The Regulation of Immunological Processes by Peripheral Neurons

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The Regulation of Immunological Processes by Peripheral Neurons

A dissertation presented

by

Jose Manuel Ordovas-Montanes

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

Harvard University

Cambridge, Massachusetts

September 2015
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The Regulation of Immunological Processes by Peripheral Neurons

Abstract

The nervous system and the immune system are the primary sensory interfaces between the internal and external environment. They are responsible for recognizing, integrating, and responding to stimuli with the appropriate valence and magnitude to optimize host fitness. Furthermore, an alluring parallel concept is that both systems have the capacity to form memories of these encounters leading to optimized and adaptive future responses. Recent work in the fields of neuroscience and immunology has led to a high-resolution map of cell subsets within both systems. Here, we start to leverage these advances to explore the relationship between these two sensory systems at the level of discrete cell subsets in immunological disease and homeostasis with a focus on interactions at barrier tissues. This work stems from the initial hypothesis that sensory neurons for noxious stimuli, nociceptors, regulate inflammation by controlling production of key instructive and effector cytokines derived from tissue-resident immune cells.

In our first investigation focused on the skin, we identified that NaV1.8+ TRPV1+ nociceptors, via interactions with dDCs, are essential in vivo regulators of interleukin-(IL)-23/IL-17 pathway cutaneous immune responses. This set of studies raised several intriguing questions including determining what the inputs and outputs of nociceptors are that regulate IL-23 production from dDCs and whether nociceptors play a role in distinct inflammatory contexts. In further experiments we determined that while TRPV1 itself is dispensable, nociceptor activity is essential to promote disease and local cytokine production. As we found that nociceptors are critical for cutaneous IL-23
production, we tested the effect of nociceptor ablation during a bacterial infection that depends on IL-23 for clearance. Surprisingly, the absence of NaV1.8+ nociceptors led to more severe skin pathology and bacterial dissemination suggesting that NaV1.8+ nociceptors are important for tissue protection and bacterial containment. In complementary studies assessing the role of nociceptors in atopic dermatitis (Type 2 inflammation), we found that TRPV1+ neurons did not regulate gross disease or the instructive cytokine thymic stromal lymphopoietin (TSLP), yet were required for full effector (IL-4) cytokine production. This suggests that nociceptors exert contextual specificity and are not exclusively pro-inflammatory in all cutaneous pathologies.

Based on the role for nociceptors in regulating cutaneous IL-23 and subsequent inflammation, we hypothesized that TRPV1+ nociceptors would be important in regulating IL-23-driven pathologies in the gut. However, we found no appreciable role for nociceptors in either an acute innate colitis or a spontaneous microbiota-driven colitis. While colitis is characterized by its gastrointestinal pathology, it affects the body systemically and we noted that in both models, mice lacking TRPV1+ nociceptors maintained healthier weights than their nerve-replete counterparts. This opens future avenues for exploration on how sensing of inflammation by nociceptors influences microbial communities, systemic metabolism, and sickness behavior.

In summary, we propose that sensory perception of inflammation by peripheral neurons exerts context-dependent effects on immunological processes. We speculate that there is the potential to uncover a modular design framework by which distinct lines of sensory neurons are triggered in specific settings and may contribute to regulating different aspects of inflammation through interactions with immune cell subsets.
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List of abbreviations

3D three-dimensional
6-OHDA 6-hydroxydopamine
APC antigen presenting cells
ATP adenosine triphosphate
BCR B cell receptor
cAMP cyclic AMP
CD cluster of differentiation
ChAT choline acetyltransferase
CFU colony forming units
CGRP calcitonin gene related peptide
CNS central nervous system
C(X)CL chemokine
C(X)CR chemokine receptor
CSF colony stimulating factor
DC dendritic cell
DDC dermal dendritic cell
dLN draining lymph node
DNFB dinitro-fluoro benzene
DRG dorsal root ganglion
DSS dextran sodium sulfate
DTR diphtheria toxin receptor
DTX diphtheria toxin
γδT17 dermal γδ T cell, Type 17 subset
GFAP glial fibrillary acidic protein
GFP green fluorescent protein
G-CSF granulocyte colony stimulating factor
HEV high endothelial venule
HSC hematopoietic stem cell
HSV herpes simplex virus
Ig immunoglobulin
IFN interferon
IL interleukin
ILC innate lymphoid cell
ICAM1 intercellular adhesion molecule 1
i.p. intraperitoneal
i.v. intravenous
IMQ imiquimod
IVM intravital microscopy
LN lymph node
LFA-1 lymphocyte function associated antigen type 1
LTα lymphotoxin α
LPS lipopolysaccharide
M-CSF macrophage colony stimulating factor
MHC major histocompatibility complex
MSC mesenchymal stem cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MPM</td>
<td>multiphoton microscopy</td>
</tr>
<tr>
<td>MP-IVM</td>
<td>multiphoton intravital microscopy</td>
</tr>
<tr>
<td>Mrgprd</td>
<td>mas-related gpr, family D</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PLN</td>
<td>peripheral lymph node</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>recombinase activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RTX</td>
<td>resiniferatoxin</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Sub P</td>
<td>substance P</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Tmem</td>
<td>Memory T cell</td>
</tr>
<tr>
<td>Trm</td>
<td>T resident memory cell</td>
</tr>
<tr>
<td>TNBS</td>
<td>trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRK</td>
<td>tropomyosin receptor kinase</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient Receptor Potential Vanniloid 1</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoietin</td>
</tr>
<tr>
<td>UCP1</td>
<td>uncoupled protein 1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
</tbody>
</table>
Attribution of collaborator contributions

Chapter 1: Jose Ordovas-Montanes wrote the chapter with conceptual advice from Seth Rakoff-Nahoum. Siyi (Tracy) Huang provided material for the introduction to the peripheral nervous system, and Olga Barreiro assisted with graphic design taking the initial sketches to the fully illustrated versions. Ulrich H. von Andrian provided the opportunity to write the draft of this invited review before adding his inimitable style to the final version which will appear in published form.

Chapter 2: Lorena Riol-Blanco, Jose Ordovas-Montanes and Ulrich H. von Andrian designed the study. Lorena-Riol Blanco, Jose Ordovas-Montanes, Mario Perro, Elena Naval, Aude Thiriot, and David Alvarez performed and collected data from experiments and Lorena-Riol Blanco, Jose Ordovas-Montanes and Elena Naval analyzed data. John N. Wood provided reagents. Jose Ordovas-Montanes, Lorena Riol-Blanco and Ulrich H. von Andrian wrote the manuscript. Lorena-Riol Blanco and Jose Ordovas-Montanes contributed equally to this work.

Chapter 3: Jose Ordovas-Montanes derived hypotheses, designed the study, initiated collaborations, collected and analyzed data from all experiments, and wrote the chapter. Seth Rakoff-Nahoum provided conceptual advice and performed experiments related to Type 2 models of inflammation. Siyi Huang aided with DRG harvests and DC co-cultures. Wan Beom Park and Jean Claire Lee provided conceptual advice and performed bacterial infections. Tuanian Luo and Sarina Elmariah in the laboratory of Ethan Lerner performed QX-314 injections. Erika K. Williams in the laboratory of Stephen D. Liberles aided in designing and performing optogenetics experiments. John
Wood provided mice. Mark Hoon provided conceptual and technical advice including a visit to the NIH. Ulrich H. von Andrian provided the environment, conceptual advice and critiques.

Chapter 4: Jose Ordovas-Montanes wrote the chapter. Jose Ordovas-Montanes and Seth-Rakoff-Nahoum designed the study, collected data, and discussed implications. Jumpei Sasabe in the laboratory of Matthew Waldor taught and performed metagenomic analyses. Roderick Bronson interpreted pathology in a blinded fashion. Ulrich H. von Andrian provided the environment, conceptual advice, and critiques.

Chapter 5: Jose Ordovas-Montanes wrote the chapter.
Acknowledgements:

I would like to start by thanking Uli for taking a chance on me and offering a spot in the lab even when there wasn’t any room. The environment created by Uli is one that allows for creative direction to dominate over any prescribed notion of what the group should be focused on. While most ordinary people, myself included, can only see things in 3D, Uli has a way of critiquing and framing concepts in four or more dimensions that is something I aspire to incorporate into my scientific toolbox someday. The vast array of ongoing projects challenges everyone to think about the traditional immunological concepts as well as their direct and indirect relationships with other systems. I am not sure the work presented in this thesis would have been possible anywhere else. Science is about much more than experimentation, and Uli has allowed me to play a key role in the manuscript writing and critique process as well as presenting this work as an invited speaker at scientific meetings. Furthermore, I’ve also had the chance to partake in rights of scientific passage that are generally above my pay-grade as in the case of drafting a successful R01 new submission.

Lorena was my gracious host in the lab upon arrival; teaching me both the scientific and social ins and outs of the group. Without the solid foundation she provided and her tenacious determination to initiate the neuro-immunology (and Spanish) subgroup of the UvA lab, this thesis would also not have been possible. I learned a lot from her and am certainly a better person for it.

Many other members of the lab both past and present have contributed. During my time in the lab, I received advice from the cadre of senior post-docs to the undergraduate and high school students who reminded me how frustrating I must have
been to deal with as a young scientist. Yasu, Hyota, Mario, Aude, Armon, Georg, Harald, Carmen, and Shaida were all important resources during my tenure here. In particular, Guiying Cheng, Michael Flynn, and Lauren Jones made sure that with a few keystrokes or words we could have anything our heart desired ready for an experiment. Meghan Perdue was not only the administrative assistant but also the lab psychologist, providing support to the bunch during times of apparent crisis. Libby Nigro stepped flawlessly into that role as well. David Alvarez (coach) did his best to convert me from a cyclist to a soccer player for one day a year for the UvA vs. CBDM soccer game. I’d like to thank him for reminding me what it’s like to be young for 90 minutes, and then feel old for the next week. My skin and muscles resented him for this. Ira Mazo, whose experience led her to avoid this soccer confrontation, provided a consistency in the lab that was always appreciated and could recall protocol intricacies from over 15 years ago while I couldn’t even remember what I had for breakfast that day.

I overlapped with Lilly, Jung, Scott, and Ashley as the other graduate students in the lab. Lilly was another fearless biker. Jung showed us the most direct route to a thesis. Ashley perhaps the most indirect. Ashley also taught me what hobby science was all about. I’ll always remember him fixing and processing a maple leaf for microscopy. Not to mention I can thank him for this great thesis template to work from. Scott provided someone to commiserate with over scientific and non-scientific matters alike. He also bolstered the American minority in the lab.

The neuro-immunology subgroup has grown recently with Olga, and Tracy joining the lab. Olga has provided great insight from her experience in imaging immunology, and Tracy has fact-checked my neuroscience since I used to be able to get
away with most anything in the lab. She also made DRG isolations something that can happen on a regular basis.

Seth has provided a source of constant intellectual and conceptual conversations inside and outside of the lab. I don’t think I drank coffee while at lab until he started treating me with his Starbucks monopoly money, and together we abused the cafeteria discount system meant for graduate students. Were it not for him, I doubt I would be using words and phrases like “modular” and “organizing principles” in this thesis. In a way, through the transitive property, I feel that he provided an additive PhD mentor and connected me to the Medzhitov-lineage. He also sold me on the virtues of moving to East Cambridge.

I would like to thank my DAC: Florian Winau, Nir Hacohen, and Amy Wagers for somehow keeping up with all the different directions I was stretching this project. Also, my Thesis Exam Committee: Florian, Jon Kagan, David Ginty, and Dorian McGavern for taking the time read through this work. Carl Novina, Shannon Turley, Florian, and Shiv Pillai all taught me a great amount about teaching. My formative years weren’t restricted just to graduate school of course, and my Bill-Nye-the-Science-guy-esque 6th grade science teacher Mr. Rasmussen, 7th grade teacher Mr. Hansen, and high school substitute biology turned-permanent Harvard PhD Dr. Langdon were all critical in getting me where I am today. At Tufts, Harry Bernheim provided my first introduction to immunology, and allowed me to break from traditional coursework and academic rules by pursuing research in London and at Children’s Hospital where I worked with Luigi Notarangelo and Itai Pessach. I hope one day to be able to emulate an amalgam of their
teaching and mentorship styles. My classmates provided some great discussions in courses during the first year which pushed me to solidify my immunological thinking.

Sue Fahlbeck/Perkins (sometimes I still try to email Sue Fahlbeck, so I hope she has email forwarding setup) makes the Immunology program run. I think she is as strong a selling point as any faculty member here for the program. Kenji Kono has to be up for Boston’s best IT guy after winning every award there is at Harvard. Alex Shimada-Brand made the F31 application as simple as those things can be, and some of those monies have been used here (5 F31 AR 63546-3).

Outside of the lab, biking and food have been a constant source of sustenance, challenge, and connections. Chris Borges was too similar to be friends with during our first few months in graduate school. But, after some meals and bike rides together, we ended up being not only training partners for a while but also started PhaDe Food Labs to take the scientific approach into something that is immediately rewarding: results you can eat now. When I started my PhD I owned one bike with one gear, and I will graduate with four. Somewhere along the way, I got the competitive bug, perhaps inspired by people asking me, “have you ever raced?” and I decided to line up for some. The ups and downs of cycling seemed to balance out those of the PhD for the most part. With a particular penchant for going uphill, I got a few good results and I ended up being a part of Harvard Cycling and Green Line Velo towards the end of my PhD. The best thing that happened from my joining GLV was meeting Lane, who has been supportive and understanding through the home stretch.

Finally, I’d like to thank my Mom (Soledad), Dad (Jose), and Sister (Maria). From before I can remember, my parents have fully supported me in everything I wanted,
and gently steered me in the right direction at the right times. My sense of scientific
curiosity and approach to how I try and take the world in is definitely thanks to the
environment they created at home. One of the best things they did for me was to make
sure that I spoke English funny through age 8, as they raised me and my sister speaking
both English and Spanish. My mom’s cooking, care, and cooking, provided great
nourishment as I grew up, and still continues to do so today. I think based on my patterns
of moving this may be one of the reasons why it is difficult to escape the Boston area.
Many have asked me if I wanted to be a scientist because my dad is a scientist as well
(and more recently if I don’t want to be a PI because he is a PI), but I actually never felt
pressured in any way to go down this path. Somehow the early memories of being in the
lab, dinner table conversations, and general curiosity about how biological systems work
got me into this whole mess…and I really couldn’t see it any other way. I honestly still
don’t know what it means to have a real job and I intend to keep it that way.
Chapter 1: Background

Modified from pending publication:

1.1 Preface

In this work, we will review the interactions between the peripheral nervous system and the immune system. While over recent years there has been an accumulation of data functionally linking these two systems, the original discovery of key molecules and cells in both these systems points to commonalities at inception. A reading of the convoluted process of scientific inquiry reveals that many of the canonical molecules and cell types of the nervous and immune systems were actually discovered in tissues whose primary function was considered the domain of the other field. A prime example is the description of his namesake epidermal cell by Paul Langerhans in 1868, which he hypothesized to function as a sensory neuron (Jolles, 2002). Perhaps due to the initial description of Langerhans cells as cells of the nervous system, it took over a century for the hematopoietic origin and immunological functions of this prototypical antigen-presenting cell (APC) to be recognized (Malissen et al., 2014; Merad et al., 2002).

Interestingly, acetylcholine, the hallmark neurotransmitter of cholinergic neurons was originally isolated from bovine and equine spleens (Andersson and Tracey, 2012). While acetylcholine was rapidly understood to have a critical role as a neurotransmitter of the parasympathetic nervous system, it’s source and role in regulating splenic immunity took decades to understand (Nance and Sanders, 2007; Pena et al., 2011; Rosas-Ballina et al., 2011; Williams et al., 1981). It was only recently described that a subset of memory T cells (Tmem) in the spleen provides a critical source of acetylcholine in the spleen since splenic innervation is predominantly adrenergic in nature (Felten et al., 1987; Pena et al., 2011; Rosas-Ballina et al., 2011).
The history of discovery for the molecules and cells involved in a biological process often dictates their perceived relevance to a specific field (Maestroni, 2004; Silverstein, 2004). So, does acetylcholine belong to the nervous system and a Langerhans cell to the immune system? For the purposes of discussion, these classifications are helpful (Serafeim and Gordon, 2001; Shepherd et al., 2005; Steinman, 1993). However, it is imperative for biologists trying to understand complex physiologic processes to attempt to avoid anchor bias and instead consider their discoveries in physiological and evolutionary context.

In this chapter, we aim to integrate classical work and thinking in the field of neuro-immunology (Ader et al., 1987) with our contemporary understanding of recently described cellular subsets in both systems (Iwasaki and Medzhitov, 2015; Le Pichon and Chesler, 2014). Along the way we will try to translate the often esoteric and intimidating vocabulary of each field, which often stimulates an allergic or “fight-or-flight” response when each discipline is confronted with the litany of interleukins, CD antigens or the nomenclature of neurotransmitters. We will focus on regulation of immunological processes by the peripheral nervous system which is composed of the autonomic nervous system and the somatosensory nervous system. We will discuss how the peripheral nervous system regulates the development and deployment of the immune system and also its effects on maintaining and resetting homeostasis. We will then focus on contribution of the somatosensory system in the context of autoimmune, autoinflammatory, allergic, and infectious processes primarily at barrier tissues.
1.2 Overview of the Peripheral Nervous System

1.2.1 Basics

The PNS represents the part of the nervous system that is outside of the brain and spinal cord and provides a channel of communication between the central nervous system (CNS) and peripheral tissues. The PNS is broadly divided into the somatic system and the autonomic system, which interface with the external and internal environment, respectively (Figure 1.1). The somatic system enables us to sense and respond to the outside world, while the autonomic system functions to maintain homeostasis.
The peripheral nervous system can be broadly divided into the autonomic and somatosensory nervous systems. It is important to note that both systems have efferent and afferent function, meaning that the autonomic nervous system has both motor and sensory neurons. While the motor neurons of the autonomic nervous system are fairly homogenous and can be subdivided into sympathetic and parasympathetic based on the post-ganglionic neurotransmitters, there is considerable sensory heterogeneity. This sensory heterogeneity is also exhibited by the somatosensory PNS which encodes specific subsets of neurons whose cell bodies reside in dorsal root ganglia (DRG) and are responsive to distinct physical, thermal, or chemical stimuli.

The names of the major ion channels that are characteristic markers of discrete subpopulations of somatosensory neurons with shared function are shown.

**Figure 1.1: Efferent and Afferent Branches of the Autonomic and Somatosensory Nervous Systems**

The peripheral nervous system can be broadly divided into the autonomic and somatosensory nervous systems. It is important to note that both systems have efferent and afferent function, meaning that the autonomic nervous system has both motor and sensory neurons. While the motor neurons of the autonomic nervous system are fairly homogenous and can be subdivided into sympathetic and parasympathetic based on the post-ganglionic neurotransmitters, there is considerable sensory heterogeneity. This sensory heterogeneity is also exhibited by the somatosensory PNS which encodes specific subsets of neurons whose cell bodies reside in dorsal root ganglia (DRG) and are responsive to distinct physical, thermal, or chemical stimuli. The names of the major ion channels that are characteristic markers of discrete subpopulations of somatosensory neurons with shared function are shown.
There are two arms to each system with sensory/afferent neurons carrying information from the periphery to the CNS and motor/efferent neurons sending commands outwards to effector tissues (Figure 1.1). Both autonomic and somatic sensory neurons have bifurcated axons with cell bodies organized into discrete clusters termed sensory ganglia. Almost all sensory information from anatomic regions caudal to the neck passes through dorsal root ganglia (DRG) or vagal sensory ganglia. Somatosensory information from above the neck including the face and brain is transmitted through the trigeminal ganglia. DRG sensory neurons are considered part of the somatic system, with vagal sensory neurons being their counterpart in the autonomic system.

There are fundamental differences between the organizational principles of the efferent components of the two systems. The efferent branch of the autonomic nervous system is comprised of two anatomically, biochemically and functionally distinct subsystems: the sympathetic and parasympathetic nervous systems. The preganglionic neurons for both systems arise in the CNS, either from the thoracic and lumbar regions of the spinal cord for sympathetic neurons, or from several nuclei of the brainstem and the sacral region of the spinal cord for parasympathetic neurons. The two systems remain spatially segregated as preganglionic neurons synapse with postganglionic neurons in a ganglion that then projects to peripheral effector tissues. The specific effect induced by sympathetic and parasympathetic systems on a particular target organ depends on a multitude of factors, including the pattern and density of innervation, the type of neurotransmitter used and the type of receptor(s) on a target cell. In a nutshell, effects in
different organs elicited by the sympathetic and parasympathetic systems are coordinated at a whole body level to support either a fight-or-flight response or a rest-and-digest function (McCorry, 2007).

The sympathetic and parasympathetic systems are composed of relatively homogenous populations of pre- and post-ganglionic neurons (Figure 1.1). For instance, preganglionic neurons of both systems, postganglionic parasympathetic neurons and a subset of postganglionic sympathetic neurons, such as the ones innervating sweat glands, are cholinergic (acetylcholine-synthesizing), while the majority of postganglionic sympathetic neurons are adrenergic (norepinephrine-synthesizing). The sympathetic and parasympathetic innervation of peripheral tissues is therefore conveniently identified based on expression of enzymes critical for biosynthesis and vesicle loading of the corresponding neurotransmitter, e.g. tyrosine hydroxylase (TH), and dopamine beta-hydroxylase (DBH) for sympathetic neurons, and choline acetyltransferase (ChAT) for parasympathetic neurons (McCorry, 2007). Efferent neurons of the autonomic nervous system can also express neuropeptides, such as neuropeptide Y (NPY), yet their functional relevance is still unclear.

In contrast, the molecular characteristics of autonomic sensory and somatosensory neurons are extremely diverse (Chang et al., 2015; Usoskin et al., 2015), and deciphering this heterogeneity is a topic of ongoing investigation (Lallemand and Ernfors, 2012; Marmigere and Ernfors, 2007). The conventional definition of sensory neurons is based on their function: nociceptors preferentially respond to noxious and pruritogenic stimuli as well as temperature; mechanoreceptors respond to innocuous tactile sensations and vibration; and proprioceptors detect joint and muscle position (Figure 1.1). Several
systems have been proposed to classify sensory neurons based on their molecular traits. For instance, within nociceptors, peptidergic nociceptors are defined by expression of neuropeptides, such as calcitonin gene-related peptide (CGRP) and Substance P, while the majority of nonpeptidergic nociceptors express binding sites for the lectin IB4 (ref. (Mulderry et al., 1988; Silverman and Kruger, 1990)). Alternatively, these same neurons can also be distinguished by their differential expression of hallmark receptors and ion channels. According to this nomenclature, the two nociceptor subsets defined by TRPV1 (transient receptor potential cation channel, subfamily V, member 1) and MrgprD (MAS-related GPR, member D) preferentially mediate thermal and mechanical nociception, respectively (Cavanaugh et al., 2009; Mishra and Hoon, 2010).

### 1.2.2 Nociceptor “Subsets”

NaV1.8 is a sodium channel expressed on the majority of sensory neurons in both the somatic and autonomic nervous systems (Abrahamsen et al., 2008; Gautron et al., 2011). The NaV1.8+ population of somatic sensory neurons includes those involved in thermosensation (TRPV1: heat, TRPM8: cold), mechanosensation (Mrgprd), chemical nociception (TRPA1), and pruriception (Mrgpra3, Nppb) but excludes proprioceptors and most mechanoreceptors (Chiu et al., 2014; Usoskin et al., 2015). Developmental deletion of TRPV1+ sensory afferents using the genetic expression of diphtheria toxin in TRPV1+ neurons has demonstrated considerable developmental overlap in subsets as these mice lose both heat and cold sensation (Mishra et al., 2011). However, adult ablation of either TRPV1 or TRPM8 neurons with diphtheria toxin injection yields mice in which either heat or cold sensation are specifically lost (Pogorzala et al., 2013). Furthermore, despite the fact that the neuronal subset expressing TRPV1 is required for thermosensation, loss
of the ion channel itself does not fully impair noxious heat sensation implying that other mechanisms can compensate (Caterina, 2000; Mishra and Hoon, 2010; Woodbury et al., 2004). While expression of some of these ion channels can be mutually exclusive, there is considerable overlap as in the case of TRPV1 and TRPA1 (Le Pichon and Chesler, 2014).

A recent study that employed a single-cell RNA-Seq approach and unbiased transcriptome clustering to identify eleven distinct subtypes of DRG neurons revealed even greater diversity (Usoskin et al., 2015). Regardless of the classification scheme, different sensory modalities are generally mediated by different populations of sensory neurons each endowed with unique morphological and physiological properties (Basbaum et al., 2009; Moayedi and Davis, 2013). There are, however, notable exceptions where the sensory modality represented by a given neuronal population depends on that population's interactions with other neurons (Duan et al., 2014; Roberson et al., 2013).

1.2.3 Experimental Strategies to Study Neuroimmune Interactions

The standard techniques to investigate neuro-immune interactions include several experimental strategies. The most reductionist approach relies on exposing leukocyte subsets in vitro to specific neurotransmitters or neurotransmitter receptor agonists and/or antagonists. This strategy provides high mechanistic resolution and allows exacting investigations of the underlying cellular biology, but it typically cannot identify a neuronal source, location or physiological role. A related approach employs receptor agonists or antagonists in vivo. This strategy can provide clues that a candidate pathway is active in a physiological setting, but it can be difficult to assess if the observed effects on the immune system are direct or indirect. One approach to address this question
involves technically demanding co-cultures of isolated DRG neurons with purified leukocyte subsets (Chiu et al., 2013). A more widely used in vivo strategy makes use of selective elimination of specific neuronal activities. For example, pharmacological agents, such as capsaicin and resiniferatoxin (RTX) can ablate TRPV1+ heat-sensing neurons and 6-hydroxydopamine (6-OHDA) can transiently deplete catecholamine stores by interfering with neurotransmitter recycling (Karai et al., 2004; Kostrzewa and Jacobowitz, 1974). These agents enable pharmacological interrogation of specific neuronal subsets but are typically used on a systemic level. More recently, investigators have implemented genetic models of neural ablation based on the diphtheria toxin system to either constitutively or conditionally ablate neurons based on targeted expression of diphtheria toxin or its receptor in neuronal populations that are defined by expression of lineage specific ion channels and other markers (Abrahamsen et al., 2008; Gautron et al., 2011; Mishra et al., 2011; Shields et al., 2012). These systems allow for the cleanest genetic interrogation of specific neuro-immune interactions but their interpretation can be challenging in light of the complex expression patterns of ion channels during neuronal development.

