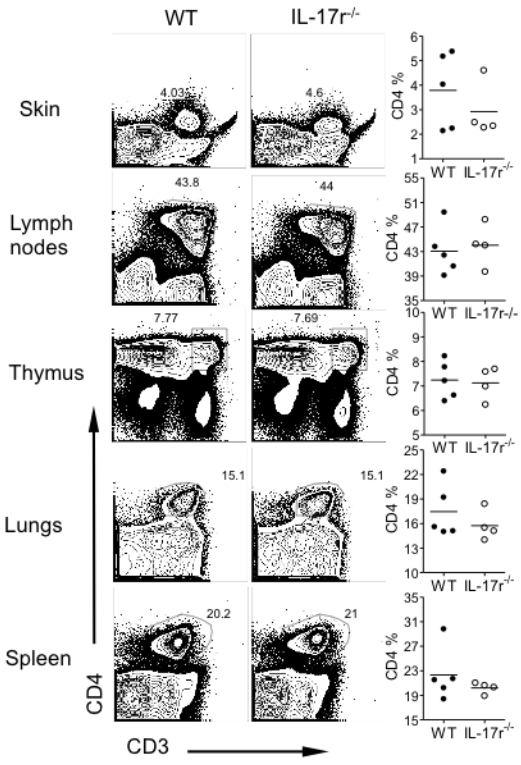


Figure 16. No expansion in CD4 T cell population in absence of IL-17 signaling

Flow cytometry studies staining homogenous tissue samples from different organs from IL-17r$^{-/-}$ mice and WT mice of similar age and sex from the same facility.

(A) Gating from the total live lymphocyte population then gated CD3 versus CD4 for total CD4 T cell percentage in each tissue. Plots representative of 7-8 experiments.

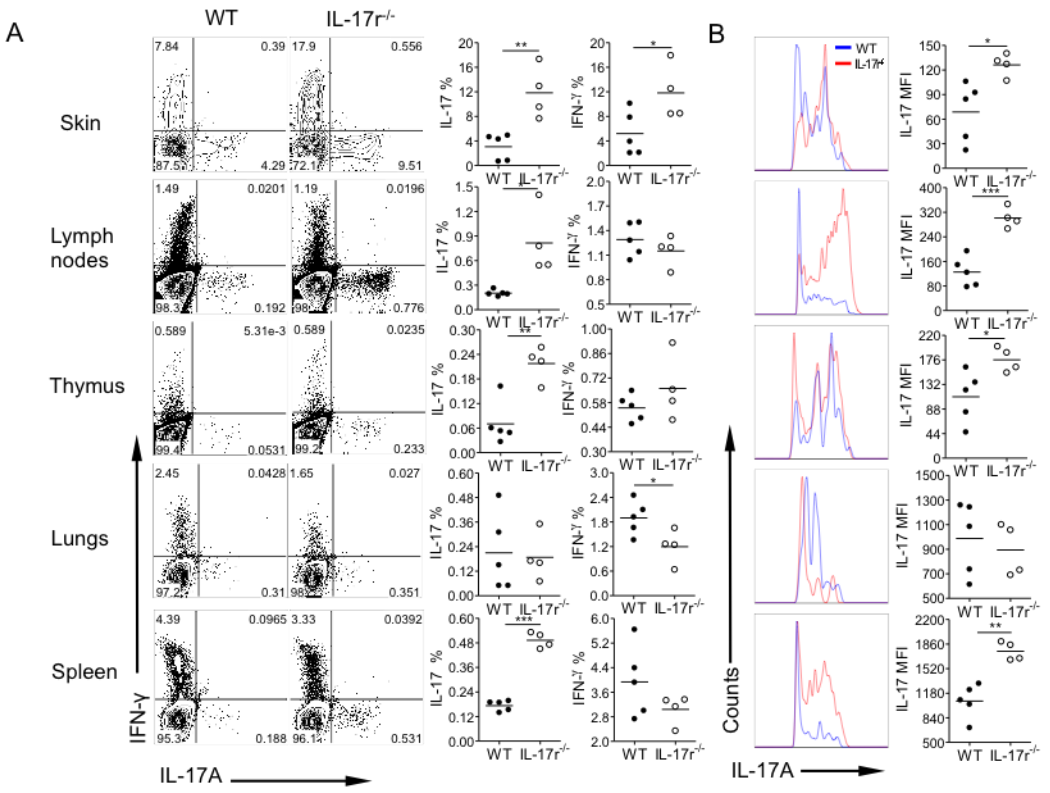


Figure 17. Th17 polarization increased with deficient IL-17 signaling

Flow cytometry studies staining homogenous tissue samples from different organs from IL-17r$^{-/-}$ mice and WT mice of similar age and sex from the same facility.

(A) Gated on total CD4 T cells stimulated with PMA/Ionomycin for 5 h to calculate IL-17 or IFN-γ-producing cells. Figures representative of 7-8 experiments. *p<0.05, **p<0.01, ***p<0.001

(B) Gated on total IL-17 producing CD4 T cells from WT (blue) and IL-17r$^{-/-}$ (red) mice and looking at mean fluorescence intensity (MFI) to calculate IL-17 production on a per cell basis. Histograms representative of 7-8 experiments. *p<0.05, **p<0.01, ***p<0.001

Cervical LNs in the neck are specifically enlarged in the absence of IL-17 signaling with monoclonal expansion of γδT17 cells

When harvesting tissues for *ex vivo* staining, we noticed in IL-17r deficient mice that all tissues were of the same size and absolute cell number except the cervical LN (cLNs). cLN weight and total cell number were tripled but not inguinal (iLNs) and mesenteric LNs (mLNs) (Figure 18). In addition, a specific and drastic 8-fold expansion of γδ T cells in the cLNs was revealed by flow cytometric analysis (Figure 19). Interestingly, the expanded γδ T cells had an increased expression of CD3 and γδTCR (Figure 20). Stimulating the total LN homogenate from the three different LN locations showed a higher frequency and MFI of IL-17 production in the cLNs and iLNs but not mLNs (Figure 21). It was previously reported that Vγ6δ1 γδ T cells have a higher expression of CD3 compared to other subsets (Paget et al., 2015), suggesting that a change in the γδ T cell composition in the LN niche might occur in the absence of IL-17 signaling. By investigating the compositions of γδ T cell subsets in the three different LN locations, we found an extreme increase of Vγ6 γδ T cells in cLN and iLNs but not in the mLNs (Figure 22). The cLNs showed a drastic increase of Vγ6 γδ T cells from 5% to 71%. Utilizing a bar code enabled high throughput *ex vivo* single-cell TCR sequence analysis to determine specific γδ TCR repertoire in the expanded cLN Vγ6 population, we found that approximately 95% of these γδ T cells in both wild type (WT) and IL-17r$^{-/-}$ cLNs expressed a single pair of TCR sequences encoded by Vd1Dd2Jd1 and Vg6Jg1 with no N region diversity (Figure 23). Since Vγ6γδT17 cells are drastically increased in cLNs, we hypothesized that the Vγ6γδT17 cells expanded in the cLNs may be then trafficking to

other areas leading to a systemic expansion phenotype. We used the LN depleting fusion protein lymphotoxin β receptor-Ig (LTBR-Ig) to treat ~E13-14 pregnant IL-17r$^{-/-}$ females to examine whether partial LN depletion and/or reduction in size would affect the Vγ6γδT17 expansion in the cLNs and other peripheral tissues. We found that partial depletion of the LNs was sufficient to abrogate gdT expansion and proliferation of the rare Vγ6 population in the cLNs with decreased numbers in the spleen as well (Figure 24). No difference was observed in mLNs. Corresponding with the decrease in the Vγ6 population we also saw a decrease in total IL-17 responses as well as specifically a decrease in Vγ6 γδT17 in all tissues studies except the mLNs (Figure 25). This data suggests that the environment, surrounding the cLNs, is highly conducive to γδT17 cell expansion. Without IL-17 signaling, γδT17 homeostatic maintenance becomes dysregulated resulting in the specific expansion of a rare lymphatic Vγ6γδT17 subset.

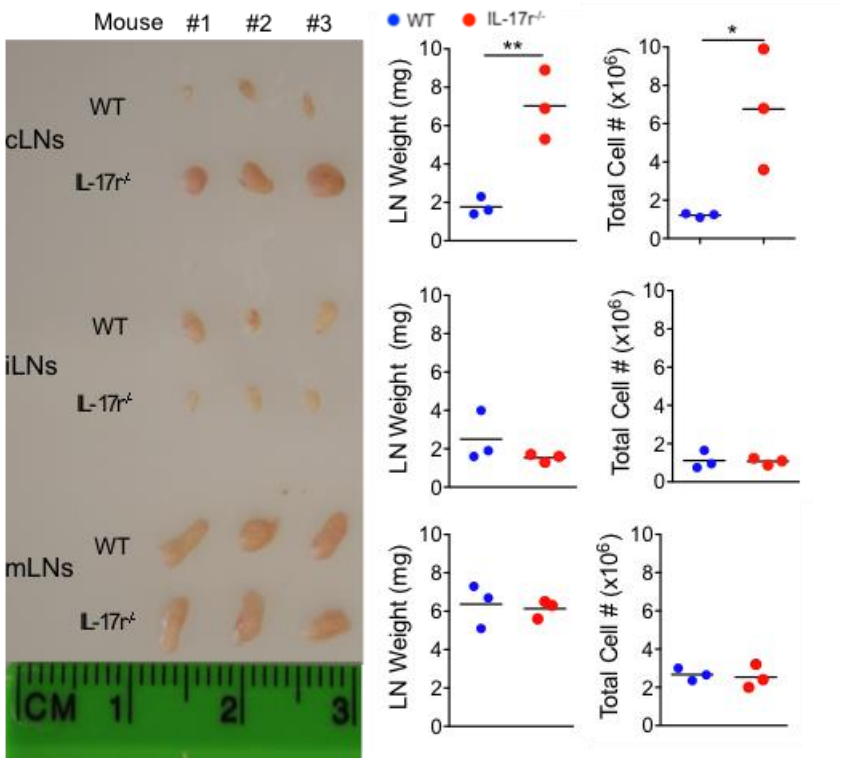


Figure 18. cLNs in the neck are specifically enlarged in the absence of IL-17 signaling

(A) Photograph showing the difference in size (scale shown in cm) of LNs between WT and IL-17r$^{-/-}$ mice from three different locations with corresponding weight and total cell count from each LN. Plots representative of 3 different experiments. *p<0.05, **p<0.01

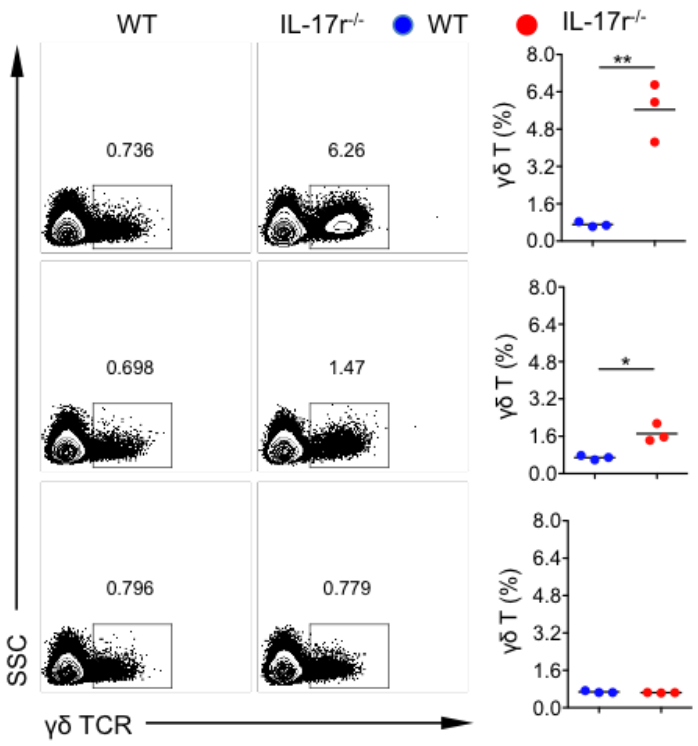


Figure 19. γδT cells specifically expand in the cLNs with less expansion in LNs further away and no expansion in mLNs

(A) Flow cytometry staining for γδ TCR from corresponding LN locations in (A) to calculate difference in total γδ T cell % between WT and IL-17r$^{-/-}$ mice. Representative of 4-5 experiments. *p<0.05, **p<0.01

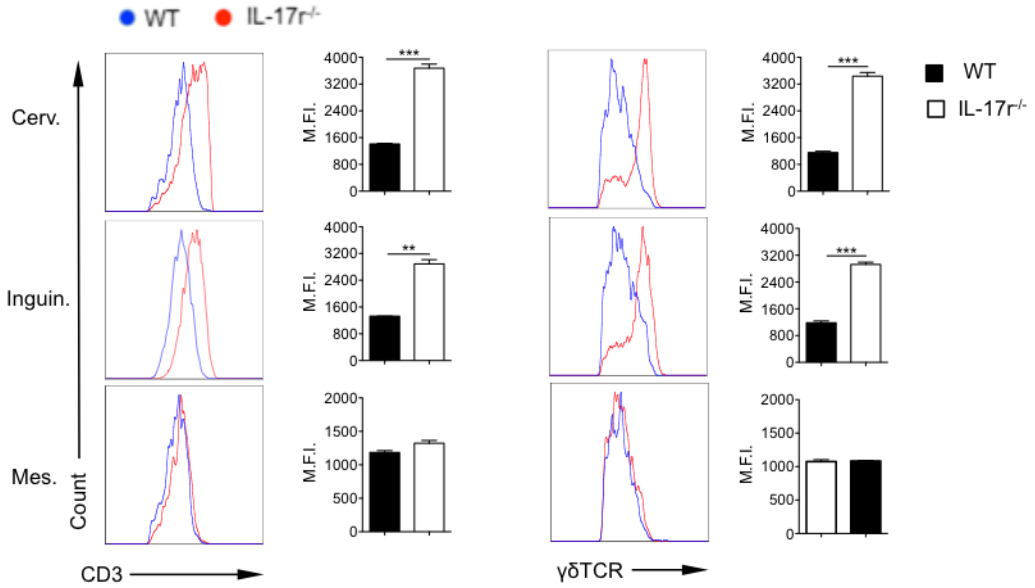


Figure 20. Expanded γδT cells in cLNs and iLNs express higher levels of CD3 and γδTCR

(A) Gated on total γδ T cells in the different LN locations and analyzed for CD3 and γδTCR surface expression intensity using MFI. Representative of 3 experiments. **p<0.01, ***p<0.001

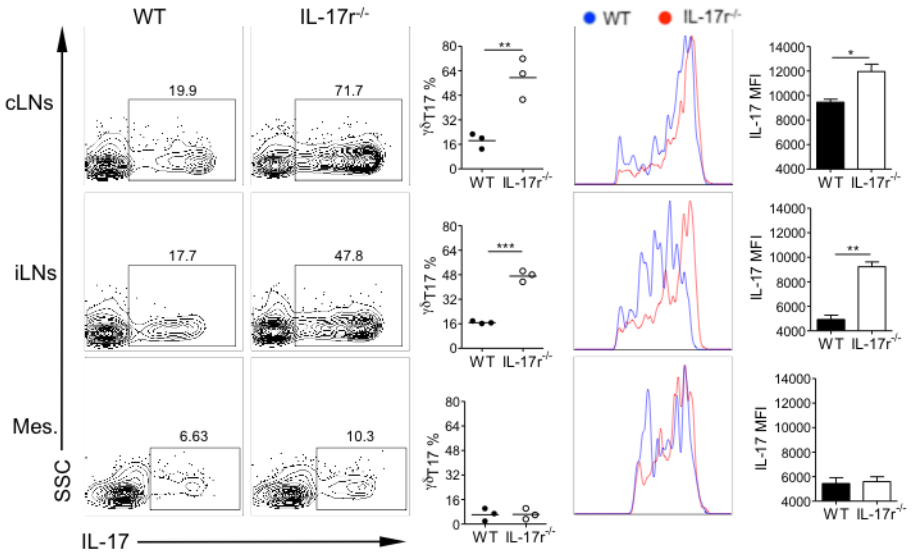


Figure 21. γδT17 cells expand most in the cLNs with less expansion in LNs further away and γδT17 cells show increase IL-17 production per cell

(A) Gated on total γδ T cells after 5 h PMA/Ionomycin stimulation to analyze γδT17 frequencies. **p<0.01, ***p<0.001

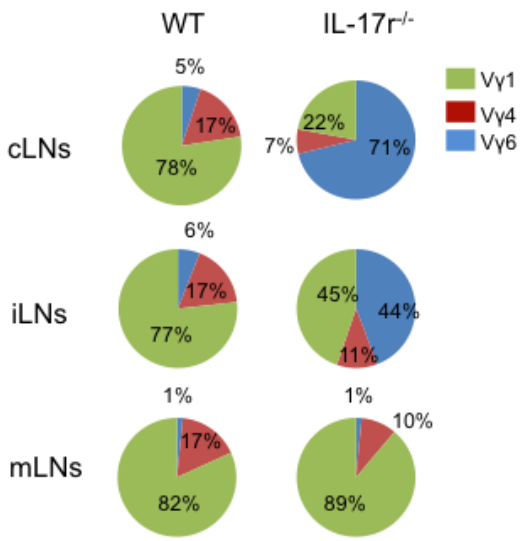
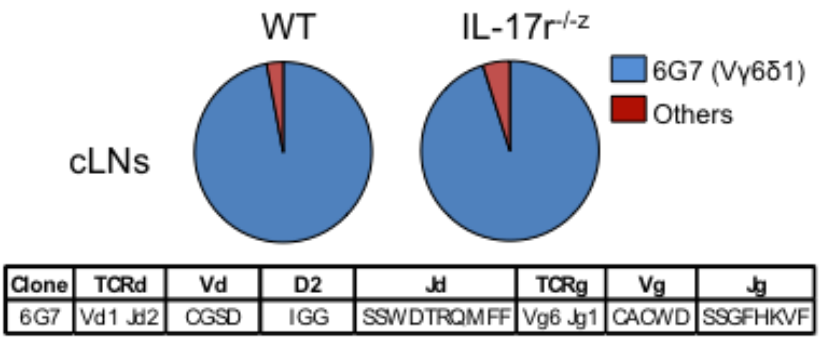


Figure 22. The Rare LN population Vγ6 γδT17 cells are specifically expanded in cLNs with less expansion in LNs further away and no expansion in mLNs

(A) Frequencies of the three subsets of γδT cells Vγ6 (Blue), Vγ1 (green) and Vγ4 (red). Pie charts representative of three different experiments.



