Exploring the potential contribution of inflammation to altered pain behavior in the aging mice

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ABSTRACT

More than 50% of elders suffer from chronic pain. As society ages, this number increases. Chronic post-surgery pain is a critical concern in the aging population considering its poor outcomes including delayed recovery and deteriorated quality of life. In general, pain in the elderly is poorly understood, not adequately treated. While aging-associated systemic chronic inflammation, also called inflammaging, has been considered as a driver for many chronic diseases, its role in chronic pain remains elusive.

In this study, we aim to understand whether inflammation could be a contributing factor to altered pain behavior in aging mice. We first characterized inflammaging profile in 19–22-month-old female mice where altered number and phenotype of circulating immune cells and altered expression of inflammatory markers were detected. Transferring serum from aging mice to young mice triggered mechanical and cold allodynia in young mice. We then investigated the role of inflammation in surgical pain by using an animal model of incision pain on C57BL6 female mice. 19–22-month-old aging mice suffered twice longer post-operative pain than 3–5-month-old young mice in both von Frey and acetone tests. To better understand the contribution of inflammation to the prolonged post-incision pain of aging mice, we collected the skin samples from both age groups. At the first period (day 1-5 post-incision) where both young and aging mice displayed significant post-incision pain, both groups of mice showed an increase in the number of leukocytes, especially macrophages; and elevated mRNA expression of inflammatory molecules on their injured skins compared to uninjured ones. At the second period (day 6-12 post-incision) where young mice recovered from post-incision pain, but aging mice remained in painful condition, only aging mice showed increased numbers of immune cells and altered inflammatory markers on the injured paw skin.

These data display a specific inflammation profile in aging mice, including systemic inflammation and local skin inflammation following incision, and indicate the potential involvement of inflammation in altered pain of the aging population. Further investigations of the underlying mechanisms and inflammaging modulation could help us to identify effective strategies to alleviate chronic pain in the elderly and to improve their quality of life.

ABSTRACT

Plus de 50% des personnes âgées souffrent de douleur chronique. Avec le vieillissement de la société, ce nombre augmente. La douleur chronique post-opératoire est une préoccupation majeure dans la population vieillissante, considérant les résultats médiocres telles la récupération retardée et la détérioration de la qualité de vie. Cependant, la douleur chez les aînés est peu comprise et traitée inadéquatement. Alors que l'inflammation systémique chronique associée au vieillissement, aussi appelée «inflammaging», joue un rôle dans plusieurs maladies chroniques, son importance dans la douleur chronique reste peu comprise.

Dans la présente étude, nous visons à comprendre si l'inflammation contribue au comportement de douleur altérée chez les souris âgées. Nous avons caractérisé en premier le profil inflammaging chez les souris femelles C57BL/6 de 19-22 mois. En comparaison avec les souris de 2-4 mois, nous avons détecté un nombre et phénotype différent chez leurs cellules immunitaires et une expression altérée des marqueurs d'inflammation dans le sang des souris âgées. Le transfert de sérum des souris âgées aux jeunes souris déclencha l'allodynie mécanique et froide chez les jeunes souris. Nous avons ensuite examiné le rôle de l'inflammation dans la douleur opératoire en utilisant un modèle animal de la douleur d'incision. Les souris âgées ont souffert de douleur post-opératoire doublement le temps des jeunes souris de 2-3 mois dans les tests von Frey et d'acétone. Pour mieux comprendre la contribution de l'inflammation dans la douleur post-incision prolongée chez les souris âgées, nous avons recueilli des échantillons de peau des deux groupes d'âge. Dans la première période (jour 1-5 post-incision), les souris jeunes et âgées ont démontré un niveau de douleur post-incision importante, une augmentation dans le nombre de cellules immunitaires et un niveau élevé d'ARNm des marqueurs d'inflammation dans la peau lésée comparé à la peau saine. Dans la seconde période (jour 6-12 post-incision,

lorsque les jeunes souris ont récupéré de la douleur post-incision, mais que les souris âgées ressentent encore la douleur, seuls les souris âgées avaient un nombre accru des cellules immunitaires et un profil altéré des marqueurs d'inflammation dans la peau de patte lésée.