In-depth molecular understanding of the PNS together with the recent advent of chemogenic and optogenetic approaches affords an unprecedented opportunity to genetically target neuronal populations of interest for functional studies (Farrell and Roth, 2013; Fenno et al., 2011). So far, these tools have been utilized mainly to map neural circuits in fine detail by activating or inhibiting specific populations of neurons in a genetically and regionally defined fashion (Carter et al., 2013). Their use should also be very informative in studying neuro-immune interactions.
1.3 Neuroimmune regulation – a historical perspective

A dichotomy between sympathetic nerves dampening and sensory nerves promoting inflammation was proposed as early as 1909. It was noted that resection of the sympathetic nerves to a rabbit ear allowed for more robust inflammation while resection of sensory nerves prevented the ordinary manifestation of inflammation (Chapman and Goodell, 1964). When challenged with Streptococcus pyogenes, rabbits lacking sensory innervation could not control replication of the bacteria presumably due to a delayed and blunted inflammatory response (Chapman and Goodell, 1964). While this work did not elucidate the cellular or molecular mechanisms, it laid the foundation for a rich literature which has followed investigating the neural regulation of inflammation.

The historical characterization of neuro-immune interactions extends beyond the peripheral regulation of inflammation into primary and secondary lymphoid organs. In Nils Jerne’s Nobel Prize acceptance speech, he mentioned how, “when we place a population of lymphocytes from such an animal in appropriate tissue culture fluid, and when we add an antigen, the lymphocytes will produce specific antibody molecules in the absence of any nerve cells” (Jerne, 1985; Mishell and Dutton, 1967). He further goes on to note that despite the complexity of the immune system, which is on par with that of the nervous system, that this reaction occurs independent of the brain (Jerne, 1985). Antigen-specific activation, proliferation, and effector functions of lymphocytes can be well studied in vitro. However, even in the original description of a cell-culture system for studying antibody responses, the authors noted that the main difference between in vivo and in vitro immunization was that the, “in vitro response does not appear to be limited
by whatever mechanisms regulate the *in vivo* response.”(Mishell and Dutton, 1967) Of course, *in vitro* splenic cultures are devoid of the neural network, primarily of sympathetic but also of sensory origin, which regulate immune responses(Downing and Miyan, 2000; Felten et al., 1985; Straub et al., 1998).

Even before the discovery of the two main cell lineages of the adaptive immune system, the innervation of primary and secondary lymphoid tissue had been recognized through classical histological techniques(Elenkov et al., 2000; Von Euler and Hillarp, 1956). In the 1980s, a pioneering body of work by Felten and others using anterograde and retrograde labeling techniques led to the circuitry of sympathetic and peptidergic innervation being mapped to primary, secondary, and mucosal-associated lymphoid tissue(Bellinger et al., 1989; Felten et al., 1987; Felten et al., 1985; Weihe et al., 1991; Williams et al., 1981). This work not only established the potential conduits for the regulation of immune responses by the peripheral nervous system, but functional studies using chemical denervation described how norepinephrine released from sympathetic nerve terminals in spleen negatively regulated antibody responses(Williams et al., 1981). Thus, this demonstrated neural regulation over the initiation of adaptive immunity(Williams et al., 1981), and the regulation of one complex system by another.
1.4 Role of the PNS in the development, deployment and homeostatic regulation of the immune system

1.4.1 Hematopoiesis

The production, distribution, and activation state of leukocytes are all subject to variation and alterations during immune responses but typically remain within a definable range, akin to many homeostatic processes (Kotas and Medzhitov, 2015). Recent studies have begun to characterize the specific subsets of cells in the peripheral nervous system that regulate hematopoiesis and the numbers of leukocytes present in circulation or in tissues. Peripheral neurons also play a role in determining the activation state and polarization of lymphocytes during immune responses. Furthermore, immune cells have been found to complete circuits that were previously thought to be the sole domain of the peripheral nervous system.

The majority of leukocytes arise in the bone marrow during adult hematopoiesis from hematopoietic stem cells (HSCs). The study of HSCs has illuminated various aspects that are generalizable to other aspects of developmental and regenerative biology such as the importance of the stem cell niche in regulating the quiescence and migration of stem cells (Scadden, 2014). HSCs contribute to the repopulation of lymphoid, myeloid, and erythroid cells as well as platelets through progressive differentiation into restricted progenitors. The balance of cellular output is sensitive to environmental perturbations such as bleeding and infection which alter the output of HSCs (Ueda et al., 2005).

The sympathetic nervous system has emerged as a key component and regulator of the HSC niche (Figure 1.2) (Hanoun et al., 2015). Early studies characterizing the innervation of lymphoid organs identified sympathetic nerves as the primary neural
constituent in bone marrow (Felten et al., 1985; Webber et al., 1970; Weihe et al., 1991). These observations stimulated an interest in determining how sympathetic tone could affect the production of leukocytes as well as their release from marrow (Webber et al., 1970).
Figure 1.2: Neural signals that regulate hematopoiesis, priming and migration of immune cells

**Development (Bone Marrow):** Hematopoiesis is regulated by the sympathetic nervous system. Sympathetic neural outflow leads to increased norepinephrine release which acts on nestin+ mesenchymal stem cells (MSCs) to reduce levels of CXCL12, which normally keeps HSCs resident in the niche. When CXCL12 levels drop, HSCs can egress from the niche. Furthermore, Schwann cells which surround autonomic neurons provide a source of TGF-β which keeps HSCs in a quiescent state.

**Priming (Lymph Node):** Dendritic cell priming of T cells requires Signal 1 through the TCR, Signal 2 via co-stimulatory molecules, and Signal 3 which is provided via soluble cytokines. Sensory neurons, via Substance P and CGRP, can regulate the production of Signal 3 cytokines from DCs, which subsequently impacts T cell polarization and effector cytokine production. Furthermore, sympathetic neurons can produce norepinephrine, which negatively regulates Type 1 responses and promotes Type 2 responses by increasing levels of intracellular cAMP.

**Deployment (Peripheral Tissue):** Peripheral neurons associated with vessels can impact leukocyte recruitment into tissues. Neuropeptides from sensory neurons can act on endothelial cells to increase vascular permeability (classically termed neurogenic inflammation) but also regulate translocation of P-selectin and expression of E-selectin which are important in leukocyte rolling. Once leukocytes are rolling within vessels, they receive chemotactic signals that trigger integrin activation. ICAM-1, an important endothelial ligand for the integrin LFA-1, is regulated by norepinephrine from sympathetic neurons in some vascular beds. Finally, firmly adherent leukocytes extravasate into the surrounding tissue. Norepinephrine from sympathetic neurons is chemotactic for macrophages and sensory neuron-derived Substance P and CGRP can bias cytokine production by dendritic cells.
Seminal work from Paul Frenette’s group converged on this line of research and identified how norepinephrine released by sympathetic neurons can trigger HSC mobilization (Katayama et al., 2006). They found an unexpected role for factors important for nerve myelination in HSC mobilization in response to granulocyte colony-stimulating factor (G-CSF) administration (Katayama et al., 2006). Release from marrow is regulated by CXCL12 levels on stromal constituents, whose cognate receptor CXCR4 is expressed on HSCs. Using both β2 adrenergic agonists and mice rendered pharmacologically or genetically deficient in catecholamine production from sympathetic neurons, they identified that intact adrenergic signaling via norepinephrine release in marrow is required for a reduction in CXCL12 produced by stromal cells which results in HSC mobilization (Katayama et al., 2006). This established a role for sympathetic nerves in the regulation of HSCs with important implications for bone marrow transplantation (BMT).

Recent work has demonstrated the importance of sympathetic neuronal regulation of HSCs via CXCL12 in the context of psychosocial stress (Heidt et al., 2014).

Circadian rhythms are critical in regulating the sympathetic nervous system. This relationship suggested that normal diurnal fluctuations in sympathetic tone could regulate HSC mobilization (Mendez-Ferrer et al., 2008). Indeed, HSCs were noted to be released from marrow and peak in circulation during periods of inactivity in mice (e.g. daytime) and that these circadian oscillations are entrained via central signals transmitted through norepinephrine secretion from sympathetic nerves (Mendez-Ferrer et al., 2008). One potential reason for HSC release and mobilization is to provide a source for “on-the-spot” hematopoiesis during infection as HSCs not only enter blood but also survey secondary lymphoid organs (Massberg et al., 2007).
Beyond mobilization, cycling of HSCs is also regulated by niche components which are composed of a variety of cell types, including some of neural origin. Recently, a relationship between nestin+ mesenchymal stem cells (MSCs) and HSCs was identified (Mendez-Ferrer et al., 2010). The niche is not homogenous as three-dimensional image analysis of bone has revealed heterogeneous distribution of HSCs (Kunisaki et al., 2013; Nombela-Arrieta et al., 2013). Nestin+ MSCs relay signals from sympathetic nerves to HSCs which regulate their maintenance in marrow. Sympathetic nerves in the bone marrow are ensheathed by non-myelinating Schwann cells which have been postulated to provide a critical source of TGF-β to maintain HSC quiescence (Yamazaki et al., 2011). Loss of sympathetic neurons, nestin+ MSCs or Schwann cells all lead to a decrease in HSCs in marrow due to enhanced mobilization and reduced quiescence (Mendez-Ferrer et al., 2010; Yamazaki et al., 2011). Regulation of hematopoiesis is not the sole domain of the sympathetic nervous system as genetic loss of NK-1R from hematopoietic stem cells (tachykinin receptor, for substance P and hemokinin-1) is important for lymphocyte generation after bone marrow transplantation (Berger et al., 2013).

While the HSC niche has been intensively studied, more committed progenitors of lymphoid and myeloid lineages inhabit distinct areas of the bone marrow (Ding and Morrison, 2013). Recent experiments studying native, non-transplant hematopoiesis have uncovered that the contribution of HSCs to steady-state hematopoiesis may actually be minimal (Sun et al., 2014). Instead, a larger number of restricted clones contributes to lymphoid, myeloid, or erythroid lineages (Sun et al., 2014). Based on this study, it will be
imperative to understand how neural regulation of niches for more committed progenitors influences their activity.

Adrenergic signaling is important in modulating myelopoiesis. Chemical sympathectomy in mice was found to result in an increase in myeloid cells after bone marrow transplantation suggesting that signals from the sympathetic nervous system typically suppress myelopoiesis (Maestroni and Conti, 1994). However, systemic sympathectomy interferes with the production of catecholamines to all lymphoid organs and the spleen has recently been identified as a key reservoir for inflammatory monocytes (Swirski et al., 2009b). The release of monocytes from spleen requires angiotensin signaling through the AT-1 receptor and is also controlled by noradrenergic neurons (Seeley et al., 2013; Swirski et al., 2009b). After peripheral catecholamine depletion, monocyte recruitment was enhanced to a site of bacterial infection resulting in enhanced survival (Seeley et al., 2013). Identifying the intersection between the renin-angiotensin system and the sympathetic nervous system in regulating the release of inflammatory monocytes will be of interest.

1.4.2 Priming of Adaptive Immunity

The detection of distinct microbial patterns and effectors by specialized antigen-presenting cells instructs the quantity and quality of a subsequent adaptive immune response (Iwasaki and Medzhitov, 2015). Type 1 immunity is primed in response to viruses, bacteria, and fungi while Type 2 immunity is engaged by helminth infections, envenomation, and encounters with allergens. Typically after a pathogen has breached an epithelial barrier, DCs are the first APCs to encounter the microbe. After pathogen
detection and phagocytosis, DCs migrate to regional lymph nodes via afferent lymphatics draining the tissue (Alvarez et al., 2008). The two main determinants of the type of immune response which will be initiated are the PRRs engaged and the specific DC subset involved (Igyártó et al., 2011; Iwasaki and Medzhitov, 2015).

DC migration and motility is influenced by sympathetic and sensory neural input. Using confocal and two-photon microscopy, studies in skin, lung, and gut have found DCs located in close proximity to sensory neurons (de Jong et al., 2015; Hosoi et al., 1993; Kradin et al., 1997; Riol-Blanco et al., 2014; Veres et al., 2007). Immature dendritic cells are responsive to both norepinephrine and neuropeptides (Dunzendorfer et al., 2001; Maestroni, 2000). While neuropeptides such as CGRP have been shown to have directional chemotactic activity on immature DCs in vitro, norepinephrine appears to enhance the generalized migratory capacity of DCs, perhaps aiding in their sampling and acquisition of antigen in tissue (Figure 1.2) (Maestroni, 2000).

In addition to PRR signaling, DCs integrate signals from peripheral neuropeptides, which in turn influence their cytokine production profile (Figure 1.2) (Madva and Granstein, 2013). The pro-inflammatory activities of substance P were first mechanistically linked to enhanced production of IL-1, TNF-α, and IL-6 from monocytes in vitro (Lotz et al., 1988). The activity of CGRP on Langerhans cells was initially described as immunosuppressive, however, a closer investigation by the same group actually led to the finding that CGRP-treated Langerhans cells stimulate Type 2 immunity (Ding et al., 2008; Hosoi et al., 1993). Deficiency in the receptor for CGRP leads to enhanced CD80 and IL-12 expression on DCs in dLNs and subsequently a more robust DTH response (Holzmann, 2013; Mikami et al., 2014). Conversely, Substance P
and signaling through the neurokinin-1 receptor *in vitro* leads to enhanced Type 1 immunity through production of IL-12 and IL-23 (Cunin et al., 2011; Janselsins et al., 2013). While peptidergic sensory neurons typically co-express CGRP and substance P, the mechanisms that preferentially lead to the release of none, one, or both are not currently understood. Since these neuropeptides have distinct actions on DC production of key instructive cytokines for T-cell polarization, understanding this fundamental mechanism of selective neuropeptide release is of interest.

Another important way by which neuropeptides modify T cell responses is by acting on T cells themselves. The priming of antigen-specific T cells involves serial contacts with DCs in lymph node which will integrate Signal 1 (through the TCR), Signal 2 (co-stimulatory molecules), and Signal 3 (cytokines) to determine the magnitude and quality of the response (Grossman and Paul, 2015; Iwasaki and Medzhitov, 2015; Mempel et al., 2004). In the polarization of CD4+ T cells, it seems that the strength of signaling through the TCR dictates the receptiveness of T cells to the inflammatory milieu (van Panhuys et al., 2014). These distinct signals converge on common signaling and metabolic pathways within T cells, which are also under regulation of neurotransmitter and neuropeptides (Pollizzi and Powell, 2014). Sympathetic outflow and norepinephrine production negatively regulate Type 1 responses by acting both to reduce IL-12 production from APCs as well as on T cells directly, which express the β2-adrenergic receptor *(Figure 1.2)* (Panina-Bordignon et al., 1997; Sanders et al., 1997). The mechanism by which this occurs is elevation of cyclic-AMP (cAMP) levels in T cells (Sanders et al., 1997; Straub et al., 1998). Levels of cAMP consequently influence mTOR function which is a key integrator of cellular metabolic state and has significant
influence over the polarization of Th1, Th2, Th17 and Treg responses with elevated cAMP levels favoring the generation of Tregs and CD8+ memory T cells by inhibiting mTOR activity (Pollizzi and Powell, 2014). Sympathetic neurons also negatively regulate DC-priming of CD8+ T cells during a primary viral infection (Grebe et al., 2009).

Most of the mechanistic experiments regarding neuropeptide effects on dendritic cells and T cells have been performed in vitro, or using neurotransmitter receptor agonists/antagonists in vivo. The in vivo neural subsets involved and anatomical location of these interactions remains to be elucidated. A challenge to the field will be to determine how the combinatorial complexity of signals from sympathetic and sensory neurons is integrated at the level of individual antigen presenting cells and T cells to influence their polarization in vivo. It will be of interest to explain how neuropeptides, which have some regioanatomical specificity, influence the ability of DC to imprint homing patterns onto T cells akin to what is seen with environmental ligands such as retinoic acid which is present at high concentrations in gut-draining LNs (Mora et al., 2003).

1.4.3 Immune Cell Trafficking

Sympathetic neurons also regulate the egress of T cells from lymphoid tissue. One of the distinctly beautiful and unique design principles of the immune system is its migratory capacity (von Andrian and Mackay, 2000). This allows for the deployment of recently-primed T cells to the appropriate anatomical location to eliminate pathogen-bearing cells. The first step in this process is exit from a lymph node into efferent lymph which eventually drains into circulation. This process is critically regulated by S1P
receptor (S1PR) signaling as cells follow a gradient of S1P which is higher in efferent lymph (Matloubian et al., 2004). Recently, β2-adrenergic receptors on lymphocytes were identified as inhibiting the egress of T cells from lymph nodes by enhancing the activity of CCR7 and CXCR4, whose ligands are highly expressed in the T-cell and B-cell zone respectively, and sequestering T cells (Nakai et al., 2014).

Peripheral neurons and the vasculature are intimately associated. Functionally, the classical studies of neurogenic inflammation highlighted the involvement of “vaso-dilators” (sensory) and “vaso-constrictors” (sympathetic) and their role in regulating tissue edema (Figure 1.2) (Chiu et al., 2012). However there is also a developmental link between these two cell types. Intriguingly, sensory nerves produce CXCL12 and VEGF-a which are required for arteriogenesis and branching thus laying the groundwork for the highways that lymphocytes use to patrol the body (Li et al., 2013; Mukouyama et al., 2002). Lymphocytes flow through vessels of progressively decreasing diameter before entering capillary beds and extravasating at post-capillary venules (von Andrian and Mackay, 2000). They express receptors which allow them to roll, stick, and transmigrate through vascular endothelium. This step-wise model allows for a tissue-specific “area-code” to recruit lymphocytes within the appropriate vascular bed (Katayama et al., 2006).

Beyond regulating generalized vascular permeability, adrenergic nerves have been identified as regulators of specific leukocyte recruitment to tissues (Figure 1.2). Sympathetic nerves transmitting circadian cues from the CNS entrain circadian rhythmicity on leukocyte numbers in circulation or in tissue by regulating levels of ICAM-1 on endothelial cells in skeletal muscle and P/E-selectin on bone marrow endothelium (Scheiermann et al., 2012). Neuropeptides can also regulate P-selectin
translocation and E-selectin expression on cutaneous vessels which influences leukocyte rolling and neutrophilic infiltration (Smith et al., 1993). The circadian regulation of leukocyte recruitment has functional consequences during inflammation, as mice were more susceptible to septic shock during periods of peak ICAM-1 expression in liver, which corresponded with an enhanced number of parenchymal neutrophils (Scheiermann et al., 2012). While the circadian regulation of physiological processes through sympathetic neural outflow has been well characterized, recent work also highlights that expression of the key clock gene BMAL1 in inflammatory monocytes intrinsically regulates diurnal rhythms (Nguyen et al., 2013). It will be of interest to determine how extrinsic and intrinsic circadian factors converge to regulate the distribution of leukocytes. Once in a tissue, norepinephrine is chemotactic for monocyte and macrophage migration (Straub et al., 2000). Presumably the importance of this leukocyte redistribution is to provide anticipatory immunity in barrier tissues during periods of peak activity where animals are at a greater risk for pathogen exposure (Nguyen et al., 2013; Pacheco-Lopez and Bermudez-Rattoni, 2011; Scheiermann et al., 2012).

1.4.4 Sharing of homeostatic circuits by the nervous and immune systems

Processes such as metabolic thermogenesis, the regulation of blood pressure, and gut motility are typically considered to involve an interaction between central neural regulators, the autonomic nervous system and parenchymal cells in the respective
tissue (Kotas and Medzhitov, 2015). However, recent studies have uncovered a key role for distinct subsets of immune cells in the regulation of these homeostatic processes.

Somatosensory and autonomic neurons are important in maintaining temperature for endotherms. A classic example is the effector responses of sympathetic innervation on end organ tissues via catecholamine release leading to shivering and sweating. However, recent work has described how catecholamines derived from macrophages are also important in thermoregulation (Nguyen et al., 2011). In mammals, thermogenesis is driven by the metabolic activity of uncoupled mitochondrial respiration in brown adipose tissue (BAT) by altering levels of UCP1 (Morrison et al., 2014). Afferent peripheral sensation from the skin of warmth and cooling is transmitted via TRPV1+ and TRPM8+ fibers, respectively and converges on the hypothalamus (Morrison et al., 2014; Pogorzala et al., 2013). The balance of these signals determines the level of efferent sympathetic outflow to WAT and BAT. While efferent sympathetic nerves in BAT produce norepinephrine which can act directly on adipocyte adrenergic receptors, alternatively activated macrophages were found to be required for thermogenesis in response to cold environments (Nguyen et al., 2011). IL-4 signaling in macrophages is essential for their alternative activation and expression of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (Nguyen et al., 2011). Norepinephrine production by macrophages in adipose tissue was shown to increase UCP1 levels and stimulates lipolysis in WAT. Due to the importance of alternative activation via the IL-4/IL-13 receptor of macrophages, future studies pursued the source of IL-4 in adipose tissue.

In addition to BAT, thermogenesis can also be regulated by the beiging of WAT, a process also controlled by immune cells (Brestoff et al., 2015; Lee et al., 2015). Both
eosinophil production of IL-4 and ILC2 production of IL-5 and IL-13 influence the
differentiation of pre-adipocytes to beige adipocytes and the catecholamine production by
macrophages (Brestoff et al., 2015; Lee et al., 2015; Qiu et al., 2014). Thus, eosinophils,
ILC2s, and alternatively activated macrophages have been identified as forming a key
efferent loop linking thermosensation with thermogenesis (Brestoff and Artis, 2015). The
specific neural circuitry that stimulate eosinophil and ILC2 cytokine production in
adipose tissue remain to be determined but evidence from the gut suggests that
neurotransmitters can act directly on ILC2 to stimulate IL-5/IL-13 production and
subsequently influence eosinophil numbers and activity (Nussbaum et al., 2013). One
fascinating area of neuro-immune connections outside of the scope of this chapter is the
resetting of the internal core temperature to the febrile range and the downstream
implications on host defense demonstrating bi-directional communication between these
two systems (Berkenbosch et al., 1987; Evans et al., 2015)

Another neuro-immune interaction which mediates a physiologic circuit is the
role for T cells in regulating vascular tension (Tracey, 2014). Evidence for the role of T
cells stemmed from experiments in Rag1-/- mice which were resistant to a model of
chronic hypertension. Repletion of the T cell compartment was sufficient to render the
mice hypertensive again (Guzik et al., 2007). A very recent study identifies the link
between the nervous system and a splenic reservoir of T cells in the model of chronic
hypertension based on angiotensin-II infusion (Carnevale et al., 2014). Angiotensin II acts
to increase sympathetic drive which acts through the celiac ganglion to drive
norepinephrine release from the splenic nerve. This acts on marginal zone macrophages
to produce placental growth factor (PIGF) which is essential for T cell mobilization to
sites of damage during the hypertensive process (Carnevale et al., 2014). Sympathectomy inhibited the release of PIGF and subsequent mobilization of T cells. Mice deficient in the receptor for CGRP are spontaneously hypertensive which suggests that the sympathetic and sensory nervous systems play opposing roles in regulating vascular tension although the source of CGRP that mediates vascular relaxation is unknown (Tsujikawa et al., 2007). Intriguingly, angiotensin-II was also identified as the factor which regulates the release of a subset of splenic inflammatory monocytes that can mediate atherosclerotic progression (Swirski et al., 2009b). This ties regulation of two immune cell subsets in the spleen by adrenergic neurons emanating from the celiac ganglion in processes which fall under the umbrella of the metabolic syndrome.

Therapies targeting both neural and immune aspects of chronic metabolic diseases may synergize in helping to restore homeostasis.

Metabolism in the host is monitored via the nervous and immune systems (Brestoff and Artis, 2015; Gautron et al., 2015; Mansuy-Aubert et al., 2015). Many metabolic diseases initiate from an imbalance in caloric intake relative to expenditure and have fundamental impacts on lifespan. Intriguingly, TRPV1-/- mice have enhanced lifespan and improved metabolic parameters due to decreased CGRP release and enhanced pancreatic insulin production (Riera et al., 2014). The neuronal or non-neuronal cell types that express TRPV1 which are instrumental in regulating systemic metabolism and longevity are not well understood, but in the NOD mouse model of Type 1 diabetes it appears that a precise balance of TRPV1 signaling in sensory neurons is essential for pancreatic cell health (Razavi et al., 2006).
The enteric nervous system, acts primarily to sense and respond to changes in luminal composition to optimally absorb nutrients. Its development and function is not only entrained by intrinsic genetic programs but is sensitive to the inflammatory state and microbial communities that inhabit the gut similar to what is seen for the immune system(Kabouridis and Pachnis, 2015; Rakoff-Nahoum et al., 2015). A recent study has shown how the presence of the microbiota is important for the development of enteric glial cells after weaning and for the continued repopulation of glial cells in villi(Kabouridis et al., 2015).

Recently, macrophages have been shown to play an important role in completing an enteric nervous system circuit involved in colonic muscular contraction(Muller et al., 2014). This study highlighted how bacterial products can actually translocate across the epithelium and lamina propria into the myenteric plexus with profound impacts on peristalsis(Muller et al., 2014). Surprisingly, microbial products did not act on glial cells but on enteric neurons themselves to stimulate CSF1 secretion(Muller et al., 2014). In mice treated with broad-spectrum antibiotics, levels of Csf1 were significantly decreased which subsequently led to a reduction in numbers of muscularis macrophages and dysregulated peristalsis. These macrophages produce BMP2 which feeds back onto enteric neurons to regulate motility via neuronal regulation of muscular contractility(Muller et al., 2014).

1.4.5 Neuroimmune circuits that sense inflammation

Peripheral inflammatory signals are important in regulating host defense responses orchestrated by the central nervous system. Hugo Besedovsky and colleagues
led pioneering work which mapped how peripheral inflammation, resulting in enhanced levels of circulating IL-1β, could be perceived by the CNS in the hypothalamus (Berkenbosch et al., 1987; Besedovsky et al., 1983). Several years later, experiments from Linda Watkins revealed that the vagus was required for fever in response to low doses of intraperitoneal IL-1β, suggesting that in settings of localized inflammation, vagal sensory afferents are important to report on peripheral immune responses (Hansen et al., 2001).

