The Presence of Pain Related Cytokines and Chemokines in Schwannomas and Their Potential Association with Chronic Pain in Schwannomatosis

2019

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The Presence of Pain Related Cytokines and Chemokines in Schwannomas and Their Potential Association with Chronic Pain in Schwannomatosis

By

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A thesis submitted in partial fulfillment of the requirements
For the Honors in the Major Program in Biomedical Sciences
In the College of Medicine
And in the Burnett Honors College
At the University of Central Florida
Orlando, Florida

Spring Term 2019

Thesis Chair: Cristina Fernandez-Valle, Ph.D.

Abstract

Schwannomatosis (SWN) is a genetic disorder that predisposes affected individuals to develop multiple Schwannomas anywhere in the peripheral nervous system. This can be due to a mutation in the *LZTR1* or *SMARCB1* genes on chromosome 22. SWN has the defining clinical symptom of chronic pain and a lack of vestibular schwannomas, which sets it apart from other, related disorders such as Neurofibromatosis Type II (NF2). Currently, it is unknown what causes the chronic pain of SWN patients but it is hypothesized that cytokines may have promote the neuropathic pain experienced by patients. This study investigates the presence of the chemokine CCL2 and the cytokine IL6 in human SWN schwannomas and non-SWN schwannomas to determine if there is a difference in the presence of these cytokines between the two tumor types. It was demonstrated that all of the SWN schwannomas expressed both CCL2 and IL6 whereas the non-SWN schwannomas expressed only one or the other protein if either. These results indicate that the presence of these cytokines within the SWN schwannomas is different from non-SWN schwannomas and could be a potential contributing factor in the occurrence of neuropathic pain experienced by SWN which is part of the differential diagnosis for NF2 and SWN.

Acknowledgements

I would like to first thank my thesis chair, Dr. Cristina Fernandez-Valle, for her expert advice and all the support she has given me in the process of carrying out the research and writing this thesis. She provided me with an opportunity in her lab three years ago and allowed me to transform into a budding researcher and prepared me for a career in medicine. She has unfalteringly supported me and I am extremely grateful.

I would also like to thank Dr. Alejandra Petrilli and Dr. Alicja Copik for supporting me as my other committee members. Without their knowledge, guidance, and support the completion of this thesis would not have been possible.

I would like to thank my research mentors in the lab, Dr. Marisa Fuse, Dr. Alejandra Petrilli, Dr. Berta Victoria-Martinez, and Rosa Rosario, for all their patience, instruction and training that allowed me to become the scientist I am today.

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List of Acronyms/Abbreviations

SC	Schwann Cell
SWN	Scwhannomatosis
NF2	Neurofibromatosis Type Two
NMDA	N-methyl-D-aspartate
OCT	Optimal Cutting Temperature

Introduction

Schwannomas are tumors that develop from Schwann Cells (SC) in the peripheral nervous system[1,2,3]. SCs are support cells for neurons of the peripheral nervous system[4] that develop from the neural crest and wrap around nerve axons[5] as myelinating or nonmyelinating cells[6]. Schwannomas are typically benign[1,2,3] and can be found associated with any peripheral nerve in the body and head.[1] Typically, vestibular schwannomas, those growing on cranial nerve eight, do not form and thus distinguish SWN from a related disorder, Neurofibromatosis Type 2.[3]

The diagnostic histological patterns of a schwannoma is the Antoni A and Antoni B areas. There are also rarer schwannomas that are histologically different[7]. Antoni A areas are highly cellular with an elongated appearing tissue type that also contains "palisades"[8]. Antoni B areas are hypocellular and are more loosely arranged with varying volume; these can also be completely absent from a schwannoma[8].