Ces résultats présentent l'inflammation de façon systématique, incluant l'inflammation systémique et post-opératoire locale dans les souris âgées, et indiquent le rôle potentiel de l'inflammation dans la douleur altérée de la population vieillissante. Plus de recherche sur les mécanismes sous-jacent et la modulation de l'inflammaging pourrait nous aider à identifier des stratégies efficaces pour réduire la douleur chronique chez les personnes âgées et améliorer leur qualité de vie.

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PREFACE AND CONTRIBUTION OF AUTHORS

von Frey and acetone behavioral tests, incision surgery, animal tissue collection and perfusion were done by Xiang Shi Qun.

I coordinated the experiments, performed RT-qPCR and flow cytometry. I also analyzed all of the data and wrote the thesis.

Dr. Ji Zhang, as the principal investigator, provided conceptual and technical guidance, and supervised all aspects of this project.

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KEY TO ABBREVIATIONS

ACK……Ammonium-Chloride-Potassium

BMSC…….Bone Marrow stromal Stem Cell

CCL2……...C-C motif Chemokine Ligand 2

CD……...Cluster of Differentiation

CCR2……...C-C Chemokine Receptor type 2

CFA…….complete Freund's adjuvant

CNS……..Central Nervous System

CPSP……Chronic Postsurgical Pain

CX3CR1……...C-X3-C Motif Chemokine Receptor 1

DAMP……..Damage-associated molecular patterns

DMEM…….Dulbecco's Modified Eagle Medium

ECM…….Extracellular Matrix

GDF11……..Growth Differentiation Factor 11

hsCRP……...high-sensitivity C-reactive protein

IL1β……...Interleukin 1 Beta

IL1RA……...Interleukin 1 Receptor Antagonist

IL6…….Interleukin 6

IL8…….Interleukin 8

IL10…….Interleukin 10

IL12…….Interleukin 12

IL21…….Interleukin 21

IL18…….Interleukin 18, also known as interferon-gamma inducing factor

MAP……Mitogen-Activated Protein

MCP……Monocyte Chemoattractant Protein

MyD88……Myeloid Differentiation Primary Response 88

NF- κB……Nuclear Factor kappa-light-chain-enhancer of activated B cells.

NK……..Natural killer

PCA……Principal Component Analysis

PNS……Peripheral Nervous System

SASP……..Senescence-Associated Secretory Phenotype

TNFα……..Tumor Necrosis Factor Alpha

TGF-β…….Transforming Growth Factor Beta

TLR……Toll-like Receptor

INTRODUCTION

Life expectancy has been increasing within the global aging population in recent decades. In Canada, the percentage of individuals aged 65 years and above increased from 17% to 18.5% within five years (Census Canada 2016 and 2021, https://www150.statcan.gc.ca/n1/daily-quotidien/210929/cg-d003-eng.htm). This proportion is projected to increase to 23% by 2030 (Government of Canada, Action for seniors report, https://www.canada.ca/en/employment-social-development/programs/seniors-action-report.html), which imposes enormous pressure on government finances, economic growth, and lack of labor force. More importantly, there is a great discrepancy between the rising life expectancy and healthspan. Where life expectancy is the time an individual is expected to live, healthspan is the period of life spent disease-free. The aging population has greater demand for health care due to the increasing prevalence of chronic diseases and complex medical conditions. The risk of mortality increases exponentially with age in individuals with age-related diseases, such as atherosclerosis, hypertension, diabetes, Alzheimer's, and Parkinson's diseases (Belikov, 2019). Chronic pain in the aging population is also associated with impaired physical function and psychological distress (Turk et al., 2016). Chronic pain is defined as pain that persists or recurs for longer than three months, clinically affecting an estimated 20% of people worldwide (Goldberg & McGee, 2011). According to Schopflocher et al., the prevalence of chronic pain in Canada is greater in older adults (above 65 years), and more prevalent in females (31.5%) than males (22.2%) (Schopflocher et al., 2011). It is reported that women have a higher risk of suffering from the comorbidity of chronic pain and depression (Scherer et al., 2016). In elderly people, chronic pain has been drawing empirical and clinical attention since the 1990s by Dr. Melding (Gagliese & Melzack, 1997; Melding, 1991).