Efferent arms stemming from the CNS have been shown to be critical in regulating inflammatory responses (Andersson and Tracey, 2012). In many cases, sepsis is the result of an over-exuberant immune response rather than uncontrolled replication of a microbe due to an immune-suppressed state. Kevin Tracey’s group made the observation that electrical stimulation of the vagus could suppress cytokine responses in the spleen and liver to a high-dose of LPS via acetylcholine acting on macrophages (Borovikova et al., 2000). A synthesis of this work led to the proposal of the inflammatory reflex in which the vagus nerve could mediate both an afferent sensing and efferent suppressive arc (Andersson and Tracey, 2012). Understanding the neuronal circuits that reset homeostasis in the immune system has the capacity to fundamentally alter critical care (Deutschman and Tracey, 2014)

The spleen does not receive vagal innervation and instead is innervated primarily by noradrenergic efferents from the superior mesenteric celiac ganglion which led to the question: where does the acetylcholine come from? (Bellinger et al., 1989). Work from Luis Ulloa’s group and Kevin Tracey’s lab identified an acetylcholine-producing splenic memory T cell subset that, in response to norepinephrine, produces acetylcholine which
can act on macrophages to suppress TNF-α production (Pena et al., 2011; Rosas-Ballina et al., 2011). The originally proposed inflammatory reflex has served as an excellent model to continually revise and refine the specifics of the neuro-immune circuitry involved (Bratton et al., 2012; Martelli et al., 2014). Unexpected circuits have been discovered with therapeutic ramifications such as a link between electroacupuncture in the sciatic nerve leading to vagal activation and adrenal synthesis of dopamine, which has the capacity to negatively regulate the NLRP3 inflammasome by enhancing cAMP levels in cells expressing the dopamine D1 receptor (Torres-Rosas et al., 2014; Yan et al., 2015). Dopamine release was found to be protective in both polymicrobial and LPS-mediated sepsis (Torres-Rosas et al., 2014; Yan et al., 2015). The recent characterization of specific subsets of vagal sensory afferents and the generation of optogenetic techniques to manipulate their activity will undoubtedly help to trace the specifics of the afferent and efferent arcs in this and other circuits which help to maintain inflammation in check (Andersson and Tracey, 2012; Chang et al., 2015)

1.5 Role of the PNS in Barrier Tissues

1.5.1 Detection

The function of the sensory nervous system is to allow for the host to interact with the external environment. Nociceptors are a specialized population of sensory neurons which detect potential harmful levels of stimuli and can provoke behavioral responses in the host to avoid or reduce potential damage. Cells of the immune system have evolved to mount innate and adaptive immune responses to noxious challenge and are strategically
positioned in barrier tissues to mount a protective inflammatory response. These protective defense mechanisms can become dysregulated in the context of autoimmune, autoinflammatory, or allergic inflammation. A growing body of literature suggests a key role of nociceptors in driving these pathological processes. Furthermore, a direct dialogue between microbes and neurons exists with profound implications for infectious disease progression.

Hosts can utilize three distinct and complementary strategies to mitigate disease which include avoidance, resistance, and tolerance (Medzhitov et al., 2012; Pacheco-Lopez and Bermudez-Rattoni, 2011; Rivas et al., 2014). The first way that the sensory nervous system can protect a host is via avoiding an environment that is potentially injurious or with high levels of pathogenic microorganisms (Pacheco-Lopez and Bermudez-Rattoni, 2011). Seminal work in *C. elegans* has described the importance of neural circuits in detecting pathogenic bacteria relative to those species that can be utilized as a harmless food source (Meisel and Kim, 2014; Reddy et al., 2009). Avoidance behavior in humans can be mediated by both innate and learned behavioral cues mediated by olfactory and gustatory cues (Choi et al., 2011; Pacheco-Lopez and Bermudez-Rattoni, 2011). Interestingly, it has been speculated that Type 2 host defense in the form of IgE-mediated mast-cell degranulation may be a way of storing “memories” to potentially injurious substances that would otherwise not trigger nociceptors (Costa-Pinto et al., 2007; Palm et al., 2012). However, in a modern environment, this form of anticipatory host defense can result in asthma or anaphylaxis triggered in response to innocuous or sub-threshold levels of allergens (Palm et al., 2012).
1.5.2 Lung

Both sensory and sympathetic nerves, by interacting with immune and parenchymal cells, have profound influence on lung pathology in disease states. In this chapter, we will focus on neural influences during asthmatic responses but refer the reader to recent reviews on the subject which integrate neural regulation of airway and lung function for a comprehensive overview (McGovern and Mazzone, 2014).

Visualizing the interactions of immune cells in tissue can be incredibly illuminating in determining putative partners-in-crime. In a series of elegant imaging studies utilizing confocal-microscopy on fixed tissue and also two-photon microscopy on ex-vivo lung slices, Braun and co-workers identified that around 10% of dendritic cells in lung are found within 0.3 microns of sensory neurons which stain positive for CGRP (Veres et al., 2007; Veres et al., 2009). Activation of sensory neurons by electrical field stimulation triggered increased motility of DCs although the mechanism of this altered motility is still unclear (Voedisch et al., 2012). During acute OVA-induced asthma, DC motility slowed significantly, and appeared in contact with DCs and proliferating T cells (Veres et al., 2009; Voedisch et al., 2012).

What is the nature of the neurons which mediate an asthmatic response? Asthma is a combination of a pulmonary inflammatory infiltrate and enhanced strength of airway constriction. Combinations of pharmacological, ion channel knockouts, and genetic tools to delete subsets of neurons have been used to address this question. In isolation, the vagus nerve, the TRPA1 but not TRPV1 ion channel, and TRPV1+ vagal sensory afferents have all been implicated (Caceres et al., 2009; Cyphert et al., 2009; Trankner et
A synthesis of these studies suggests a pathway by which the triggering of antigen-specific IgE-bearing mast cells induces serotonin release which acts on 5-HT receptors on TRPA1+TRPV1+ vagal sensory afferents (Figure 1.3) (Bautista et al., 2014; Cyphert et al., 2009). Additionally, IL-5 from CD4+ Th2 cells can also act on TRPA1+TRPV1+ vagal afferents and stimulate VIP release which forms a positive feed-forward loop (Talbot et al., 2015).

TRPA1−/− but not TRPV1−/− mice showed a diminished inflammatory infiltrate after OVA challenge as well as decreased airway hyperreactivity which could not be explained by diminished OVA-specific IgE but was associated with reduced levels of Type 2 cytokines and chemokines (Caceres et al., 2009). Specific developmental (TRPV1-DTA) or post-developmental ablation (TRPV1-DTR) of TRPV1-expressing neurons in mice led to loss of both TRPV1 and TRPA1 vagal sensory neurons and ameliorated airway hyperreactivity (Pogorzala et al., 2013; Trankner et al., 2014). Surprisingly, this was not associated with a reduced inflammatory infiltrate but only with a reduction in hyperreactivity (Trankner et al., 2014). These authors harnessed optogenetic tools, for the first time, to investigate neural activity in an inflammatory model by activating TRPV1+ vagal sensory neurons using optogenetics which led to enhanced airway hyperreactivity only in the presence of an inflamed airway (Trankner et al., 2014).

Conversely, a recent study from the Woolf lab elucidates a potential cascade of neuro-immune interactions with influences over both airway and inflammatory parameters. Either pharmacological silencing using a charged derivative of lidocaine or ablation of NaV1.8-lineage neurons (which targets TRPV1+ and TRPA1+ neurons
amongst others) decreased eosinophilia and macrophage accumulation in bronchoalveolar lavage fluid (Talbot et al., 2015). The study also reported that IL-5, a cytokine produced by ILC2s, can act directly on TRPA1+ vagal sensory afferents triggering the release of VIP, which stimulates further IL-5 and IL-13 production from ILC2s and CD4+ Th2 cells (Talbot et al., 2015). Further work will be required to understand how complete absence of NaV1.8-lineage neurons leads to reduced inflammation and hyperreactivity while specific loss of TRPV1+TRPA1+ vagal sensory afferents uncouples airway hyperreactivity from inflammation (Caceres et al., 2009; Talbot et al., 2015; Trankner et al., 2014).
Figure 1.3: An integrative view of neural signals in regulation of immune responses at barrier tissues.

Certain commonalities have emerged in the neural regulation of immune responses at barrier tissues, such as the skin, gut and lung. This input/output diagram highlights some of the recent discoveries and uncharted territory. Neuronal activation can occur via inflammatory cytokines, itch mediators, mast-cell derived products, metabolites, canonical ion channel ligands, lipid mediators, microbial products, and as-yet characterized stimuli. The available evidence suggests that primarily TRPV1+ TRPA1+ sensory neurons (depicted in red) promote Type 1 inflammation in the skin and gut, and Type 2 inflammation in the lung. In the skin, NaV1.8+ neurons, which encompass TRPV1+ TRPA1+ neurons, appear to have contextual anti-inflammatory properties during microbial infections, particularly with S. aureus. The mechanisms of neuronal activation and whether the regulation of immune cells occurs via single and/or combinatorial neuropeptides as well as other pathways remains to be firmly determined for most of these conditions. However, recent discoveries shed light on cytokines such as TSLP and IL-5 playing a key role in the skin and lung, respectively, in triggering sensory neurons. TRPM8+ (depicted in green) sensory neurons have been identified as serving anti-inflammatory roles in the gut via CGRP release, however, the role of these neurons in skin and lung inflammation remains to be determined. Sensory neurons exert their effects via direct communication with several target immune cells of which only a few are highlighted here.
1.5.3 Gut

The gut receives various sources of innervation including the pseudo-autonomous enteric nervous system that is partially tuned by the sympathetic nervous, as well as separate vagal and spinal sensory inputs (Udit and Gautron, 2013). Neurons with sensory function exist in the enteric, autonomic, and DRG-derived nerves that innervate the gut and oftentimes their precise source is not identified (Ben-Horin and Chowers, 2008; Engel et al., 2011a). There is a gradient of TRPV1+ CGRP+ nerve fiber density which increases along the cephalocaudal axis of the colon (Engel et al., 2012). Enteric innervation has been demonstrated to modify intestinal inflammation and has been implicated in the pathogenesis of the inflammatory bowel diseases, Crohn’s disease and ulcerative colitis. Early suggestions of this connection came from the finding that the acute ablation of GFAP+ enteric glial cells in adult mice leads to a jejuno-ileitis (Bush et al., 1998). One major challenge for the field will be to determine which sensory branch is activated as this determines the efferent motor circuitry engaged and neuropeptides released.

TRP channels and the neuronal subsets that express them have been shown to both promote and inhibit intestinal inflammation. Several studies have employed either capsaicin or RTX-mediated denervation which target both vagal sensory afferent and spinal TRPV1-expressing sensory neurons but the effects of these compounds on the enteric nervous system remains equivocal (Berthoud et al., 1997; Mishra and Hoon, 2010). In a model of TNBS-colitis, capsaicin-denervation promoted disease while sympathectomy was protective. These studies suggested that TRPV1+ vagal or spinal sensory afferents are net anti-inflammatory while adrenergic neural transmission is detrimental (McCafferty et al., 1997). Furthermore, TRPV1-/- mice subjected to DNBS
colitis had increased inflammation relative to littermate controls suggesting that TRPV1 activation on sensory neurons is protective in DNBS-colitis (Massa et al., 2006). However, there are also reports using RTX to ablate TRPV1+ sensory neurons that demonstrate amelioration of TNBS- and DSS-colitis, which was attributed to loss of pro-inflammatory Substance P (Engel et al., 2011b) and in a T cell transfer model of colitis (Gad et al., 2009). In these studies, TRPV1/- mice did not show differences in TNBS-colitis relative to controls (Massa et al., 2006). Clearly, the role of the TRPV1 ion channel and TRPV1 expressing cells in colitis deserves further investigation. The distinct outcomes across publications may of course be due to alterations in the microbiota across facilities (Belkaid and Naik, 2013).

Both TRPA1 and the cold-sensing TRPM8 ion channel, which is expressed on a distinct subset of neurons from TRPV1, have been implicated in regulating intestinal inflammation (Figure 1.3). TRPA1/- mice showed protection from TNBS colitis compared to WT controls (Engel et al., 2011b). Interestingly, TNBS, in addition to other haptens, has been found to bind or activate TRPA1 directly and to stimulate the production of lipid inflammatory lipid mediators which can secondarily be sensed through TRPA1 (Engel et al., 2011b; Liu et al., 2013). Two recent studies investigate how the presence of the TRPM8 ion channel inhibits colitis (Pogorzala et al., 2013). In one study, a TRPM8 agonist ameliorated both TNBS- and DSS-colitis, but TRPM8/- mice had a disease course comparable to control mice (Ramachandran et al., 2013). However, a subsequent study by a different group found that TRPM8-deficiency in nonhematopoietic cells (presumably sensory neurons) resulted in worse DSS-induced colitis due to enhanced production of pro-inflammatory cytokines (de Jong et al., 2015).
The authors identify that TRPM8-/ mice cannot release CGRP from afferent sensory neurons during DSS-colitis. As a result, closely associated CD11c+ DCs produce more pro-inflammatory cytokines which drives disease and is in agreement with the preponderance of evidence suggesting that CGRP is anti-inflammatory (de Jong et al., 2015; Holzmann, 2013).

The current evidence implicates that TRPA1+ TRPV1+ vagal/spinal sensory neurons mediate net pro-inflammatory effects during murine models of colitis via release of Substance P, and that CGRP from TRPM8+ spinal afferent sensory neurons appears to be protective (Figure 1.3) (Assas et al., 2014). Even those neurons which may not be actively driving the disease process are sensitive to the inflammatory state of the gut. Colitis often leads to gastrointestinal dysfunction due to death of enteric neurons and in an elegant study, Keith Sharkey’s group unraveled a mechanism by which elevated levels of ATP in colitis trigger direct neuronal cell death via P2X7 and pannexin-1 (Gulbransen et al., 2012). Therapies with neuro-protective capacity in conjunction with the standard anti-inflammatory medications should be considered.

One major mechanism by which the immune system regulates the microbial communities in the gut and keeps pathogens at bay is via mucosal IgA secretion (Macpherson et al., 2012). Some of the initial papers describing effects of gut-associated neuropeptides found that Substance P could increase IgA secretion from gut-associated lymphocytes while VIP had differential effects depending on the location (Stanisz et al., 1986). In an in vitro class-switch reaction, VIP drove full class switching to IgA in synergy with CD40-activation (Fujieda et al., 1996). Furthermore, IgA+ B cells are found in close proximity to peptidergic sensory neurons in gut lamina
propria suggesting that switching to and synthesis of IgA may be regulated by sensory neurons \textit{in vivo}(Shibata et al., 2008).

\section*{1.5.4 Skin}

Perhaps the most extensively studied tissue to date with regards to neuro-immune interactions is the skin(Roosterman et al., 2006). The skin is the body’s largest sensory interface with the environment and discriminates between harmless and noxious stimuli using specialized somatosensory neurons(Le Pichon and Chesler, 2014; Lumpkin and Caterina, 2007). The skin-resident immune system also provides a diverse repertoire of cell subsets with dedicated roles in host defense(Malissen et al., 2014; Mueller et al., 2013). Autoinflammatory and allergic diseases of the skin such as atopic dermatitis and psoriasis are characterized by symptoms of pain and itch which are transmitted via dedicated nociceptive or pruriceptive neurons, yet intriguingly autoimmune diseases such as vitiligo are generally reported to be painless(Bautista et al., 2014; Mishra and Hoon, 2013; Pogorzala et al., 2013; Rashighi et al., 2014).

Before the molecular genetic characterization of neuronal subsets in the skin, much of the literature focused on the modulation of classic delayed type hypersensitivity reactions through capsaicin denervation with a focus on neuropeptides(Roosterman et al., 2006). The overall conclusion of these studies mirrors that seen at other barrier tissues in that Substance P is pro-inflammatory and CGRP is anti-inflammatory(Madva and Granstein, 2013). In the interest of space and simplicity, we refer the reader to some of the original literature and comprehensive reviews on this topic(Banvolgyi et al., 2005;
Psoriasis is a painful and pruritic disease characterized by keratinocyte hyperplasia and a characteristic neutrophilic infiltrate which is driven by the IL-23/IL-17 axis (Lowe et al., 2013; Perera et al., 2012). An active role for neurons in psoriasis was suggested by the finding that enhanced neuronal turnover was observed in pre-psoriatic lesions (Weddell et al., 1965). In this seminal study, Weddell et al., applied novel histological techniques to study the innervation pattern in uninvolved and involved psoriatic skin noting enhanced neural turnover in healthy skin before the development of a plaque from patients and increased axonal entry into the epidermis accompanied by Schwann cells (Weddell et al., 1965). The authors speculated that the neurites and Schwann cells are capable of promoting the development of lesions (Weddell et al., 1965). This line of thinking was substantiated by several clinical reports indicating that interruption of innervation to affected skin led to remission and that the return of sensation was accompanied by plaque progression (Dewing, 1971; Farber et al., 1990b; Joseph et al., 2005). In a genetic mouse model of chronic psoriasiform dermatitis accompanied by enhanced cutaneous innervation, Ward and colleagues showed that surgical denervation ameliorated epidermal hyperplasia and some inflammatory parameters though it did not have substantial effects on IL-23/IL-17 axis cytokines (Ostrowski et al., 2011). They found that Substance P promoted CD11c+ cell accumulation, CGRP was important for epidermal thickening and that blockade of neuropeptide release using locally injected botulinum neurotoxin could ameliorate disease (Ostrowski et al., 2011; Ward et al., 2012).
This line of clinical observation and laboratory experimentation prompted us to pursue the specific nature of innervation and neuro-immune interactions which regulate the initiation of a psoriatic lesion. In recent work utilizing both RTX-mediated ablation of TRPV1+ nociceptors and genetic deletion of NaV1.8+ sensory neurons, we identified that an overlapping population of TRPV1+ and NaV1.8+ nociceptors regulate the production of the key instructive cytokine IL-23 and effector cytokines IL-17 and IL-22 in the imiquimod model of psoriasiform dermatitis (Figure 1.3) (Riol-Blanco et al., 2014). Confocal and two-photon in vivo microscopy showed that dermal dendritic cells, the principal source of IL-23, were in close and dynamic contact with nociceptors (Riol-Blanco et al., 2014). In the absence of TRPV1+ nociceptors, DDC-derived IL-23 and γδT17-derived IL-17 and IL-22 were significantly reduced which could be rescued by the direct injection of IL-23 (Riol-Blanco et al., 2014; Sumaria et al., 2011; Wohn et al., 2013). While this study did not identify a specific neuropeptide responsible for the effect, the description of the key cellular players responsible for the initiation of an acute psoriasiform lesion including TRPV1+NaV1.8+ nociceptors, CD11b+ DDCs, and γδT17 cells provides a foundation for future work to dissect this regulation. The characterization of populations of cells at a single cell level in both the nervous and immune system may help to clarify some of these underlying mechanisms based on the signaling modules present or absent from cell types (Satija and Shalek, 2014; Usoskin et al., 2015).

Atopic dermatitis is a disease colloquially referred to as the, “itch that rashes.” It is driven by Type 2 inflammatory processes and results from a combination of epidermal barrier breakdown and the synthesis of TSLP, the key instructive cytokine, in keratinocytes. The most bothersome symptom for patients is the intractable itch (Garibyan
et al., 2013). Chronicity in atopic dermatitis can result from a severely disrupted epidermal barrier which leads to sensitization to a variety of allergens and also altered microbial communities and translocation (Nakamura et al., 2013; Schommer and Gallo, 2013).

Clinical studies have also documented an increase in innervation within atopic dermatitis lesions and amelioration of disease in areas of skin with diminished neural input (Azimi et al., 2015; Tobin et al., 1992). In a comprehensive study, Jordt and colleagues showed that the TRPA1 channel, but not TRPV1, on cutaneous nociceptors is required for the development of both inflammation and itch in allergic contact dermatitis to oxazolone and urushiol (poison ivy) (Liu et al., 2013). TSLP acts on dendritic cells, local effector T cells, ILC2s and prurireceptors in order to contribute to disease progression and itch (Figure 1.3) (He et al., 2008; Kim et al., 2013; Leyva-Castillo et al., 2013; Wilson et al., 2013). The activity of TSLP stimulates IL-4 and IL-13 production from CD4+ Th2 cells and IL-5 and IL-13 from ILC2s (He et al., 2008; Kim et al., 2013; Leyva-Castillo et al., 2013). Diana Bautista’s group made the connection between TSLP and TRPA1 in atopic dermatitis in a study describing how the TSLP-receptor (TSLPR and IL7Ra) is expressed on a subset of sensory neurons (Wilson et al., 2013). The expression of TRPA1 was required to induce itch while TRPV1 was dispensable, yet RTX pre-treatment also ablated TSLP-evoked itch suggesting that TRPV1+TRPA1+ sensory neurons conduct itch in this system in a TRPA1 dependent fashion (Wilson et al., 2013). These sensory neurons were largely distinct from the traditionally described histamine and chloroquine responsive prurireceptors (Bautista et al., 2014; Roberson et al., 2013).
While lymphocytes and mast cells were not required for acute TSLP-driven itch (Wilson et al., 2013), the complexity of this pathway is likely to increase in chronic conditions. A functional role for TRPA1 on mast cells and keratinocytes in chronic atopic dermatitis driven by elevated levels of IL-13 has been proposed (Oh et al., 2013). Furthermore, Th2-derived IL-31 acts on IL-31RA present on TRPA1+TRPV1+ sensory neurons which can produce a “memory” of itch independent of TSLP (Cevikbas et al., 2014). Unlike the proposed linear model for TRPV1+NaV1.8+ nociceptors in regulating the instructive cytokine IL-23, TSLP is derived from epithelial cells and is likely to act simultaneously on immune cells and sensory neurons. Of course both proposed models will certainly be refined to include as yet uncharacterized positive and negative feedback loops,

One of the originally described neuro-immune interfaces in barrier tissues is between peptidergic neurons and mast cells (Forsythe and Bienenstock, 2012; Naukkarinen et al., 1991; Stead et al., 1987). The presence of cutaneous sensory nerves is essential for mediating classic reactions such as IgE-driven passive-cutaneous anaphylaxis (Siebenhaar et al., 2008). Recent characterization of the three-dimensional localization of skin-resident leukocytes has highlighted that mast cells are actually closely apposed to ILC2s (Roediger et al., 2013; Tong et al., 2014). It will be of interest to determine how the “mast cell-nerve functional unit” extends its influence to ILC2s in cutaneous immunity (Forsythe and Bienenstock, 2012; Roediger et al., 2013).
1.6 Neuroimmune interactions during infection and disease

1.6.1 Bacterial and viral infection

Some bacterial and viral infections trigger intense pain in the host. In this section, we will discuss how neuropeptides can function in host defense, the mechanisms through which bacterial infections can cause or modulate pain, and how viral infections are counteracted by the intrinsic immune capacity of neurons. We will also discuss recent evidence suggesting that sensory and autonomic neuropathies can result in immunodeficiency.

In the African clawed frog (*Xenopus laevis*), specialized neuroepithelial glands are present in the skin which, upon norepinephrine signaling, discharge a cocktail of neuropeptides with antimicrobial properties onto the skin of the frog (Brogden et al., 2005). Neuropeptides exhibit commonalities with canonical antimicrobial peptides in their size, cationic charge, and amphipathic nature. Recent evidence suggests this mechanism may not be restricted to amphibians but may be a defense strategy utilized by mammals as well (Brogden et al., 2005; El Karim et al., 2008). In fact, chemosensory cells in the upper respiratory tract can release antimicrobial peptides in response to sensing secreted bacterial products via bitter taste receptors (Lee et al., 2014).

What are the afferent neural signaling circuits that could mediate neuropeptide release during an infection? Infection associated inflammatory pain has long been considered to be secondary to signals derived from immune cells acting on nociceptors (Binshtok et al., 2008; Chiu et al., 2012; Iwasaki and Medzhitov, 2015).
However recent evidence suggests that infection associated pain may be caused by direct action of bacteria on nociceptors.

Adding to the observation that bacterial cell lysates can directly trigger calcium flux in DRG neurons (Ochoa-Cortes et al., 2010), pioneering work by Isaac Chiu and Clifford Woolf extended this work on bacterial activation of nociceptors and demonstrated in vivo that pain in response to S. aureus infection is not secondary to inflammation mediated by pattern recognition (TLR2/MyD88), lymphocytes (T and B cells), or myeloid cells (neutrophils and monocytes), but rather directly triggered by N-formylated peptides and α-haemolysin, a pore-forming toxin (Chiu et al., 2013). They further characterized that these bacterial products act on TRPV1+NaV1.8+ nociceptors (Figure 1.3) (Chiu et al., 2013). Interestingly, LPS can activate TRPA1+ sensory neurons in vitro in a TLR4-independent but TRPA1-dependent fashion suggesting that the recognition of bacterial products by nociceptors during infection may be a generalized phenomenon (Meseguer et al., 2014).

Despite the generalized view of nociceptors as pro-inflammatory, in the absence of NaV1.8-lineage nociceptors, paw swelling and lymphadenopathy after S. aureus challenge were increased compared to WT mice (Figure 1.3) (Chiu et al., 2013). While the authors propose a direct anti-inflammatory effect of CGRP acting to suppress macrophage-derived TNF-α and draining LN hypertrophy, NaV1.8 is not restricted to cutaneous nociceptors and is also broadly expressed on sensory neurons within the vagus nerve (Gautron et al., 2011). It will be of interest to determine the precise circuits modulating immune cell proliferation in response to S. aureus infection, if there are any shared components with the prototypical anti-inflammatory reflex, and how the presence
or absence of bacterial-sensing nociceptors affects bacterial clearance and viability at different stages of infection (Andersson and Tracey, 2012; Chiu et al., 2013).

While assumed that pruritis in atopic dermatitis was primarily due to IgE mediated histamine release, new evidence suggests that certain bacteria may more directly trigger itch. S. aureus, which is commonly found in eczematous lesions, was recently found to trigger itch and inflammation via direct delta-toxin induction of mast cell degranulation (Nakamura et al., 2013). Due to the capacity for S. aureus to directly activate nociceptors and mast cells, and the important role for both cell types in atopic dermatitis, it would not be surprising if direct activation of neural and immune components by S. aureus precipitates and sustains eczematous lesions.

Bacteria can also hijack elements of the nervous system to avoid detection and establish permanent residence by diminishing pain. Mycobacterium leprae and Mycobacterium ulcerans both lead to alterations in pain sensation either by causing nerve demyelination through infecting Schwann cells or by secreted mycolactone which hyperpolarizes neurons (Marion et al., 2014; Rambukkana et al., 2002). Furthermore, M. leprae actually reprograms Schwann cells to a mesenchymal stem cell-like state in order to promote its permanence and dissemination (Masaki et al., 2013).