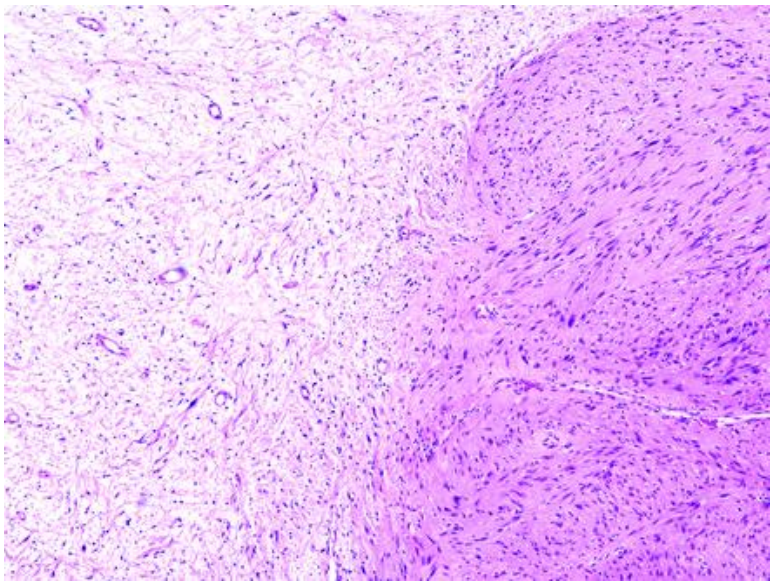

Figure 1 Pictomicrograph of a hematoxylin-eosin stained schwannoma with an Antoni A area on the right and an Antoni B area on the left. Taken from Wippold, F.j., et al. "Neuropathology for the Neuroradiologist: Antoni A and Antoni B Tissue Patterns."

A genetic condition causing the development of schwannomas is Schwannomatosis (SWN)[1,9,10]. SWN is presently considered a third type of Neurofibromatosis[1,10] and is defined by the development of multiple peripheral schwannomas[1]. Currently reclassification of SWN and NF2 as Schwannoma Predisposition Syndromes is being considered because neurofibroma tumors do not form in either condition. SWN can also be associated with development of meningiomas, albeit less frequently than for NF2[12]. The most common clinical symptom of schwannomatosis patients is localized or diffuse pain not necessarily at the site of the schwannoma[12]. Tumor volume is associated with the amount of pain (I.e. larger tumor, more pain) and the pain is usually chronic; causing anxiety and depression in some patients[12]. Despite this association, tumor volume does not provide an adequate explanation for chronic, radiating pain. Mutations in *SMARCB1* (also called *INI1*)[17] and the *LZTR1* genes[18] have been found to predispose patients toward acquiring SWN.

Neurofibromatosis Type 2 (NF2) can also cause the development of Schwannomas and is caused by a mutation to the *NF2* gene which codes for the tumor suppressant protein Merlin[9,10]. NF2 is characterized by the development of bilateral vestibular schwannomas[13,14]. A clinical diagnosis of SWN requires the patient to not have a diagnosis of NF2 and not have vestibular schwannomas develop[12,15].

The genetic mutations underlying these conditions that lead to the development of schwannomas has been studied and is still the focus of intense research. However, there is also a difference in the pain experienced. The bilateral schwannomas that are pathognomic for NF2 cause hearing and balance deficits[14]. But other schwannomas that develop in NF2 patients do not generally cause pain other than irritation pain due to touching the tumor[16]. The unrelenting

neuropathic pain and lack of bilateral VS are the key distinguishing features of SWN as opposed to NF2.

It is hypothesized that cytokines and chemokines mediate this pathological pain in SWN. Pro-inflammatory cytokines that mediate pain include IL-1ß, IL6, and tissue necrosis factor α (TNF- α).[27] A chemokine that can potentially mediate neuropathic pain is CCL2. Therefore, the presence of these cytokines could be related to the pain experienced by SWN patients.

Literature Review

In order to understand the rationale behind investigating chemokine presence in SWN solid tumors, the difference in pain experienced as well as causes and sensations of pain should be addressed. Pain in patients with *LZTR1* mutations is greater than pain in patients with *SMARCB1* mutations[21]. The exact function of the *LZTR1* gene is unknown however, it is thought that the protein functions within the golgi apparatus.[19] The *SMARCB1* gene codes for the molecular machinery that allows transcriptional access to the DNA.[20] About 86% of familial SWN patients and 40% of sporadic SWN patients have mutations to *LZTR1* or *SMARCB1*[15]. However, only 13-25% of SWN patients are familial cases[15]. Both *SMARCB1* and *LZTR1* are located on chromosome 22[22,23] near the *NF2* gene that is located on chromosome 22q12[13,14].