The reluctance of the elder population to report painful symptoms, limited access to diagnostic facilities or pharmacy services, and inconsistent definitions of chronic pain make chronic pain assessment and management more complicated in the aging population (Chodosh et al., 2004). Due to the lack of understanding of characteristics and specific treatments of geriatric pain in the past several decades, the pain and aging subfield emerged (Gagliese, 2009). The concept that pain in older and younger adults is different in both theoretical and clinical aspects is supported by the evidence of several unique characteristics of geriatric pain such as unwillingness of reporting pain, increased vulnerability to neuropathic pain, prolonged sensitization after injury, and different psychosocial factor involvements (Ashcroft et al., 2002; Noroozian et al., 2018; Smith & Torrance, 2012; Stein & Miech, 1993).

In the 11th revision of the International Classification of Disease, the cause of chronic pain is classified into seven groups based on underlying etiology: cancer, neuropathic, musculoskeletal, post-traumatic or postsurgical, visceral, headache and orofacial (Treede et al., 2015). Chronic postsurgical pain (CPSP) was firstly described in 1999 by Crombie et al. The patients with persistent pain beyond normal healing after surgery and some types of injuries amounted to 22.5% of total 5130 post-surgical patients (Crombie et al., 1998). Later in 2001, the concept of CPSP was expanded by Macrae, as “pain that develops after surgical intervention and lasts at least 2 months with other causes of pain being excluded” (Macrae, 2001a). Although the incidence of CPSP varies in different operations from a low of 5% to a high of 85% at one year, higher rates (more than 40%) were observed after major thoracic and breast surgery (Bayman et al., 2017; Haroutiunian et al., 2013; Jin et al., 2016; Macrae, 2001b; Steegers et al., 2008). Publications on specific or general surgical procedures reflect an acceptance of chronic pain as an important outcome of surgery, adversely affects long-term recovery and overall quality of life

(Fuzier et al., 2017; Kehlet et al., 2006; Reddi & Curran, 2014). CPSP could be contributed by multivariate predictors: capacity overload, local preoperative pain, chronic preoperative pain, post-surgery acute pain and co-morbid stress symptoms (Althaus et al., 2012; Katz et al., 1996). In this decade, the incidence of surgical procedures performed in the elderly population (above 65 years) is continuously rising, which accounts for more than 30% of total surgeries (Hall et al., 2017). In addition to significantly higher morbidity and mortality post-surgery (Turrentine et al., 2006), the elderly is also facing chronic post-surgery pain as a long-term concern. Frailty syndrome and the existence of pre-surgical pain in older adults predispose a larger risk for the development of CPSP in the aging population (Ali et al., 2018; Esses et al., 2019). As the number of older adults undergoing surgical intervention is rising, CPSP management is critical for their long-lasting physical recovery and cognitive healing. Although CPSP has been considered as a neuropathic condition which strongly associates with nerve injury, there is evidence suggesting potential mechanisms like inflammation and central sensitization (Bande et al., 2020; Johansen et al., 2012; Pluijms et al., 2006; Van de Ven & John Hsia, 2012). Despite the increasing number of studies on CPSP in recent years, understanding the mechanism and potential risk factors of CPSP in the aging population is a field that remains unclear.

Inflammation is a physiological protective process in response to endogenous and exogenous dangers. The immune system consists of innate and adaptive immunity. The innate immune is the first line of nonspecific host defense against pathogens, mediated by leucocytes including basophils, eosinophils, mast cells, monocytes, NK cells and phagocytes (i.e. macrophages, neutrophils and dendritic cells). The level of CX3CR1 expression defines the two major subsets of monocytes in both human and mice. As a marker of homeostasis, CX3CR1 prevents monocytes from apoptosis by binding to its ligand Fractalkine and facilitates monocyte

migration to tissues in the absence of inflammation (Geissmann et al., 2003; White & Greaves, 2012). CCR2 is the receptor of chemoattractant protein 1 and 3 (MCP-1/CCL2 and MCP-3), attracting mononuclear cells to the sites of chronic inflammation (Charo & Ransohoff, 2006; Lu et al., 1998; Palframan et al., 2001). The expression of CCR2 is critical for monocytes egress and necessary for the recruitment of circulating monocytes to the inflamed tissues (Tsou et al., 2007). The adaptive immune response is highly specific to a particular pathogen and provides long-lasting protection. Adaptive immunity is carried out by two classes of lymphocytes, B cell for antibody response and T cell for cell-mediated immune response. The trafficking of naïve T cells to and from peripheral lymph nodes is regulated by CD62L (L-selectin). The recognition of antigen by T cells sheds CD62L from the cell membrane and rapidly upregulates surface marker CD44 in effector cells. The expression of CD44 is associated with cellular activation status, which is also maintained in memory cells (Schumann et al., 2015; Yang et al., 2011).