Clone	TCRd	Vd	D2	Jd	TCRg	Vg	Jg
6G7	Vd1 Jd2	CGSD	IGG	SSWDTRQMFF	Vg6 Jg1	CACWD	SSGFHKVF

Figure 23. Monoclonal Expansion of the highly invariant 6G7 clone of Vγ6 γδT17 cells in cLNs

(A) Deep sequencing of individual clonal γδTCR expression on the Vγ6 γδ T cells sorted from cLNs of WT and IL-17r$^{-/-}$ mice. Pie charts are representative of 192 clones from WT and 192 clones from IL-17r$^{-/-}$.

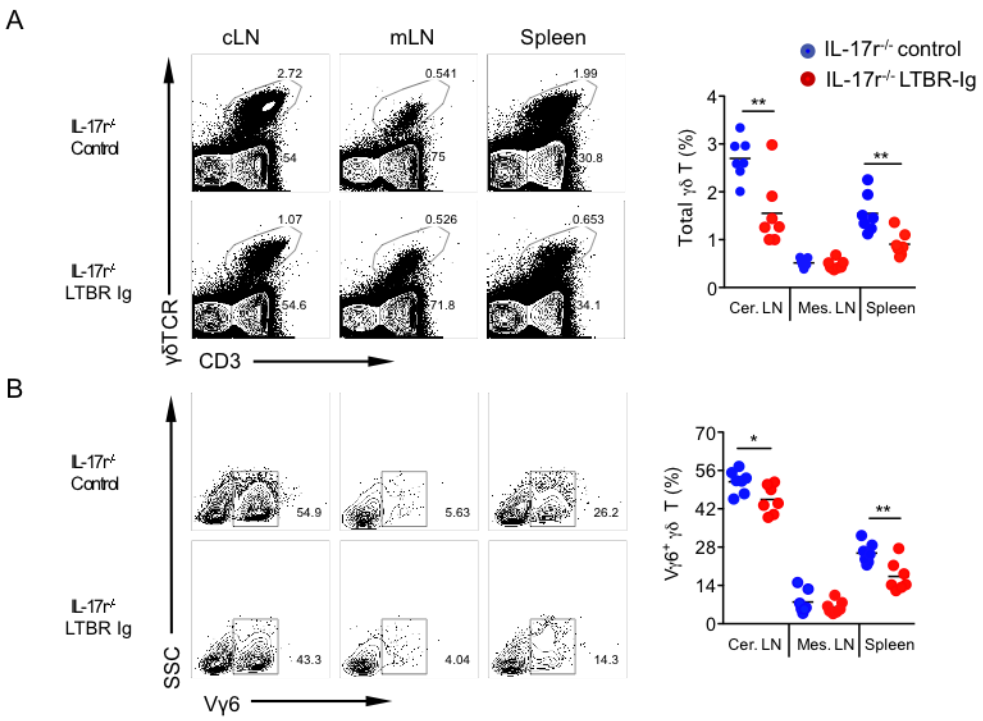


Figure 24. Partial LN depletion with LTBR-Ig decreases expansion of Vγ6 γδT in cLN and spleen but not mLN

LTBR-Ig depleting protein was injected I.P. into D20 post-conception pregnant mice which results in partial depletion of some lymph nodes (Inguinal) and reduction in size of other LNs (Cervical/Mesenteric) in 6-8 week old adult mice.

(A) Gating from total lymphocyte population then gated CD3 vs. γδTCR to analyze total γδ T %. Representative of 2 experiments. **p<0.01.

(B) Gating from total γδ T population (A) then gated for Vγ6 γδ T %. Representative of 2 experiments. *p<0.05, **p<0.01

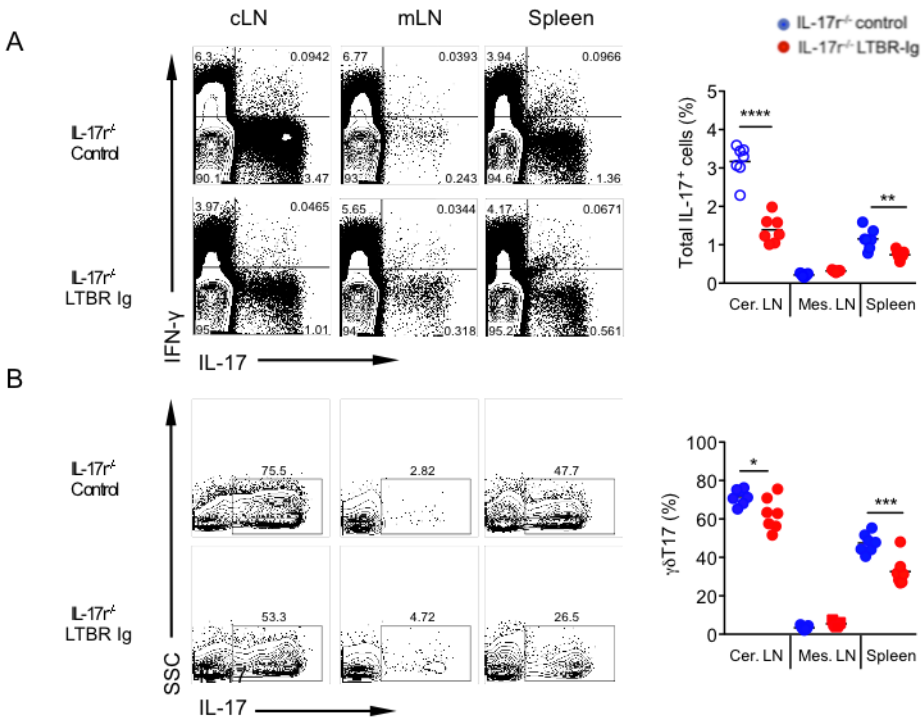


Figure 25. Partial LN depletion with LTBR-Ig decreases expansion of Vγ6 γδT17 in cLN and spleen but not mLN

LTBR-Ig depleting protein was injected I.P. into D20 post-conception pregnant mice which results in partial depletion of some lymph nodes (Inguinal) and reduction in size of other LNs (Cervical/Mesenteric) in 6-8 week old adult mice.

(A) Gating on total lymphocyte population then gated for IL-17 and IFN-γ-producing cells. Representative of 2 experiments. **p<0.01, ***p<0.001

(B) Gating from total Vγ6 γδ T population (B) then gated for IL-17-producing Vγ6 γδT cell %. Representative of 2 experiments. *p<0.01, ***p<0.001

Deficiency in IL-17r signaling leads to upregulation of RORγτ, suppression of BTLA and expansion of IL-17^{+} γδ T cells in some but not all tissues

IL-7 has been shown to specifically expand γδT17 cells in mice and humans and B- and T- lymphocyte attenuator (BTLA) directly regulates γδ T cell expansion through suppression of IL-7 signaling (Bekiaris et al., 2013; Michel et al., 2012). The master transcriptional regulator of IL-17 production, RORγτ, inhibits the transcription of BTLA. We thus hypothesized that lack of IL-17 signaling through its receptor would increase IL-17 mRNA levels by increased levels of RORγt thus decreasing BTLA regulation of IL-7 signaling. Indeed, we found increased RORγt in IL-17r$^{-/-}$ CD27^{-}γδT cells from cLNs and iLNs (Figure 26). Knowing that RORγτ inhibits the transcription of BTLA, we stained for extracellular surface expression of BTLA and found a decrease in expression (Figure 27). An increase in RORγτ and a decrease in BTLA levels suggest a decrease inhibition of IL-7 signaling leading to a γδT17 specific expansion. However, IL-7r expression on γδT cells or levels of IL-7 mRNA in various tissues in the absence of IL-17 signaling was comparable with WT mice (data not shown). To investigate whether decreased expression of BTLA would lead to intrinsic expansion of IL-17r$^{-/-}$ Vγ6 γδ T cells over WT Vγ6 γδ T cells, we irradiated δTCR$^{-/-}$ mice and reconstituted their γδT cells with a 1:1 ratio of IL-17r$^{-/-}$ and WT Vγ6 γδ T cells from neonatal thymocytes. Eight weeks after reconstitution, the data suggested a slight advantage of IL-17r$^{-/-}$ γδ T cells but only in certain tissues such as LNs and spleen but not in the skin and lungs (Figure 28). Interestingly, this intrinsic advantage was seen strongest in the cLNs specifically but only in total γδ T cells and not γδT17 frequency. If gating on total Vγ6 γδT cells the percentage is similar between WT and IL-17r$^{-/-}$ (data not shown) since this population is

centrally preprogrammed in the thymus produce IL-17 so in this case it is more accurate to look at the total Vγ6 rather than more specifically the IL-17 producing in order to determine any intrinsic advantage after reconstitution. These data suggest that γδT17 cell expansion is partly regulated by the presence of IL-17R signaling through the intrinsic RORγt-BTLA regulatory pathway. However, this modest intrinsic defect advantage does not explain a monoclonal expansion of Vγ6 γδT17 cells in all peripheral tissues besides the thymus, colon and mLNs with a specific and drastic expansion noted in the cLNs.

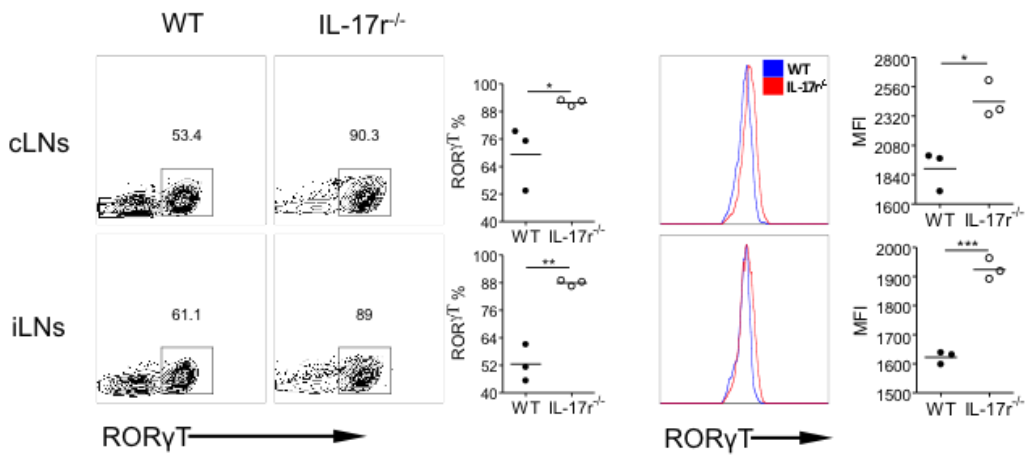


Figure 26. IL-17r$^{-/-}$ CD27^{-} γδT17 cells have increased RORγt expression level.

(A) Gated on total CD27^{-} γδT from IL-17r$^{-/-}$ (red) and WT (blue) then analyzing for % and MFI of RORγt. Representative of 3 experiments. *p<0.05, **p<0.01, ***p<0.001

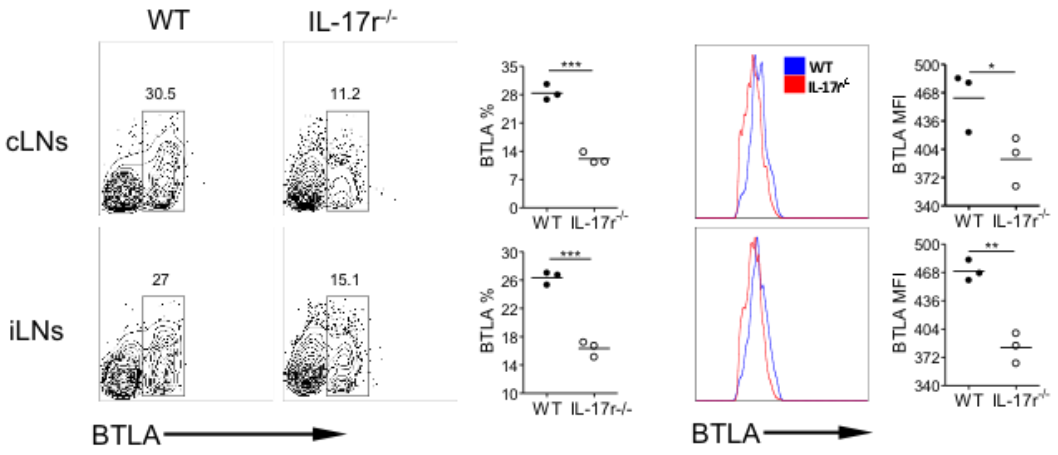


Figure 27. IL-17r$^{-/-}$ CD27^{-} γδT17 cells have decreased BTLA expression.

(A) Gated on total CD27^{-} γδT from IL-17r$^{-/-}$ (red) and WT (blue) then analyzing for % and MFI of BTLA. Representative of 3 experiments. *p<0.05, **p<0.01, ***p<0.001

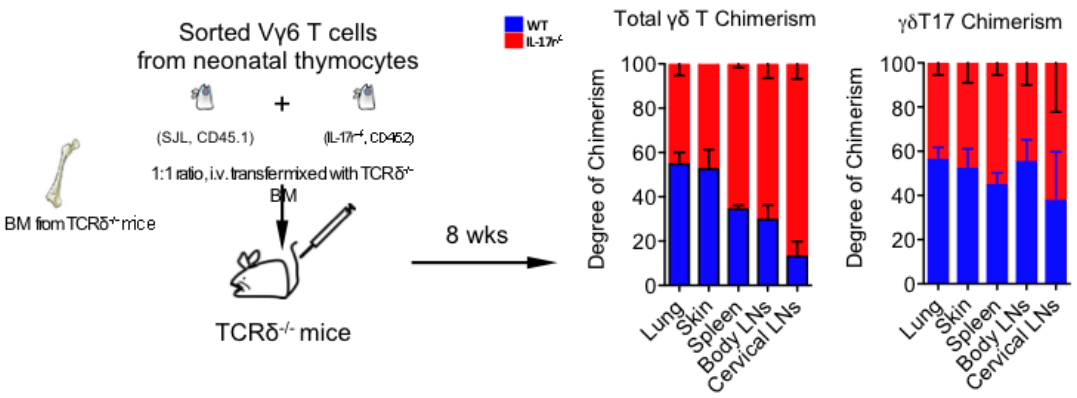


Figure 28. Neonatal IL-17$^{-/-}$ Vγ6 γδT from thymocytes show some reconstitutive advantage over their WT counterparts particularly in the spleen and LNs.

(A) Diagram of the neonatal thymocyte and adult BM reconstitution protocol implemented. Thymic Vγ6 γδT cells from neonatal WT SJL (CD45.1) and IL-17r$^{-/-}$ mice were mixed at 1:1 ratio and then adoptively transferred along with BM cells from *Tcrd*$^{-/-}$ mice into lethally irradiated *Tcrd*$^{-/-}$ recipient mice. Mice were reconstituted for 8 weeks and tissues from the lungs, skin, spleen, body LN, and cLN were harvested and stimulated with PMA/ionomycin. Total γδT cells and γδT17 cells from WT or IL-17r$^{-/-}$ were differentiated using congenic markers CD45.1 versus CD45.2. The bar graphs, combined data from 2 experiments with 3 mice each, show the degree of chimerism in the lungs, skin, spleen, body LNs (all LNs except cLN and mLN) and cLNs. Degree of chimerism was calculated using the before and after reconstitution ratio of WT:IL-17r$^{-/-}$ Vγ6 γδ T.