How a patient feels pain is also critical to understanding the link between a disease state and the sensation of pain. The sensation of pain is transmitted by nerves from the periphery to the spinal cord where the axons synapse with neurons in a specialized region known as the spinal cord dorsal horns[24]. In the spinal cord, this pain message can be amplified, delayed, suppressed, or unaltered[24]. There are two main tracts that carry pain information from the spinal cord to the brain. These are the spinothalamic and the spinoreticular tracts[25]. The spinothalamic tract ascends contralaterally to nuclei within the thalamus which then transmits the signal to the somatosensory cortex via third order neurons. This pathway helps localize pain within the body[25]. The spinoreticular tract also ascends contralaterally to the reticular formation in the brainstem and then connects to the thalamus and hypothalamus[25]. This tract is involved in emotional aspects of pain[25]. Glutamate and Substance P are the classic neurotransmitters for transmission of pain signals in the spinal cord[24]. Pain can be amplified by

the N-methyl-D-Aspartate (NMDA) mediated production of prostaglandins and nitric oxide in spinal cord neurons[24].

One type of pain is neuropathic pain. Neuropathic pain results from an injury to the somatosensory system or a dysfunction of the system[26]. Neuropathic pain bypasses the need for transduction by nociceptors because tissue damage directly affects the nervous system[26]. Neuropathic pain can occur due to peripheral sensitization from inflammatory responses due to injury and repeated simulation[26]. Ectopic discharges and hyperalgesia can also cause the maladaptive pain of neuropathic pain[26].

Small proteins secreted by cells that influence interaction and communication between cells are known as cytokines[27]. A single cytokine can act on many different cell types[27]. Typically, they are produced by immune cells such as macrophages[27]. Inflammatory responses, mediated by cytokines, can be a source of persistent pathologic pain states[27]. Pro-inflammatory cytokines such as Interleukins and chemokines can modulate and up-regulate neuronal pain pathways[27]. Chemokines have been demonstrated to be involved in neuropathic pain via the dorsal horn spinal cord in rat models[28]. Cytokines and chemokines have been implicated in neuronal-microglial signaling after a nerve injury and has been shown to increase the activity of NMDA receptors in the spinal cord[28]. Interleukins have been identified as proteins that may be involved in chronic pain[29].

According to unpublished proteomic data shared from the laboratory of Dr. Larry Sherman from Oregon Health and Science University, IL6 and CCL2 are the most prevalent cytokines present in schwannomas from schwannomatosis models. IL6 is a proinflammatory cytokine and has been previously demonstrated to play a role in neuronal reaction to injury.[29] The effects of

IL6 can be seen when it binds its receptor (IL6R) and interacts with the membrane bound protein gp130. This complex allows IL6 to propagate its intracellular signal.[30] Studies have also shown that increased IL6 in afferent neurons and the spinal cord can contribute to the development of neuropathic pain.[31] CCL2 is a chemokine and has a G-protein coupled receptor in cells.[32] The interactions of chemokines, like CCL2, and their receptors can localize leukocytes such as monocytes, natural killer cells, and memory T lymphocytes to specific locations within a tissue compartment.[32] Studies in mice have demonstrated that when the CCL2 receptor is knocked out, the mice felt less pain than the wild type.[32] CCL2 has also been demonstrated to have the characteristics of a neuronal mediator in nociceptive signal processing.[33]

Objectives

The purpose of this study is as follows:

1. To determine if pain inducing Cytokine IL6 and Chemokine CCL2 are present in the schwannomas resected from patients diagnosed with Schwannomatosis.
2. To assess if schwannomas from Schwannomatosis patients express more Cytokine IL6 and Chemokine CCL2 than schwannomas from non-Schwannomatosis patients.