Many viruses exhibit exquisite neurotropism and utilize the peripheral nervous system to access and establish latent reservoirs in ganglia (Koyuncu et al., 2013; Swanson and McGavern, 2015). Herpes and rabies viruses are two classes that are particularly insidious in human populations. Utilizing vesicular stomatitis virus (VSV), a relative of rabies viruses, we showed that VSV gains access to the CNS by infecting peripheral lymph node-innervating neurons (Iannacone et al., 2010). Typically, subcapsular sinus
macrophages, which function as a “flypaper” in lymph nodes, trap VSV and produce type I IFN which acts on neighboring nerves to prevent viral spread (Iannacone et al., 2010). In the absence of this key macrophage subset, VSV gains entry to peripheral neurons and ascends to disseminate within the CNS and manifests as a lethal infection.

While the nervous system is less of an immune privileged site than originally thought, neurons do not have the luxury of being cytolytically eliminated by type I IFN, CD8+ T cells or NK cells in the same fashion that an epithelial cell does which can rapidly be replaced from an adjacent stem cell. As a result, neurons utilize a fascinating array of intrinsic innate immune defense mechanisms to combat viral infections while maintaining cell health (Lafaille et al., 2012; Swanson and McGavern, 2015). In the case of HSV-1, DRG neurons exhibit low sensitivity to the antiviral and cytotoxic effects of type I IFN (Yordy et al., 2012). Instead, DRG neurons also employ the autophagy pathway in parallel to control viral replication and attempt to resist cell death (Yordy et al., 2012).

1.6.2 Primary and secondary immunodeficiency

Neurons can resist viral infection by PRRs expressed within cells as well as strategically located T cells. In most individuals if HSV-1 reaches the CNS, it will establish a latent reservoir which can become reactivated to produce what at most amounts to an unsightly cold sore. However, in a small fraction of the population, HSV-1 infection can result in herpes simplex encephalitis (HSE) (Lafaille et al., 2012). HSE is a Mendelian primary immune deficiency which has been genetically linked to defects in TLR3, UNC-93B, TRIF, TBK1, and STAT1 leading to the hypothesis that TLR3
production of IFN-α/β or IFN-λ is essential to restrict CNS replication (Lafaille et al., 2012). In a pioneering study harnessing patient-derived induced pluripotent stem cells and neural differentiation to neural stem cells, neurons, astrocytes and oligodendrocytes, Notarangelo and colleagues showed that functional TLR3 signaling in neurons and oligodendrocytes is essential to restrict HSV-1 infection while astrocytes and NSCs can utilize other pathways (Lafaille et al., 2012). This study showed how canonical PRRs of the immune system mediate intrinsic immunity in the CNS (Full disclosure: I was involved in this work). Novel differentiation protocols to obtain nociceptors opens the door to study how peripheral sensory neurons respond to HSV-1 infection (Wainger et al., 2015). Furthermore, a newly described subset of T resident memory cells provides a critical local layer of control to prevent viral reactivation in chronically infected individuals by localizing to skin and sensory ganglia (Gebhardt et al., 2009).

Stroke-induced neurological defects can manifest as transient systemic immunodeficiency predisposing individuals to bacterial infection (Wong et al., 2011). In a mouse model, the group of Paul Kubes showed how norepinephrine signaling to hepatic iNKT cells was important for the bacterial susceptibility after a stroke event. This occurred through induced immunosuppressive production of IL-10 and an alteration in the systemic balance of immunity away from a protective Type 1 response (Wong et al., 2011).

Are there immunodeficiencies which result from defects in neural development? Patients with mutations in SCN9A (NaV1.7) suffer from congenital insensitivity to pain due to the impaired generation of action potentials in nociceptors (Cox et al., 2006) and mutations in NGFB or TRKA (the NGF receptor) lead to hereditary sensory and
autonomic neuropathy (HSAN 4 or 5)(Hepburn et al., 2014). A patient with a mutation in TRKA presented with recurrent infections which were secondary to hypogammaglobulinemia(Kilic et al., 2009). This may be due to the role for NGF signaling as a survival factor for memory B cells(Torcia et al., 1996). However, an elegant study by Floto and colleagues recently identified a key role for NGF-B in human immunity to *S. aureus* infection(Hepburn et al., 2014). NGF-B has significant structural homology with drosophila Spaetzle, a Toll ligand, and patients with mutations in NGF-B and TRKA had frequent severe *S. aureus* infections. NGF-B was found to be released from macrophages in response to viable *S. aureus* infection in an NLR-dependent fashion, which mediated autocrine effects such as enhanced phagocytosis as well as promoting neutrophil recruitment(Hepburn et al., 2014). As the spectrum of genetically described primary immunodeficiencies rapidly expands beyond defects in immune cell subsets, it will be of utmost clinical importance to recognize how alterations in neural development or function influence immunity.

One might imagine that decreased pain sensation would not be evolutionarily selected for as the host could not attend appropriately to otherwise injurious tissue damage. Furthermore, some painful insect toxins can cause lethal anaphylaxis, so it is in the benefit of the host to avoid exposure. To mitigate these effects, rodents induce protective Type 2 allergic responses in order to mitigate their spread from the site of injection(Marichal et al., 2013; Palm et al., 2012; Palm et al., 2013). However, in a beautiful example of co-evolution, grasshopper mice have a unique mutation in NaV1.8 not shared with other rodents that binds bark scorpion toxins and blocks action potential propagation in response to the toxin activating NaV1.7(Rowe et al., 2013). As a result,
the toxin functions as both an agonist and antagonist of pain in these mice allowing them to consume their otherwise painful prey.

1.6.3 Other Diseases

In this chapter we focus on the regulation of the immune system at barrier tissues during disease and infection. Two fascinating areas of neuro-immune interactions which we cannot expound on for space and time limitations are those of rheumatoid arthritis and cancer so we refer the reader to other reviews on the topic (Hanoun et al., 2015; Pongratz and Straub, 2013). In brief, both sensory afferents and sympathetic efferents play complex, temporally dependent roles in the regulation of arthritis by interacting with endothelial, immune, and parenchymal cells in affected joints. Sympathetic and TRPV1+ sensory neurons are pro-inflammatory in the initiation of disease while established disease results in a loss of immunomodulatory sympathetic fibers and outgrowth of sensory innervation which may contribute to the chronicity of disease (Borbely et al., 2015; Harle et al., 2005; Levine et al., 1984; Levine et al., 1986; Levine et al., 1985; Miller et al., 2000; Stangenberg et al., 2014).

The outgrowth or regression of autonomic neurons has also been described to influence the oncogenic process (Hanoun et al., 2015). In the case of prostate cancer, a solid tumor, autonomic nerves were found to influence both tumor development and metastasis with stromal β2 and β3 adrenergic receptors promoting tumor development and cholinergic signaling via type 1 muscarinic receptors leading to metastasis (Magnon et al., 2013). Conversely, sympathetic neuropathy results in the progression of myeloproliferative malignancy due to the importance of sympathetic neurons in maintaining nestin+ MSCs and Schwann cells in order to form a stable niche for
HSCs (Arranz et al., 2014; Hanoun et al., 2014; Mendez-Ferrer et al., 2008; Mendez-Ferrer et al., 2010). Current standard of care for leukemia includes the use of neurotoxic chemotherapeutics such as cisplatin and vincristine which reduce the ability of bone marrow transplants to engraft and repopulate a patient due to a loss of sympathetic neurons and niche constituents (Lucas et al., 2013). The inclusion of neuroprotective agents during chemotherapy could improve the clinical recovery from bone marrow transplantation by preserving the niche for HSCs (Lucas et al., 2013).
Chapter 2: Nociceptive sensory neurons drive interleukin-23-mediated psoriasiform skin inflammation

Modified from original publication:

2.1 Abstract

The skin has a dual function as a barrier and a sensory interface between the body and the environment. To protect against invading pathogens, the skin harbors specialized immune cells, including dermal dendritic cells (DDCs) and interleukin (IL)-17 producing γδ T cells (γδT17), whose aberrant activation by IL-23 can provoke psoriasis-like inflammation (Cai et al., 2011a; Gray et al., 2011; Nestle et al., 2009; Perera et al., 2012). The skin is also innervated by a meshwork of peripheral nerves consisting of relatively sparse autonomic and abundant sensory fibers. Interactions between the autonomic nervous system and immune cells in lymphoid organs are known to contribute to systemic immunity, but how peripheral nerves regulate cutaneous immune responses remains unclear (Chiu et al., 2012; Rosas-Ballina et al., 2011). Here, we have exposed the skin of mice to imiquimod (IMQ), which induces IL-23 dependent psoriasis-like inflammation (Flutter and Nestle, 2013; van der Fits et al., 2009). We show that a subset of sensory neurons expressing the ion channels TRPV1 and NaV1.8 is essential to drive this inflammatory response. Imaging of intact skin revealed that a large fraction of DDCs, the principal source of IL-23, is in close contact with these nociceptors. Upon selective pharmacological or genetic ablation of nociceptors (Abrahamsen et al., 2008; Lumpkin and Caterina, 2007; Sandor et al., 2009b), DDCs failed to produce IL-23 in IMQ exposed skin. Consequently, the local production of IL-23 dependent inflammatory cytokines by dermal γδT17 cells and the subsequent recruitment of inflammatory cells to the skin were dramatically reduced. Intradermal injection of IL-23 bypassed the requirement for nociceptor communication with DDCs and restored the inflammatory response (Zheng et
These findings indicate that TRPV1⁺NaV1.8⁺ nociceptors, by interacting with DDCs, regulate the IL-23/IL-17 pathway and control cutaneous immune responses.

2.2 Introduction

Repeated topical application of imiquimod (IMQ) to murine skin provokes inflammatory lesions that resemble human psoriasis (Flutter and Nestle, 2013; van der Fits et al., 2009). This response is mediated by IL-23, which stimulates skin-resident γδ T cells to secrete IL-17 and IL-22, cytokines that induce inflammatory leukocyte recruitment and acanthosis (Lowes et al., 2013). Indeed, antibodies targeting the shared p40 subunit of IL-12 and IL-23 inhibit both IMQ-induced murine dermatitis and human psoriasis (Flutter and Nestle, 2013; Lowes et al., 2013). Frequent symptoms in human psoriasis, aside from the prominent skin lesions, include the sensations of itch, pain and discomfort in affected areas (Rapp et al., 1999). Clinical reports suggest that intralesionally administered anesthetics or surgical denervation of psoriatic lesions not only abrogate local sensation, but also ameliorate local inflammation (Farber et al., 1990a). Similarly, in mutant mice with disseminated psoriasiform dermatitis, peripheral nerve dissection attenuated skin inflammation (Ostrowski et al., 2011); however, cutaneous nerves are composed of sympathetic and several types of sensory fibers, and the role of individual types of nerve fibers remains unclear (Chiu et al., 2012). Here, using the IMQ-model, we have investigated whether and how specific subsets of peripheral nerves contribute to the formation of psoriasiform skin lesions.
2.3 Results:

2.3.1 TRPV1+ nociceptors promote skin inflammation in the IMQ model

Skin sensations perceived as inflammatory pain, noxious heat and some forms of itch are transmitted by sensory fibers that express the cation channel TRPV1. Most TRPV1+ fibers co-express the sodium channel NaV1.8 (ref. (Abrahamsen et al., 2008; Lumpkin and Caterina, 2007; Sandor et al., 2009b)). NaV1.8+ nociceptors can be identified in the dermis of NaV1.8-TdTomato (TdT) mice by their red fluorescence (Figure 2.1) (Abrahamsen et al., 2008). Confocal microscopy of skin samples from NaV1.8-TdT mice co-stained for tyrosine hydroxylase (TH), which identifies sympathetic fibers, and for β3-tubulin, a pan-neuronal marker, revealed that NaV1.8+TH- nociceptors represent the vast majority of cutaneous nerve fibers, while NaV1.8 TH+ sympathetic fibers are rare.

To dissect the roles of sympathetic fibers and nociceptors in the IMQ-model, mice were treated systemically with either 6-hydroxydopamine (6OHDA) or resiniferatoxin (RTX) to ablate TH+ sympathetic neurons or TRPV1+ nociceptors, respectively (Figure 2.1, 2.2 and 2.3) (Grebe et al., 2009; Sandor et al., 2009a). Subsequently, IMQ was applied topically to one ear and the ensuing inflammatory response was assessed based on the change in ear thickness, size of the myeloid infiltrate (Figure 2.4) and tissue contents of inflammatory cytokines.
Following sympathetic denervation, IMQ-induced ear swelling was reduced compared to controls (Figure 2.2); however, the inflammatory infiltrate was increased, while IL-17A, IL-17F, IL-22 and IL-23-p40 production remained unchanged (Figure 2.2). Thus, sympathetic innervation exerts little or no direct local control over the inflammatory skin response. The observed changes were likely due to cardiovascular effects and/or global immune dysregulation following systemic sympathectomy (Rosas-Ballina et al., 2011). By contrast, in RTX-treated mice both ear swelling and inflammatory infiltrates in IMQ-exposed ears were profoundly reduced (Figure 2.1 and 2.5). RTX treatment did not alter the systemic supply of inflammatory cells (Figure 2.5) (Swirski et al., 2009a). Moreover, intravital microscopy of ear skin revealed similar leukocyte rolling in RTX-treated and control mice (data not shown) indicating that the absence of nociceptors did not affect the baseline adhesiveness of dermal microvessels (Weninger et al., 2000). More likely, ablation of TRPV1+ sensory nerves reduced IMQ-induced inflammation via local, extravascular mechanisms. However, the attenuated inflammatory response was not limited to the skin, as the IMQ-induced enhancement in cellularity of the draining auricular lymph node (LN) was also blunted by RTX (Figure 2.1).

LN s have a critical function in dermal antigen presentation to naive T-cells and generation of migratory effector cells. LN s also possess peripheral innervation (Iannacone et al., 2010), which could have been altered by RTX; however, the role of skin-draining LN s during psoriatic inflammation is unclear. To address this issue, we tested the effect of IMQ in lymphotoxin-α-deficient (LTα−/−) mice, which are devoid of LN s. Compared to
WT mice, there was no statistical difference in ear thickness, frequency, or composition of the inflammatory infiltrate in ears of LTα−/− mice (Figure 2.1). Additionally, we treated WT mice with FTY720, which blocks T cell egress from LNs preventing trafficking of effector cells to peripheral tissues (Matloubian et al., 2004). Again, IMQ elicited full-fledged inflammation in exposed ears (Figure 2.1), indicating that T cell priming in skin draining LNs is dispensable for the acute induction of psoriasiform inflammation. Together, these findings imply that TRPV1+ nociceptors promote local immune responses directly in the skin.
Figure 2.1: TRPV1⁺ nociceptor ablation attenuates skin inflammation and draining lymph node hypertrophy in the IMQ model.

(a) Representative whole-mount confocal micrograph of normal ear skin from NaV1.8-TdT reporter mice (NaV1.8⁺ nociceptors, red) stained for β3-tubulin (peripheral nerves, blue) and tyrosine hydroxylase (TH, sympathetic nerves, green). (b-f) The ear skin of vehicle treated controls (DMSO) or TRPV1⁺ nociceptor ablated (RTX) mice was treated with topical IMQ cream daily. (b) Ear thickness was measured relative to the contralateral ear at indicated time points (n=10-15 mice per time point; *, P < 0.02). (c) Representative histological sections of IMQ treated ears at day 6 stained by H&E. (d) Total inflammatory monocytes (n=10) and (e) total neutrophils in skin at day 3 (n=10; **, P < 0.005). (f) Total cell number in auricular lymph nodes at day 3 (n=20; *, P = 0.01; ***, P < 0.001). (g-j) IMQ was applied daily to (g,h) WT (n=10) and LTα⁻/⁻ mice (n=6) or (i,j) vehicle treated (n=10) and FTY720 treated mice (n=10) and (g,i) ear swelling was measured at indicated time points. (h,j) The percentage of CD45⁺ leukocytes was determined in ear skin digests on day 6.
Figure 2.2: 6-Hydroxydopamine (6OHDA) treatment ablates sympathetic nerve function and reduces ear swelling, but does not ameliorate the inflammatory response to IMQ treatment.

a, Experimental protocol: Mice were injected intraperitoneally with 6OHDA, resulting in a reversible chemical sympathectomy lasting ~two weeks. After a rest period of 3 days animals were challenged topically on the ear with IMQ. b, Representative section of splenic white pulp showing B cells (B220, white), T cells (CD3, red), and tyrosine hydroxylase+ (TH, green) nerve fibers in vehicle (ascorbic acid) treated and sympathectomized (6OHDA) mice. c-i. Analysis of the inflammatory response in ears of vehicle (ascorbic acid) treated and sympathectomized (6OHDA) mice after daily topical IMQ challenge: (c) timecourse of change in ear thickness of IMQ treated ear relative to the contralateral ear (n=10; *, P < 0.05; **, P < 0.01) and (d) total number of infiltrating monocytes and (e) neutrophils, and the amount of (f) IL-17A, (g) IL-17F, (h) IL-22 and (i) IL-23-p40 in protein extracts of IMQ exposed ears at day 3 (*, P < 0.05; n=5).
Figure 2.3: Resiniferatoxin (RTX) treatment diminishes noxious heat sensation and decreases the expression of nociceptor markers on dorsal root ganglia (DRGs).

a, Schematic protocol of nociceptor ablation and induction of psoriasisform skin inflammation. RTX was injected subcutaneously into the back in three escalating doses (30 µg/kg, 70 µg/kg and 100 µg/kg) on consecutive days and mice were allowed to rest for at least 4 weeks before IMQ treatment. b, Denervation was confirmed by immersing the tail of mice into a temperature controlled water bath maintained at 52°C and the latency to the first tail movement to avoid water was measured (n=6). c, Total RNA was isolated from dorsal root ganglia (DRG; level C1-C7) of vehicle (DMSO) and RTX-treated mice and the levels of trpv1, scn10a (NaV1.8), tac1 (Substance P), trpm8 and trpa1 mRNA relative to gapdh were determined (n=3).
Figure 2.4: Gating strategy for T cell subsets and myeloid cells from digested ear skin.

a, The ear skin of mice challenged for 3 days with IMQ was digested as described in methods and, after doublet exclusion and gating on defined FSC-A, SSC-A parameters, infiltrating myeloid cells were gated as CD45+ I-Ab (Class-II)-, CD11b+ CD11c-, and then subdivided into inflammatory monocytes and neutrophils based on Ly6C and Ly6G staining. b, The ear skin of naïve mice was digested as described in methods and, after doublet exclusion and gating on defined FSC-A, SSC-A parameters, cutaneous T cells were gated on CD45+, Thy1+, and then divided into subsets based on staining for δ-TCR and β-TCR.
Figure 2.5: RTX treatment reduces the immune cell infiltrate upon IMQ treatment in the skin but does not affect reservoirs of inflammatory monocytes and neutrophils at steady-state.

a, The ear skin of vehicle (DMSO) or sensory denervated (RTX) mice was treated with topical IMQ cream daily and the total numbers of CD45^+ cells were determined on day 3 as explained in methods (n=10). b, Representative histological sections of untreated and IMQ treated ears at day 3 stained by H&E (20x) (n=5 per condition). c,d, Total inflammatory monocytes (CD45^+, CD11b^+, Ly6C^{high}) and neutrophils (CD45^+, CD11b^+, Ly6G^{high}) were determined by flow cytometry (n=5-10 mice per time point). Two-way ANOVA was run to compare total numbers of inflammatory monocytes and neutrophils between DMSO and RTX conditions over days 3-6 (****, P<0.0001). One-way ANOVA was run to compare total inflammatory monocytes and neutrophil numbers over days 3-6 within DMSO or RTX conditions (**, P<0.003). e, Bone marrow was isolated from WT and RTX mice from one femur and the frequency of inflammatory monocytes (CD45^+, CD11b^+, Ly6C^{high}, Ly6G^{high}) and neutrophils (CD45^+, CD11b^+, Ly6C^{high}, Ly6G^{high}, Ly6C^{mid}) relative to CD45^+ cells was determined by flow cytometry (n=5). f, Spleens from WT and RTX mice were processed for flow cytometry and the frequency of inflammatory monocytes (CD45^+, CD11b^+, Ly6C^{high}, Ly6G^{high}) and neutrophils (CD45^+, CD11b^+, Ly6G^{high}, Ly6C^{mid}) relative to CD45^+ cells was determined (n=5).
2.3.2 TRPV1+ nociceptors regulate cytokine production from tissue-resident lymphocytes

Neutrophil recruitment to the skin and keratinocyte hyperproliferation, hallmarks of IMQ-induced inflammation, are driven by IL-17 and IL-22, respectively (Perera et al., 2012; van der Fits et al., 2009; Zheng et al., 2007) so we asked whether nociceptors regulate the production of these cytokines. Indeed, after IMQ treatment, protein levels of IL-17A, IL-17F, and IL-22 had dramatically increased in ears of control mice, while in RTX-treated mice IL-17A was very low and IL-17F and IL-22 remained below the detection limit (Figure 2.6). Thus, TRPV1+ nociceptors control the generation of several key effector cytokines in psoriasiform dermatitis.

Since our findings in FTY720-treated mice suggested that recruitment of LN derived effector T-cells is not needed for IMQ-induced skin inflammation, we asked whether nociceptors regulate cytokine production by skin-resident lymphocytes, particularly γδT17 cells, which produce IL-17F and IL-22 (ref. (Cai et al., 2011b; Gray et al., 2013; Pantelyushin et al., 2012)). In ears of IMQ-treated control mice, most IL17F+ lymphocytes were dermal γδ T cells, while few conventional αβ T cells expressed IL-17F (Figure 2.4, 2.6 and 2.7). In RTX-treated mice, both IL-17F+ and IL-22+ dermal γδ T cells were significantly reduced, indicating that TRPV1+ neurons drive the local production of IL-17F and IL-22 primarily by γδT17 cells (Figure 2.6 and 2.7). These T-cells seed the skin in early life and are poised for rapid IL-17 production upon stimulation by IL-23 (ref. (Guo et al., 2012)). Indeed, in ears of IL-23R<sup>GFP/+</sup> mice(Awasthi et al., 2009), ~60% of dermal γδT17 and ~20% of αβ T-cells expressed IL-23R (Figure 2.6),
whereas T-cells in auricular LNs and epidermal γδ cells expressed little or no IL-23R (Figure 2.7).
Figure 2.6: TRPV1+ nociceptors control IL-17F and IL-22 production by IL-23R+ dermal γδ T cells.

a-c, After 3 days of IMQ challenge, ears from vehicle- (DMSO; n=5) or RTX-treated mice (n=5) were harvested to perform ELISA for (a) IL-17A, (b) IL-17F, and (c) IL-22. (**, P < 0.01; ***, P < 0.001).

d-g, Flow cytometry on digested ear skin was performed on day 6 of IMQ challenge. (d) Relative frequency of IL-17F+ or IL-22+ dermal γδ T cells and αβ T cells in IMQ treated control mice (n=15). (e) Representative FACS plots of IL-17F staining in dermal γδ T cells from vehicle (DMSO) and RTX treated mice. (f) Quantification of frequency of IL-17F+ amongst dermal γδ T cells in DMSO and RTX mice (n=5/group; *, P < 0.05; **, P = 0.01).

h, Representative FACS plots of normal ear skin from an IL-23R<sup>GFP<sup>+</sup> mouse and i, quantification of frequency of IL-23R-GFP<sup>+</sup> cells among skin resident Thy1<sup>+</sup> T cell subsets (n=8).
Figure 2.7: Dermal γδ T cells represent a major source of IL-17F and IL-22 in skin during IMQ challenge and already express IL-23R+ at steady state

a, WT mice were challenged with IMQ and the total numbers of IL17F+ dermal γδ T cells and αβ T cells at 3 days (n=15) or 6 days (n=10) were quantified. b, Representative flow plots related to (Figure 2g) of gating for IL-22+ cells within dermal γδ T cells after 6 days of IMQ treatment. c, Ears of DMSO or RTX treated mice were exposed for 6 days to IMQ and the frequency of IL-17F+ and IL-22+ cells within αβ T cells was determined (n=5). d, Auricular lymph node (aLN) cells from IL-23R<sup>GFP/+</sup> mice were analyzed by flow cytometry for expression of IL-23R-GFP+ cells within the γδ T cells and αβ T cell compartment at steady state (representative FACS plot from 8 mice analyzed). e, The ear skin from IL-23R<sup>GFP/+</sup> mice was digested and analyzed by flow cytometry and the distribution of T cells subsets within IL-23R-GFP<sup>+</sup> and IL-23R-GFP<sup>-</sup> fractions of Thy1<sup>+</sup> cells determined (representative FACS plot from 8 mice analyzed).
2.3.3 TRPV1+ nociceptors regulate dermal DC IL-23 production

In light of the preferential expression of IL-23R on dermal γδT17 cells, the known role of IL-23 as a driver of IL-17 and IL-22 generation in psoriasiform inflammation (Lowes et al., 2013) and our finding that RTX pretreatment profoundly diminished IMQ-induced cytokine production by γδT17 cells, we hypothesized that TRPV1+ nociceptors might control dermal IL-23 production. Topical IMQ treatment of control mice dramatically increased p40 protein levels, as well as mRNA levels of il12b (IL-12/23p40) and il23a (IL-23p19), but not il12a (IL-12p35). These effects were nearly abolished after RTX treatment (Figure 2.8 and 2.9), suggesting that TRPV1+ nociceptors are essential for cutaneous IL-23 production. It seems unlikely that IL-12 had a major impact in the IMQ model, because IMQ-induced inflammatory parameters were markedly reduced in IL-23R<sup>GFP/GFP</sup> mice, which respond to IL-12 but not IL-23, (Figure 2.8). However, IL-12-dependent skin inflammation induced using a chemical irritant, DNFB (Muller et al., 1995), was profoundly reduced in RTX-treated mice, suggesting that RTX-sensitive fibers also play a role in IL-12-driven dermatitis (Figure 2.9).

Of note, while IL-17F and IL-22 production as well as myeloid infiltrates were virtually abolished in IL-23R<sup>GFP/GFP</sup> mice, ear swelling was only partially reduced (Figure 2.8), suggesting that topical IMQ promotes modest tissue edema through an IL-23 independent pathway. This activity was independent of nociceptors since RTX treatment of IL-23R<sup>GFP/GFP</sup> mice had no effect on the IMQ-induced swelling (Figure 2.9).