γδT17 cells expand due to DC-dependent proliferation

Different mechanisms could lead to a population's expansion such as increased survival, less apoptosis or proliferation. We searched to find in what way the Vγ6 γδ T cells were expanding especially in the cLNs where the phenotype was most prominent. *Ex vivo* immunostaining for the proliferation protein Ki-67 confirmed specific and significant proliferation in $CD27^{-}CCR6^{+}$ γδ T cells (Figure 29). These are drastic changes considering in WT mice the predominant γδT proliferative cells were $CD27^{+}CCR6^{-}$ γδT cells. A previous study showed that IL-7 specifically drives γδT17 expansion in the LNs (Michel et al., 2012). Therefore we examined whether γδT17 in IL-$17r^{-/-}$ LNs responded more to IL-7 stimulation. Surprisingly, we observed that using whole cells from IL-$17r^{-/-}$ mice after 5 days of culture led to spontaneous endogenous proliferation of γδ T cells without any stimulation, mainly γδT17 cells (Figure 30). Accounting for the endogenous proliferation, we did not see an advantage of γδT17 from IL-$17r^{-/-}$ mice over WT when stimulated with LPS or IL-7. LPS was used as a control because its been shown that $CD27^{+}$ (IFN-γ capable) γδT cells express TLR4 preferentially while $CCR6^{+}$ (IL-17 capable) γδT cells express other receptors such as IL-7r, TLR2, and dectin-1 (Martin et al. 2009). Our data supports Martin et al's conclusions showing that LPS indeed induces the proliferation of IL-17^{-} γδT cells with much higher IL-17^{-} γδT proliferation shown in the WT mice due to the higher frequency of $CD27^{+}$ γδT cells. Endogenous proliferation was not observed in other tissues such as lungs, spleen or bone marrow (Figure 31). It was also noted that γδT cell percentage in the bone marrow was very low similar to WT again confirming that γδT17 regulation through IL-17 signaling is peripheral and not centrally regulated either in the thymus or bone marrow.

This *in vitro* spontaneous proliferation system allowed us to specifically identify the mechanism of γδT17 proliferation. Endothelial cells have been shown to be the major producers of IL-7 (Iolyeva et al., 2013). However, by sorting out $CD45^-$ cells from total cLN homogenate then culturing for 5 days, we observed no difference in proliferation of γδT17 cells *in vitro* (Figure 32). We have previously shown that DCs are important inducers of γδT17 proliferation through the production of IL-1β/IL-23 (Cai et al., 2011; Wu et al., 2014). Therefore, we next examined whether γδ T proliferation in this system was dependent on DCs. Indeed, by depleting $CD11c^+$ DCs, γδT17 cell proliferation was significantly reduced and when adding DCs back we restored γδT17 proliferation (Figure 33). Next, we examined γδ T cell and DC interaction *in situ* in the IL-$17r^{-/-}$ cLNs using immunofluorescence and confocal microscopy. We saw increased total γδ T cells as well as $CD11c^+$ DCs validating our flow cytometry findings. At lower magnification we did see increased co-localization between γδ T cells and DCs not seen in the WT control cLNs. At higher magnification in the IL-$17r^{-/-}$ cLNs, close and intimate interactions between γδ T cells and DCs were readily seen (Figure 34). Thus, DCs from cLNs may directly interact and induce γδT17 proliferation and expansion.

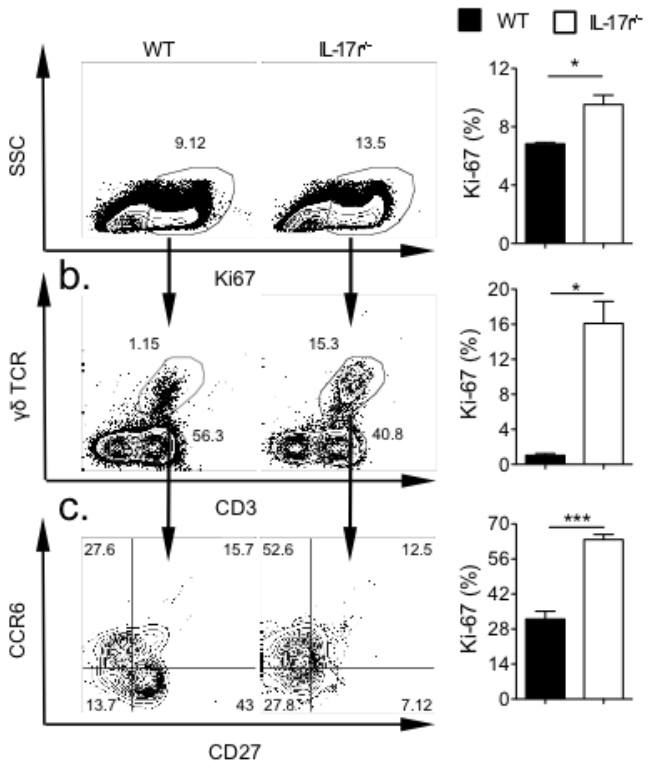


Figure 29. Expansion of γδT population in the lack of IL-17 signaling is due to cell proliferation

(A) *Ex vivo* staining of WT and IL-17r$^{-/-}$ cLN cells for proliferation marker, Ki-67, to calculate total proliferating cells and contribution of γδ T and specifically CCR6^{+}CD27^{-} γδ T to proliferation. Representative of 2-3 experiments. *p<0.05, ***p<0.001

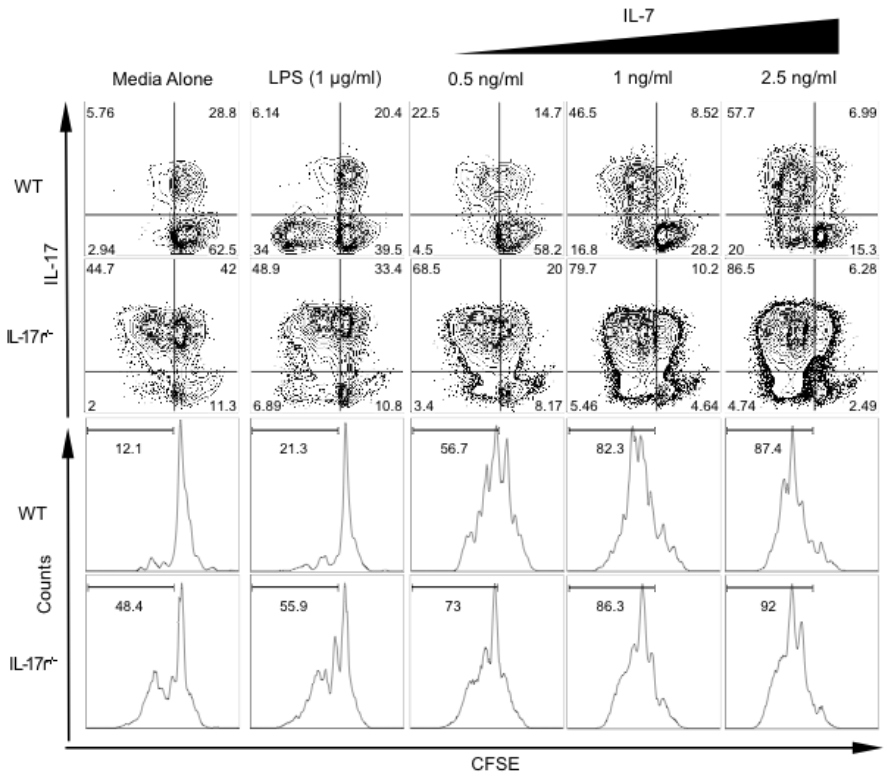


Figure 30. Endogenous proliferation of γδT17 cells in the absence of IL-17r *in vitro*

(A) CFSE *in vitro* assay with no stimulation, LPS stimulation and IL-7 titrated stimulation of total cLN cells from WT and IL-17r$^{-/-}$ mice for 5 days then re-stimulated for 5 h with PMA/Ionomycin to examine proliferation of γδT17. Gated on total γδ T cells. Dot plots and histograms representative of 5 experiments.

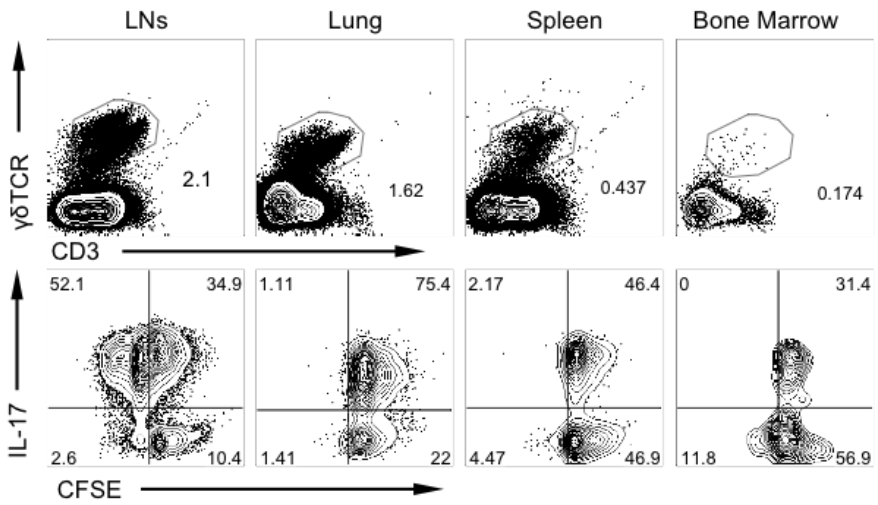


Figure 31. Endogenous proliferation of γδT17 as seen in only the LNs and not other tissues.

(A) Gating on total live lymphocyte population in different tissues then gated on total γδT cells to analyze the γδT17 proliferation using IL-17 vs CFSE. Representative of 2-3 experiments.

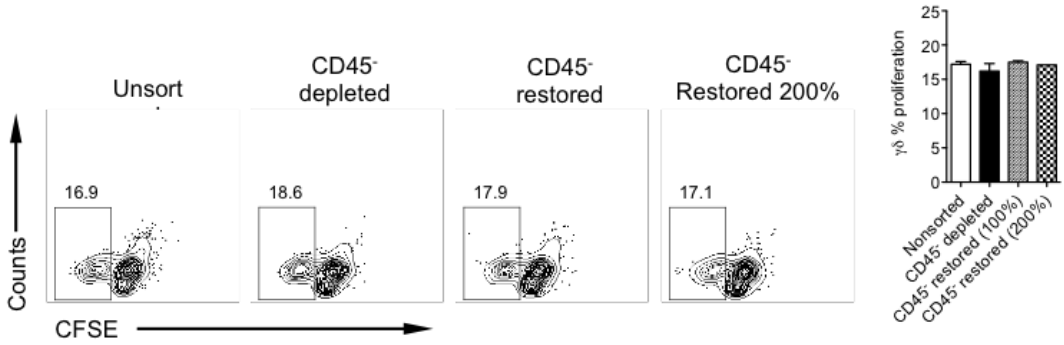


Figure 32. Endogenous proliferation of γδT17 is independent of CD45$^-$ cells

(A) Depleted CD45$^-$ cells from IL-17r$^{-/-}$ LNs using FacsAria to access CD45$^-$ cell contribution to endogenous proliferation after CFSE labeling and 5 days culture. Representative of 3 experiments.

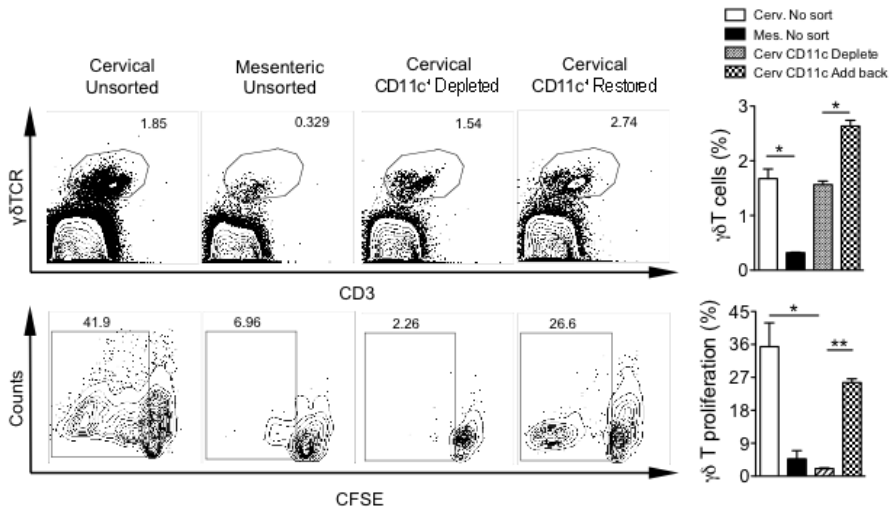


Figure 33. γδT17 cells expand due to DC-dependent proliferation

(A) Depletion of $CD11c^{+}$ cells from IL-$17r^{-/-}$ cLNs or adding back of $CD11c^{+}$ cells to examine γδT cell proliferation after 5 days culture. Cells were gated on $7AAD^{-}$ $CD3^{+}\gamma\delta TCR^{+}$ cells. Representative of 5 experiments. *$p<0.05$, **$p<0.01$

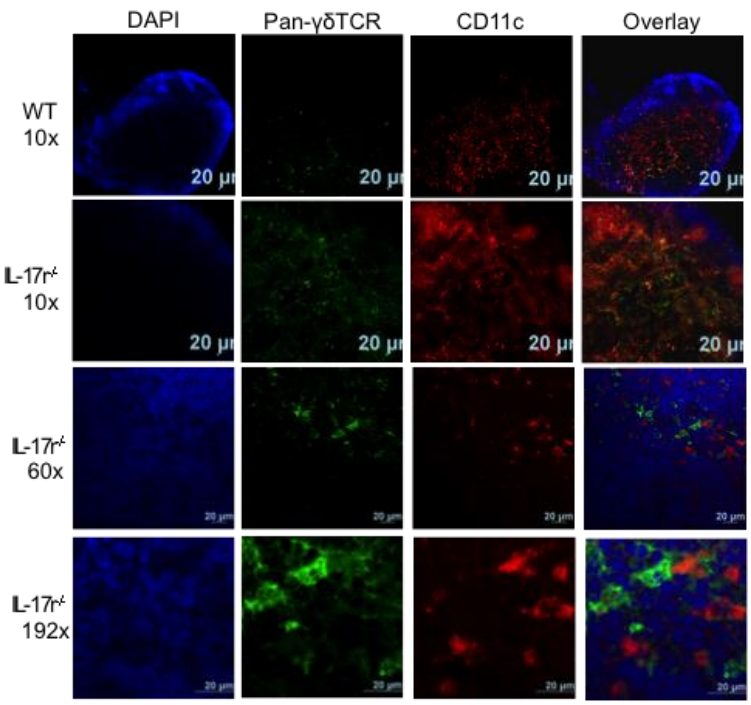


Figure 34. γδT and DC expanded and co-localized in situ with close interaction of two populations

(A) Immunohistochemistry staining of WT and IL-17r$^{-/-}$ cLNs with pan-γδTCR (green), CD11c (red) and DAPI (blue) to visually show increase in γδ T cells and DCs (left) and to show γδ T cell and DC interaction *in situ* in IL17r$^{-/-}$ cLNs (right). Representative of cLNs from 3 different mice. Scale bar: 20 μm.

CD103$^+$ DCs specifically induce Vγ6 γδT17 cell proliferation

DCs in LNs are composed of various subsets with different functions. We noticed that in cLNs CD103$^+$ DCs (CD11b^{lo}CD11c^{hi}CD103^{hi}) were increased in frequency within total CD11c$^+$ DCs (Figure 35). Not only was frequency increased but the side-scatter (SSC) for IL-17r$^{-/-}$ CD103$^+$ DCs was increased which is correlative with increased activation (Crawford et al., 2003). The increased CD103$^+$ DCs were in fact more activated with upregulated CD80 and CD86 expression levels but not MHC class II (Figure 36). cLNs in the IL-17r$^{-/-}$ mice under H&E show hypertrophy and germinal center reactivity suggesting there might be bacterial activation of the immune system (Figure 37). To determine whether these DCs could be activated by bacterial Ags, we utilized 16s rRNA FISH hybridization and confocal microscopy to probe for bacterial 16s rRNA in the cLNs. The enlarged IL-17r$^{-/-}$ cLNs showed a drastic increase in the presence of 16s rRNA compared to WT counterparts, suggesting that CD103$^+$ DC activation could be due to bacterial product exposure (Figure 38).

To investigate whether CD103$^+$DCs drive γδT17 cell proliferation, we depleted this subset from whole cLN and cultured the cells without stimulation for 5 days. γδT17 cell proliferation was reduced by 75% after CD103$^+$DCs depletion and restored by adding back CD103$^+$ DCs to culture (Figure 39). In addition, IL-1β Ab blockade did not reduce γδT17 proliferation. To further determine whether γδT17 expansion can be induced by cLN CD103$^+$DCs, WT γδ T cells were co-cultured with different DC populations including CD103$^+$ DCs either from cLNs or mLNs of IL-17r$^{-/-}$ mice (Figure 40). Only CD103$^+$ DCs from the cLNs were able to induce proliferation of the WT γδ T and this CFSE^{low} population being negative for Vγ4 and Vγ1 thus Vγ6$^+$ is consistent with *ex vivo*

cLN staining. Consistent with the IL-1β blockade results, γδT cell from IL-1r$^{-/-}$ proliferate well when co-cultured with cLN CD103^{+} DCs (Figure 41), suggesting that γδT17 proliferation is not dependent on IL-1r signaling. To examine whether CD103^{+} DCs drive γδT17 proliferation *in vitro* either through cell-to-cell contact or soluble factors, WT γδ T cells were co-cultured with CD103^{+} DCs from IL-17r$^{-/-}$ mice in a transwell system. The co-cultured γδ T cells with the CD103^{+} DCs showed strong proliferation, predominately Vγ6. However, when separated from the CD103^{+} DCs, the WT γδ T cells did not proliferate (Figure 42), suggesting that a cell-to-cell interaction between DCs, specifically CD103^{+}DCs and γδT17 cells is required for the induction of their proliferation.