A major type of inflammatory stimulation includes the microorganism-derived pathogen-associated molecule patterns (PAMPs), and host cell-derived damage-associated molecule patterns (DAMPs). The recognition of PAMP and DAMP signal is best-described by Toll-like receptor (TLR) family. Stimulation by the ligands of TLRs recruits its adaptor MyD88, leading to nuclear translocation of NF-kB and activation of MAP kinase cascades, in turn, initiate the expression of pro-inflammatory cytokine genes (Korneev et al., 2017; Sun et al., 2011). The release of cytokines and chemokines includes both pro-and anti-inflammatory proteins such as IL1B, IL6, TNFa, MCP-1 and IL10, which could lead to local activation of macrophages and neutrophils by autocrine and paracrine effects (Zhang & An, 2007). Chemokines function as a chemoattractant to recruit immune cells to the site of inflammation, where phagocytes could exert a crucial role in killing pathogens. Active monocytes and neutrophils release large amounts

of cytokines and chemokines in the circulation, which in turn stimulate the release of other molecules mediating the inflammatory signals and symptoms (fatigue and fever) by the endocrine effect (Shattuck & Muehlenbein, 2015).

Inflammation is viewed as a driving factor for various diseases, as well as a major contributor to age-related conditions. The basal inflammatory response rises with age, characterized by a chronic, low-grade systemic inflammation, in the absence of overt infection, known as inflammaging (Franceschi, Bonafè, Valensin, et al., 2000). The evolutionary perspective of inflammaging theory was firstly proposed by Franceschi et al., which implicated the confluence of the evolution of immune responses and the aging of the immune system. A major source of inflammaging is the continuous stimuli of endogenous damaged molecules, due to the impaired balance of proteasome disposal with age. Inflammaging has been listed as one of the seven pillars that promote aging, highly intertwining with other factors such as metabolism, adaption to stress and proteostasis (Kennedy et al., 2014). Cellular senescence is a significant contributor to inflammaging, which is an irreversible growth arrest expressed at the cellular level by intrinsic factors. Immunosenescence results from life-long exposure to a variety of antigens. The naïve T-cell pool shrinks, accompanied by the accumulation of effector T cells. In compensation, innate immune cells, monocytes and NK cells refill the immunological pool (Franceschi, Bonafè, & Valensin, 2000; Márquez et al., 2020). The limited number of senescent cells broaden their effects by secreting pro-inflammatory factors, which is characterized as "senescence-associated secretory phenotype (SASP) (Coppe et al., 2008; Lasry & Ben-Neriah, 2015). SASP is constituted by proinflammatory cytokines, CXCL/CCL family chemokines, growth factors and matrix-remodeling enzymes (Acosta et al., 2008; Coppé et al., 2010; Kuilman et al., 2008). These inflammatory molecules have been used to assess inflammaging. In a

prospective population-based study of the elderly, 19 inflammatory biomarkers were measured multidimensionally by principal component analysis (PCA) to reveal the relationships among the markers. The first axis was largely driven by both pro-and anti-inflammatory markers (e.g. IL6, TNFa, hsCRP, IL18, IL1RA) and was strongly correlated with age, indicating a more active immune system. The second axis was explained by innate immune markers MCP, IL12 and IL8 (Morrisette-Thomas et al., 2014). The soluble inflammatory molecules are strong predictors of morbidity, mortality, and multiple chronic diseases in the elderly (Howcroft et al., 2013; Michaud et al., 2013). However, the extent of their involvement to drive such diseases remain elusive. Mounting evidence demonstrated that the blood-circulating molecules hold great promise of counteracting the aging phenotypes and pathologies. Serum from young donors rejuvenated the differentiation capacity of muscle stem cells (satellite cells) in both aging humans and mice (Barberi et al., 2013; Conboy et al., 2005). Systemic GDF11 (growth differentiation factor 11) had been proved to reverse age-related cardiac hypertrophy and improve cerebral vasculature and neurogenesis in aging mice (Katsimpardi et al., 2014; Loffredo et al., 2013). Treatment with extracellular vesicles extracted from young mice serum attenuated age-related chronic inflammation in the periphery and CNS, by partial rejuvenation of aged thymus (Wang et al., 2018).