While the above findings clearly demonstrate that nociceptors are indispensable for IMQ-induced dermal IL-23 production, it remained possible that nociceptive fibers exert additional proinflammatory functions, e.g. by directly regulating γδT17 cells. To
address this, we performed intradermal IL-23 injections, which trigger psoriasiform skin inflammation in murine skin (Zheng et al., 2007). Regardless of whether nociceptors were ablated or left intact, IL-23 administration resulted in profound ear swelling and fully rescued IL-17 and IL-22 production by Thy1+ cells (Figure 2.8) and γδT17 cells (Figure 2.9). Together, these results suggest that the proinflammatory function of TRPV1+ neurons is rooted exclusively in the promotion of IL-23 production, at least in this experimental setting.

Next, we sought to identify the IL-23 producing cell type(s) regulated by TRPV1+ nociceptors. In intestinal barrier tissues, Ly-6Chigh inflammatory monocytes were identified as a major source of IL-23 in a colitis model (Zigmond et al., 2012). However, depletion of neutrophils and inflammatory monocytes with anti-Gr-1 did not affect IMQ-induced dermal IL-23-p40 or IL-17F levels (Figure 2.8 and 2.10), suggesting that skin-resident DCs and/or macrophages rather than migratory myeloid cells were the critical source of IL-23. Thus, we injected CD11c-diptheria toxin receptor (DTR) mice with diphtheria toxin (DTX), which depleted dermal DCs (DDCs) and Langerhans cells (LCs), but not macrophages (Figure 2.10). Following DTX treatment, IMQ-induced expression of il23a mRNA was dramatically reduced in treated ears (Figure 2.8). Although these results do not discriminate between the relative contributions of LCs and dDCs to IL-23 production, IL-34 deficient mice, which selectively lack LCs are not impaired in IMQ-induced skin inflammation (Flutter and Nestle, 2013). We focused our analysis on DDCs which can be further subdivided into CD103+ and CD11b+ subsets (Guilliams et al., 2010). Both DC subsets, as well as macrophages, were FACS sorted from IMQ-treated and control ears to measure mRNA for il23a and il12a. Although CD103+ DDCs showed the most
dramatic upregulation of $il23a$ mRNA, taking into consideration that CD11b$^+$ DDCs are more abundant, we estimate that the latter subset produced ~75% of the $il23a$ mRNA, consistent with a recent report (Figure 2.10 and 2.11)(Wohn et al., 2013).
Figure 2.8: Dermal DC-derived IL-23 is critical to drive psoriasiform skin inflammation and acts downstream of RTX sensitive nociceptors.

a. After 3 days of IMQ challenge of vehicle (DMSO) or RTX treated mice, ears were harvested and total protein was prepared to quantify IL-23p40 by ELISA (n=5/experiment; ***, \( P < 0.001 \)).
b. Ears of WT or IL-23R\(^{GFP/GFP}\) mice (n=5/group) were treated daily with IMQ and ear thickness was measured relative to the contralateral ear at indicated time points (***, \( P < 0.001 \)).
c-F After 3 days of IMQ challenge in WT (n=5) or IL-23R\(^{GFP/GFP}\) mice (n=4) total protein was prepared from ear skin and (c) IL-17F and (d) IL-22 were quantified by ELISA (*, \( P < 0.05 \); ***, \( P < 0.001 \)). Cell suspensions from exposed ears stained for (e) inflammatory monocytes and (f) neutrophils to assess total numbers (n=5/experiment; *, \( P < 0.05 \); **, \( P < 0.01 \)).
g-i, IL-23 was injected intradermally every other day into ears of DMSO or RTX treated mice (n=8/group). (g) Ear thickness was measured as indicated. After 3 days, (h) IL-17A and (i) IL-17F producing Thy1\(^+\) cells per ear were quantified by FACS (n=5).
j,k, Mice were treated with anti-Gr1 to deplete neutrophils and inflammatory monocytes or isotype-matched control mAb and challenged with IMQ for 3 days. Ear skin protein lysates were analyzed for (j) IL-23p40 and (k) IL-17F by ELISA (n=5).
l, CD11c-DTR mice were treated with diphtheria toxin (DTX) or PBS 12 h prior to IMQ challenge. After 6h, ears were harvested and processed for total RNA isolation and il23a mRNA levels analyzed by qPCR (n=4; ***, \( P = 0.001 \)).
Figure 2.9: TRPV1+ nociceptors regulate the expression of *il12b* and *il23a* upon IMQ challenge, the inflammatory response to DNFB application, and IL-23 injection can bypass their contribution to activate γδ T cells.

a-c, After 3 days of IMQ challenge, ears were harvested and processed for total RNA isolation and (a) *il12b* (b) *il23a* and (c) *il12a* mRNA levels were analyzed by qPCR (n=5). d, DNFB (0.5% in acetone) was applied topically to DMSO and RTX mice. Time course of change in ear thickness of IMQ treated ear relative to the contralateral ear is represented (n=10). Two-way ANOVA was run to compare ear swelling under DMSO and RTX conditions over time (****, *P* < 0.0001). e, Representative FACS plots from ears harvested after 24h of DNFB application. f, IL-23R−/− mice were treated with RTX and then compared to WT and IL-23R*GFP/GFP* littermate controls during IMQ treatment. Ear thickness was calculated relative to the contralateral ear (n=5). g, After two IL-23 injections into the ear skin of WT and IL-23R*GFP/GFP* mice, the frequency of IL-17F+ cells within dermal γδ T cells was determined by flow cytometry (n=5). h, IL-23 was injected twice into the ear skin of Vehicle- and RTX-treated mice and the total numbers of IL17A+ or IL-17F+ dermal γδ T cells per ear were determined by flow cytometry (n=5).
Figure 2.10: Selective depletion of migratory and skin resident myeloid cell subsets in ear skin and gating strategy used for sorting to isolate RNA from MHC-II+ cells in skin.

a, WT mice were treated with anti-Gr1 (clone RB6-8C5 to deplete neutrophils and inflammatory monocytes) or matched isotype control, challenged with IMQ for 3 days and skin was digested to quantify the total numbers of inflammatory monocytes and neutrophils per ear. Shown are representative plots pre-gated on CD45+ cells and quantification of cell numbers from n=3 mice. b, DTX treatment resulted in depletion of both subsets of dermal DCs (DDCs) as well as Langerhans cells (LCs) but not macrophages. Cells were gated as shown in (Extended Data Fig. 8c) and normalized to levels in WT mice based on the frequency within the CD45+ population from n=4 mice. c, Ear skin from naïve mice was digested and analyzed by flow cytometry for the indicated subsets. Shown is a representative plot pre-gated on CD45+ Class II+ cells from which further subsets were divided based on CD11b and CD11c expression and then F4/80 and CD103 as indicated. d, Total RNA from sorted cells was isolated and qPCR for il12a relative to gapdh was performed from naïve and IMQ treated ears after 6 hours from n=20 pooled mice.
2.3.4 Interaction of NaV1.8+ nociceptors and dermal DCs in vivo

Having identified DDCs as the principal source of IMQ-induced IL-23, we sought to characterize the spatial relationship between DDCs and cutaneous nerves. Remarkably, confocal microscopy of skin whole mounts revealed that at steady state ~75% of DDCs were either in direct contact or in close proximity to sensory nerves (Figure 2.11 and 2.12). Interactions were apparent along the entire length of nerves suggesting that DDCs may receive signals from unmyelinated nociceptor axons and not just from nerve terminals. However, given the high density of peripheral nerves in the skin, it was difficult to judge whether the association with DDCs occurred merely by chance or reflected a biased distribution. To address this possibility, we compared DDC localization relative to two other dense anatomical structures: blood and lymph vessels. In resting tissues contacts of DDCs with these microvascular networks were only about half as frequent as with peripheral nerves (Figure 2.11).

Of note, even though inflammation enhances DC motility and egress into draining lymphatics, IMQ-challenge did not alter the frequency of DDCs contacting nerves (Figure 2.11), suggesting that dDCs engaged in dynamic interactions with nociceptors. We reconstituted NaV1.8-TdT mice with CD11c-YFP bone marrow and performed time-lapse multiphoton–intravital microscopy (MP-IVM) in ear skin to generate three-dimensional time-lapse movies of interactions between YFP+ DDCs and TdT+ nociceptors. These interactions were highly diverse (Suppl. Video 1); some DDCs seemed to be anchored on nerves and sometimes extended protrusions to probe the surrounding tissue, while others appeared to use nerve fibers as a scaffold for directional migration (Suppl. Video 2).
Together, our results support the idea that DDCs can physically interact with a subset of nociceptors that regulate the production of IL-23. However, it should be cautioned that the pharmacological target of RTX, TRPV1, is also expressed on some non-neuronal cells (Lumpkin and Caterina, 2007) so we sought an alternative to confirm the role of nociceptors in an experimental system that does not rely on pharmacologically targeting TRPV1. To this end, we employed NaV1.8-diptheria toxin (DTA) mice in which NaV1.8+ fibers, which respond to mechanical pressure, inflammatory pain and noxious cold, are selectively deleted (Abrahamsen et al., 2008). Although nociceptor-associated transcripts in dorsal root ganglia were more profoundly reduced in this genetic model than after RTX treatment, trpv1 mRNA levels were only reduced by ~80%, consistent with the fact that noxious heat sensing fibers express TRPV1, but not NaV1.8 (Figure 2.3 and 2.12)(Abrahamsen et al., 2008). Following IMQ challenge, ears of NaV1.8-DTA mice contained very low protein levels of IL-17A, IL-17F, IL-22 and IL-23-p40 as compared to littermate controls (Figure 2.11). We conclude that NaV1.8-TRPV1+ neurons are insufficient to induce IL-23 production; rather, NaV1.8+TRPV1+ nociceptors are driving the response.

In light of these results, we propose a model of cutaneous neuroimmune interactions (Figure 1.3) whereby dermal NaV1.8+TRPV1+ nociceptors are essential to induce IL-23 production by nearby DDCs. IL-23 then acts on IL-23R+ γδT17 cells to induce IL-17F and IL-22 secretion, which precipitates the recruitment of circulating neutrophils and monocytes driving psoriasiform skin inflammation. The fact that both RTX and NaV1.8-DTA mice largely preserve a dense meshwork of dermal nerves (Figure 2.12) implies that DDCs do not simply rely on nerves as a scaffold from which...
to produce IL-23. More likely, NaV1.8$^{+}$TRPV1$^{+}$ nociceptors actively induce and control IL-23 production.
**Figure 2.11:** Dermal DCs (DDCs) are closely associated with cutaneous nerves and depend on NaV1.8+ nociceptors for IMQ induced IL-23 production.

**a,** Mice were challenged with IMQ (n=20 pooled mice/condition) and after 6h, myeloid cell populations comprising dermal macrophages and two subsets of DDCs were FACS sorted (Figure 2.10) from cell suspensions to measure il23a mRNA by qPCR. **b,** Representative confocal micrographs of ear skin whole mounts from CD11c-YFP mice stained for β3-tubulin (peripheral nerves, red) and Lyve-1 (collecting lymphatics, blue) or CD31 (blood and lymphatic endothelial cells, blue). Original magnification was 200X. **c,** Close-up confocal micrograph of a CD11c-YFP cell in contact with a nerve (see also Suppl. Videos 1&2). **d,** Quantification of 3D DDC proximity to peripheral nerves, lymphatics, and blood vessels in normal ear skin. The frequency of DDCs (n=330) in contact, proximal (0-7µm) and distal (>7µm) to nerve fibers was determined as described in Methods and a Chi-square test showed bias of DDC to nerves relative to lymphatics and blood vessels (**P< 0.0001). **e-h,** Ears of NaV1.8-DTA or control littermates were treated daily with IMQ. Total protein was prepared from ear skin after 3 days and (e) IL-17A, (f) IL-17F, (g) IL-22 and (h) IL-23p40 were quantified by ELISA (n=4 ears/condition; *, P < 0.05; **, P < 0.01).
Dermal DCs (DDCs) are found in close apposition to NaV1.8+ nociceptors in skin, NaV-DTA mice express reduced levels of key nociceptor markers, yet nociceptor deletion does not grossly affect the peripheral neural network in skin.

a, Representative confocal micrographs of CD11c-YFP mice stained for β3-tubulin, Lyve-1 (collecting lymphatics), and CD31 (blood and lymphatic endothelial cells). b, 3D quantification of DDC proximity to peripheral nerves in naïve and 6 hours post-IMQ treatment binned into contact (<0 um), proximal (0-7 um) and distal (>7 um) fractions as explained in the methods (n of DCs = 200). c, Total RNA from dorsal root ganglia (DRGs) (C1-C4) of littermate control and NaV1.8-DTA mice was isolated and levels of mRNA for trpv1 (TRPV1), scn10a (NaV1.8), tac1 (Substance P) and trpa1 (TRPA1) were determined relative to gapdh. This demonstrates the efficacy of the NaV1.8-DTA system and combined with the original reference characterizing the pain phenotype of these mice illustrates that a subset of peptidergic TRPV1+ nerve fibers is spared. d, Representative confocal micrograph of whole mount ear skin of Vehicle- and RTX-treated mice showing preserved nerve scaffold and e, representative confocal micrographs of whole mount ear skin of Control and NaV1.8-DTA mice showing preserved nerve scaffold. While DRGs showed a loss of the hallmark ion channels of these nerve subsets (Figure 2.2 and 2.12), surprisingly we still observed that RTX mice and NaV1.8-DTA mice maintain a meshwork of nerves in the skin.


2.4 Conclusions

While further studies will be needed to dissect the precise molecular underpinnings of neuroimmune communication in the skin, the present findings indicating nociceptor mediated control of DDCs and the IL-23/IL-17 axis open new avenues for the treatment of inflammatory diseases in the skin and perhaps elsewhere. Intriguingly, recent work has shown that NaV1.8+ nerve fibers exert immunosuppressive activity during *S. aureus* infection (Chiu et al., 2013), raising the possibility that some pathogens have evolved mechanisms to subvert the proinflammatory function of nociceptors. Together, this recent work and the present study suggest an emerging new paradigm whereby TRPV1+NaV1.8+ nociceptive fibers integrate environmental signals to modulate local immune responses to a variety of infectious and inflammatory stimuli.
Chapter 3: Nociceptors promote IL-23 mediated skin inflammation via neuronal activity, regulate cutaneous microbial pathogenesis, and are dispensable for Type 2 skin inflammation

3.1 Abstract:

The experimental results from Chapter 2 raised several intriguing questions including determining what the inputs and outputs of nociceptors are that regulate IL-23 production from dDCs and whether nociceptors play a role in distinct inflammatory contexts. In further experiments we determined that while the TRPV1 ion channel and TLR7 are not required for psoriasiform skin inflammation, nociceptor activity is essential to promote disease and local cytokine production. Future work will aim to characterize the signal(s) from nociceptors to dDCs using simplified in vitro co-culture approaches.

As we found that nociceptors are critical for cutaneous IL-23 production, we tested the effect of nociceptor ablation during Staphylococcus aureus infection, a bacterial infection which depends on IL-23 for clearance. Surprisingly, the absence of NaV1.8+ nociceptors promoted an exuberant inflammatory response characterized by draining lymph node (dLN) hypertrophy. While viable bacteria were decreased in the skin of denervated mice, there was more severe skin pathology and higher numbers were recovered from the dLN suggesting that NaV1.8+ nociceptors may play a role in tissue protection and bacterial containment. Furthermore, despite the role for nociceptors of both instructive (IL-23) and effector (IL-17) cytokines in psoriasiform skin inflammation, we found that in a model of atopic dermatitis (Type 2 inflammation), TRPV1+ nociceptors did not regulate gross disease or the instructive cytokine thymic stromal lymphopoitin (TSLP) yet were required for full effector (IL-4) cytokine production. This suggests that nociceptors exert contextual specificity and are not exclusively pro-inflammatory in all cutaneous pathologies.
3.2 Introduction:

This chapter includes a series of experiments which are thematically related to the initial observation that TRPV1+ nociceptors regulate Type 17 inflammation (Riol-Blanco et al., 2014). Despite being linked by this common thread, they branch in different directions and require an introduction to somatosensory subsets, accessory cells, tools to modulate neural activity, bacterial infections, and Type 2 skin inflammation. As a result, I have provided a brief introduction within each corresponding results section.

3.3 Results:

3.3.1 Congenital vs. adult NaV1.8+ neuronal ablation leads to disparate inflammatory outcomes

Based on our experiments utilizing RTX to ablate TRPV1+ nociceptors, we became interested in assessing the role of defined nociceptor subsets using genetic approaches of ablation. In the NaV1.8-DTA model, the majority of nociceptors and mechanoreceptors are developmentally ablated leaving intact proprioception and a few sparse non-peptidergic TRPV1+ fibers (Abrahamsen et al., 2008; Chiu et al., 2014). Surprisingly, despite the profound effects we observed when using NaV-DTA mice to ablate NaV1.8+ sensory neurons on IL-23/IL-17 axis cytokines (Riol-Blanco et al., 2014), we observed that ear swelling was identical between littermate controls and NaV1.8-DTA mice (Figure 3.1).

Neuronal development is accompanied by incredible plasticity such that removal of a defined subset of neurons can be compensated by the outgrowth of others. As a result, we crossed NaV1.8-Cre mice with Rosa-DTR mice in order to generate an
inducible system of nociceptor depletion (NaV-DTR). This was based on the hypothesis that a compensatory outgrowth of a particular nerve subset maintained the ear swelling phenotype independently of IL-23/IL-17 axis cytokines. We tested the efficacy of nociceptor subset ablation by performing qPCR on isolated DRGs with distinct DTX dose schedules centered on published literature (Cavanaugh et al., 2009; McCoy et al., 2013) determining that DTX treatment of NaV-DTR mice resulted in an almost equivalent loss of TRPV1 signal to NaV-DTA mice and an equivalent reduction in TRPA1 to RTX (data not shown).

When we assembled co-housed cohorts of NaV-DTR mice +/- DTX and treated daily with IMQ, we observed that in these experiments ear swelling was significantly reduced in NaV-DTR + DTX mice (Figure 3.1). However, when we performed analyses of cytokine levels in ear skin lysates at Day 3 or Day 7, IL-23p40 and IL-17A were either the same or if anything enhanced in NaV-DTR + DTX mice over controls (Figure 3.1).

While these data are difficult to reconcile, it is consistent with the sensory heterogeneity that is encompassed within the NaV1.8+ subset of neurons (Chiu et al., 2014; Usoskin et al., 2015). In summary, our data shows that a subset of sensory neurons that is within the TRPV1+ subset and is ablated by the NaV-DTA approach and not the NaV-DTR approach regulates the IL-23/IL-17 axis. However, a sensory neuronal subset within the TRPV1+ subset that is ablated by NaV-DTR and not NaV-DTA appears to regulate the ear swelling phenotype. This could mean that non-peptidergic TRPV1+ sensory neurons, which are spared by the NaV-DTA approach, directly regulate keratinocyte proliferation and that peptidergic TRPV1+ nociceptors are responsible for the regulation of the cytokine networks. Alternatively, compensatory outgrowth of other
fibers provides distinct pro-inflammatory mediators that we have not investigated in the NaV-DTA model. A fine characterization of the neuronal subsets which are present in each of these nerve-ablated mice still remains to be determined. In chapter 3.4 we discuss proximal experiments leveraging novel genetic approaches of nociceptor ablation to unravel the data collecting using RTX, NaV-DTA and NaV-DTR mice.

![Diagram](image)

**Figure 3.1: Congenital vs. adult NaV1.8+ neuronal ablation leads to disparate inflammatory outcomes.**

a-b, Ears from control (n=12) or NaV-DTA (n=15) mice were treated with IMQ for six days and a) ear swelling was assessed from days 0-6 and b) cytokine levels were measured at day 4 for IL-23p40 and IL-17A. c-d, NaV-DTR mice were either treated with PBS (controls, n=10) or DTX (NaVDTR+DTX n=10), treated daily with IMQ, and c) ear swelling was assessed from days 0-7 and d) cytokine levels of IL-23p40 and IL-17A were measured at day 3.
3.3.2 How do nociceptors sense inflammation?

Sensory neurons are, as the name suggests, geared towards sensing an array of distinct stimuli. While these can be canonical activators of ion channels, such as capsaicin acting on TRPV1 to induce the sensation of heat, nociceptors can also be triggered by inflammatory mediators, pruritogens, and even directly by microbial products (Figure 1.3)(Bautista et al., 2014; Binshtok et al., 2008; Caterina, 2000; Chiu et al., 2013). Based on our results that implicated the TRPV1-expressing neuronal subset in providing pro-inflammatory cues during the IMQ model, we wanted to understand the signals acting on TRPV1+ nociceptors for them to provide this message.

We started our investigations by asking whether the TRPV1 ion channel itself was important in this process. When we applied IMQ to littermate controls and TRPV1-deficient mice, we were not able to find appreciable differences in either ear swelling parameters or IL-23/IL-17 axis cytokines (Figure 3.2). This suggests that while the TRPV1-expressing neuronal subset is important in promoting inflammation during psoriasiform dermatitis, that the TRPV1 ion channel itself is dispensable. While this may seem surprising at first, it is not without precedent as even the heat-sensing function of TRPV1-neurons is not impaired in the absence of the channel(Woodbury et al., 2004).

Shortly after our initial findings that TRPV1+ nociceptors are important in regulating IMQ-mediated inflammation, several groups published on the expression of Toll-like receptor (TLR) 7 in TRPV1 neurons with distinct conclusions on its functional role in pruritus(Kim et al., 2011; Liu et al., 2010; Park et al., 2014). As IMQ is a TLR7 ligand, we first began by asking if TLR7 is required for psoriasiform skin inflammation in vivo(Hemmi et al., 2002). When we compared TLR7-deficient mice to littermate
controls, we surprisingly observed that TLR7 was not required for either ear swelling or inflammatory cell recruitment to IMQ-treated skin (Figure 3.2). This means that IMQ induces inflammation in a TLR7-independent fashion in vivo and suggests that a sensing role for TLR7 in TRPV1+ neurons is unlikely in this disease setting. So, what could IMQ be acting through to trigger psoriasiform dermatitis? Besides acting on TLR7, small antiviral compounds including IMQ can also act through the inflammasome to release IL-1β in a caspase-1 dependent manner (Kanneganti et al., 2006). Furthermore after our initial observations, a publication reported that in addition to IMQ, other compounds within IMQ creams, such as isostearic acid, can induce IL-1β release from keratinocytes (Walter et al., 2013).

In an attempt to ask the question of whether TLR- and/or IL-1R-signaling on sensory neurons is important in IMQ-mediated inflammation, we utilized NaV1.8-Cre mice crossed with MyD88-fl/fl mice to conditionally delete MyD88 from NaV1.8+ nociceptors (Abrahamsen et al., 2008; Hou et al., 2008). However, we ran into the unanticipated genetic issue that NaV1.8 and MyD88 are not only present on the same chromosome but only two genes apart, meaning that using NaV1.8-Cre would not be a feasible strategy to remove MyD88 in nociceptors as tight genetic linkage precludes this approach (data not shown). We have since imported TRPV1-Cre mice and can utilize these in the future to address the question of what nociceptors are sensing in vivo during psoriasiform dermatitis (Cavanaugh et al., 2011).

Even though Schwann cells are traditionally associated with myelination and nociceptors in the skin are small unmyelinated c-fibers, there is a subset of non-myelinating Schwann cells present at high density in the skin and tightly associated to
nociceptors (Figure 3.3). These Schwann cells, as well as hepatic stellate cells in the liver, are marked by glial fibrillary acidic protein (GFAP) and as such we could utilize GFAP-Cre mice to visualize or manipulate their function (Garcia et al., 2004; Winau et al., 2007). By crossing GFAP-Cre mice with Rosa26-fl-STOP-fl-TdTomato animals we were able to visualize the Schwann cell network in vivo (GFAP-TdTomato) (Figure 3.3). In preliminary experiments, we wanted to see if DDCs in close proximity to NaV1.8+ nociceptors as seen in Supplementary Video 1 in ref. (Riol-Blanco et al., 2014) were also interacting with Schwann cells. We generated bone marrow chimeras using CD11c-YFP marrow to reconstitute GFAP-TdTomato mice and performed in vivo 2P-IVM (Supplementary Video 2 in ref. (Riol-Blanco et al., 2014)) which shows that DDCs and Schwann cells tightly interact.

Based on observing this interaction, we then wanted to understand whether Schwann cells produce inflammatory mediators during IMQ-mediated inflammation. In order to ask this we sorted GFAP+ cells from inflamed ear skin and performed Nanostring analysis on Schwann cells at 0, 6 and 24 hours of IMQ-treatment for an array of inflammatory cytokines and chemokines (Figure 3.3). We found that Schwann cells produced a limited set of chemokines and cytokines including CXCL1 and IL-6 (Figure 3.3) which are important in neutrophil recruitment and in generalized inflammation. Intriguingly, the receptor for IL-6 which is formed by the common single transducer gp130/IL6ST and IL-6Rα is functionally expressed on nociceptors where it can sensitize them during inflammatory settings (Malsch et al., 2014).

In order to test if TLR and/or IL-1R signaling is important in Schwann cells to detect and amplify inflammation by acting as a member of a triad with nociceptors and
dendritic cells, we utilized GFAP-Cre mice crossed to MyD88-fl/fl mice (Garcia et al., 2004; Hou et al., 2008). We tested for deletion of MyD88 in glial cells by performing qPCR on brain, skin, spleen and LN and only observed a reduction in signal within the brain (Figure 3.4). Furthermore, we ensured that TLR responses to canonical ligands were not impaired in immune cells (data not shown). We performed in vivo experiments assessing the role of MyD88 in Schwann cells by applying IMQ daily and assessing various inflammatory parameters. When comparing littermate controls to GFAP-MyD88fl/fl mice, we observed that ear swelling was slightly but significantly and reproducibly reduced (Figure 3.4). However, this did not correlate with a reduction in inflammatory monocytes, neutrophils, IL-23p40, or effector cytokine production by skin-resident γδT17 cells (Figure 3.4).