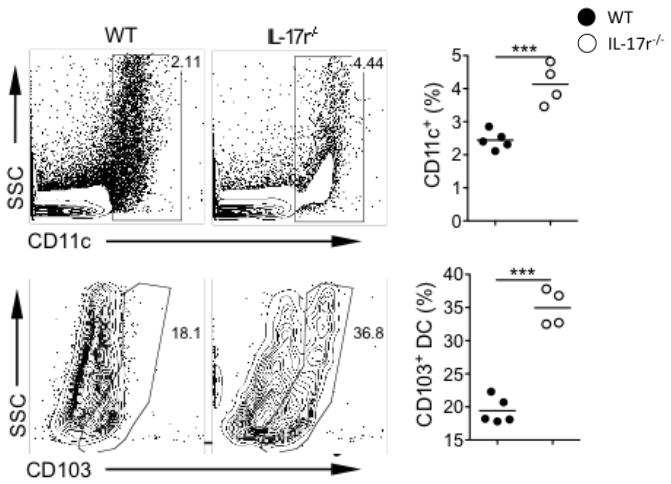


Figure 35. CD11c+ DCs and specifically CD103$^+$ DCs expanded in cLNs

(A) After *ex vivo* staining of cLN cells and gating from total live cells, CD11c$^+$ cells were gated for calculating total DCs (top) then from this population CD103$^+$ DCs were gated (bottom). Representative of 2-3 experiments. ***p<0.001

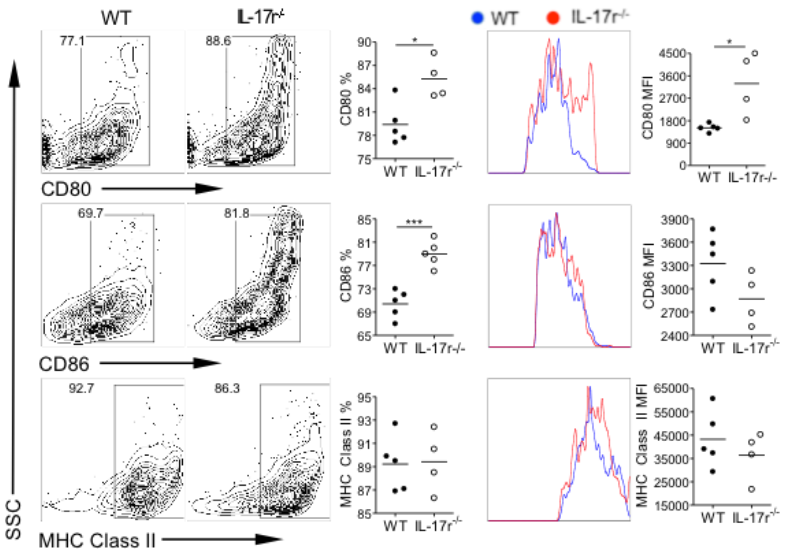


Figure 36. CD103^{+} DCs in cLNs activated with increased expression of CD80 and CD86

(A) Gated on total CD103^{+} DCs from WT and IL-17r$^{-/-}$ cLNs, staining of surface activation markers CD80, CD86 and MHC Class II was performed. Both percentages and MFI are shown. Representative of 2-3 experiments. *p<0.05, ***p<0.001

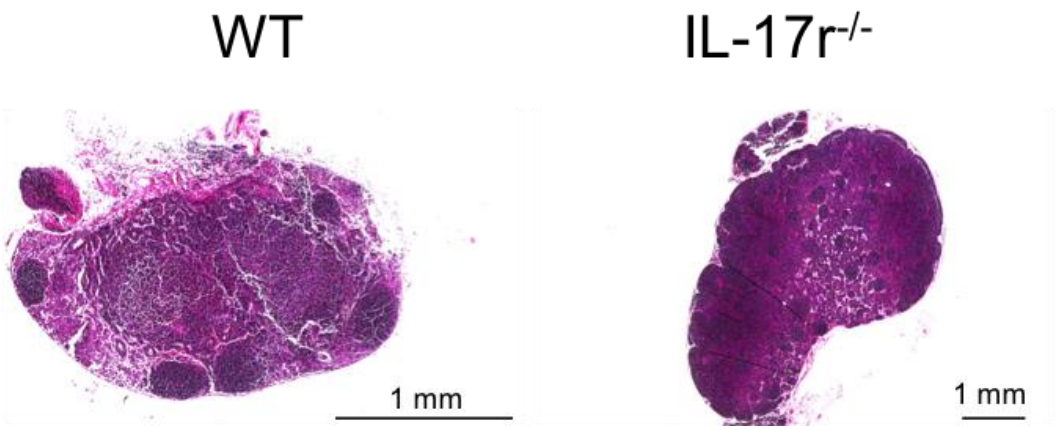


Figure 37. Hypertrophy and increased germinal center reactions in IL-17r$^{-/-}$ oral draining cLN

(A) Sectioning and H&E staining was performed by the University of Louisville Department of Pathology. Sections were analyzed and photographed using Asperio ScanScope. Images representative of 5 cLNs from WT and IL-17r$^{-/-}$.

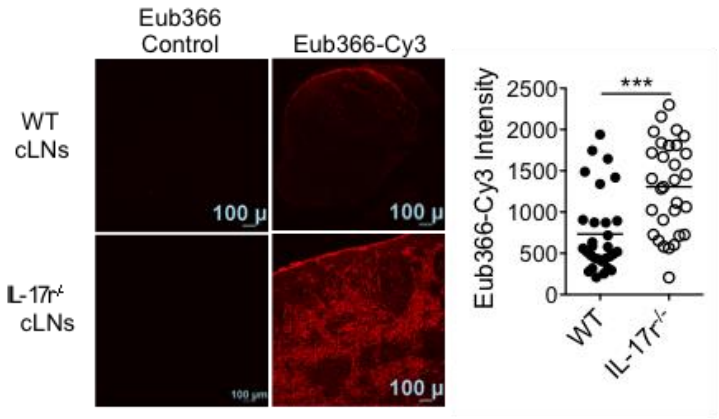


Figure 38. 16s rRNA drastically increased in cLNs of mice lacking IL-17 signaling

(A) 16s rRNA FISH hybridization on frozen tissue sections from WT and IL-17r$^{-/-}$ cLNs. Probe Eub336-cy3 was used and TRIC signal intensity at random yet the same locations for WT and IL-17r$^{-/-}$ cLN was used to quantify the amount of bacterial RNA in LNs. Representative of 3 different cLNs from 3 mice. ***p<0.001

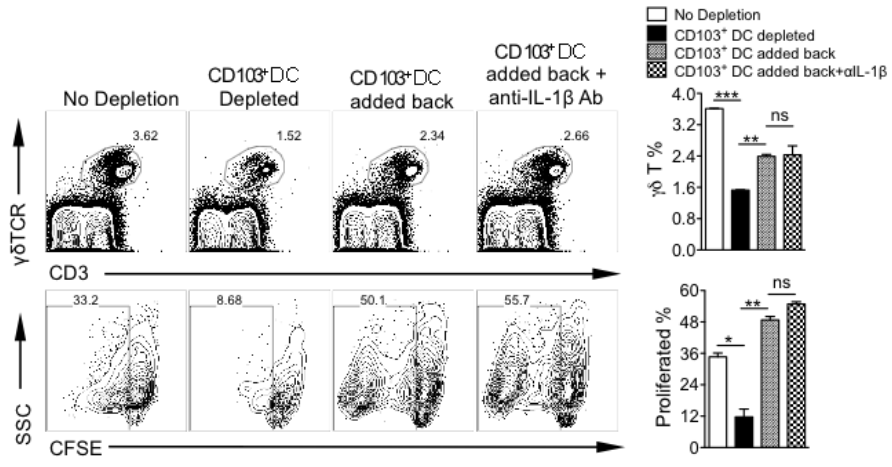


Figure 39. CD103$^+$ DC depletion reduces proliferation of γδT cell *in vitro*

(A) CFSE-labeled whole cLN cells were depleted of CD103$^+$ DCs and cultured for 5 days. In conditions of whole cLN cells depleted with CD103$^+$DCs, sorted CD103$^+$DCs were added back in the presence or absence of anti-IL-1β neutralizing mAb (2 μg/ml). γδT cell proliferation was shown. Cells were gated on 7AAD$^-$CD3$^+$γδTCR$^+$ cells. Representative of 5 experiments. *p<0.05, **p<0.01, ***p<0.001. n.s., not significant

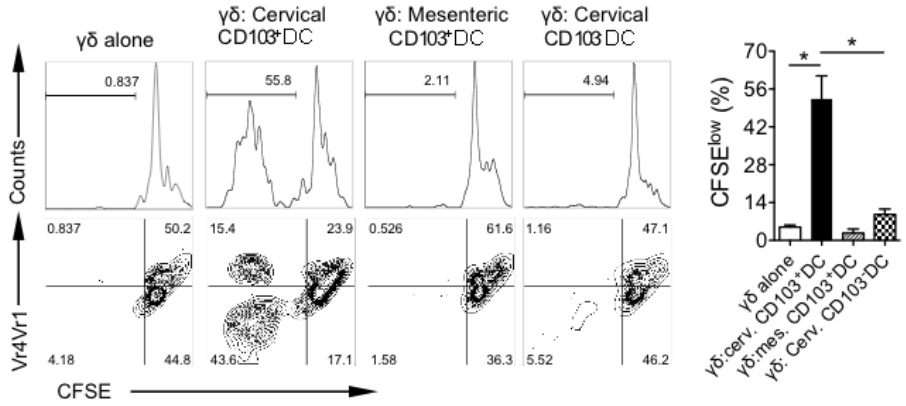


Figure 40. CD103$^+$ DCs specifically from cLNs not mLNs induce Vγ6 γδT17 cell proliferation

(A) Using MoFlo sorter, WT γδ T cells from LNs and spleens were purified and CFSE labeled then co-cultured with three different IL-17r$^{-/-}$ DC populations (CD103$^+$ cLNs, CD103$^+$ mLNs, and CD103$^-$ cLNs) for 5 days. Representative histograms of γδT cell proliferation and summarized percentages of proliferated cells are shown (upper panel). Representative dot plots of proliferated γδT cells with Vγ4/Vγ1 staining are shown (bottom panel). Cells were gated on 7AAD$^-$ CD3$^+$γδTCR$^+$ cells. Representative of three experiments. *p<0.05

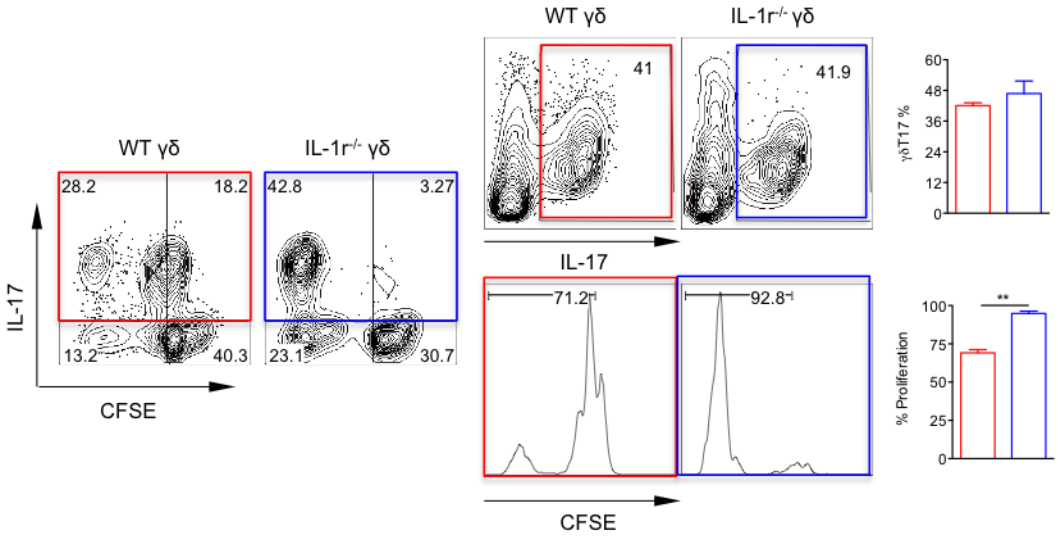


Figure 41. CD103+ DC induction of γδT17 proliferation is IL-1r independent

(A) $CD103^{+}$ DCs co-cultured with WT and $IL\text{-}1r^{-/-}$ γδ T cells (1:5) to determine the role of γδT IL-1r expression in the proliferation of γδT17 (left panel). Gated on total γδT population and then showed total $IL\text{-}17^{+}$ cells after 5 days co-culture. Gated on total γδT17 and then gated for CFSE to analyze the % of proliferated γδT17 from co-cultures. Representative of 3 experiments. **p<0.01

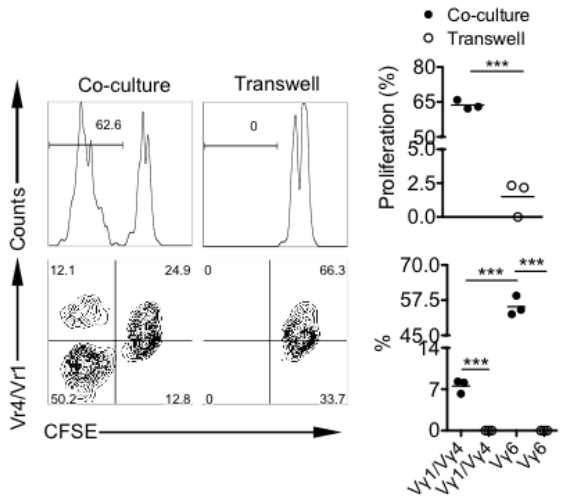


Figure 42. CD103^{+} DCs induction of Vγ6 γδT17 cell proliferation is cell-to-cell contact dependent

(A) 96-well transwell plate was utilized to separate CD103^{+} DCs from cLNs of IL-17r$^{-/-}$ mice from WT γδ T cells to determine if cell-to-cell contact is required. Representative histograms of γδT cell proliferation and summarized percentages of proliferated cells are shown (upper panel). Representative dot plots of proliferated γδT cells with Vγ4/Vγ1 staining and summarized data are shown (bottom panel). Cells were gated on 7AAD^{-}CD3^{+}γδTCR^{+} cells. Representative of 3 experiments. ***p<0.001

Oral but not gut microbiota influences the expansion of γδT17 cells in the draining cLNs

Previous studies have shown an important role for microbiota in the expansion of γδT17 cells during various disease settings (Cheng et al., 2014; Duan et al., 2010; Wu et al., 2014). In the gut, the increase of SFB leads to increased frequency of Th17 cells (Goto et al., 2014). The 16s rRNA gene sequencing data of fecal gut microbiota indeed showed that WT and IL-17r$^{-/-}$ mice have distinct gut microbiota (Figure 43). In addition, IL-17r$^{-/-}$ mice had increased SFB in the gut that correlated with increased Th17 cells in the lamina propria of the colon (Figure 43). However, we did not see a difference in γδ %, Vγ usage composition or an increased γδT17 cells in the colon (Figure 44). To further examine the effect of gut microbiota on γδT17 cells, we transferred fecal samples from IL-17r$^{-/-}$ mice to WT mice via gavage needle injection to avoid any oral cavity contamination. However, we did not observe γδT or Vγ6 γδT expansion in the WT mice transplanted with IL-17r$^{-/-}$ gut microbiota (Figure 45). Staining for CCR6 and CD27 we were able to determine that IL-17-capable (CCR6+CD27-) γδT cells were not expanded as well as CCR6+ (chemokine specific for IL-17 producing Th17 cells) CD4 T cells (Figure 46). Even though not significant we observed an increasing trend in CCR6^{+} CD4 T cells in the colon LP after transfer with IL-17r$^{-/-}$ fecal transplant which is consistent with increased Th17 in the colon (Figure 44) most likely due to increase SFB levels in IL-17r$^{-/-}$ gut microbiota (Figure 43).