Methods

Immunofluoresence Staining

Schwannomas from de-identified patients and Rat Stomach tissue are frozen in Optimal Cutting Temperature (OCT) compound. The Rat Stomach is used for positive control tissue. The tissue is sectioned at 10-14 microns using the LEICA Cryostat Microtome. All sections are placed on PTFE slides in a 14mm well printed on the slide. Slides are stored at 4°C in a slide box after sectioning until staining begins. Sections are incubated in a blocking solution (NGS, Triton X-100, 1XPBS) for one hour at room temperature to prevent non-specific binding. Following the blocking, sections are incubated in a primary antibody solution. The primary antibody dilutions are made in fresh blocking solution with a concentration of 1:200 for chemokine A and 1:100 for Cytokine B. Rat Stomach sections serve as a positive control for both antibodies. Negative controls are incubated again in blocking solution instead of a primary antibody solution. Secondary antibody staining is initiated after the completion of the primary antibody staining overnight at 4°C in a humidified chamber and four washes of 15 minutes each in PBS. The incubation at 4°C is done to reduce the amount of non-specific interactions with the primary antibody. The secondary antibody used is a goat anti mouse AlexaFluor568 or goat anti rabbit AlexaFluor568 and is diluted at 1:250 in fresh blocking solution. The secondary antibody solution also contains DAPI diluted at 1:36,000. The secondary antibody staining takes place in the dark for 45 minutes at room temperature. Sections ate then washed four times with PBS for ten minutes each still in the dark. Slides are mounted with a coverslip using Fluorogel after the last wash. The sections are imaged with the Zeiss 710 Confocal Microscope.

Results and Discussion

Frozen sections of tumors are processed for indirect immuno-detection of CCL2 and IL6 using the primary and secondary antibodies listed in the methods section. Six de-identified patient Schwannomas were studied; three Schwannomas were from SWN patients and three were vestibular schwannomas. The three SWN schwannomas were labelled SP06, SP01, and SP05 in Figures 2 and 3. The three non-SWN schwannomas were labelled SP02, SP03, and SP04 in Figures 4 and 5.