While we were encouraged by our preliminary data and this evidence does not discount an active role for Schwann cells in psoriasiform skin inflammation as proposed by Weddell in his original neural characterization of psoriatic skin (Weddell et al., 1965), we decided to pursue other mechanisms of neural sensing of inflammation which can contribute to psoriasiform dermatitis (Chapter 3.3.3 and Chapter 3.4). The role of Schwann cells may be particularly intriguing in the context of bacterial infection as they are the targets of *Mycobacterium leprae*, the causative agent of leprosy (Masaki et al., 2013; Rambukkana et al., 2002).
Figure 3.2: TRPV1 and TLR7 are not required for psoriasiform skin inflammation.

a-b, Ears from WT (n=5) or TRPV1−/− (n=5) mice were treated with IMQ for six days and a) ear swelling was assessed from days 0-6 and b) cytokine levels were measured at day 4 for IL-17A and IL-17F. c-d, WT (n=5) or TLR7-KO (n=5) mice were treated daily with IMQ, and c) ear swelling was assessed from days 0-6 and d) the frequency of neutrophils and inflammatory monocytes of CD45+ cells in digested ear skin was measured by flow cytometry.
Figure 3.3: Schwann cells produce inflammatory mediators during psoriasiform skin inflammation.

a) Skin from GFAP-ttdTomato mice was harvested and processed for whole-mount confocal immunofluorescence and stained for B3-tubulin to mark all neurons. b-c, Schwann cells were sorted from the digested skin of GFAP-ttdTomato mice for nanostring analysis of inflammatory mediators after at 0, 6, and 24 hours after IMQ treatment and b) a representative plot and c) absolute chemokine and cytokine expression counts in Schwann cells are presented.
Figure 3.4: Removing the key TLR and IL1R adaptor MyD88 from Schwann cells does not reduce immunological outcomes of IMQ treatment.

a) Indicated tissues from GFAPCre+MyD88fl/fl and GFAPCre-MyD88fl/fl mice were harvested and processed for RNA in order to quantify relative MyD88 expression levels.  
b-e, Control (GFAPCre- MyD88fl/fl, n=20) and GFAPCre+ MyD88fl/fl (n=20) mice were treated with IMQ and ear swelling assessed over indicated time periods. c) total numbers of inflammatory monocytes and neutrophils were quantified by flow cytometry, d) IL-23p40 was measured in ear skin lysates, and e) total numbers of IL-17A+ dermal gD T cells quantified by intracellular cytokine staining.
3.3.3 Modulating nociceptor activity influences skin inflammation

One outstanding question from the approaches we have taken so far to dissect the contribution of nociceptors to IMQ-mediated inflammation is whether neuronal activity (e.g. action potentials) are important in mediating neuronal-DC communication. Our data generated to date has relied on two methods of neuronal ablation, however, we have yet to identify if electrical activity in TRPV1+ or NaV1.8+ fibers is important in regulating cytokine production by DCs. The compound QX-314 is a cell-impermeable lidocaine derivative which internally blocks sodium channels, thus inhibiting propagation of action potentials (Binshtok et al., 2007; Brenneis et al., 2013). The way that it enters cells is via open TRP channels so it will only enter and silence neurons with open TRP channels. When we administered IMQ concomitantly with daily injections of either saline or QX-314, the local inflammatory response as measured by ear-swelling and recruitment of neutrophils and inflammatory monocytes was significantly blunted by QX-314 treatment (Figure 3.5). A very recent study published during preparation of this thesis utilized QX-314 to silence nociceptors in the lung which revealed a role for inflammatory-sensitization of nociceptors contributing to Type 2 inflammation ((Talbot et al., 2015) and Chapter 2). This provides evidence that action potentials are required for the pro-inflammatory activity of nociceptors in the IMQ model and allergic asthma.

To further explore this idea, we employed optogenetic approaches, in which specific cell populations are either activated or inhibited by stimulation of light-sensitive channelrhodopsins (Madisen et al., 2012). This technology has been used extensively by neuroscientists to turn specific neuronal subsets “on” and “off” in a temporally and spatially defined manner. Using an activating transgenic channelrhodopsin mouse-strain
TRPV1-Chr2 (Table 1) obtained by crossing commercially available TRPV1-Cre mice with commercially available Rosa26-fl-STOP-fl-Ai32 (Channelrhodopsin-2 (Chr2)) mice, Dr. Hoon has developed a system to selectively activate TRPV1+ nociceptors resulting in pain behavior after cold blue 473nm light to the skin of transgenic mice but not control mice (Mark Hoon, unpublished observation) and references (Daou et al., 2013; Iyer et al., 2014). Using this approach, we administered cold blue light concurrent with IMQ administration to isoflurane-anesthetized mice maintained at 37°C and observed increased levels of il23a and ccl20 compared to contralateral ears that had also been treated with IMQ but were not exposed to blue light (Figure 3.6). Furthermore, TRPV1-Cre mice lacking the transgenic channelrhodopsin did not exhibit a preferential enhancement of cytokine production on the blue-light exposed ear (Figure 3.6). Thus, enforcing action potentials in local TRPV1+ fibers potentiates local cytokine and chemokine production by DDCs, suggesting that we can regulate dermal cytokine production using optogenetic control of neurons. Intriguingly il12b was not affected in the same manner by blue light suggesting that perhaps there is some level of specificity to the transcriptional effects on DDCs from TRPV1+ nociceptor firing.

These experiments were particularly informative in pursuing further characterization of a potential, direct, local signal produced by nociceptors that acts on DDCs. All data presented to date had previously relied on systemic ablation of neural subsets which means we had not confirmed that local nociceptor activity was, in fact, the driving mechanism in DDC IL-23 production. While this data set is still preliminary in
nature, it implicates that nociceptor activity is important (QX-314) and that potentiating nociceptor activity can rapidly enhance DDC cytokine production (optogenetics).

Figure 3.5: Silencing nociceptors diminishes psoriasiform skin inflammation. 

a) Schematic of the charged lidocaine derivative QX-314 enters neurons via TRP-channels to silence neurons by internally blocking Na+ channels. 

b-c, Mice were treated daily with saline or QX-314 by intradermal injection and IMQ was applied daily for four days and 

b) ear swelling and 

c) CD45+ total cell numbers were quantified in indicated groups.
Figure 3.6: Artificially enforcing nociceptor activity using optogenetics promotes local cytokine production. 

*a-e*, optogenetic activation of TRPV1+ nociceptors whereby 

1. TRPV1-Ai32 Mouse or TRPV1-Cre (Control) 
2. IMQ to both ears 
3. 20 Hz Blue light to one ear for 4 Hours

Ear skin was harvested and indicated cytokines/chemokines were assessed by qPCR in

- b-c)  blue-light vs. no light skin of TRPV1-Ai32 mice relative to housekeeping genes or
- d-e) expressed as a ratio of the blue-light vs. no light skin in TRPV1-Ai32 and TRPV1-Cre mice.
3.3.4 Potential mechanisms of immunoregulation

During our characterization of RTX-treated mice, we made several intriguing observations about the immune compartment at steady-state. While there were no significant alterations in the levels of principal leukocyte subsets in various lymphoid organs (data not shown), when we carefully examined cell numbers in skin we observed reductions in both epidermal γδT cells and dermal γδT17 cells but not conventional αβT cells in RTX-treated mice (Figure 3.7). While there was also a decrease in frequency of cytokine-producing γδT17 cells during IMQ-mediated inflammation, total numbers showed a more drastic effect than frequency alone (Riol-Blanco et al., 2014). This suggests that perhaps an altered steady-state environment could affect inflammatory settings in the absence of TRPV1+ nociceptors.

There was also a slight but significant decrease in the total number of MHC-II+CD11b+CD11c+ myeloid cells present in the skin which encompass langerin- DDCs and Langerhans cells (Malissen et al., 2014) (Figure 3.7). This suggests that TRPV1+ nociceptors may be important in the persistence of skin-resident innate-like lymphocyte subsets potentially through effects on DDCs. It will be of interest to characterize DDC transcriptomes in steady state from RTX-treated mice to determine if survival factors for T cells, such as IL-15, are diminished by loss of TRPV1+ neurons, or if total levels of TGF-β in the skin decrease (Mackay et al., 2013). Perhaps sensory neurons and/or Schwann cells contribute to the formation of the niche for T resident memory (Trm) cells (Schenkel and Masopust, 2014).

One important question left to answer is what is the nature of the molecular signal between TRPV1+ nociceptors and DCs that regulates IL-23 production? The two most
prominent neuropeptides produced by TRPV1+ nociceptors are CGRP and Substance P. In preliminary experiments, we have observed that both CGRP and Substance P can synergize with R848 in vitro to induce a slight increase in IL-23 production by DCs which was not reliably reproduced across experiments (data not shown). Furthermore, our in vivo experiments to date have been equivocal, with CGRP and Substance P receptor antagonists having no effect on levels of IL-23 detected by ELISA (data not shown).

Of course the attempt to find one, key, molecular mediator for a biological process may be naïve to the underlying complexity. Nociceptors express an array of neuropeptides that can be released in a spatiotemporally regulated manner and perhaps one individual neuropeptide is not responsible for the effect we are observing. Nociceptors can also release ATP, a molecule which can induce IL-23 production, in non-synaptic manners via volume activated anion channels (Fields, 2011; Paustian et al., 2013). In order to identify the nature of the molecular mechanism(s) at play we will utilize an in vitro system with both DRGs and DCs. In initial experiments, we isolated CD11c+ DCs from lymphoid organs and DRG neurons from wild-type mice. We then culture DCs alone with IMQ in a range of doses and assessed for il12b transcript levels (Figure 3.8). We observed that IMQ alone is a poor inducer of il12b. However, when DCs were co-cultured with DRG neurons, il12b expression was greatly enhanced at each dose, even at low doses where IMQ alone had no effect on DCs (Figure 3.8). This suggests a synergistic effect of DRG neurons on DC IL-12/23 production. Setting up these culture conditions required extensive troubleshooting and optimization at various
steps but now provides an assay to test various iterations discussed further in Chapter 3.4.

Figure 3.7: TRPV1+ neurons regulate numbers of DCs and γδ T cells in skin at steady state.

a-d, Naïve ear skin from WT or RTX-mice was enzymatically digested and analyzed by flow cytometry to quantify total numbers of a,b) T cell subsets and c,d) CD11b+ CD11c+ dendritic cells.
Figure 3.8: In vitro culture of DCs with DRG neurons promotes IL-23 production

a) DRG neurons were harvested, enzymatically dissociated, and plated on laminin-coated V-bottom 96 well plates with ~1,000 DRG neurons per well allowing them to extend neurites for 3 days. Dendritic cells were harvested from spleen and LNs of FLT3-L tumor bearing mice and seeded in each well at ~10,000 DCs per well. Neurobasal media with indicated amounts of IMQ was added to either DCs alone or DCs with DRG neurons and cells were lysed and analyzed for *il12b* expression relative to *gapdh*. 
3.3.5 Nociceptor function during cutaneous infection

Using RTX treatment (Karai et al., 2004; Mishra and Hoon, 2010) to ablate TRPV1+ nociceptors and the NaV1.8-Cre x Rosa26-fl-STOP-fl-DTA (NaV-DTA) model (Abrahamsen et al., 2008) to developmentally ablate NaV1.8+ nociceptors, we identified a role for both subsets in regulating IL-23 and IL-17 in the skin during psoriasiform inflammation (Riol-Blanco et al., 2014). While these two cytokines are important drivers of autoinflammatory diseases, they also have critical roles in providing host defense against cutaneous infections as exemplified by patients who have genetic deficiencies in this pathway (Di Cesare et al., 2009; Lowes et al., 2013; Marodi et al., 2012). As a result, we hypothesized that since TRPV1+ NaV1.8+ nociceptors regulate the IL-23/IL-17 pathway during psoriasiform dermatitis, that they would also play an important role in controlling a cutaneous extracellular bacterial infection (Cho et al., 2010).

In collaboration with Jean Lee at the Channing Labs, we developed a model of epicutaneous *S. aureus* infection utilizing Morrow-Brown Allergy Test needles to inoculate mice on dorsal ear skin with the LAC USA-300 strain (Prabhakara et al., 2013). As we were initiating pilot experiments to investigate the role of NaV1.8+ nociceptors on inflammatory parameters and bacterial burden in this infectious model, Clifford Woolf’s group published an article in *Nature* on this subject (Chiu et al., 2013). In their paper, they also utilized the NaV-DTA mouse model to identify that NaV1.8+ nociceptors directly sense Staphylococcal toxins. These toxins trigger pain during a bacterial infection does not rely on the host inflammatory responses but rather direct activation of sensory neurons by *S. aureus* (Chiu et al., 2013). Furthermore, they noted the interesting
observation that in the absence of NaV1.8+ nociceptors, mice developed severe lymphadenopathy and increased skin inflammation after subcutaneous challenge with USA300. As a result, they concluded that NaV1.8+ nociceptors had an anti-inflammatory role during *S. aureus* challenge (Chiu et al., 2013). However, they only assessed a limited panel of inflammatory markers and did not quantify bacterial burden until resolution of infection in skin or dLNs after bacterial challenge in the presence or absence of NaV1.8+ nociceptors.

Since we already had a pipeline of experiments ongoing and in preparation, we decided to continue investigating our original hypothesis with an interest in determining if inflammation was in fact deregulated directly as a result of deficiency in NaV1.8+ nociceptors or secondary to increased bacterial burden. After epicutaneous challenge with *S. aureus* in NaV-DTA mice and control littermates, we also observed tremendous lymphadenopathy at Day 1 and also at Day 4 confirming and extending the observations from the recent publication (Figure 3.9). This increase in LN cellularity was seen across all types of lymphocytes and leukocytes but in particular B cells (data not shown). Furthermore, large numbers of neutrophils were recruited to the dLN of NaV-DTA mice potentially indicating the presence of disseminated bacteria (Figure 3.9).

When we assessed the bacterial burden in the skin at Day 1, both NaV-DTA and littermate controls had identical CFUs (Figure 3.10). However, at this timepoint, NaV-DTA mice had significantly increased CFU in the dLN (Figure 3.10). At day 4, CFUs in the skin of NaV-DTA mice were paradoxically decreased but the ears showed gross signs of necrosis relative to control mice (Figure 3.10). When we measured levels of IL-23, IL-17, and TNF-α in the skin of control and NaV-DTA mice at both Day 1 and Day 4, we
did not note any significant differences in cytokine levels indicating that the lymphadenopathy and tissue necrosis may be occurring for distinct reasons than what was speculated by Chiu et al(Chiu et al., 2013) (Figure 3.11). In particular, NaV1.8+ nociceptors may be regulating early bacterial dissemination from the skin to dLN. It will be of interest to characterize the role of NaV1.8+ nociceptors in disease tolerance mechanisms during bacterial infection(Medzhitov et al., 2012).

What about the role of TRPV1+ nociceptors during S. aureus infection? Using the RTX system, we did not observe any differences in lymph node cellularity or CFU in the skin or dLN of RTX-treated mice relative to controls (Figure 3.12). These results suggest that TRPV1- but NaV1.8+ nociceptors play a role in controlling S. aureus infection. However, an alternative explanation is that in one model we are ablating nociceptors post-developmentally (RTX) while the NaV-DTA system relies on continuous developmental ablation of NaV1.8+ lineage neurons. This could impact lymphatic architecture and vessel patterning during development(Mukouyama et al., 2002). These and other observations led to the search for alternative models of denervation to investigate the role of nociceptors during autoinflammatory and infectious settings.
Figure 3.9: The absence of NaV1.8+ nociceptors promotes dLN hypertrophy during cutaneous S. aureus infection.

a-b, NaVWT or NaVDTA mice were inoculated with $10^9$ CFU of LAC USA300 S. aureus in 10uL administered by 10 pinpricks of Morrow-Brown allergy test needles and a) draining lymph nodes were harvested to quantify total cell numbers and b) numbers of neutrophils at Day 1 or Day 4
Figure 3.10: NaV1.8+ nociceptors are required for dermal tissue protection and bacterial containment.

a-c, NaVWT or NaVDTA mice were inoculated with $10^9$ CFU of LAC USA300 *S. aureus* in 10uL administered by 10 pinpricks of Morrow-Brown allergy test needles and a) ear skin CFU and b) dLN CFU were quantified in mice of indicated genotypes. c) macroscopic tissue damage was visualized in NaV-DTA mice at Day 4 after *S. aureus* infection.
Figure 3.11: Nociceptors do not significantly regulate cytokine levels during S. aureus infection.

a-b, NaVWT or NaVDTA mice were inoculated with $10^9$ CFU of LAC USA300 S. aureus in 10uL administered by 10 pinpricks of Morrow-Brown allergy test needles and a) TNF-α and b) IL-12/23p40 were quantified in ear skin lysates by ELISA at Day 1 and Day 4.
Figure 3.12: TRPV1+ neurons do not play an appreciable role during S. aureus infection.

a-d, WT or RTX mice were inoculated with $10^9$ CFU of LAC USA300 S. aureus in 10uL administered by 10 pinpricks of Morrow-Brown allergy test needles and a) draining lymph nodes were harvested to quantify total cell numbers and b) numbers of neutrophils and c) skin and d) dLN CFU were quantified.
3.3.6 Nociceptors in Type 2 inflammation

Type 17 responses, such as the IMQ model, only represent one of the branches of inflammation which can become dysregulated and cause skin disease. Type 2 inflammation is predominantly driven by the instructive cytokines TSLP, IL-33, and IL-25 (Palm et al., 2012). One common skin condition in which dysregulation of TSLP provokes disease is atopic dermatitis (AD).

As a result, we sought a system to investigate the role of TRPV1+ fibers in an AD-like condition. The topical application of MC903 (a Vitamin D3 analogue) to skin results in TSLP production, itching behavior (Diana Bautista, unpublished observation), and a TSLP-dependent dermatitis (Kim et al., 2013; Leyva-Castillo et al., 2013; Li et al., 2006). When we applied MC903 to vehicle and RTX-treated mice, we observed no difference in ear swelling over the course of 6 days (Figure 3.13). However, after MC903 treatment, dLN size was slightly but significantly increased at Day 5 and Day 6 in RTX-treated mice (data not shown). When we assessed levels of cytokines by qPCR in whole skin, we noted that there was no difference in the induction of Tslp in WT and RTX mice (Figure 3.13). However, there was a significant decrease in Il4 in skin from RTX mice and also at the protein level in cultures of dLN cells after ex vivo stimulation (Figure 3.13).

This indicates that while RTX-treatment did not affect the gross pathology of the disease, it did lead to decreased IL-4 in the skin. It will be of interest to characterize the source of IL-4 (e.g. T cell, ILC, basophil derived) in particular because IL-4 production in the MC903 model is dependent on DCs (Leyva-Castillo et al., 2013). While it is still
highly speculative, it could be that TRPV1+ fibers regulate DC-dependent processes in both psoriasiform and AD-like dermatitis but not cytokine production by epithelial cells.

**Figure 3.13: Nociceptors do not regulate disease pathogenesis during an acute model of atopic dermatitis.**

**a-d,** MC903 dissolved in 20 uL EtOH was applied to the ear skin of WT or RTX mice and **a**) ear swelling was measured relative to EtOH treated ears. **b,c**) Ear skin was harvested at day 5 and day 6 to determine gene expression of TSLP and IL4 and **d**) dLNs were re-stimulated ex vivo with PMA/Ionomycin and supernatants assayed for IL-4 protein levels.
3.4 Conclusion and Future Directions

Our results implicate TRPV1+ nociceptors in regulating ear swelling, inflammatory cell recruitment, and cytokine production in a Type 17 model of inflammation. Despite their role in regulating the IL-23/IL-17 axis, they did not seem to be important for production of these cytokines during S. aureus infection. However, they were important for tissue tolerance of infection and containment within the skin to prevent dissemination of bacteria to local dLNs. Furthermore, TRPV1+ nociceptors were not required for gross inflammation in a Type 2 model, but did contribute to IL-4 production.

When we investigated the role of NaV1.8-lineage sensory neurons (NaV1.8 is expressed on approximately 85% of nociceptors) in Type 17 inflammation, we identified that developmental ablation results in decreased IL-23/IL-17 axis cytokines yet ear swelling responses were normal in denervated mice. Since the NaV-DTA system ablates several classes of nociceptors whereas TRPV1+ only ablates heat-sensing fibers, this raises the possibility that distinct subsets of nociceptors provide distinctive and potentially counteracting roles. Furthermore, compensatory mechanisms present during development may account for discrepancies between the NaV-DTR and NaV-DTA strains.

Mrgprd+ (mechanical) and TRPM8+ (cold) fibers represent the other predominant populations of nociceptors beyond TRPV1+ fibers (Le Pichon and Chesler, 2014). In order to investigate the specific contributions of each nociceptor subset to Type 17 (IMQ), Type 2 (MC903), and also Type 1 (DNFB) inflammation, we propose to take a systematic approach using novel tools to deplete each nociceptor subset either in isolation.
or in combination using DTX. Through our collaboration with Dr. Mark Hoon at NIH, we have obtained three mouse strains, which he has characterized: TRPV1-DTR, TRPM8-DTR, and Mrgprd-DTR (Cavanaugh et al., 2009; Pogorzala et al., 2013). We will then assess the inflammatory responses in nociceptor ablated mice (+DTX) and littermate controls (+saline) to each inflammatory stimulus at the level of tissue pathology, inflammatory cell recruitment and cytokine production. This will provide a comprehensive understanding on the role of distinct nociceptor subsets (and the pain modalities which they convey) and specific inflammatory modules in skin.

Regarding the molecular mechanism of nociceptor action we will extend experiments presented in chapter 3.3.3 and 3.3.4 regarding neural activity and in vitro co-cultures. The molecular signals that are sensed by nociceptors during IMQ-inflammation are still unclear and will also be important to characterize. Future experiments will extend this data set by assessing protracted timepoints utilizing both activating and inhibitory optogenetic approaches as well as chemicogenic models (Farrell and Roth, 2013; Fenno et al., 2011). This will allow us to turn defined nociceptor subsets on/off in vivo in a spatiotemporally defined fashion for short durations (<6 hours) or, systemically over longer periods of time (>12 hours).

To investigate contact-independent mechanisms in vitro, we will take conditioned media from DRG neurons treated with capsaicin (TRPV1), allyl-isothiocyanate (AITC) and menthol (TRPM8) in order to activate distinct subsets of neurons. We will also incubate neurons with R837 to test if they directly respond to and produce an inflammatory mediator which can then activate DCs. Our main readout will be il23a and il12b production by DCs isolated from FLT3L-treated mice. Since neuronal signals alone
may be insufficient to trigger cytokine production, we will also incubate DCs with a suboptimal dose of IMQ +/- conditioned media in order to identify synergistic effects.

We will then isolate DRGs from RTX-treated mice to identify if the nature of the molecular signal is unique to TRPV1+ neurons and not present in the preserved Mrgprd or TRPM8 subset. We can take an analogous approach using Mrgprd-DTR and TRPM8-DTR mice to deplete those neurons before isolating DRG neurons. Contact dependent mechanisms will be more difficult to isolate as capsaicin, AITC, menthol, and R837 have the potential to activate DCs directly. If we obtain promising data from co-culture experiments, we can then take DRGs and DCs from TLR7/-/-, TRPV1/-/- TRPA1/-/- and TRPM8/-/- mice in order to identify the principal cell type on which the stimuli are acting on.

Lastly, optogenetic tools can also be utilized to activate DRG neurons in vitro with the caveat that they do not release measureable levels of neuropeptides but do fire action potentials and flux Ca^{2+} (Daou et al., 2013). We will take DRG neurons from TRPV1-Chr2 mice cultured with DCs and stimulate them with blue light in vitro. If we identify that action potentials are sufficient to drive *il23a* and *il12b* production from DCs, this will allow us to determine that neuropeptide-independent mechanisms are a main method of communication between nociceptors and DCs.
Chapter 4: Nociceptors are not essential for innate and adaptive colitis yet promote systemic wasting disease

Jose Ordovas-Montanes, Seth Rakoff-Nahoum, Jumpei Sasabe, Matthew Waldor, Roderick Bronson, Ulrich H. von Andrian.
4.1 Abstract

Based on the role for nociceptors in regulating cutaneous IL-23 and subsequent inflammation, we hypothesized that TRPV1+ nociceptors would be important in regulating IL-23-driven pathologies in the gut. However, we found no appreciable role for nociceptors in either an acute innate colitis or a spontaneous microbiota-driven colitis. While colitis is characterized by its gastrointestinal pathology, it affects the body systemically and we noted that in both models, mice lacking TRPV1+ nociceptors maintained healthier weights than their nerve-replete counterparts. This opens future avenues for exploration of how sensing of inflammation by nociceptors influences microbial communities, systemic metabolism, and sickness behavior.
4.2 Introduction

The immune system has been implicated as a key contributor to several inflammatory diseases of the gut including Crohn’s disease and ulcerative colitis. As was highlighted in chapter 1.4.4, the maintenance of gut homeostasis requires interactions between the peripheral nervous system and resident immune cells (Sharkey, 2015). In a disease-state, these neuro-immune interactions regulate the magnitude and quality of inflammation present in the intestine. The gut receives various sources of innervation including the pseudo-autonomous enteric nervous system that is partially tuned by the sympathetic nervous as well as separate vagal and spinal sensory inputs (Udit and Gautron, 2013). The literature can be complex to follow as neurons with sensory function exist in the enteric, autonomic, and DRG-derived nerves that innervate the gut and oftentimes their precise source is not identified (Ben-Horin and Chowers, 2008; Engel et al., 2011a). One major challenge for the field will be to determine which sensory branch is activated as this determines the efferent motor circuitry engaged and neuropeptides released.

Mouse models of colitis generally rely on the disruption of the epithelial barrier using supplementation of dextran sodium sulfate (DSS) in the drinking water, the transfer of activated T cells, or immunization and challenge with haptens. Several studies have employed either capsaicin or RTX-mediated desensitization which target both vagal sensory afferent and spinal TRPV1-expressing sensory neurons but the effects of these compounds on the enteric nervous system remains equivocal (Berthoud et al., 1997; Mishra and Hoon, 2010).
In our initial foray into the gut, we selected a model of colitis which is dependent on IL-23 and provoked by systemic administration of one dose of agonistic anti-CD40 antibody to recombination activating gene 1 (Rag1) deficient mice (Uhlig et al., 2006). We chose this system due to its dependence on dendritic cell-derived IL-23 to induce mucosal immunopathology (Uhlig et al., 2006; Visekruna et al., 2015). IL-23 acts on T cells and ILC3s to induce colitis via IL-22 (Buonocore et al., 2010; Eken et al., 2014). Besides an IL-23 dependent colitis, anti-CD40 administration also induces IL-12 production from myeloid cells which triggers a systemic wasting disease characterized by weight loss (Uhlig et al., 2006). Intriguingly, the two phenotypes are separable as demonstrated by neutralizing antibodies to p40, the shared subunit, inhibiting both wasting and colitis, while antibodies to p35 or p19 only inhibit one aspect of disease, respectively (Uhlig et al., 2006). Depletion of ILCs preferentially ameliorated colitis with little to no effect on weight loss (Buonocore et al., 2010; Fuchs et al., 2013). Sickness behavior and systemic inflammation are also a component of septic-like disease models as is the case with endotoxemia (Deutschman and Tracey, 2014). We used LPS administration to complement our observations from the anti-CD40 model regarding the role of TRPV1+ neurons in regulating aspects of systemic inflammation.