Considering the drastic phenotype in the cLNs and the increase in migratory CD103^{+} DCs and bacterial products there, we investigated the effect of oral microbiota on γδT17

cell regulation. Unlike the gut microbiota composition, the overall oral microbiota composition at phylum and genus levels was significantly altered in IL-17r$^{-/-}$ mice compared to WT (Figure 47). Specifically, *Lactobacillus* genus (Firmicutes phylum) were significantly increased in IL-17r$^{-/-}$ mice compared to WT. In contrast, *Aggregatibacter* genus was significantly decreased in IL-17r$^{-/-}$ mice. The statistical analysis of these distance matrices was performed using ANOSIM test with 999 permutations. The results demonstrated that both weighted and unweighted UniFrac distances were significantly different compared to each other indicating definitive differential microbiota composition between WT and IL-17r$^{-/-}$ mice. We next utilized broad spectrum antibiotic treatment (ampicillin, neomycin, vancomycin and metronidazole) from prior to birth all the way to 6 weeks old to determine whether γδT17 expansion could be abrogated. Treatment with the oral antibiotic regimen prevented the increase in size and absolute cell number of the cLNs but did not affect the size and absolute number of cells in other LNs (Figure 48). γδ T cell %, Vγ6 % and γδT17 % and absolute numbers were all decreased in the cLNs (Figure 49) as well as other peripheral tissues including the spleen and lungs but importantly not in the colon (Figure 50). Together these data suggest that microbiota in the oral cavity and not the colon play an important role in the monoclonal proliferation of Vγ6 γδT17 cells leading to systemic expansion of γδT17 cells.

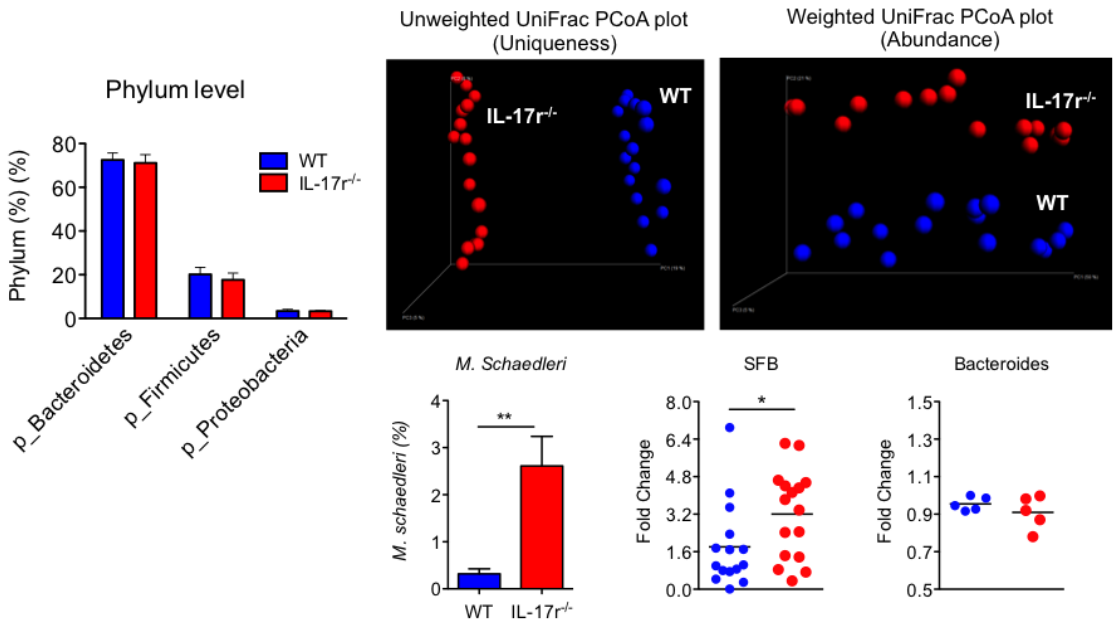


Figure 43. Gut microbiota composition is different in the absence of IL-17 signaling

(A) Bacterial genomic DNA was extracted from the fecal droppings of 15 WT and 15 IL-17r$^{-/-}$ then using deep sequencing to analyze for differences in microbiota populations. RT-PCR was used in order to confirm SFB and Bacteroides findings. *p<0.05, **p<0.01

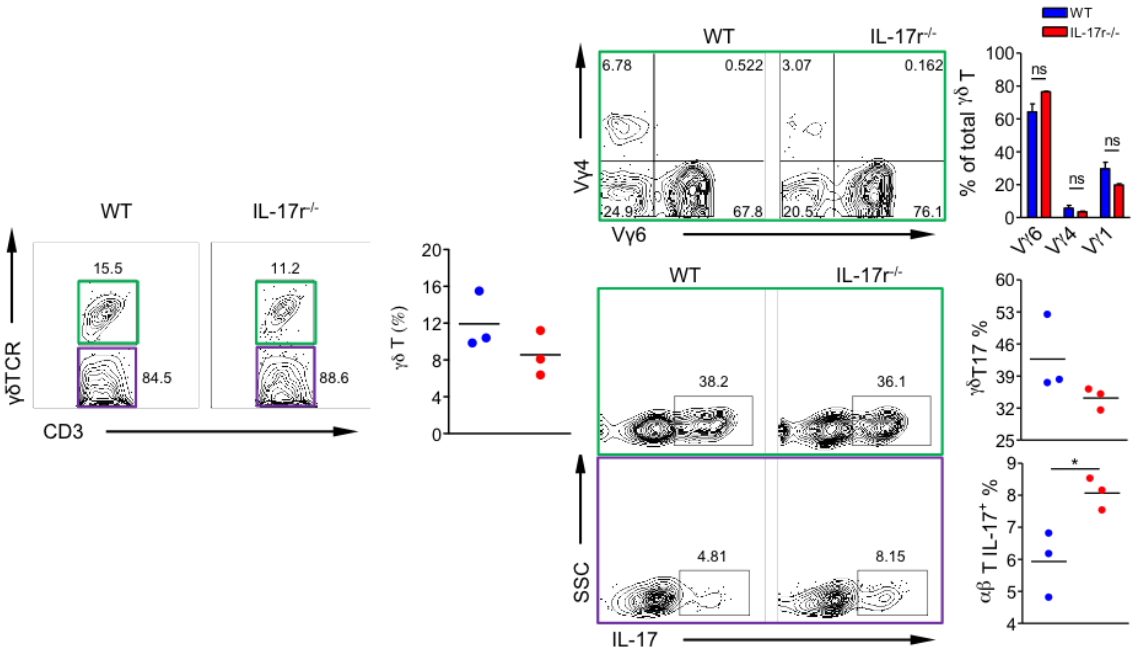


Figure 44. Differences in gut microbiota in IL-17r$^{-/-}$ mice does not lead to a difference in lamina propria γδ T populations but does lead to increase in Th17 responses.

(A) Gated on total live CD45^{+} cells in the lamina propria of the colon after tissue processing and cell homogenization. From live CD45^{+} cells gated CD3 vs γδTCR to analyze γδT % and αβ T %. From these two populations gated further looking at Vγ expression of Vγ4 and Vγ6 in γδ % population as well as IL-17^{+} cells in the γδT and αβ T populations. 5 h PMA/I stimulation was used to look at IL-17 responses. Representative of 3 experiments. *p<0.05

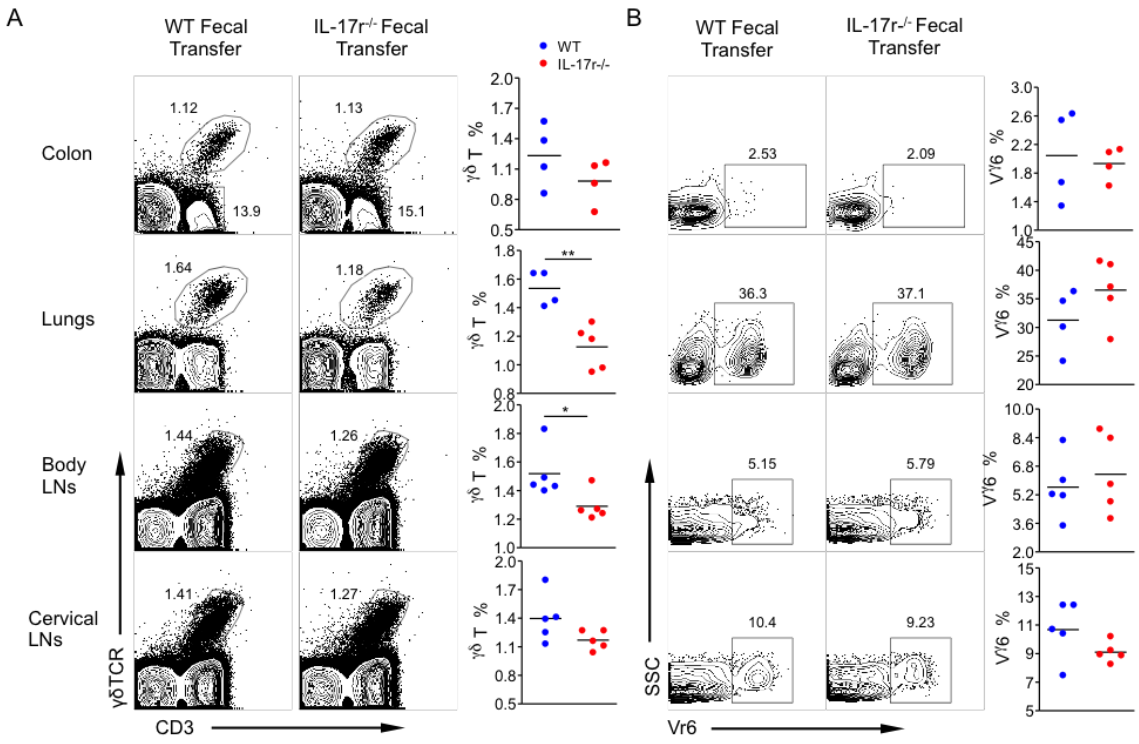


Figure 45. Fecal transfer from IL-17r$^{-/-}$ mice to WT mice does not induce γδT or Vγ6 expansion

Gastric gavage needle injection was used to do 3 times treatment of 12 mg IL-17r$^{-/-}$ or WT fecal matter to 10 WT mice in order to determine whether IL-17r$^{-/-}$ gut microbiota directly induces a change in γδT populations and γδT17 expansion.

(A) Gating on total live lymphocyte population and then gated on CD3 vs. γδTCR to quantify γδ T %. *p<0.05, **p<0.01.

(B) Gating on the total γδT population and then gated for Vγ6^{+} %.

(C) Gating on total γδT cells and then gated for CD27 vs CCR6^{+} in order to analyze the CCR6^{+}CD27^{-} potential γδT17 %.

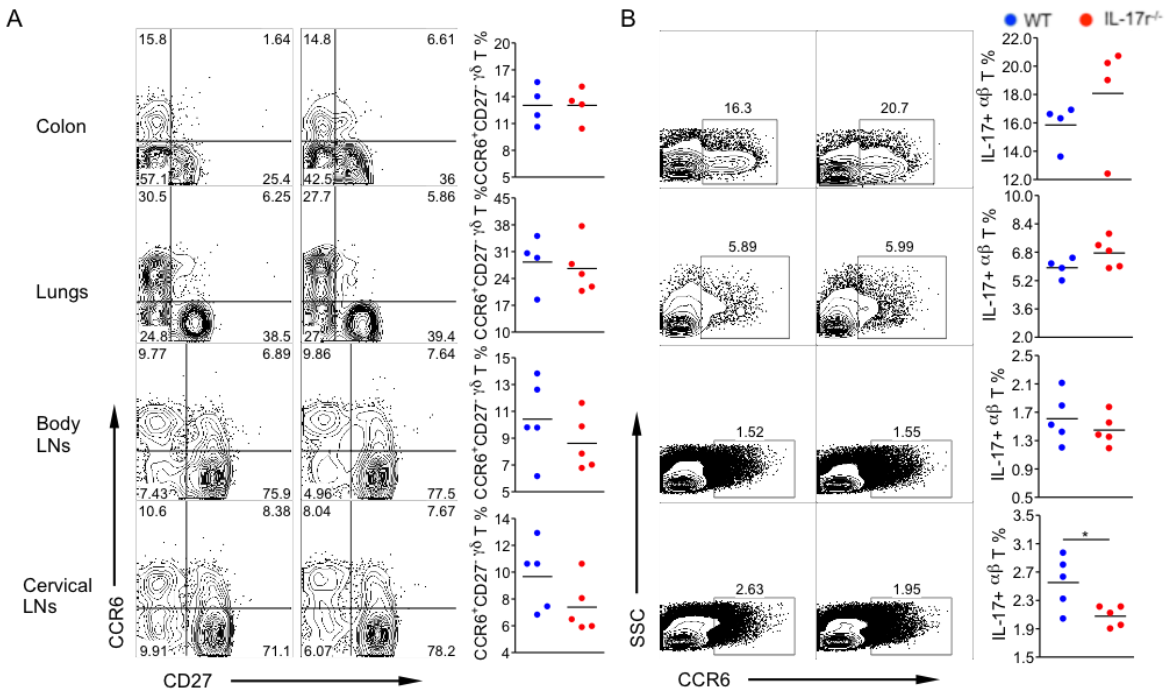


Figure 46. Fecal transfer from IL-17r$^{-/-}$ mice to WT mice does not induce CCR6^{+}CD27^{-} γδT cells or CCR6^{+} CD4 T cells

Gastric gavage needle injection was used to do 3 times treatment of 12 mg IL-17r$^{-/-}$ or WT fecal matter to 10 WT mice in order to determine whether IL-17r$^{-/-}$ gut microbiota directly induces a change in γδT populations and γδT17 expansion.

(A) Gating on total γδT cells and then gated for CD27 vs CCR6^{+} in order to analyze the CCR6^{+}CD27^{-} potential γδT17 %.

(B) Gating on total CD4 and then gated for CCR6^{+} in order to analyze the CCR6^{+} (critical Th17 chemokine receptor) CD4 %.

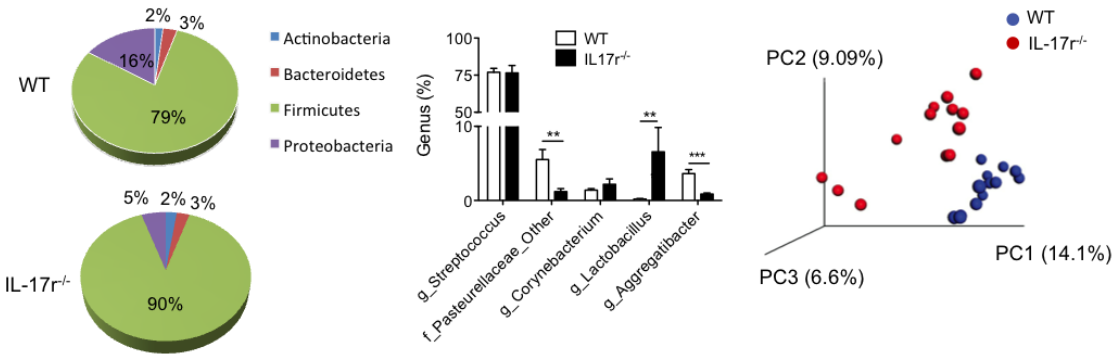


Figure 47. Oral Microbiota is different in the lack of IL-17 signaling

(A) The oral microbiota from WT and IL-17r$^{-/-}$ mice was analyzed by sequencing 16s rRNA gene (v1-v3 region) and analyzed using QIIME 1.9.0 software platform. The abundant phylum and genus distribution between WT and IL-17r$^{-/-}$ suggest alterations in the microbial composition. Principle coordinate analysis of unweighted UniFrac distance matrix showing the distinct clusters for WT (blue) and IL-17r$^{-/-}$ (red) mice. ** $p<0.001$; *** $p<0.0001$

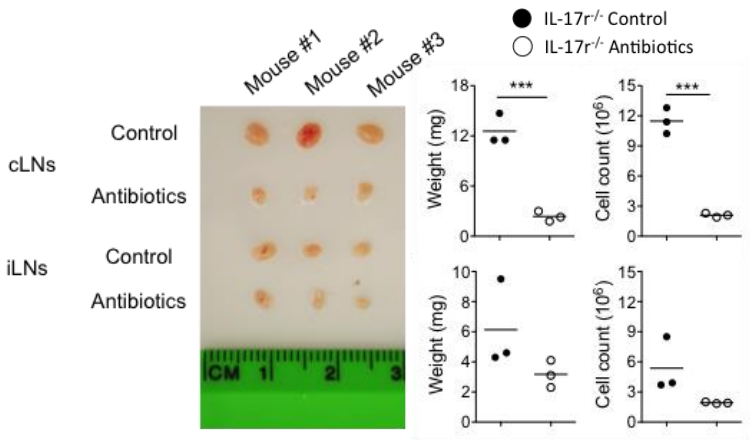


Figure 48. Oral Broad Spectrum Antibiotics reduces the size of cLNs specifically

(A) cLNs (upper) and iLNs (lower) after oral antibiotics treatment for 6 weeks along with the corresponding weight of each node and the total cell number from that LNs. Representative of 2-3 experiments. ***$p<0.001$

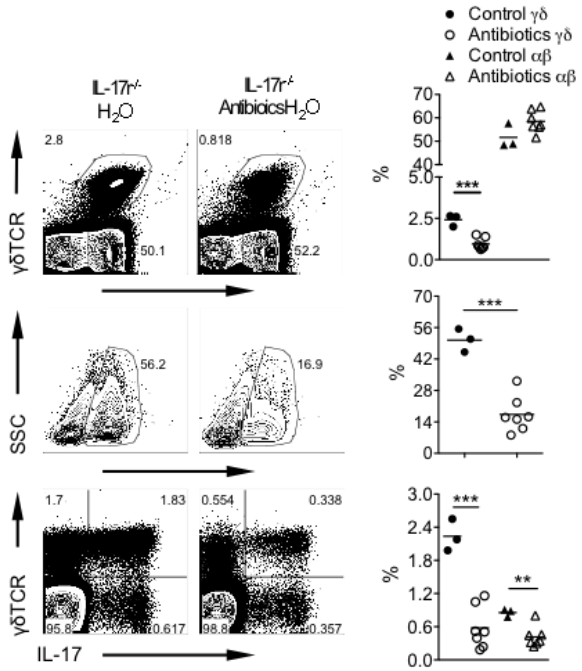


Figure 49. Oral Broad Spectrum Antibiotics Lead to Drastic Decrease in Vγ6 γδT17 population in the cLNs