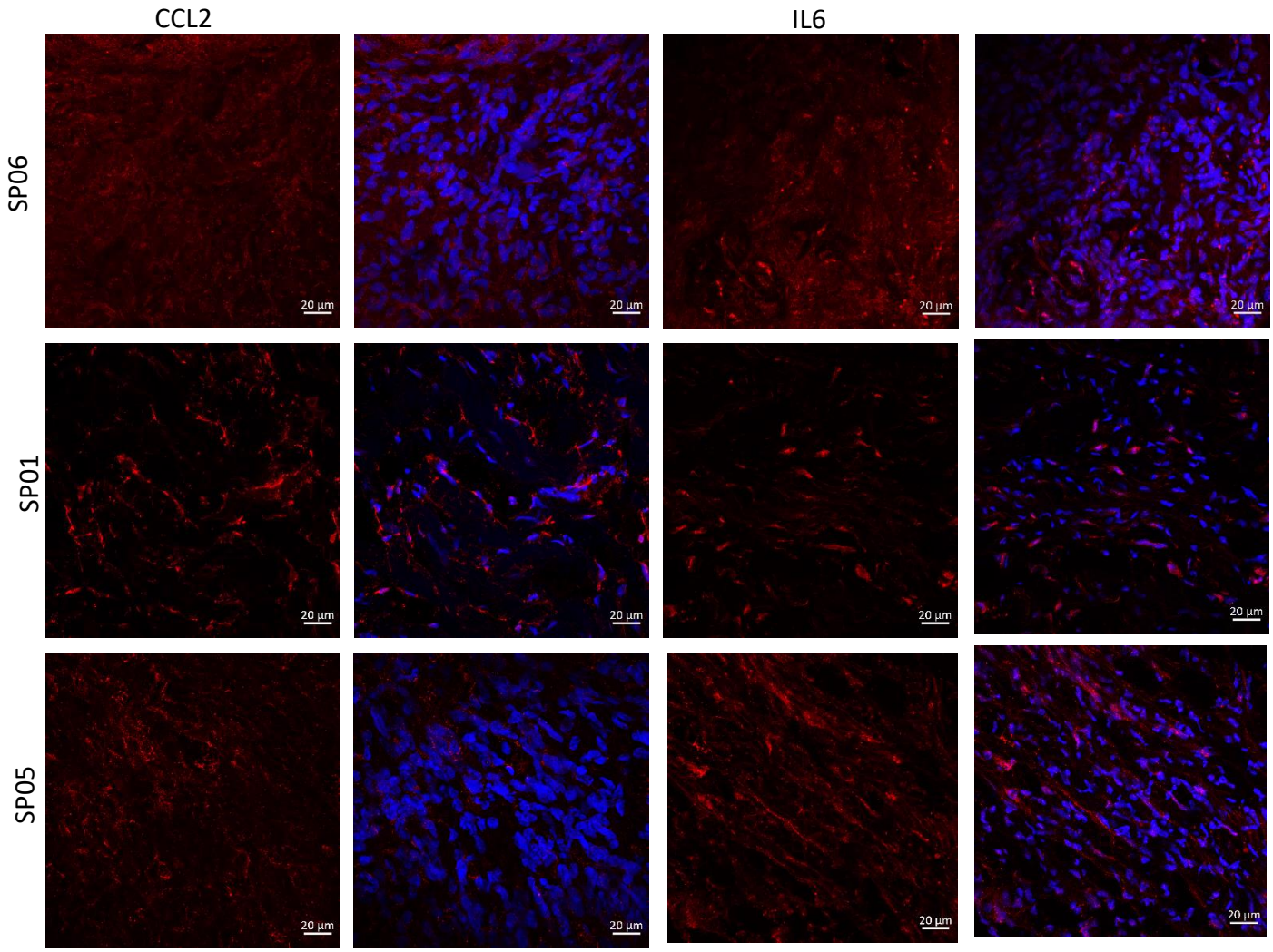

Figure 2 Immunohistochemistry of SWN schwannomas SP06, SP01, and SP05 stained for CCL2 and IL6.