While inducible models are convenient from an experimental standpoint, they do not accurately parallel the spontaneous and heterogeneous presentation of human inflammatory bowel disease where it is likely that distinct etiological agents can converge on similar disease outcomes. To complement our investigation of the role of TRPV1+ nociceptors in an acute/innate-driven colitis, we also utilized the IL-10-KO mouse model which develops an IL-12/23 and IFNγ mediated T-cell dependent colitis (Berg et al.,...
1996; Davidson et al., 1998; Kuhn et al., 1993). IL-10 is an essential cytokine that can be produced by leukocytes of the innate and adaptive immune systems with targets in both the immune system and parenchymal cells (Moore et al., 2001). Inflammation in IL-10-KO mice varies across animal facilities and requires the presence of an intestinal microbiota (Sellon et al., 1998).

The host constantly senses intestinal microbiota via TLRs expressed on mucosal epithelial cell and these interactions are essential for intestinal homeostasis (Rakoff-Nahoum et al., 2004). However, during settings in which the balance towards inflammation is shifted, as in the absence of IL-10, TLR signaling drives the colitogenic process (Rakoff-Nahoum et al., 2006). Are nociceptors in the gut also sensitive to the presence of pathogenic or commensal microbes? Intriguingly, the development of inflammatory hyperalgesia depends on the presence of commensal bacteria (Amaral et al., 2008). Furthermore, the application of Bacteroides fragilis, a model commensal microorganism, to the gut epithelium triggers primary sensory afferent excitability in a polysaccharide A dependent fashion (Mao et al., 2013). Pathogenic colonization by Campylobacter jejuni or Salmonella typhimurium activated TRPV1+ vagal afferents, which the authors speculate regulates the release of efferent neuronal inflammatory mediators (Riley et al., 2013).
4.3 Results:

4.3.1 TRPV1+ nociceptors in innate colitis and systemic wasting disease

In order to assess the role of TRPV1+ nociceptors in an innate IL-23 dependent colitis, we treated control Rag1-/- or RTX Rag1-/- mice with anti-CD40 and assessed colitis histologically. In this analysis, we noted that colitis was mild, patchy and not fully penetrant with foci of inflammation observed throughout the colon (Figure 4.1). Cytokines implicated in driving the disease process such as IL-23 and IL-22 were expressed at equivalent levels in colons of control or RTX-treated mice suggesting that TRPV1+ nociceptors do not regulate anti-CD40 driven colitis (Figure 4.1).

However, despite a lack of an effect on the colitogenic phenotype, we reproducibly observed that RTX-treated mice fared better with diminished weight loss and enhanced recovery from disease re-establishing a weight closer to baseline more rapidly than control mice (Figure 4.2). Control mice showed a phase of great inactivity during day 2 of the disease leading to a lack of grooming while RTX-treated mice generally maintained a healthier appearance (data not shown). We hypothesized that since IL-12 is important in driving systemic aspects of immunopathology during this disease course, that perhaps TRPV1+ nociceptors were regulating systemic IL-12 levels and other early pro-inflammatory cytokines. While both control and RTX-treated mice showed greatly elevated levels of serum IL-12p40, TNF, and IL-6, there were no differences between the two groups that were correlated with diminished weight loss or the observed behavioral differences (Figure 4.2).
While TRPV1+ nociceptors may not regulate levels of these cytokines, we speculated that perhaps TRPV1+ vagal sensory neurons reduced by RTX-treatment may not transmit inflammatory signals to key regions of the brain to decrease appetite during settings of inflammation (Carter et al., 2013; Gautron et al., 2015). Specifically, signals from the vagus can be transmitted through the parabrachial nucleus to the amygdala via CGRP+ neurons (Carter et al., 2013). These can override central regulators of appetite in the hypothalamus (Gautron et al., 2015). While our initial experiment measuring daily eating behavior in mice was promising, further experiments to solidify these findings provided equivocal data and a composite of eating behavior revealed no significant differences between control and RTX-treated mice both at baseline and also during anti-CD40 inflammation (Figure 4.2). Food deprivation for 12 hours also induced equivalent weight loss, compensatory food intake, and weight gain in control and RTX mice suggesting that basal metabolic and appetite sensing circuits are maintained (data not shown). We also assessed the principal anorexogenic hormone leptin and found no differences between control and RTX mice. While so far the mechanism by which TRPV1+ sensory neurons promotes systemic cachexia eludes us, we will discuss potential future directions in chapter 5.

When we noted a systemic phenotype in the anti-CD40 model, we initiated experiments using intraperitoneal administration of LPS to induce endotoxemia which is characterized by intense inflammation and inactivity. High doses of LPS can trigger mortality within a few hours. We again used Rag1/-/- mice to maintain consistency with our previous experiments in the anti-CD40 model and used a dose of 10 mg/kg of LPS based on ref. (Rosas-Ballina et al., 2011). Of course, one of the most prominent neuro-
immune connections highlighted in the literature is that of the anti-inflammatory reflex arc in which vagal stimulation results in a decrease in TNF production by splenic and hepatic macrophages (Andersson and Tracey, 2012; Borovikova et al., 2000). However, the nature of the vagal afferent or efferent neurons involved in this reflex are not currently well characterized and functional outcomes of vagal activation on physiological parameters such as temperature and behavior have not been reported. We monitored the temperature of control and RTX-treated mice over a 24 hour period and observed that, while the initial temperature drop was identical up to 8 hours, all RTX-treated mice recovered to almost normal core temperatures by 24 hours after injection while control mice continued a precipitous drop (Figure 4.3). At this timepoint, all control mice were completely sessile while RTX-mice behaved normally (Figure 4.3). While we did not confirm lethality, the temperature trend was suggestive that all control mice would have died and RTX-mice survived. While perhaps earlier cytokine levels when the temperature diverged would have been more telling, a 24 hour timepoint analysis revealed significantly elevated levels of TNF in both cohorts which did not correlate with the severity of disease (Figure 4.3). However, the canonical inducer of the acute phase response, IL-6, was significantly reduced in RTX-treated mice (Figure 4.3).

From these experiments we conclude that TRPV1+ vagal sensory afferents are not responsible for the afferent arm of the reflex arc as loss of sensation of LPS-induced endotoxemia would have precluded the anti-inflammatory effects of the efferent arm mediated by norepinephrine and acetylcholine. These experiments instead suggest that TRPV1+ sensory afferents, in an as-yet-to-be determined location, are important for
sustained elevation of systemic inflammatory cytokines leading to murine septic-like responses and sickness behavior.

Figure 4.1: TRPV1+ nociceptors do not regulate innate IL-23 mediated colitis. a-b, PBS or RTX mice were treated with anti-CD40 agonist antibody on day 0 and sacrificed on day 7 to assess colitis a) histologically and b) by gene expression of indicated cytokines and chemokines.
Figure 4.2: TRPV1+ nociceptors promote systemic wasting disease independent on regulation of appetite and key cytokines.

a-d, PBS- or RTX-treated mice (n=23 each) were injected with anti-CD40 agonist antibody on Day 0 and disease was a) monitored by tracking weight of individual mice b) serum cytokine levels at day 7 c) daily food intake behavior and d) serum leptin concentrations.
Figure 4.3: LPS endotoxemia requires TRPV1+ nociceptors for systemic sickness behavior.

a-c, PBS- or RTX-treated mice were injected intraperitoneally with 10 mg/kg of LPS and a) temperature was monitored rectally in mice for 24 hours b) grooming behavior documented and c) terminal 24 hour serum cytokine levels quantified by ELISA.
4.3.2 TRPV1+ nociceptors in intestinal disease and homeostasis

While TRPV1+ nociceptors have been implicated in various acute models of colitis, in particular those utilizing DSS to cause epithelial barrier breakdown, no studies to date have been published in spontaneous models which are dependent on the microbiota (Sellon et al., 1998). As such, we wanted to ask the question whether TRPV1+ nociceptors are important in regulating chronic intestinal immunopathology. To do so, we either PBS- or RTX-treated IL-10-deficient littermates one week after weaning, separated them into distinct cages, and monitored for the development of colitis. Despite the pro-inflammatory role attributed to TRPV1+ TRPA1+ nociceptors in DSS colitis (Engel et al., 2011b), we did not observe any significant differences in gross colitis scores when examining colons of mice harvested at 28 weeks (Figure 4.4). However, we again noted that RTX-treated mice maintained significantly elevated weights relative to control IL-10-/- mice, though this observation was restricted to the male cohort (Figure 4.4). We also observed a significant decrease in serum TNF levels in RTX-treated mice suggesting that systemic inflammation is decreased (Figure 4.4). While gross histopathology was not affected, more careful analyses of colonic inflammation by qPCR and of intestinal dysbiosis by metagenomic analyses are pending to see if TRPV1+ fibers regulate some aspects of spontaneous colitis.

Coming back full circle to the start of this thesis work, we wanted to address if in fact nociceptors are a part of the immunological sensory interface with the external environment by asking the question: do nociceptors have an effect on barrier tissue homeostasis at steady-state? Alterations in intestinal homeostasis at steady-state frequently lead to dysbiosis of microbial communities. As such, we started a set of pilot
experiments to determine if nociceptors are important regulators of intestinal microbial communities. We first took the broadest approach by inducibly deleting NaV1.8-lineage sensory neurons in NaV-DTR mice by treating co-housed NaV-DTR mice with either PBS or DTX (Figure 4.5). We then collected fecal stool and mucosal samples for metagenomic analyses five days after nociceptor ablation to see if we could catch any short-term alterations in communities. While this experiment was underpowered as a pilot experiment, we received encouraging data in that the predominant polysaccharide-metabolizing commensal phyla of Bacteroidales (Rakoff-Nahoum et al., 2014) was depleted in NaV-DTR mice (Figure 4.5). It is too early to conclude anything from these experiments, but it is tempting to speculate that sensing of commensal bacteria or bacterial-derived metabolites provides signals which can regulate colonization either directly through release of neuropeptides or indirectly via epithelial cells (Brogden et al., 2005; Rakoff-Nahoum et al., 2004).
Figure 4.4: TRPV1+ nociceptors do not regulate a microbiota-driven colitis in the absence of IL-10.

a-d, IL-10−/− littermates were weaned and either treated with PBS or RTX to form experimental groups. a) Photograph of cecum and colon of PBS and RTX mice demonstrating transmural inflammation and colonic thickening and shortening and b) gross colitis score at 28 weeks. c) Weights were measured from 12-28 weeks and d) serum TNF concentrations measured by ELISA at 28 weeks.
Figure 4.5: NaV1.8+ nociceptors influence intestinal microbial communities. a) Experimental schematic of NaV1.8+ nociceptor ablation and fecal collection and b) LEFSE analysis of microbial phyla in PBS (green) or DTX-(red)-treated NaV-DTR mice.
4.4 Conclusions

We started this set of experiments to address the role of TRPV1+ sensory neurons in gut inflammation with the simplistic and naïve notion that we would identify a similar chain of communication as we had in the skin (Riol-Blanco et al., 2014). We hypothesized that if we biased the experiments in our favor by choosing two models dependent on IL-23 for colitis, that we would identify a TRPV1-dependent regulation of dendritic cell cytokines with a concomitant decrease in effector cytokine production by ILCs and/or T cells. Instead we observed that TRPV1+ sensory neurons do not appear to play a significant role in either an acute/innate colitis or a chronic/adaptive colitis. However, these experiments did not leave us empty-handed as we noted in three distinct models cases that TRPV1+ nociceptors regulate systemic aspects of inflammation including temperature, body weight, and sickness behavior. These are three very relevant parameters to the perception of inflammatory disease states by individuals.

From our results in the experiment designed to test the influence of NaV1.8-lineage nociceptors on intestinal homeostasis, we have now designed and completed a well-powered experiment to detect alterations on immunological parameters and microbial communities. We utilized TRPV1-WT or TRPV1-DTR mice provided by Mark Hoon in order to inducibly and robustly deplete TRPV1+ nociceptors in matched littermates (Pogorzala et al., 2013). We followed these co-housed mice for 8 weeks after finalizing DTX treatment collecting fecal samples at regular intervals before separating them into cages by genotype to further collect fecal samples for 4 weeks. We then harvested gut, gut-associated, and peripheral lymphoid tissues to analyze alterations in immune cell subsets by flow cytometry and banked tissue for RNA extraction and
correlation with pending metagenomic analyses. While the work in this dissertation has focused on the role of TRPV1+ nociceptors in inflammatory settings, we are eagerly awaiting to see how the cross-talk between sensory neurons, immune cells, and epithelial cells influences intestinal microbial communities at steady-state.
Chapter 5: Summary and Perspective

Jose Ordovas-Montanes
5.1 Personal Summary:

The work presented in this thesis stemmed from a desire to work on immunology at the interfaces between the host and environment. These skin and mucosal epithelial tissues have been colloquially referred to as barrier tissues in immunological parlance (Belkaid and Artis, 2013). My initial informal thesis proposal was actually to characterize the signals which create the niche and regulate the tissue permanence of T cells in the skin borrowing concepts from the HSC field (Scadden, 2014). The hypothesis here was that if we understood these signals, we would be able to both induce them by vaccination or inhibit them in disease. Several recent publications had highlighted the presence of T cells in human skin at steady-state or after murine viral infection that seemed to hang around longer than your traditional “effector-memory” cells (Clark et al., 2006; Gebhardt et al., 2009). The term resident-memory T (Trm) cell was not yet a household name and their existence was met with some skepticism in informal discussions within the lab (Schenkel and Masopust, 2014). Perhaps, in a fortuitous twist, my experimental path drew me away from this initial aim and towards several others leaving me to be an enthused spectator of the Trm field rather than an active participant. (Let’s just say I might have been scooped once or twice (Jiang et al., 2012; Mackay et al., 2013; Steinert et al., 2015)).

Instead, I was struck by an unexplained finding in the lab where, in the absence of heat-sensing neurons, psoriasis-like skin inflammation was reduced. From here, I began my exploration of the immune system in the skin, but also unwittingly entered into the world of sensory neurobiology. Through the experimental path discussed above, I gained the appreciation for a well-characterized model, that if we don’t know what the
relevant cells are playing a role in a disease process, it’s quite hard to understand what they may be saying amongst each other, and that there really is no such thing as a well-characterized model. Armed with my current knowledge and present understanding of the sensory nervous system, the proposal for this thesis would have commenced where I am leaving it.

The principal finding explaining our initial observation was the identification of a chain of communication involving NaV1.8+TRPV1+ nociceptors, CD11b+ CD11c+ dermal DCs, γδT17 cells, neutrophils, and inflammatory monocytes. Based on these results the most proximal question would be to identify the immediate message being relayed from nociceptor to dendritic cell. However, through my reading about the sensory and immune systems and discussions in the lab, in particular with Seth Rakoff-Nahoum, I started to become interested in the organizing principles of neuro-immune interactions. Before getting specific, it was important to go broad. We had discovered that heat-sensing nociceptors regulate Type 17 inflammation. Was this a specific phenomenon or something more generalizable (Figure 5.1)?
Figure 5.1: A modular logic of neuroimmune interactions
A synthesis of our own experimental (orange) data and those of other groups which have characterized the role of the sensory nervous system across distinct barrier tissues, types of inflammation, and infection. The use of NaV1.8 to interrogate the sensory nervous system has provided a useful foundation but left many questions about the specific relationship between subsets of neurons and inflammation. (+++ Strong link, ++ link, +/- distinct effects, ? open)

5.2 Generalizability or specificity: a modular logic in skin neuroimmune interactions?

What exactly do I mean by this? In the same way that immunologists have contributed to the field by practicing subsetology(Grossman and Paul, 2015; Satija and Shalek, 2014; Spits et al., 2013), I observed the development in our understanding of the sensory nervous system from a classification scheme based on size and conduction velocity(Lumpkin and Caterina, 2007), to one also involving an ever-increasing number of cell subtypes(Le Pichon and Chesler, 2014; Usoskin et al., 2015). The molecular and genetic understanding of unique cell populations associated with particular modalities has led to the generation of tools to interrogate the function of these neurons(Pogorzala et al., 2013), instead of genetically ablating the majority of sensory neurons(Abrahamsen et al.,
2008) as had been the only choice when we initiated this project. Given the different types of inflammation (e.g. Type 1 and Type 2) and the distinct modalities of peripheral nervous sensation, do different types of sensory neurons instruct different arms of immune effector responses (Figure 5.1)?

As is often the case in science, the investigation that occurred was not a neatly organized process but instead one guided by puzzling outcomes, serendipitous observations, chance discussions, failed experiments, and the published progress of others. In my preliminary qualifying exam, I hypothesized that if TRPV1+ nociceptors did in fact exert their pro-inflammatory effect via acting to enhance cutaneous IL-23/IL-17 production, it would follow that immune resistance against a pathogen that requires these cytokines for clearance would be impaired. I wanted to begin the expansion into this modular thinking by staying close to our initial observation and asking: is Type 17 immunity, promoted by nociceptors in a deleterious autoinflammatory disease, also regulated by nociceptors during bacterial infection? This was partly fueled by my reading of countless reviews on the subject of neuro-immunology where they introduce the relationship between the two saying that while neuro-immune interactions can be beneficial in host defense, that they could be detrimental in disease (Andersson and Tracey, 2012; Chiu et al., 2012; Downing and Miyan, 2000). While the latter link had been extensively shown experimentally, and avoidance behavior is definitively regulated by the nervous system (Choi et al., 2011; Pacheco-Lopez and Bermudez-Rattoni, 2011), the evidence that nociceptors somehow regulate a concerted form of host defense against infectious challenge was and is lacking, at best.
When we were optimizing our infectious protocol for such a pathogen, *Staph aureus*, a neighboring lab published that nociceptors could not only directly sense the presence of these bacteria but that they in fact exerted a paradoxically anti-inflammatory effect (Chiu et al., 2013). Was my hypothesis wrong? We pushed on with our experimental pipeline and using the same genetic model of nociceptor ablation noted the same drastic lymph node hypercellularity in the absence of NaV1.8-lineage nociceptors (chapter 3.3.5). However, when we carried out experiments longer than 24 hours, we noted that the ears of mice lacking NaV1.8-lineage neurons had severe necrosis, and furthermore that greater numbers of bacteria had drained from the site of infection to the local lymph node. The story was seemingly more complex than the purported anti-inflammatory effect and perhaps NaV1.8+ neurons, while not important to promote host resistance factors in *S. aureus* infection, were instead important for tissue tolerance (Medzhitov et al., 2012). This idea remains to be formally tested.

While we and many others have utilized both pharmacological RTX and the genetic NaV-DTA approach, particularly within the neuroscience community, I thought one way to begin to reconcile these paradoxical effects was to move away from these relatively crude tools and again dig deeper into the expanding toolbox of inducible genetic models. Even the NaV-DTA system leaves one wondering about what neurons could be compensating in the absence of those which are gone. In the same way that the immune system can exhibit tremendous plasticity and some subsets may only be a phenomenon that can be observed *in vitro*, neural-loss during development is a powerful driver for those cells that remain to overgrow. As a result, I was not sure if the effects
observed in the *S. aureus* system was a caveat of compensatory outgrowth of proprioceptors, or, in fact due to loss of nociceptors.

While the system had not been previously published, I optimized a protocol to acutely deplete nociceptors in NaV-DTR mice hopefully circumventing the caveats of the NaV-DTA system. Furthermore, in conjunction with the previously available Mrgrpdr-DTR mice, TRPV1-DTR, and TRPM8-DTR mice were also generated and characterized around this time period (Cavanaugh et al., 2009; Pogorzala et al., 2013). I reached out to the investigators who had generated these strains in order to import them but unfortunately was met with a harsh lesson in animal facility bureaucracy (apparently if mice come from NIH to Harvard, they are too dirty. But if these same mice have a stopover through Dana-Farber, they are okay). It took one year from the time that these conversations were initiated until we had a set of TRPV1-DTR mice with which to do an experiment and unfortunately the Mrgrpdr-DTR and TRPM8-DTR mice remain frozen by red tape and in a literal cryopreserved state. A formal test assessing all sensory modalities in distinct models of inflammation is still pending (Figure 5.1).

This led to the series of experiments using the RTX method to assess the role of TRPV1+ nociceptors in Type 2 inflammation in skin, as well as in Type1/17 in the gut. Ongoing experiments have begun to leverage the benefits of using TRPV1-DTR mice but are still too preliminary to present in this setting. Nonetheless, filling in just one column, that represented by TRPV1+ nociceptors, across one tissue in the skin, has provided glimpses that there may be contextual specificity in the inflammatory responses regulated by TRPV1+ nociceptors. To date, we have found TRPV1+ nociceptors to regulate Type 1 (DNFB) and Type 17 (IMQ) inflammation in the skin, but they were largely dispensable.
for Type 2 (MC903) driven inflammation and control of *S. aureus* infection (Figure 5.1). This suggests that TRPV1+ sensory neurons promote IL-12/23 mediated sterile inflammation and are dispensable for TSLP-driven inflammation or Type 17 inflammation during pathogen challenge. Despite the lack of an inflammatory phenotype in the MC903 model, TRPV1+ nociceptors were still required for skin and dLN IL-4 production. The cascade of known cellular players has been characterized in this system and IL-4 production is known to be a DC-dependent process(Leyva-Castillo et al., 2013). As a result, we think that TRPV1+ nociceptors may more globally affect DC-priming events initiated in the skin (Figure 1.2).

Intriguingly, despite the requirement for IL-23/IL-17 axis cytokines in clearance of *S. aureus*, low levels were induced relative to IMQ. *S. aureus* is known to have a repertoire of toxins which act to kill, disable, or distract almost any immune cell population so this is not without precedent(DuMont and Torres, 2014; Miller and Cho, 2011). In the absence of almost all nociceptors (and with the caveat of outgrowth of other neuronal subsets), *S. aureus* infection was not adequately contained and the tissue could not tolerate bacterial burden. In order to further investigate this mechanism, I wanted to profile the transcriptome of the bacteria during an infectious course in the presence and absence of nociceptors. Furthermore, we could use infections using attenuated mutants incapable of inducing toxin expression to identify if it was an over-exuberant immune response or the lack of appropriate countermeasures that must be deployed by the host. Some have speculated that neuropeptides may function as antimicrobial peptides(Brogden et al., 2005).
5.3 One Barrier Tissue is not like the Other

Based on the literature assessing TRPV1+ sensory neurons in colitis, one might assume that nociceptors in the gut would once again be important to promote inflammation (Engel et al., 2011a). However, all prior published investigations utilized irritant or detergent-based regiments to initiate an allergic response or damage the epithelial barrier. As such, it is in line with what we and others have seen in the skin but perhaps of limited relevance to human colitis where dysbiosis is an important component and potential driving force (Liu et al., 2013; Rakoff-Nahoum et al., 2006; Riol-Blanco et al., 2014). We chose to utilize the IL-10/- model of spontaneous microbiota-driven colitis, and the anti-CD40 agonist model of acute colitis as both models have been shown to be dependent on IL-12/23 (Davidson et al., 1998; Uhlig et al., 2006). This would give us the ability to draw parallels to our observations in the IMQ model over a short time course, while also assess the functional relevance during loss of homeostasis. However, we observed that TRPV1+ sensory neurons do not appear to play a significant role in either an acute/innate colitis or a chronic/adaptive colitis. However, despite the lack of notable differences in the colitogenic phenotype, we noted in three distinct models (including LPS) that TRPV1+ nociceptors regulate systemic aspects of inflammation including temperature, body weight, and sickness behavior. It will be of interest to determine what subset of TRPV1+ neurons (somatosensory emanating from DRGs, vagal sensory, or enteric) is responsible for linking colonic inflammation with weight loss.

Based on these observations, we are focusing future experiments on the role of TRPV1+ sensory neurons in regulating metabolism with a specific focus on white, brown, and beige adipose tissue. Recent data implicates ILCs, eosinophils, and
macrophages as key regulators of adipose tissue state completing the efferent arm of the thermogenic circuit linking the autonomic nervous system to adipocyte function (Brestoff et al., 2015; Lee et al., 2015; Morrison et al., 2014; Nguyen et al., 2011). While these circuits have been well characterized during homeostatic conditions or thermal challenge, how they respond during inflammation is currently unknown. As a result, our preliminary findings presented in this chapter provide a convergent course onto this line of investigation to delineate the key neuro-immune players in metabolic circuits during inflammation. Based on the known immune cellular mechanisms in each disease setting we have investigated, we propose that mice lacking TRPV1+ sensory neurons may have an altered balance of Type 1/Type 2 immunity in adipose tissue, which is protective during cases of severe inflammation. This has implications in clinical settings where cachexia is a severe co-morbidity of the disease or treatment as in the case of cancer, autoimmune, and immunotherapy-induced cachexia (Gautron and Laye, 2009).

**5.4 So what’s the mechanism? Understanding Inputs and Outputs**

Returning to the skin, we sought ways to drill down into the mechanism of the regulation of IL-23 production by TRPV1+ nociceptors. We tested a variety of neuropeptides in isolation or in combination on DCs in vitro, and also agonists and antagonists in vivo (data not shown). However, these experiments provided equivocal results with no consistent effect on IL-23 production. Even while performing them, these experiments were bound to leave one unsatisfied as we did not know in vivo the frequency or duration of neural signaling to DCs. I set out to try and leverage the imaging
tools available in the lab using novel genetically encoded Ca\textsuperscript{2+} reporters to visualize neural activity during IMQ-inflammation (Chen et al., 2013). However, the commercially available versions of these reporters, which work in isolated neurons and in some tissues, did not work in skin, perhaps due to its greater autofluorescence.