(A) Flow cytometry analysis showing percentages of γδ T cells, αβT cells, Vγ6 γδ T cells and IL-17-producing γδ T and αβ T cells in the cLNs after 6 week oral antibiotics treatment from 1-2 days prior to birth to 6 weeks of age. Representative of 2-3 experiments. *p<0.05, **p<0.01, ***p<0.001

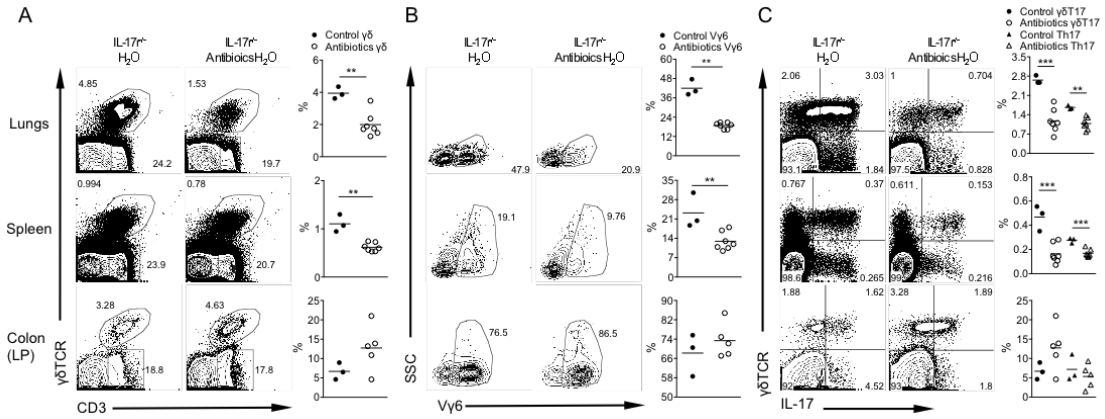


Figure 50. Oral Broad Spectrum Antibiotics Lead to Drastic Decrease in Vγ6 γδT17 population in the lungs and spleen but not the colon

(A) Flow cytometry analysis showing percentages of γδ T cells, Vγ6 γδ T cells, γδT17, and Th17 in the lungs, spleen and colon (lamina propria) after 6 week oral antibiotics treatment from 1-2 days prior to birth to 6 weeks of age. Representative of 2-3 experiments. *p<0.05, **p<0.01, ***p<0.001

Microbiota exchange induces the proliferation of γδT17 cells in the cLNs and systemic expansion of γδT17 cells

To examine the influence of microbiota on the peripheral regulation of γδT17 cells, without affecting the development of mucosal immunity, we cohoused animals together (Kabat et al., 2014; McDermott and Huffnagle, 2014). WT mice were cohoused with IL-17r$^{-/-}$ mice for 10 weeks after weaning. Co-housing resulted in expansion of T cells, γδT cells and Vγ6^{+} γδT cells in the cLNs, skin and lungs (Figure 51). After stimulation, an observed increase in γδT17 and Vγ6^{+} γδT17 cells was also seen (Figure 52). Conferral of γδT17 expansion by microbiota exchange was strongest in the cLNs and skin which also resulted in a significant increase in γδT17 cells in the spleen. This finding suggests that oral microbiota can in fact induce the specific induction of Vγ6 γδT17 systemic expansion. Th17 cells were also increased in the skin and LNs however not in the spleen (Figure 53) again suggesting that the phenotype of systemic expansion is γδT17 specific. As previously shown that microbiota led to increased expression of surface activation markers on CD103^{+} DCs (Figure 36), CD103^{+} DCs from cohoused WT mice showed increased levels of activation in cLNs with increased frequency and expression of CD80 and CD86 (Figure 54) although frequencies of CD11c^{+} or CD103^{+} DCs were not altered. These data indicate that microbiota under homeostatic conditions do in fact modulate the proliferation and expansion of γδT17 cells.

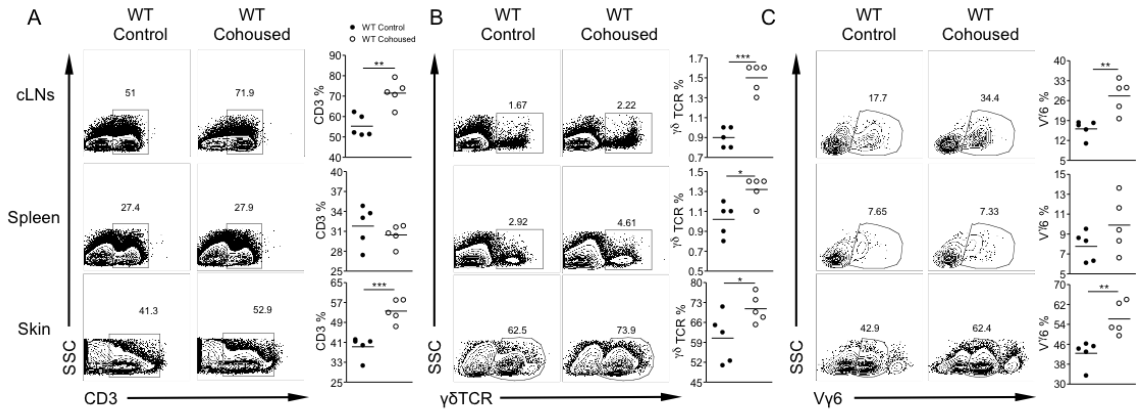


Figure 51. Cohousing of IL-17r$^{-/-}$ mice with WT mice increases CD3^{+} T cells and specifically expands Vγ6 γδT cells in cLNs, spleen and skin

(A) Flow cytometry analysis of cell homogenate from different tissues examining the surface marker percentages of CD3, γδ T cells and Vγ6 γδ T cells between control and cohoused WT mice with IL-17r$^{-/-}$ mice of the same age and sex.

Representative dot plots and summarized data showing percentages of total CD3+ T cells, γδT cells, Vγ6 T cells. *p<0.05, **p<0.01 ***p<0.001

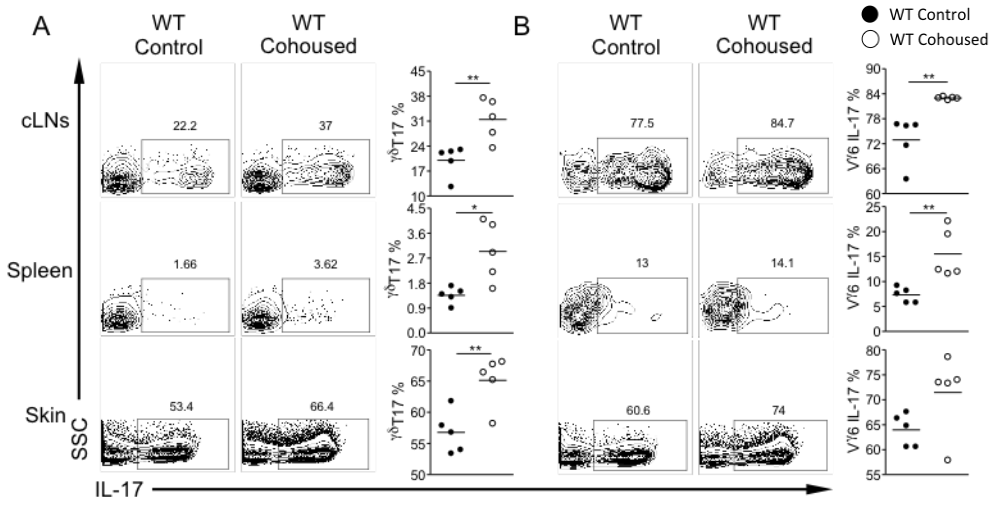


Figure 52. Cohousing of IL-17r$^{-/-}$ mice with WT mice induces the proliferation of γδT17 cells in the cLNs and systemic expansion of γδT17 cells in WT mice

(A) Flow cytometry analysis of cell homogenate from different tissues examining the intracellular IL-17 expression between control and cohoused WT mice with IL-17r$^{-/-}$ mice of the same age and sex. Representative dot plots and summarized data showing percentages of total γδT17 cells as well as IL-17-producing Vγ6 T cells are shown. *p<0.05, **p<0.01 ***p<0.001

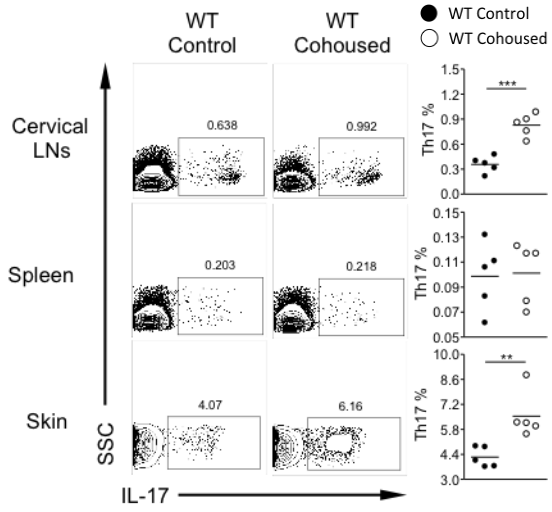


Figure 53. Cohousing of IL-17r-/- mice with WT mice leads to increase Th17 polarization in the cLNs and skin but not in the spleen

Flow cytometry analysis of cell homogenate from different tissues looking at the intracellular expression of IL-17 from CD4 T cells.

(A) Gating on total live lymphocyte population then gated on $CD3^{+}CD4^{+}$ T cells to analyze Th17 responses after 5 h PMA/Ionomycin stimulation. **p<0.01, ***p<0.001

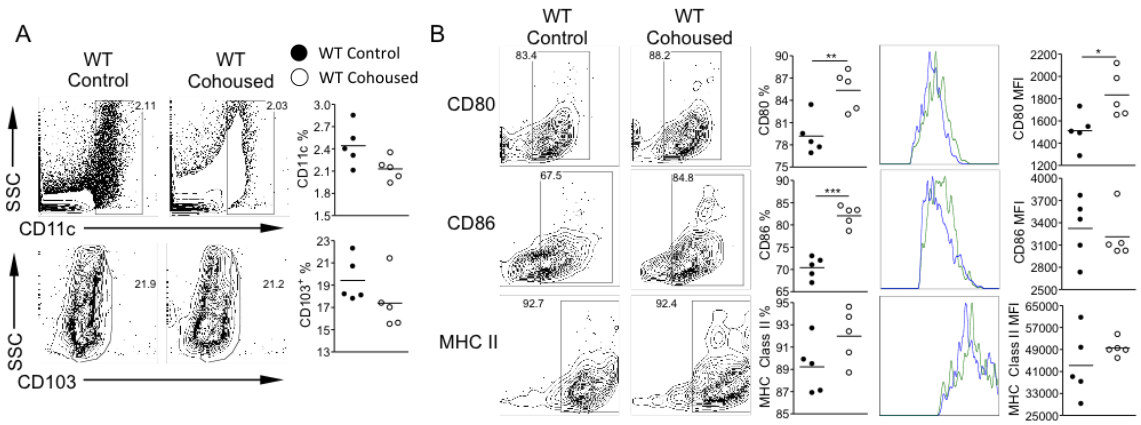


Figure 54. Cohousing of IL-17r$^{-/-}$ mice with WT mice induces the activation of CD103^{+} DCs with increase expression of CD80 and CD86

(A) Surface marker staining of DCs and CD103^{+} DCs from cLNs in control and cohoused WT mice. Cells were also stained with CD80, CD86 and MHC Class II. Representative dot plots and summarized data showing percentages of total CD11c^{+} DCs, CD103^{+}DCs, CD80^{+}CD103^{+}DCs, CD86^{+}CD103^{+}DCs, MHC classII^{+}CD103^{+}DCs are shown. MFI of CD80, CD86, and MHC class II on CD103^{+}DCs is also shown. *p<0.05, **p<0.01, ***p<0.001

Lack of oral microbiota reduces Vγ6 γδT17 population and introduction of dysbiosis-inducing oral pathogen PG leads to γδT17 expansion in cLNs

GF mice have been used extensively of late to study how immunological systems are function-dependent on microbiota-host interactions (Grover and Kashyap, 2014; Kostic et al., 2013; Yi and Li, 2012). Using antibiotics to remove the microbiota from IL-17r$^{-/-}$ mice we previously showed that γδT17 expansion beyond physiological levels in IL-17r$^{-/-}$ mice was abrogated (Figure 48-50). We also showed that dysbiotic microbiota transfer from IL-17r$^{-/-}$ to WT mice induces γδT17 expansion in the oral cavity but not the gut (Figure 51-52). We next investigated whether under physiological conditions in the absence of microbiota γδT17 population especially in the oral draining cLNs would be reduced. In Figure 55A, total IL-17 producing cells in the cLNs of GF mice were drastically lower compared to specific-pathogen-free (SPF) mice. Looking specifically at IL-17 producing γδT cells we observed a dramatic decrease in their IL-17 producing capabilities with a corresponding decrease in presence of Vγ6^{+} γδT cells. Immunostaining of dendritic cell populations showed a correlating decrease in total dendritic cells and CD103^{+} dendritic cells in the absence of microbiota (Figure 55B).

Oral pathogen PG has been used for decades in order to study the pathophysiology of periodontal disease; however, it has not been until recently that investigators have determined that periodontal disease results from PG-induced dysbiosis of oral microbiota (Maekawa et al., 2014; Zenobia and Hajishengallis, 2015). Using a chronic PG infection model, we observed expansion of total IL-17 and γδT17 cells in the cLNs of PG treated mice compared to sham controls (Figure 56A). We also observed increasing levels of Vγ6 γδT cells in the cLNs (Figure 56A) corresponding to increasing

activation of $CD103^+$ DCs using activation marker CD80 and CD86 though not statistically significant (Figure 56B, data not shown). IL-17 and γδT17 expansion seen in this model was observed specifically in the draining oral cLNs and not in other tissues including the bLNs, mLNs, spleen, skin, lungs and colon (data not shown).

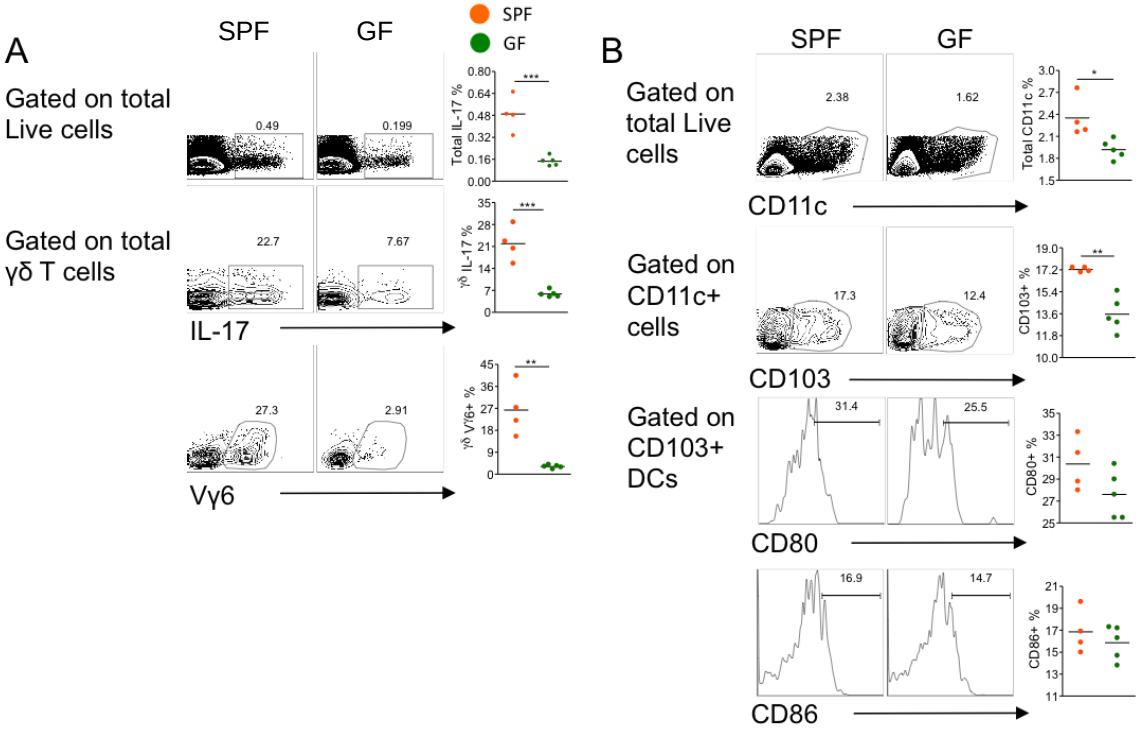


Figure 55. Lack of oral microbiota reduces Vγ6 γδT17 and CD103$^+$ DCs in Germ Free Mice