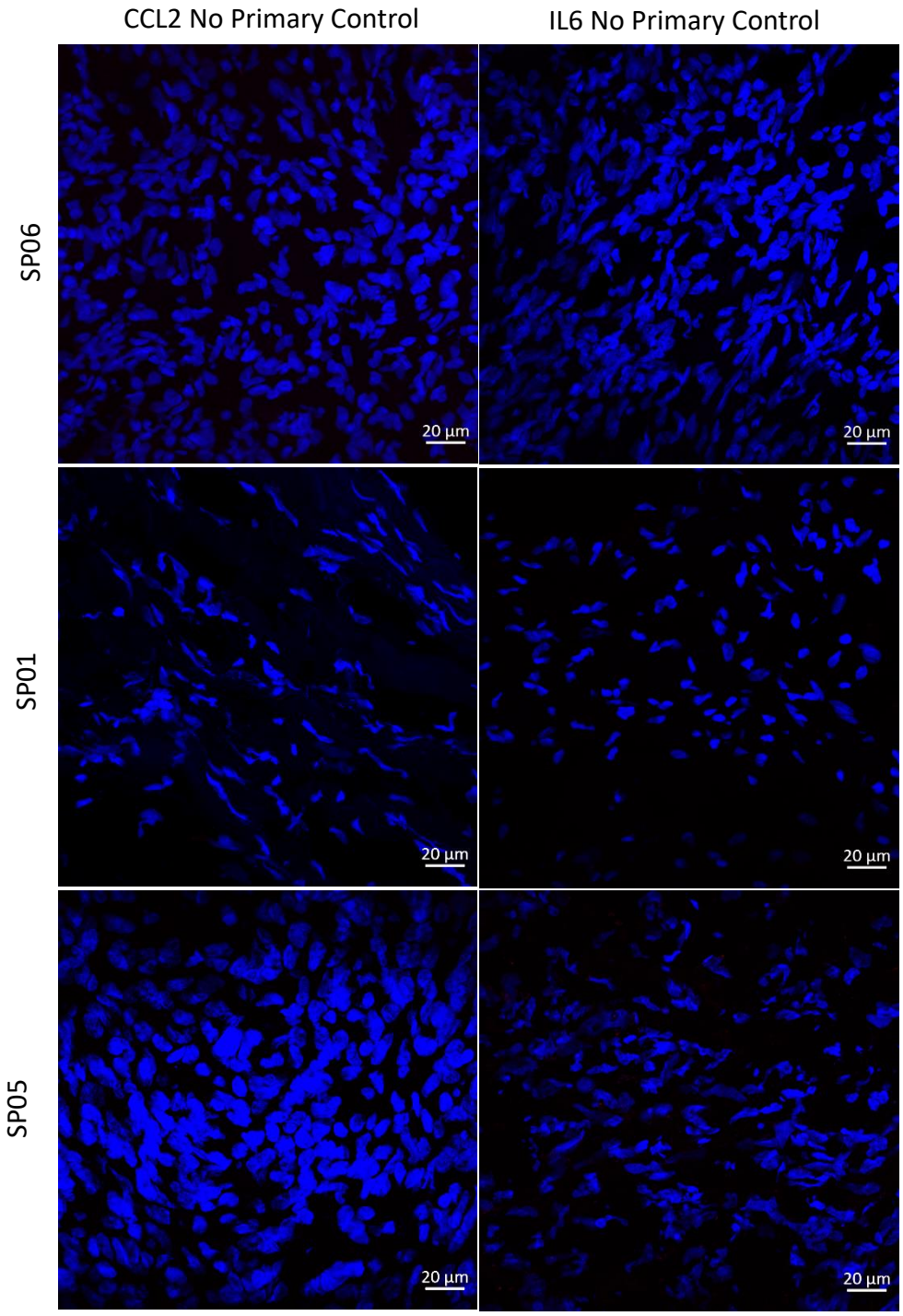

Figure 3 Negative controls for the SWN Schwannomas.

As seen in Figures 2 and 3, all three of the SWN patient derived tumors exhibit staining for both CCL2 and IL6. These molecules overall appear to be associated with the tumor

but not with individual cells. As seen in Figure 3, some cells, especially in the SP01 tumor appear to be actively producing or secreting these cytokines because the staining is closely associated with specific nuclei. These cytokines can be seen via the staining around the cells however, they do not appear to be associated with any particular nucleus of a cell.