Without the ability to visualize the firing, I turned to methods of removing the “input” that the neuron may use for it to trigger its pro-inflammatory effects. The first hypothesis of TLR7 was perhaps motivated by my previous work on a role for TLR-sensing in herpes simplex virus infection of neurons (Lafaille et al., 2012). Patients with inactivating mutations of TLR3 and UNC-93B are unable to control HSV infection, presumably due to defects in mechanisms of intrinsic anti-viral immunity in neurons and oligodendrocytes (Lafaille et al., 2012). While TLR7 did not prove uniquely responsible for IMQ-mediated inflammation, I wanted to investigate if IL-1 and/or TLR-sensing by nociceptors was the responsible input for the pro-inflammatory output. As we did not yet have TRPV1-Cre mice, I initiated these studies in NaV1.8-Cre mice crossed with MyD88fl/fl mice. In a twist of murine Mendelian genetic fate, it turns out these genes are tightly linked so my experimental approach would not pan out.

During the course of my thesis, some inputs have been identified in other disease settings. In the case of allergic skin disease, it has been shown that TSLP can be a direct trigger of TRPV1+TRPA1+ sensory neurons (Wilson et al., 2013) and in allergic asthma, that IL-5 can act on TRPA1+ sensory neurons (Talbot et al., 2015). During S. aureus infection, the pore-forming toxin alpha-hemolysin can also directly activate these neurons (Chiu et al., 2013). The question of what is being sensed in the skin during autoinflammation is still open (Figure 1.3).
With the obvious outputs (neuropeptides) seemingly irrelevant to our model, and experimental difficulties in determining the inputs and visualizing neural activity, I decided to initiate a line of experimentation to manipulate the neural activity myself. This was in an attempt to see if, beyond the presence of the neural subset, the firing of action potentials is required to communicate with DCs. Through a coincidental introduction to a group studying pruritus at MGH with expertise in utilizing QX-314, we were able to show that inhibiting neural activity does in fact ameliorate inflammation in the IMQ model. Furthermore, through conversations with Dr. Mark Hoon, a visit to the NIH, and great experimental support locally from Erika Williams, I initiated optogenetic and chemicogenetic experiments to modulate neural activity in defined subsets (Farrell and Roth, 2013; Fenno et al., 2011). While these experiments are still preliminary and ongoing, we were able to experimentally show for the first time that driving neural activity in TRPV1+ nociceptors in the presence of an inflammatory stimulus potentiates cytokine production from an immune cell (Figure 3.6). The chemicogenetic experiments are pending expansion of imported mice as our initial experiments using viral delivery of the designer receptor activated by designer drug (DREADD) constructs to neurons were unsuccessful. I am very excited about the potential to use novel Cre-driver lines in combination with acute optogenetic and chronic chemicogenetic approaches to delineate the spatiotemporal contribution of defined neuronal populations to each of the aforementioned disease settings.

5.5 Concluding Thoughts

Most of us are quite familiar with the sensations imparted by activation of our sensory nervous system when we are lamenting that something feels too hot, too cold, or
occasionally, just right. Despite the common use of the phrase, “I feel sick,” few individuals, immunologists included, probably consider that this invokes an activation of the sensory nervous system and thus a link between the nervous and immune systems. Those diseases which afflict barrier tissues manifest with acute and chronic sensations which can be debilitating such as the intractable itch of psoriasis or the chest tightness accompanying asthma. Regardless of the conscious perception of ongoing inflammation and immunological processes in these tissues, it is clear that through a concerted effort of sensing and responding by the somatosensory and autonomic nervous systems, the host aims to maintain the health of tissues and that a balance of inputs and outputs must be maintained to avoid pathological outcomes.

The recent description of cell subsets in both the nervous and immune systems combined with tools for specific ablation and modulation of function will allow investigators to map fine circuits involving cells from both systems. It will be exciting to see the research outcomes of this technical and conceptual knowledge applied to those processes and diseases discussed in this review. For example, delineating the full circuit involved in thermoregulation presumably involving TRPV1+ and TRPM8+ sensory afferents, a set of centrally integrating neurons, and sympathetic outflow to various adipose depots with target cells including ILC2s, macrophages, and adipocytes will not only provide basic biological insight but also provide treatment opportunities into metabolic diseases (Chapter 2). Determining where the findings from our investigations in models of colitis converge on this circuit will be of interest.

An understanding of the cell subsets involved will also help to assimilate our findings that NaV1.8+ nociceptors regulate the IL-23/IL-17 axis (which has previously
been implicated as an important regulator of *S. aureus* colonization) with the immunosuppression (or tissue tolerance?) provided by nociceptors during *S. aureus* (Figure 1.3). Exploring the signals that nociceptors sense during a cutaneous infection compared to psoriasisform inflammation may aid in assimilating these paradoxical observations. Furthermore, utilizing the current molecular characterization of defined nociceptor subtypes to identify and manipulate the ensembles of neurons which are activated (Figure 1.1) and contribute to each disease process will be informative not only in the skin but also at other barrier tissues (Figure 1.3).

In summary, we propose that sensory perception and regulation of inflammation by peripheral neurons exerts context-dependent effects on immunological processes. We speculate that there is the potential to uncover a modular design framework by which distinct lines of sensory neurons are triggered in specific settings and may contribute to regulating different aspects of inflammation through interactions with immune cell subsets. Furthermore, both systems have the capacity to form memories of these encounters leading to learned or 'adaptive' future responses.

I suppose instead of taking the direct route from beginning to end, as a neuron might, I stubbornly migrated like a leukocyte; trying different routes until one of them kind of worked. Despite my best attempts to learn neuroscience, I’m still an immunologist after all.
Chapter 6: Methods

Mice

C57BL/6 mice, 4-8 weeks old, were purchased from Charles River or the Jackson Laboratory and female mice were used in experiments. IL-23R<sup>GFP/GFP</sup> mice (Awasthi et al., 2009) in which GFP is knocked into the cytoplasmic tail of IL-23R and the homozygous GFP mouse acts as a functional receptor knockout were provided by M. Oukka and both male and female mice were used in experiments. CD11c-YFP mice (Lindquist et al., 2004) were a gift from M. Nussenzweig and both male and female mice were used in experiments. LTA<sup>-/-</sup> mice (De Togni et al., 1994) were purchased from the Jackson Laboratory and male mice were used in experiments. CD11c-DTR (Jung et al., 2002) mice were purchased from Jackson Laboratory and both male and female mice were used in experiments. NaV1.8-Cre mice were previously described (Abrahamsen et al., 2008). Rosa26-DTA mice and Rosa26-TdTomato mice, which express a floxed-STOP cassette upstream of the ubiquitously expressed Rosa26-Diphtheria Toxin or Rosa26-TdTomato construct respectively, were purchased from Jackson Laboratory. NaV1.8-Cre male mice were bred with Rosa26-DTA and Rosa26-TdTomato female mice in order to generate NaV1.8-DTA for functional studies and NaV.1.8-TdT for imaging experiments respectively of which both matched male and female litters were used in experiments. NaV1.8-Cre homozygous male mice were also bred with Rosa26-Diphereria Toxin Receptor (DTR) mice to generate NaV-DTR mice for inducible depletion of nociceptors. Glial fibrillary acidic protein (GFAP)-Cre-TdTomato mice were generated by crossing heterozygous GFAP-Cre mice (Garcia et al., 2004) purchased from Jackson Laboratory with Rosa26-TdTomato mice. GFAP-MyD88<sup>fl/fl</sup> mice were generated by
crossing GFAP-Cre mice with MyD88fl/fl mice (Hou et al., 2008) purchased from the Jackson Laboratory. For optogenetics experiments, TRPV1-Cre mice (Cavanaugh et al., 2011) were bred with Ai32 mice, a conditionally activating Channelrhodopsin2 mouse line (Madisen et al., 2012) both purchased from the Jackson Laboratory. Rosa26-Chr2 mice TLR7-/- mice (Hemmi et al., 2002) and TRPV1-/- mice (Caterina, 2000) were purchased from the Jackson Laboratory. MHC-II-GFP mice (Boes et al., 2002) were provided by M. Boes. Balb/c Rag1-/- mice were bred in house and utilized as they were capable of surviving in our specific pathogen free facility without the need for prophylactic antibiotics unlike our C57BL/6 Rag1-/- colony. Interleukin-10-knockout mice (Kuhn et al., 1993) were acquired from the Jackson Laboratory. Bone marrow chimeras were generated by irradiating NaV1.8-TdT mice or GFAP-Cre-TdT mice with two split doses totaling 1,300 rad and reconstituting with CD11c-YFP or MHC-II-GFP unfractionated bone marrow injected intravenously respectively. Bone marrow chimeras were allowed to rest for 12 weeks before use. Mice were all housed in specific pathogen-free conditions in accordance with the National Institutes of Health and all experimental animal protocols were approved by the IACUC at Harvard Medical School. For most animal experiments since the contralateral ear served as a control for the ear in which an inflammatory stimulus was applied, no randomization was used. Investigators were blinded for the initial ear-swelling experiments and subsequently no blinding was used.

**Denervation**

Resiniferatoxin (RTX), a capsaicin analogue, was injected subcutaneously into the flank of 4 week-old mice in three escalating (30 µg/kg, 70 µg/kg, and 100 µg/kg) doses on
consecutive days (Sandor et al., 2009b). Control mice were treated with vehicle solution (DMSO in PBS). Mice were allowed to rest for 4 weeks before denervation was confirmed by tail-flick assay. The tail-flick assay was conducted by holding mice vertically in a relaxed fashion allowing their tail to be immersed in a temperature-controlled water bath maintained at 52°C. Denervated mice exhibited a tail-flick latency of >10 seconds. Denervation was also assessed by the capsaicin eye wipe test (Craft et al., 1993) in which a 100uM solution of capsaicin is dropped onto the eye of a mouse. Wipes are then quantified over the next minute. All RTX-treated mice which received 3 doses did not respond in this test with nocifensive behavior (data not shown) and TRPV1-DTR mice after 3 weeks of DTX treatment also were insensitive (data not shown). Besides insensitivity to noxious heat stimuli, overall behavior qualitatively remained unaltered in RTX-treated mice. For chemical sympathectomy, Mice were intraperitoneally injected with 80 mg/kg 6-OHDA in 0.01% ascorbic acid in PBS at day -3. Control mice received injections of 0.01% ascorbic acid in PBS (Grebe et al., 2009). For inducible depletion of nociceptors in NaV-DTR mice, an optimized dose of diphtheria toxin was selected from pilot experiments (data not shown) based on previous studies (Cavanaugh et al., 2009; McCoy et al., 2013).

**Imiquimod Treatment, IL-23 injection, DNFB Treatment, MC903 treatment, and Ear Measurement**

Mice, 8-12 weeks old, of indicated genotypes or pharmacological treatments were treated with 5 mg of 5% imiquimod cream (IMQ) applied topically to dorsal and ventral aspects of ear skin totaling 125 µg of IMQ/day. Mice were treated starting on day 0 three times
and sacrificed for analyses on day 3 or were treated six times and sacrificed for analyses on day 6. Interleukin-23 (IL-23) was injected intradermally into the dorsal aspect of the ear skin as a 50 µg/mg solution in 10 µL PBS (500 ng/ear). Contralateral ears were injected with PBS alone. A 0.5% solution of DNFB in Acetone was applied to the dorsal and ventral aspects of ear skin without prior sensitization. Ear thickness was measured using an engineer’s micrometer (Mitutoyo) at indicated timepoints and the Δ ear thickness is calculated as the change in ear thickness from the treated ear relative to the contralateral naïve or vehicle treated ear. MC903 (1 nmol, 20uL) dissolved in EtOH was applied topically to both aspects of ear skin as described previously (Leyva-Castillo et al., 2013; Li et al., 2006).

**Infection and CFU Determination**

S. aureus infection was performed using a modified protocol adapted from ref. (Prabakara et al., 2013). Briefly, mice were inoculated with $10^9$ CFU of LAC USA300 S. aureus in 10uL administered by 10 pinpricks of Morrow-Brown allergy test needles. CFU from whole organs were determined as described in ref. (Misawa et al., 2015). Briefly, tissues were weighed, suspended in growth media for S. aureus (TSB), diluted, and plated on mannitol salt agar (MSA) before incubation for 48 hours at 37 degrees Celsius (Misawa et al., 2015). All infections were performed in BL2 animal facilities and protocols approved by the Harvard Committee on Microbiological Safety.
**Anti-CD40 Colitis and systemic LPS treatment**

Acute colitis was induced in Balb/c Rag1−/− mice by single intraperitoneal injection of anti-CD40 agonist antibody (clone: FGK4.5; BioXCell) as described previously (Uhlig et al., 2006). Mice were marked and weighed daily as individuals and collective feeding behavior of cages was monitored by dispensing 15 pre-weighed pellets of standard lab chow diet and calculating the amount consumed on a daily basis. Food intake is expressed per mouse based on the number of mice per cage in each experimental group. Endotoxic shock was induced in Balb/c Rag1−/− mice using a medium dose of LPS by intraperitoneal injection of 10mg/kg of LPS from E. coli 026:B6. This dose was selected as the one used by Tracey et al., in their studies on the anti-inflammatory reflex arc (Rosas-Ballina et al., 2011). Temperature was monitored intrarectally using a handheld thermometer.

**FTY720 treatment and in vivo depletion of dendritic cells or inflammatory monocytes/neutrophils**

FTY720 (1 mg/kg) or PBS was injected daily intraperitoneally commencing at Day 0 of IMQ challenge. Efficacy of treatment was confirmed by a dramatic reduction in circulating lymphocytes from peripheral blood (data not shown). In order to deplete dendritic cells from skin, CD11c-DTR mice (Jung et al., 2002) were injected at Day (-1) with 4ng of diphtheria toxin / g mouse, a dose which is nontoxic to murine cells not expressing the diphtheria toxin receptor (Saito et al., 2001). Depletion of dermal DCs and Langerhans cells, but not macrophages, was confirmed by flow cytometry (Suppl. Fig. 8). In order to deplete neutrophils and inflammatory monocytes, 500 µg of anti-Gr1/
mouse (clone: RB6-8C5; BioXCell), was injected intraperitoneally at Days -1, 0, 1 and 2. Depletion was confirmed by flow cytometry of peripheral blood as well as challenged ear skin showing a paucity of neutrophils and inflammatory monocytes (Suppl. Fig. 8).

**Manipulation of Neural Activity**

To inhibit conduction of action potentials in actively firing peripheral sensory neurons, mice were injected daily intradermally with 1% w/v QX-314 solution (Brenneis et al., 2013) in normal saline during experimental time course (lidocaine N-ethyl bromide; Sigma). For optogenetics experiments, TRPV1-Ai32 or control TRPV1-Cre mice were anesthetized by intraperitoneal injection with avertin, IMQ applied to ear skin, and positioned carefully in an isofluorane chamber located within an incubator maintained at 37 degrees Celsius. Cold blue light (473nm) was pulsed at 20hz for 4 hours on one ear based on the experimental approach found in ref. (Daou et al., 2013) and suggestions from Dr. Mark Hoon. Ear skin was harvested for RNA extraction and subsequent qPCR analysis.

**Histology**

Ears and colons were embedded in paraffin and submitted for histological analysis by haematoxylin and eosin staining to the Harvard Rodent Histopathology Core. Histological evaluation was carried out in a blinded fashion.
**Whole mount immunofluorescence analysis of ear skin**

Ears were harvested and split into dorsal and ventral halves. After fixation in 4% paraformaldehyde, any adherent cartilage was removed under a dissecting microscope in order to expose the dermis evenly for imaging. Tissue was blocked in blocking buffer containing PBS with 0.5% BSA, 0.3% Triton X-100, 10% goat serum and Fc Block and also stained with the following antibodies in the same buffer. Unconjugated antibodies used include: anti-neuronal class III β-tubulin (clone TUJ1; Covance), anti-tyrosine hydroxylase (clone A2B5-105; Millipore), anti-Lyve1 (clone ALY7; eBiosciences), anti-peripherin (Polyclonal; Abcam), and anti-NeuN (A60; Millipore). Alexa488 conjugated antibodies include: anti-neuronal class III β-tubulin (clone TUJ1; Covance), goat anti-mouse IgG (Invitrogen) and goat anti Rabbit IgG. Alexa 647 conjugated antibodies include: goat anti-rat IgG and goat anti-rabbit IgG. Washing steps were performed in PBS with 0.2% BSA, and 0.1% Triton X-100. Ears were mounted with the dermis facing the imaging plane in FluorSave reagent (Calbiochem).

**Confocal Microscopy**

Confocal images were acquired on an Olympus Fluoview BX50WI inverted microscope with 10X/0.4, 20X/0.5, and 40X/1.3 magnification/numerical aperture objectives. For images used in three-dimensional analysis, image planes were acquired at 0.5 μM intervals through the imaging volume.
**Intra-vital two-photon microscopy**

Anesthetized mice were placed on a custom-built stage and the ear fixed to a temperature-controlled metallic support to facilitate exposure of the dorsal aspect to a water-immersion 20X objective (0.95 numerical aperture) of an upright microscope (Prairie Technologies). A MaiTai Ti:sapphire laser (Spectra-Physics) was tuned between 870nm and 900nm for multiphoton excitation and second-harmonic generation. For dynamic analysis of cell interaction in four dimensions, several xy section (512x512) with z spacing ranging from 2µm to 4µm were acquired every 15-20 seconds with an electronic zoom varying from 1X to 3X. Emitted light and second-harmonic signals were directed through 450/80-nm, 525/50-nm and 630/120-nm bandpass filters and detected with non-descanned detectors. Post-acquisition image analysis, volume-rendering and four-dimensional time-lapse videos were performed using Imaris software (Bitplane scientific software).

**Intravital microscopy and Image Analysis**

Intravital microscopy of skin was performed as previously described (Weninger et al., 2000). In brief, control or RTX treated mice were anesthetized and the left ear was exposed and positioned for epifluorescence intravital microscopy. Preparations were transferred to an intravital microscope (IV-500; Mikron Instruments, San Diego, CA), equipped with a Rapp OptoElectronic SP-20 xenon flash lamp system (Hamburg, Germany) and QImaging Rolera-MGi EMCCD camera (Surrey, BC). The fluorescent dye rhodamine-6G (20 mg/kg in PBS) was administrated through the catheterized right
carotid artery to visualize circulating leukocytes. Cell behavior in skin venules was recorded in 10 min recordings through 10x or 20x water immersion objectives (Achroplan; Carl Zeiss). Rolling fractions in individual vessel segments were determined offline by playback of digital video files. Rolling fraction was determined as the percentage of cells interacting with skin venules in the total number of cells passing through a vessel during the observation period.

**Cytokine Quantification by ELISA**

Skin biopsies from ears were obtained using 10mm diameter skin biopsy punches (Acuderm, Inc). Samples were homogenized in Tissue Extraction Reagent I (Invitrogen) in the presence of protease inhibitor cocktail (Roche) using a gentleMACS dissociator (Miltenyi Biotec). Skin protein extracts were assayed for IL-17A, IL-22, IL-23p40, (Biolegend) and IL-17F (R&D Systems) in accordance with manufacturer’s instructions.

**RNA Isolation and qPCR**

RNA from sorted and pelleted cells was isolated using RNEasy Plus Mini Kit (Qiagen) including a gDNA elimination step. Ear skin and dorsal root ganglia (DRGs) were harvested and placed immediately in RNALater (Ambion) before homogenization and RNA isolation using Qiagen RNEasy Plus Mini Kit (Qiagen)(Abrahamsen et al., 2008). DRGs for comparison of the efficacy of denervation were harvested from equivalent anatomical locations, typically consisting of the cervical and thoracic ganglia from C1-T2. cDNA synthesis was done using Superscript Vilo cDNA synthesis kit (Invitrogen)
following the manufacturer’s instructions. Relative quantification of transcripts was done using validated Quantitect Primer Assays (Qiagen) combined with the QuantiTect SYBR Green Detection Kit (Qiagen) on a LightCycler 480 (Roche). Relative expression of genes was calculated to \textit{gapdh} using the \textit{\Delta}CT method. Nanostring analysis was performed on RLT lysates submitted to the Boston Children’s Hospital Digestive Disease Center core facility.

\textbf{Tissue Digestion}

Single-cell suspensions of lymph nodes, spleen, and bone marrow were generated as described previously (Iannacone et al., 2010). For ear-skin digestion, the dorsal and ventral aspects of the ear were mechanically separated before mincing and placing into a digestion mix modified from E. Gray and J. Cyster (Gray et al., 2011). Ears were digested for 80 minutes at 37°C in gentleMACS tubes (Miltenyi) with gentle agitation in freshly prepared digestion mix consisting of DMEM (Gibco) supplemented with HEPES (Invitrogen), 2% FCS, 100 µg/mL Liberase TM (Roche), 100 µg/mL DNase I (Roche) and 0.5 mg/mL Hyaluronidase (Sigma). After enzymatic digestion, the mixture was processed using a gentleMACS homogenizer (Miltenyi) in order to obtain a cell suspension, which was then filtered through a 70 µM cell strainer (BD). Cells were then resuspended in FACS buffer for analysis. If cell suspensions were to be analyzed for cytokine producing cells, ears were harvested and digested in the presence of Brefeldin A (Biolegend).
Flow Cytometry, Cell Sorting, and Cell Counts

Single-cell suspensions in FACS Buffer (PBS with 2 mM EDTA and 2% FCS (Invitrogen-GIBCO)) were pre-incubated with Fc-Block (clone 2.4G2) before staining for surface antigens. FITC-conjugated antibodies used include: anti-Ly-6G (Clone 1A8; BD Pharmingen), Alexa488-conjugated antibodies used include: anti-CD103 (Clone 2E7; Biolegend), PE-conjugated antibodies used include: anti-δ-TCR (Clone GL3; Biolegend), anti-Ly-6C (Clone HK1.4; Biolegend), PerCP/Cy5.5-conjugated antibodies used include: anti-CD45.2 (Clone 104; Biolegend), PE-Cy7-conjugated antibodies include: anti-TCR-B (Clone H57-597; Biolegend), anti-CD11c (Clone HL3; BD Pharmingen), Alexa647-conjugated antibodies used include: anti-CCR6 (Clone 140706; BD Pharmingen), anti-CD11b (Clone M1/70; Biolegend), APC-Cy7-conjugated antibodies used include: anti-Thy1.2/CD90.2 (Clone 30-H12; Biolegend), anti-I-A/I-E “Class-II” (Clone M5/114.15.2; Biolegend). Cells were then washed with PBS and resuspended in MACS buffer for immediate acquisition or fixed in Cytofix (BD Pharmingen) per manufacturer’s instructions for later acquisition. For analysis, cells were acquired on a BD FACS CANTO (BD Pharmingen) and analyzed using FlowJo software (Treestar Inc.). For intracellular cytokine staining, cells were not restimulated and rather harvested and digested in the presence of Brefeldin A (Biolegend). After staining for surface antigens, cells were then fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Pharmingen) as per manufacturer’s instructions. Cells were stained in Perm/Wash buffer. Alexa488-conjugated antibodies used include: anti-IL-17A (Clone TC11-18H10.1; Biolegend), anti-IL-17F (Clone 9D3.1C8; Biolegend), Alexa647-conjugated antibodies used include: anti-IL-17A (Clone TC11-18H10.1; Biolegend), anti-IL-17F (Clone
9D3.1C8; Biolegend), and anti-IL-22 (Clone Poly5164; Biolegend). For determining total counts of cell subsets, an aliquot of the same cell suspension used for flow cytometry was stained for CD45.2 and acquired on an Accuri Cytometer (BD Biosciences) with a known amount of CountBright counting beads (Invitrogen). The total CD45+ cell number was then determined in the original cell suspension and used for total quantification of the cell number of each subset of interest defined by multi-parameter flow cytometry based on that subset’s frequency relative to the CD45+ population. For cell sorting, cells were stained for indicated surface markers and sorted using a BD FACSARia (BD Biosciences) into complete DMEM media prior to RNA extraction.

**Culture of DRG Neurons**

DRG neuron isolation and culture was performed with some modifications from ref. (Chiu et al., 2013. Briefly, mice were sacrificed and spinal cords excised away from animals before longitudinally removing the dorsal side of vertebrae. A dissection scope was utilized to manually collect as many accessible DRG from each animal. DRGs were digested in Liberase TM (100 ug/mL), Dispase II (2.4 U/mL), DNase I (100 ug/mL) in DMEM supplemented with 2% FCS and HEPES for 45 minutes at 37 degrees Celsius. Digestion was quenched by addition of excess serum and EDTA before resuspending digested DRGs in media to triturate with three glass Pasteur pipettes of decreasing size. A 28% Percoll gradient was used to clean the preparation of some axonal debris. DRGs were plated on laminin coated 96 well tissue culture treated plates or laminin and Poly-D-lysine coated glass coverslips in Neurobasal B-27 Media (Invitrogen) supplemented with NGF. For short-term culture with dendritic cells, splenic and lymph node CD11c+ cells
were isolated from Flt3-L treated mice using CD11c+ microbeads (Miltenyi) (Mora, 2003 #244). CD11c+ DCs were washed and placed in culture with DRG neurons in Neurobasal B27 media.

**Image Analysis**

Images from confocal microscopy and from two-photon microscopy were analyzed on either Volocity software (Improvision) or Imaris (Bitplane). For determining the distance of CD11c-YFP cells from nerves, lymphatics, or blood vessels, the centroid of DCs was calculated by an unbiased approach, and the distance between a given DC centroid and the closest nerve, blood vessel or lymphatic vessel was calculated using Imaris software. In order to bin cells into contact (<0 µm), proximal (0-7 µm), and distal (>7 µm) fractions, the calculated average radius of a DC (7.04 µm) was subtracted from each measured distance.

**Statistical Analyses**

Precise experimental numbers of animals are reported in the figure legends. Experiments were repeated at least three times except in Figure 2e, Figure 3e, Figure 3f, and Figure 3g in which two replicates were done assessing ten mice total in each experimental group overall. Some data, such as ear-swelling curves, represent pooled averages of the sum total of animals used in experiments whereas other data consists of a representative experiment of the independent experiments. All statistical analyses were performed using Prism (GraphPad Software) and results are calculated as means with error bars representing the s.e.m. Means between two groups were compared by using a two-tailed
t-test. Means between three or more groups were compared by using a one-way or two-way ANOVA. A Chi-square statistical analysis was performed for Figure 4d comparing the total number of dendritic cells in contact, proximal and distal bins relative to nerves vs. lymphatic vessels or vs. blood vessels.
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