Flow cytometry studies of immunostained homogenized cLNs from GF and SPF mice of similar age and sex from the same facility (U. of Chicago, Chicago, IL)

(A) Gating from the total live cells stimulated with PMA/Ionomycin for 5 h then gated IL-17$^+$ population (upper panel), gating from the total γδT cell then gated on IL-17$^+$ population (middle panel) and gated on Vγ6 population (lower panel). **p<0.01, ***p<0.001

(B) Gating from total live cells then gated CD11c$^+$ population (upper panel) and gating from CD11c$^+$ population then gated CD103$^+$ population. *p<0.05, **p<0.01

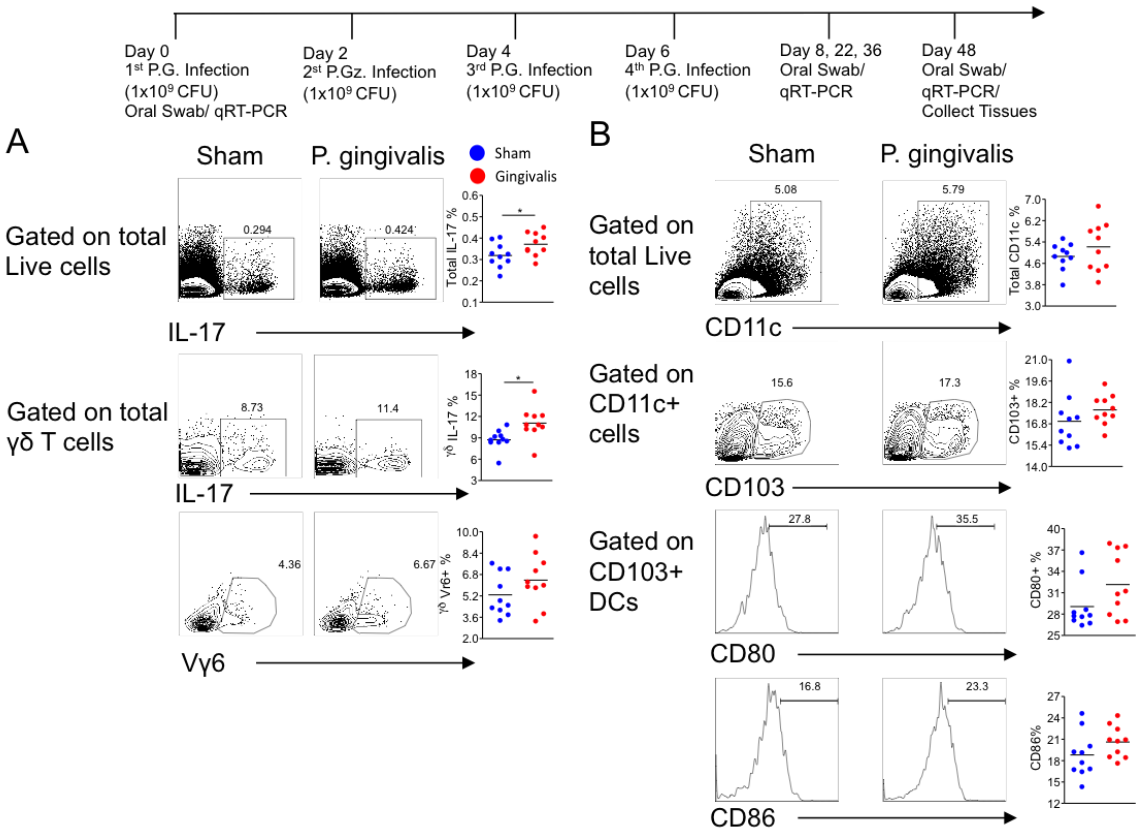


Figure 56. Introduction of dysbiosis-inducing oral pathogen PG leads to γδT17 expansion in cLNs

Flow cytometry studies of immunostained homogenized cLNs of PG infected and sham treated mice of similar age and sex from the same facility (Baxter II Vivarium, University of Louisville, Louisville, KY).

(A) Diagram of the PG infection protocol over 48 days. Gating from the total live cells stimulated with PMA/Ionomycin for 5 h then gated IL-17^{+} population (upper panel), gating from the total γδT cell then gated on IL-17^{+} population (middle panel) and gated on Vγ6 population (lower panel). Plots representative of 2 experiments. *$p<0.05$

(B) Gating from total live cells then gated $CD11c^+$ population (upper panel) and gating from $CD11c^+$ population then gated $CD103^+$ population (middle panel). Gating from $CD103^+$ population then gated for CD80 in histogram format. Plots representative of 2 experiments.

DISCUSSION

In this study, we demonstrate that γδT17 cells are regulated both directly through IL-17r intrinsic feedback signaling as well as indirectly by modulating the epithelial microbiota which in turn regulates γδT17 frequency and IL-17 producing capabilities. The major regulatory pathway appears to be location and environment specific due to disproportionate expansion of γδT17 cells in the oral draining cLNs. We show that γδT17 cells are regulated in the cLNs through microbiota activated $CD103^+$ DCs in a cell-to-cell contact dependent mechanism leading to monoclonal expansion of Vγ6 γδT17. Unlike conventional DC activation of γδT17 cells using IL-1β cytokine induced proliferation, the $CD103^+$ DCs activation in this study was IL-1β and IL-1r independent. This peripheral regulation network is initiated at the epithelial surface and through $CD103^+$ DC activation leads to Vγ6 γδT17 proliferation in the draining cLNs and subsequently to systemic expansion in tissues such as the lungs, skin and spleen but not in the colon or mLNs. Partial depletion of LNs resulted in less notable expansion of the γδT17 population systemically suggesting a watershed effect initiated at the oral draining cLNs.

A master transcription factor in the regulation of IL-17 production by γδT17 is RORγt (Malhotra et al., 2013; Ribot et al., 2009). In addition, it has recently been reported that RORγt represses BTLA transcription while IL-7 increases the surface expression of BTLA on γδ T cells (Bekiaris et al., 2013). Our results suggest that IL-17r signaling

suppresses RORγt resulting in heightened BTLA inhibition of IL-7 signaling. IL-7 is a ubiquitous survival cytokine throughout the body and as we've shown in our IL-7 stimulation *in vitro* studies, it can induce γδT17 proliferation at very low concentrations. IL-17r signaling modulating the $CD27^-$ γδT17 specific proliferative capability of IL-7 would provide a key feedback regulatory system in order to maintain γδT17 cells in the periphery. By reconstituting 1:1 WT and IL-$17r^{-/-}$ Vγ6 γδ T cells, we showed some intrinsic constitutive advantage for IL-$17r^{-/-}$. Interestingly, total IL-$17r^{-/-}$ γδ T cells showed some advantage in cLNs and iLNs; however, there was little if no advantage for IL-$17r^{-/-}$ γδT17. With the majority of Vγ6 γδT in both IL-$17r^{-/-}$ and WT mice producing IL-17 in any tissue creating such a high baseline, total Vγ6 is a better indicator of an intrinsic advantage rather than $IL\text{-}17^+$ Vγ6 γδT cells. Previously, Smith et al. described a short feedback loop capable of regulating the transcription of IL-17 through the IL-17r signaling (2008); however, our studies show how IL-17r signaling can also regulate the proliferation of γδT17 perhaps through modulation of the RORγt-BTLA regulation pathway. Zhao et al. discovered that SerpinB1 plays a role in regulating γδT17 cells intrinsically through transcriptionally pushing γδ T cells to an IL-17 prominent effector phenotype (2014). It is plausible that SerpinB1 could be the link between the IL-17r signaling pathway and RORγt transcription regulation. Indeed, a drastic expansion of γδT17 cells in many tissues in the IL-$17r^{-/-}$ mice suggests an intrinsic defect could be driving this phenotype. Kumar et al. showed using IL-$17r^{-/-}$ mice that increase levels of IL-17 in these mice is not due to lack of receptor clearance of IL-17 but instead caused by increase production of IL-17 by both Th17 cells and γδ T cells (Kumar et al., 2016). They also showed using CD4 specific IL-$17r^{-/-}$ mice that lack of IL-17r does not cause an

intrinsic defect such as an endogenous increase in Th17 polarization or higher sensitization to polarization ligands upon exposure *in vitro*. While Kumar et al. focused specifically on the lamina propria Th17 response which is most the most prominent affect in IL-17r$^{-/-}$ gut, we focused on other peripheral tissues where the major affect was seen on γδT17 cells not Th17 cells. However, we did not see γδT cell expansion in the thymus, colon or mLNs while oral draining cLNs were specifically affected with incongruous expansion. Therefore, we conclude that γδT17 peripheral regulation is mainly location and environment dependent.

Previous studies have shown the ability of microbiota to influence the expansion of γδT17 cells (Cheng et al., 2014; Wu et al., 2014). Here, we show that the expansion of γδT17 cells is transferred between mouse strains simply by co-housing WT and IL-17r$^{-/-}$ mice. Microbiota differences between the IL-17r$^{-/-}$ and WT mice can be explained by the idea of "microbiota adaptation". In the absence of IL-17 signaling, the microbiota and the immune system are trying to adapt and in essence reach a new equilibrium. After all the presence of IL-17 has been constant for millions of years therefore the lack of IL-17 is a shock to both systems. Even if the microbiota do not have an IL-17r on their own membranes, they still respond to signals downstream of endogenous IL-17 signaling such as secretion of antimicrobial products HBD2 and calgranulin by keratinocytes (Kolls et al., 2008). Cohousing WT mice with IL-17r$^{-/-}$ mice may force the WT mouse's oral microbiota to adapt and reach a new equilibrium which in turn results in an adaptation and adjustment by the WT mouse's immune system shown here as an expansion of γδT17 cells. However, an important experiment to rule out gut microbiota contamination through consumption of fecal droppings was the fecal transfer experiment. The results

shows that gut microbiota as reported previously is important in Th17 induction in the colon (Goto et al., 2014; Ivanov et al., 2009; Wu et al., 2009). However, γδT17 cells are not affected by fecal transplant. Therefore, oral but not colonic microbiota is important in the peripheral regulation of γδT17. With the treatment of IL-17r$^{-/-}$ mice with broad-spectrum antibiotics through oral feeding, we completely abrogated γδT17 expansion in the periphery bringing the size and absolute cell number of IL-17r$^{-/-}$ cLNs back to WT baseline levels, suggesting oral microbiota is critical in regulating γδT17 proliferation and maintenance. It's important to emphasize that the continued administration of oral broad-spectrum antibiotics from before birth to adulthood did not bring the cell count, size or IL-17 responses below that which is seen in WT mice. The development, trafficking and regulation of these populations remained intact; however, we observed less expansion of the Vγ6 γδT17 population beyond average numbers. These findings suggest a dichotomy in the regulation of the two major IL-17 production pathways which includes either Th17 or γδT17 cells. As mentioned previously, extensive studies have shown the important in gut microbiota to regulate Th17 responses in gut lamina propria and the mesenteric lymph nodes. Our data suggest that γδT17 cells are regulated not through gut microbiota but oral microbiota. Perhaps with gut microbiota and food antigens placing such a central tolerigenic role on the gut immune system the necessity of a more dynamic cytokine response including Th polarlization and plasticity are better suited for the special microcosm of the host-environment. However tolerance isn't a primary role of oral immunity yet the microenvironment of the oral cavity is specialized since birth when babies begin sensing the world through their mouths. The oral cavity immune system and microbiota must be well prepared for sensing and responding to

possible pathogenic microbes since this is perhaps the most important orifice for future infections. Our GF mice studies show that in the absence of microbiota when compared to SPF mice Vγ6 γδT17 cells are at a minimal almost non-existent level even though total γδT cell frequency is not changed (data not shown). This suggests that microbiota plays a specific regulatory role in the peripheral maintenance of IL-17 producing Vγ6 γδT cells but not Vγ4 and/or Vγ1 γδT cells. We also observed a correlating drop in the $CD103^+$ DC population in the cLNs in the absence of microbiota supporting our hypothesis that microbiota regulated $CD103^+$ DCs play a role in the maintenance and expansion of Vγ6 γδT17 cells. A well-known oral infection model that leads to microbiota dysbiosis is PG-induced periodontal disease. Using a chronic infection model with PG, we did in fact see increased levels of IL-17 producing cells that were specifically γδT17 cells as well as corresponding increasing levels of $CD80^+$ $CD103^+$ DCs though not statically significant. These findings suggest that oral diseases that affect the microbiota, i.e. periodontal disease, could have systemic effects on the immune system from γδT17 local expansion resulting in the initiation and/or progression of IL-17-driven inflammatory diseases.

The gut is a very distinct immune site with Th17 cells playing the dominant role in epithelial integrity and immune responses. This is understandable because it's already been shown in arthritis, transplantation and gut disease models that Th17 cells can convert to Tregs and visa-versa (Komatsu et al., 2014; Obermajer et al., 2014; Omenetti and Pizarro, 2015). The gut is such a dynamic, ever-changing environment that plasticity in the effector immune population is essential. In contrast, the majority of γδT17 cells are pre-programmed in the embryonic thymus with low plasticity ability; therefore, they are likely designated to more stable epithelial barriers such as oral, lung and vagina

where tolerance is less important. However, at these surfaces γδT17 have to respond immediately to any signs of threat with overwhelming IL-17.

In the physiological disease setting, differentiating IL-17 responses either from the gut or oral cavity could prove difficult due to fact that diseases that affect one location are also associated with simultaneous diseases that affect the other. Epidemiology studies have shown that bad oral healthcare normally have bad overall nutrition (SHIMAZAKI, 2007). These patients potentially suffer from dental caries and/or gingivalis infections plus increase fats in the GI tract and obesity. Both pathologies occurring together would result in changes to the microbiota at the oral and gut interface inducing γδT17 and Th17 responses simultaneously. Our study however shows that oral microbiota seems to play a prominent role on systemic γδT17 populations while gut microbiota plays a more specific role on local Th17 responses.

A more tangible question suggested by our study is that generic dysbiosis locally could result in increased γδT17 responses systemically. Whether antigen specificity is driving the proliferation and activation seen in our study is unknown. γδT17 cells are efficient at recognizing cell stress and danger signals therefore general inflammation in many different models leads to expansion of γδT17 responses. I think this might be why we see increase γδT17 cells in many different models including inflammation related cancers, autoimmune diseases and infections. Again trying to pinpoint the response to either the initial dysbiosis or the ensuing inflammation is difficult. In reality, these will cycle as ensuing inflammation leads to increased dysbiosis until the inflammation is finally resolved.

DC cross talk with γδT17 cells using cytokines IL-1β and IL-23 to induce proliferation and IL-17 production during inflammatory settings has been well described (Cai et al., 2011; Wu et al., 2014). Our *ex vivo* depletion and co-culture studies were designed to determine what led to the proliferation of γδT17 cells in IL-17r$^{-/-}$ mice during steady-state conditions. DCs, specifically the CD103^{+} DCs, in the cLNs of IL-17r$^{-/-}$ can drive the proliferation of γδT17. This further supports our hypothesis that the observed proliferation and systemic expansion of γδT17 cells in the IL-17r$^{-/-}$ mice is in fact location and environment dependent, perhaps due to "microbiota adaption" in the oral cavity. Mesenteric CD103^{+} DCs could not drive the proliferation of γδT17 cells but cervical CD103^{+} DCs with increased CD80 and CD86 upregulation could drive γδT17 proliferation very strongly even lacking IL-1r. Although CD103^{+} DC induction of γδT17 cell proliferation is independent of IL-1β or IL-1r signaling, a cell-to-cell contact is required for this process. These are novel results considering how well established DC cytokine induction, specifically through IL-1β/IL-23, is for γδT17 activation and proliferation. Previous studies showed that direct cell-to-cell contact between DCs and Vγ4^{-}Vγ1^{-} γδ T cells could potentially be important in regulation of γδ T cell function (Wands et al., 2005). Our findings suggest a new regulatory network independent of soluble factors, dependent on APCs showing that γδT17 are not as socially inapt as once thought when compared to their conventional brothers and sisters, the αβ T cells. Regulatory cross talk between γδT17 and CD103^{+} DCs either through cytokine or cell-to-cell interaction could be a major factor in how the immune system is able to respond and communicate with microbiota populations at the epithelial surface. Further investigation

will be needed to determine through what mechanism activated $CD103^+$ DCs induce γδT17 proliferation.

One of the most confusing issues in γδT17 biology has been what events are needed for γδT17 activation and cytokine secretion (Chien et al., 2014). While some γδ T cells responding to Ag stimulation in the periphery and undergoing Ag-specific differentiation into IL-17A-producing cells, such as phycoerythrin (PE)-specific γδ T cell response (Zeng et al., 2012), other γδ T cells make IL-17 within hours after an immune challenge. The rapid IL-17 response does not show an apparent explicit TCR engagement but requires inflammatory cytokines such as IL-1/IL-23 for its induction (Chien et al., 2013). These natural or innate like γδT17 cells have a highly focused TCR repertoire regardless of their anatomical origin (Wei et al., 2015). Wencker et al. reported previously that these γδ T cells require Ag recognition during thymic development which endows these cells to make IL-17 in response to cytokines in the periphery without engaging Ag (Wencker et al., 2014). Although the expanded γδT17 cells in IL-$17r^{-/-}$ mice that we reported here have a highly invariant TCR repertoire, their monoclonal expansion is cell-to-cell contact dependent via interaction with activated $CD103^+$ DCs. It is still probable that they can recognize an array of different molecules depending on molecular makeup and structure therefore acting more like a BCR on a B cell than the conventional αβ TCR. Further investigation is needed into determining what Ags are important for the homeostatic upkeep of γδT17 cells as well as proliferative induction of γδT17 cells during disease.