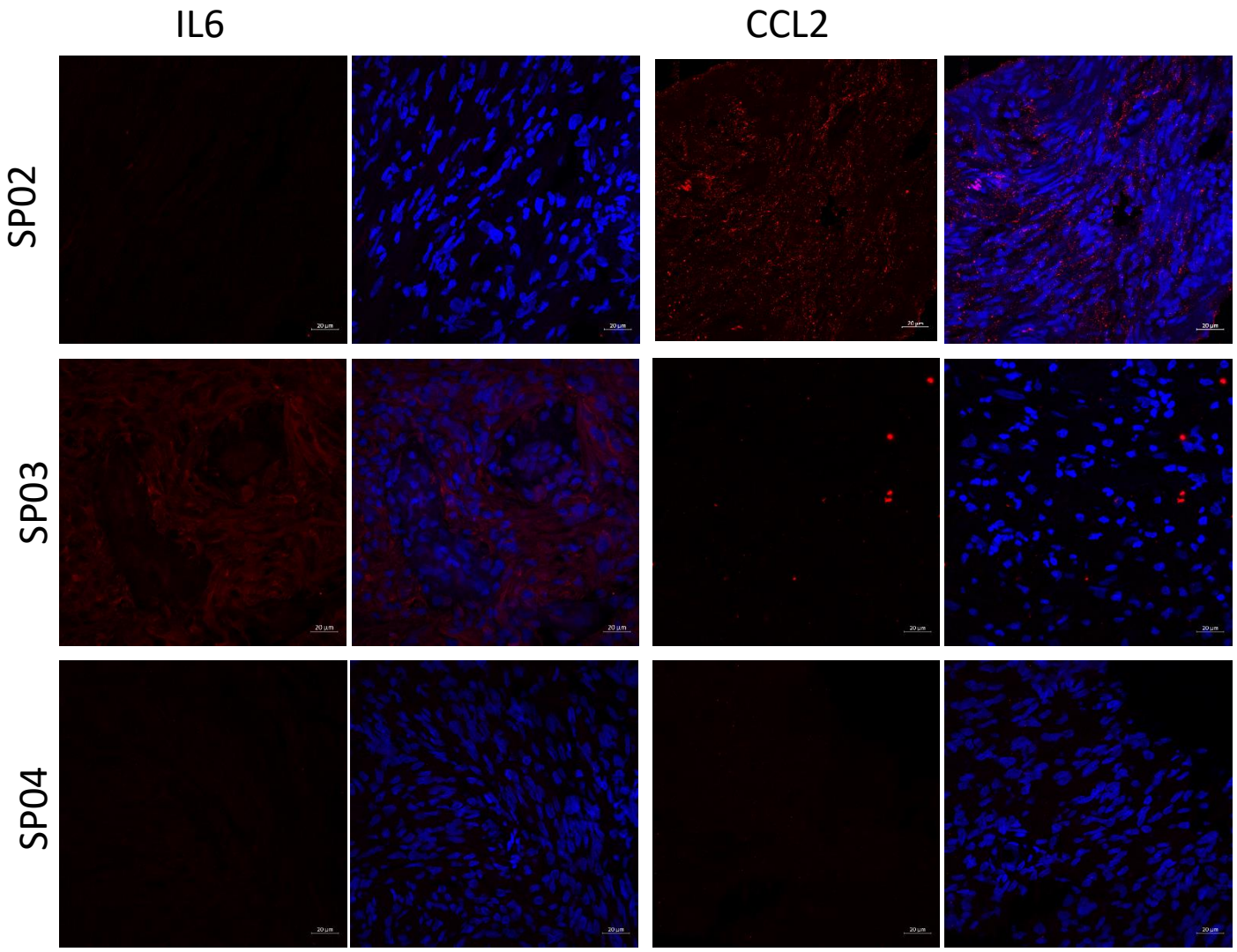

Figure 4 Immunohistochemistry of non-SWN schwannomas SP02, SP03, and SP04 stained for CCL2 and IL6.