Collectively, we propose a novel cellular pathway important in the regulation of host-microbiota interactions. γδT17 are important in the maintenance of epithelial barrier function and $CD103^+$ migratory DCs are important in microbiota Ag sampling (Duan et

al., 2010; Farache et al., 2013; O'Brien and Born, 2015; Schulz et al., 2009). Our findings connect the two processes and provide insights into how our body regulates this critical interface. Our results suggest that γδT17 and $CD103^+$ DCs play a role of maintaining healthy microbiota populations at the epithelial surface in return being regulated themselves by the signals they receive from the microbiota. As the significance of γδT17 cells continue to grow in a constantly expanding list of diseases they are associated with, therapeutic solutions targeting γδT17 will become a necessity. Finding specific targets to modulate this critical interaction could be beneficial to the homeostatic maintenance of normal microbiota populations as well as to many patients suffering from inflammation-driven diseases.

CONCLUSION, REMARKS AND FUTURE DIRECTION

γδT17 cells are now considered both friend and foe depending on what context you study them in. They are potent producers of IL-17, which is a powerful cytokine with many downstream affects mainly aimed towards inducing inflammation. Most diseases involve inflammation initiating and progressing at the locations where γδT17 cells reside. An important area of investigation should focus at how these inflammatory cells are regulated and maintained in the periphery without causing damage during homeostasis yet are rapidly capable of providing enough inflammatory signals for pathogen clearance and/or wound repair. In this study, we observed the tip of the γδT17 regulation iceberg with the discovery of a novel interaction and activation induction of γδT17 cells by oral microbiota activated $CD103^+$ DCs. As we dance and play in the macro world around us, γδT17 cells and $CD103^+$ DCs stand guard and orchestrate the micro-world helping to bring about integration between host and microbiota. Knowing that microbiota

influences the regulation of γδT17 cells and that microbiota are regulated by the primary effector cytokine produced by γδT17 cells, in the future we should aim to better understand the dance between these two populations. $CD103^+$ DCs appear to be mediators in the process but how exactly does this regulatory process tell who is friend or foe or even when too many friends begin to get out-of-control. Future studies should focus on nailing out the details of these interactions determining what is critical to the maintenance of these two population and what are the signals being sent. Super resolution microscopy and two-photon intravital microscopy can be utilized in order to get a better close-up look at how these populations interact (or "dance") with each other. We could visualize the $CD103^+$ DC network and how Vγ6 γδT17 cells interact with the network according to various stimuli applied in real time. Ultimately these studies could lead to important applications for the health of patients by using new therapeutics to increase the beneficial role of this network and decreasing the unfortunate side-effects during different disease states. Daily application of various creams for example which contain various probiotics or immunomodulators could be used to strength our immune system and prevent the onset of various diseases such as pathogen infection or autoimmune diseases. The location of γδT17 cells at the epithelial surfaces provides an opportunity for less invasive therapeutic interventions. Integration of the immune system with the microbiota and vice-a-versa is probably one of the most important least understood areas of biomedical research and the implications of the discoveries therein are too numerous to state here. However the more we understand about this interface and

how it affects our health will provide key insights into how we treat disease and even more important how we prevent disease from occurring.[1]

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Zhao, P., Hou, L., Farley, K., Sundrud, M.S., and Remold-O'Donnell, E. (2014). SerpinB1 regulates homeostatic expansion of IL-17+ gammadelta and CD4+ Th17 cells. J Leukoc Biol *95*, 521-530.

CURRICULUM VITAE

Christopher J. Fleming, M.S.
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Investigating the Regulation of γδT17 Cells in Health and Disease

Proven success in graduate and medical school classrooms as well as in the lab conducting cutting-edge tumor immunobiology research at three different cutting-edge research institutions in the U.S.

- First author of primary research paper submitted to Nature Immunology
- Co-author on three primary research papers
- Two review paper publications
- Awarded 1st Place for Midwest Regional APSA Graduate Poster Award in 2013
- Awarded 1st Place for James Graham Brown Cancer Center Graduate Research Award in 2013
- Awarded 1st place for Doctoral Students at ResearchLouisville! in 2013
- Received Masters Degree from University of Louisville in 2012
- Awarded UofL Sponsored Research Tuition Award in 2011

Education

UNIVERSITY OF LOUISVILLE – Louisville, KY
Ph.D. Candidacy in Microbiology and Immunology, 2011-Current

UNIVERSITY OF LOUISVILLE – Louisville, KY
M.D. Candidacy, School of Medicine, 2009-2011

UNIVERSITY OF LOUISVILLE – Louisville, KY

MS in Microbiology and Immunology, 2011-2012

CENTRE COLLEGE – Danville, KY
BS *Cum Laude* in Biochemistry and Molecular Biology, 2005-2009

OGLETHORPE COUNTY HIGH SCHOOL – Lexington, GA
HS Diploma, College Prep, Valedictorian Award, 2001-2005

Ph.D. Thesis

James Graham Brown Cancer Center, University of Louisville – Louisville, KY

Ph.D. Student & Research Fellow, 2011-Present

Investigating how γδT17 cells are regulated in the periphery and maintained under homeostatic conditions and in disease. γδT17 cells have been shown to play important roles in cancer and autoimmune inflammation drive pathogenesis therefore understanding how they are regulated in the periphery after development is critical to developing effective and specific immunotherapies in the future.

Research Project Highlights:

- Found a novel phenotype in the proliferation and expansion of γδT17 cells in mice lacking IL-17 receptor and that the phenotype is location and environment specific.
- Found a new cellular regulation pathway between γδT17 cells and CD103+ DCs
- Found that microbiota are essential for the peripheral regulation of γδT17 cells but not in tissues such as the colon, thymus and mesenteric lymph nodes.
- Found that dysbiosis of oral microbiota either due to transfer of microbiota from IL-17r-/- to WT mice or by the infection of *P. gingivalis* leads to expansion of γδT17 cells in cervical LNs.

Publications

Fleming, C., Yihua Cai, Feng Xue, Yu-Ling Wei, Venkatakrishna Jala, Christopher Worth, Na Liu, Yueh-Hsiu Chien, Chuanlin Ding, Jun Yan. “Microbiota Activated CD103+ DCs Stemming from Oral Microbiota Adaptation Specifically Drive γδT17 Proliferation and Activation.” **[Submitted to Nature Immunology]**

Yan, J., Kloeker, G., **Fleming, C.**, Bousamra, M., Hansen, R., Hu, X., Ding, C., Cai, Y., Dong, X., Donninger, H., Eaton, J., Clark, G.. " Human Polymorphonuclear Neutrophils Specifically Recognize Tumor Cells for Killing." Oncoimmunology. 2014 Jul 3;3(7):e950163.

Cai, Y., Xuefeng, X., **Fleming, C.**, Yan, J. "Differential Developmental Requirement for Dermal IL-17-producing Vγ4 and Vγ6 T Cells and Their Peripheral Regulation in Health and Skin Inflammation." Nat Commun. 2014 Jun 9;5:3986. doi: 10.1038/ncomms4986.

Cai, Y., **Fleming, C.**, & Yan, J. (2013). Dermal γδ T cells - A new player in the pathogenesis of psoriasis. *International immunopharmacology*. doi:10.1016/j.intimp.2013.02.018

Cai, Y., **Fleming, C.**, & Yan, J. (2012). New insights of T cells in the pathogenesis of psoriasis. *Cellular and Molecular Immunology*, *9*(4), 302–309. doi:10.1038/cmi.2012.15

Research Internships

James Graham Brown Cancer Center – Louisville, KY

Research Intern (SROP program), Dr. Jun Yan and Dr. John Eaton, Department of Medicine, June-August 2009

The purpose of this research was to ascertain whether Cisplatin treated NSCLC cancer patients are immunologically compromised due to inhibition of neutrophil killing. After getting IRB approval, a small clinical study was conducted in order to acquire blood samples from over 50 healthy donors and cancer patients. Using blood samples, neutrophils were isolated and comparison studies of their neutrophils were done using an *in vitro* cytotoxicity assay (Label-free ACEA cytolysis assay), flow cytometry for surface marker expression and an NBT assay (nitro blue tetrazolium) in order to compare difference in respiratory burst. The results of the study were that patients treated with cisplatin have less neutrophil killing and this results from an inhibition of respiratory burst function.

Barbados Wildlife Reserve – Barbados, W.I.

Research Intern (Centre Study Abroad), Drs. Brian Cusato and Melissa Burns-Cusato , Department of Psychology, January 2009

This research experience was part of Centre's study abroad program (PYB450 Research in Primate Behavior) where observations and recordings were made of the behavior and migration patterns of wild vervet monkeys as they cross in-and-out of the

Barbados Wildlife Research to avoid predators and the local communities. Different behaviors and migration patterns observed between different matriarchal families were recorded in detail using a computer then mapped out later for presentation. The title of the project was *"The Migration Patterns and Behaviors of Vervet Monkeys near the Barbados Wildlife Reserve."* Our group won a competition between 8 groups for a presentation spot at the Southwest Psychology Association Meeting in San Antonio, TX. A Powerpoint presentation was also given at Centre's R.I.C.E. symposium in April of 2009.

Medical University of South Carolina – Charleston, SC

Research Intern (SURE Program), Dr. David Cole, Department of Immunology, June-August 2008

Investigating how to optimize the killing efficiency of CD8 T cells against melanoma using Adoptive T Cell Therapy (ACT). Having acquired a proficient skill level for immunology lab techniques the previous summer at the Mayo Clinic, Drs. Cole and Montero-Diaz allowed an independent research experience in terms of experimental design and execution. The purpose of the research titled *"Therapeutic Efficacy of T Cells Specific for Human Tyrosinase in the Treatment of Melanoma"* was to examine the therapeutic efficacy of T cells from *H*uman *T*yrosinase *T*IL-derived *T*ransgenic (H3T) mice after ACT. Traditional T cell expansion for ACT against melanoma used IL-2 cytokine treatment however we found that by using IL-12 the therapeutic efficacy of ACT is increased. The conclusions from the research were that H3T mice are able to reject B16-A2 tumors that present tyrosinase epitopes in the context of human HLA-A2 molecules. ACT of IL-12 versus IL-2 *ex vivo* activated H3T T cells increases the survival of mice bearing B16-A2 melanoma tumors. IL-12 treatment increases T cell proliferation and expression of CD62L, CD127, and IFN-γ prior to ACT. H3T mice represent a good model to optimize the activation condition of T cells prior to ACT. A Powerpoint presentation was presented at the MUSC SURE Closing Banquet and at the Centre College R.I.C.E. Symposium the following Spring.

Mayo Clinic – Rochester, MN

Research Intern (SURP Program), Dr. Larry Pease, Department of Immunology, June-August 2007

First experience doing wet lab research and learned how to harvest bone marrow for DC differentiation and lymphocytes from lymph nodes, perform Cr release cytotoxicity assays, flow cytometry, ELISA and western blots and by the end of the summer I was able to do all these assays independently. The project was titled *"Phenotypic and Functional Differences of OT1 T cells activated with B7-DC Crosslinking antibody (XAb) in vitro for 24 hours."* The purpose was to look at the phenotypic differences of CD8 OT-I T cells after 24 hours co-culture with bone marrow derived dendritic cells that had been activated with melanoma lysate plus B7-DC Xab (isolated from a Mayo patient with Waldenstrom's macroglobulinemia in 2000) or melanoma lysate plus control antibody. The results were that B7-DC Xab increased cytotoxicity of B16-F10 and EG7 tumor cells

by CD8 T cells, no difference in surface activation markers or IFN-γ production was observed and tyrosine phosphorylation was increased in the stimulated CD8 T cells. A Powerpoint presentation was presented at the Mayo Clinic SURP Closing Banquet and at Centre College the following spring at the Centre R.I.C.E. Symposium.

Awards

2013- Midwest Regional American Physician Scientists Association 1st Place Graduate Poster Award

2013- James Graham Brown Cancer Center 1st Place Graduate Research Award

2013- ResearchLouisville! First Place Doctoral Student Award

2011- UofL Sponsored Research Tuition Award

2007- W. Burford Davis Memorial Scholarship

2007- Combs Achievement Award for Academic Excellence

2006- Gold Presidential Service Award (400+ annual volunteering hours)

2005-2009- Four Dean's List Recognitions, Centre College

2005- Athens Area Human Relations, Inc. – Dana and Zoe Memorial Scholarship

2005- Elk's Lodge 1st Place Most Valuable Student Award for Northeast Georgia

2005- Valedictorian of Oglethorpe County High School

Presentations

Oral

Fleming, C., Diaz-Monterro, M., Cole, D. Therapeutic Efficacy of T Cells Specific for Human Tyrosinase in the Treatment of Melanoma. **Centre RICE Symposium 2009.** Centre College, Danville, KY. April 2009.

Fleming, C., Royalty, S., Cusato, B., Cusato, M. The Migration Patterns and Behaviors of Vervet Monkeys near the Barbados Wildlife Reserve. **Centre RICE Symposium 2009.** Centre College, Danville, KY. April 2009.

Fleming, C., Royalty, S., Cusato, B., Cusato, M. The Migration Patterns and Behaviors of Vervet Monkeys near the Barbados Wildlife Reserve. **Southwest Psychology Association Annual Conference.** San Antonio, TX. April 2009.

Fleming, C., Diaz-Monterro, M., Cole, D. Therapeutic Efficacy of T Cells Specific for Human Tyrosinase in the Treatment of Melanoma. **MUSC SURE Closing Banquet.** MUSC, Charleston, SC. August 2008.

Fleming, C., Schenk, E., Radhakrishnan, S., Pease, L. Phenotypic and Functional Differences of OT1 T cells activated with B7-DC XAb *in vitro* for 24 hours. **Centre RICE Symposium 2008.** Centre College, Danville, KY. April 2008.

Fleming, C., Schenk, E., Radhakrishnan, S., Pease, L. Phenotypic and Functional Differences of OT1 T cells activated with B7-DC XAb *in vitro* for 24 hours. **Mayo Clinic SURP Closing Banquet.** Mayo Clinic, Rochester, MN. August 2007.

Poster

Fleming, C., Cai, Y., Sun, X., Jala, K., Wei, Y…. Yan, J. The Extrinsic and Intrinsic Regulation of γδT17 cells through the IL-17-IL-17r axis during homeostasis. **ResearchLouisville!** Louisville, KY. September 2015.

Fleming, C., Cai, Y., Sun, X., Jala, K., Wei, Y…. Yan, J. The Extrinsic and Intrinsic Regulation of γδT17 cells through the IL-17-IL-17r axis during homeostasis. **2015 AAI Meeting New Orleans** New Orleans, LA. May 2015.

Fleming, C., Cai, Y., Yunfeng, M., Fengling, L., Teng, T., Yan, J. Characterization of IL-17 Producing γδ T cells in the Lungs: Implications for Lung Cancer Development and Metastasis. **American Physician Scientists Association Midwest Conference.** Columbus, OH. November 2013.

Fleming, C., Cai, Y., Yunfeng, M., Fengling, L., Teng, T., Yan, J. Characterization of IL-17 Producing γδ T cells in the Lungs: Implications for Lung Cancer Development and Metastasis. **James Graham Brown Cancer Center Annual Retreat.** Louisville, KY. October 2013.

Fleming, C., Cai, Y., Yunfeng, M., Fengling, L., Teng, T., Yan, J. Characterization of IL-17 Producing γδ T cells in the Lungs: Implications for Lung Cancer Development and Metastasis. **ResearchLouisville!** Louisville, KY. September 2013.

Affiliations

Current	Past
American Association of Immunologists	American Medical Association
Microbiology and Immunology	American College of Physicians

Student Organization	Kentucky Academy of Sciences
	American Physician Scientist Association
	Phi Delta Epsilon (2009-2011)
	Drive Cancer Out (2009-2011)
	St. Jude Up Til' Dawn (2006-2009)
	Alpha Phi Omega (2006-2009)
	Beta Beta Beta (2008-2009)

Professional Skill Set

Multi-color Flow Cytometry (6 colors) and Cell Sorting (8 colors)

Mouse Husbandry, Breeding, and Development

Adoptive Cell therapy and *in vivo* mouse studies

Irradiation and Bone marrow transplants

Microscopy (confocal)

Mouse Handling

- Injections (Subcut, IV, Peritoneal)
- Anesthetics (Catamine/isofluorane)
- Euthanization
- Necropsy

Genotyping

Genomic DNA extraction from tissue

Western Blot

Northern Blot

E.L.I.S.A.

RT-PCR

Cromium Release Cytolysis Assay

ACEA Label-Free Cytolysis Assay

CFSE Proliferation Assay

Chromatography

- Gel
- Affinity
- Ion-exchange