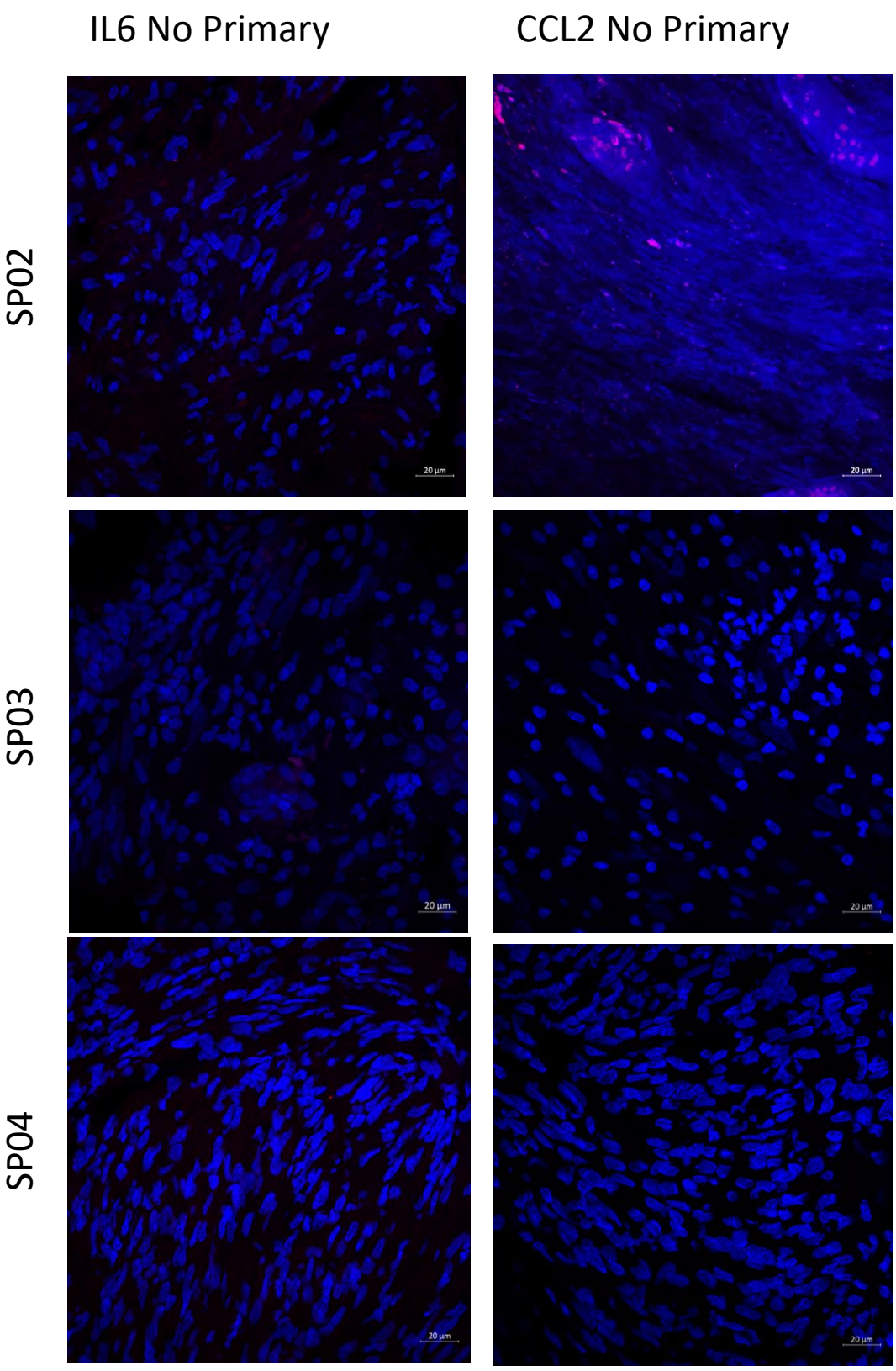

Figure 5 Negative Control for non-SWN Schwannomas.

As seen in Figures 4 and 5, the non-SWN patient derived tumors display low to no CCL2 or IL6 immunostaining. The SP03 tumor expresses IL6, and SP02 expresses CCL2 however, SP04 does not demonstrate the presence of either cytokine and none of the non-SWN schwannomas display production and/or secretion of both CCL2 and IL6. The bright staining spots of SP03 stained for CCL2 in Figure 4 appears to indicate the presence of CCL2 however, the lack of tumor-wide stains and the lack of cellular association indicates that this is non-specific binding or clumps of secondary antibody. As seen in Figure 5, SP02 appears to have some non-specific binding as well.

Conclusion

In this study of the presence of the chemokine CCL2 and the cytokine IL6 in Schwannomas, it was demonstrated that both CCL2 and IL6 were present in all of the Schwannomas from SWN patients while only one or the other cytokine was present in few cells within the non-SWN Scwhannomas. There did not appear to be a cytokine that was consistently present in all of the non-SWN Schwannomas. Cytokines that were present in the non-SWN schwannomas were only present sporadically. However, both CCL2 and IL6 were present in at least one of the non-SWN Schwannomas.

The conclusions concerning the presence or absence of these cytokines should be confirmed through repeated experimentation with more samples of SWN and Vestibular Schwannomas as well as with other techniques such as western blots and other appropriate assays. A stronger connection between pain and the presence of these cytokines must be established through further experimentation and with patient surveys as well. If a stronger connection between the cytokines and pain is found, then the mechanism of pain involving the cytokines should be investigated in order to find new therapeutic methods for SWN patients and others suffering from cytokine related chronic pain.

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