While increasing evidence strongly implies the importance of inflammaging to most chronic conditions of the elderly, its role in the development and progression of chronic pain in elders remains to be elucidated. Furthermore, although substantial evidence from the last 20 years' research strongly supports the contribution of the immune system to chronic pain, data is not available for the role of inflammation in chronic pain in geriatric patients. While the elderly

population are living constantly with inflammaging, it is imperative to refine our knowledge on the link between inflammaging and age-related deterioration of chronic pain.

In this study, we aimed to understand whether inflammation is associated to altered pain behavior in aging mice. First, we characterized inflammaging in aging mice by analyzing the circulating immune cells including their numbers, phenotypes and functions. Secondly, we investigated post-surgery pain and local inflammation in the young and aging mice by performing a 5-mm-long incision on the hind paw. The characterization of the prevailing innate immunity, diminished adaptive immunity, and altered expressions of inflammatory markers in the blood indicated an inflamed systemic environment of the aging mice, which could contribute to altered mechanical and thermal sensitivities of the young mice. Prolonged post-surgery pain was also observed in the aging mice with elevated local inflammation signals.

METHODS

2.1. Animals

Female C57BL/6 mice from Charles River Laboratories were housed on a 12/12h light/dark cycle in a temperature-controlled room. 3-5 mice were grouped in a ventilated poly- carbonate cages, with corncob bedding (7097; Teklad Corncob Bedding, Envigo, United Kingdom). Mice were given access to food (hard pelleted chow) and water *ad libitum*.

The animals were divided into young (2-3 months) and aging (18-21 months) groups based on their age. All mice were bred in house or purchased from Charles River and kept until the age planned for experiments. The life span of C57BL/6 mice is between 18 and 27 months. Only the healthy aging animals were included in our experiments.

Behavioral experiments were performed between 9:00 AM. to 3:00 PM. All procedures were in accordance with the ethical guidelines of the Canadian Council on Animal Care and approved by the animal care committee of McGill University.

2.2. Mouse model of post-surgery pain

This protocol was adapted from (Pogatzki & Raja, 2003). Mice were anesthetized with 1.5% to 2% inhaled isoflurane. The left hind paw was adhered to the surface of surgical area. A 5mm longitudinal incision was sliced through the skin. The incision was 2mm from the proximal edge of heel. The underlying muscle was lifted by a curved forceps, with the origin and insertion of the muscle intact. The skin layer was closed with suture and covered by Aluspray. The suture

was removed at the third day post-incision. Control mice underwent anesthesia and antiseptic procedures, without an incision.

2.2.1 Tissue preparation

Mice were deeply anesthetized and trans-cardiac perfused with 0.9% saline to harvest the tissue. Approximately 2.5mm x 2.5mm of skin from the walking pad, adjacent to the incision site, was collected after 0.9% saline trans-cardiac perfusion. In the post-surgical FACS and qPCR experiments (**Fig. 6** and **7**), the injured left hind paw of the mouse was tested as the ipsilateral side of incision, the intact right hind paw from the same mouse was tested as the contralateral side.

2.3. Behavioral analysis

Animals were placed on an elevated metal mesh platform and were given 40 to 60 min for habituation before testing. Baseline data was obtained by measurements made 1-2 days before the start of the experiments. A single examiner conducted all testing between 9:00 AM and 3:00 PM.

Mechanical and thermal sensitivities were assessed as perception of pain behaviours. Baseline values were obtained before incision surgery or injection. Upon surgery, animals were tested on post-incision day 1, 3 and 6 for young mice. Day 1, 3, 6, 8, 9, 10, 11, 13 and 16 for aging mice. The experimental timepoints were determined based on the pilot data. The pilot experiments were tested daily in both young and aging groups, from day 1 post-incision till the incision-induced hypersensitivity was fully recovered.

Mechanical thresholds was assessed using calibrated von Frey Hairs as described in previous literature (Chaplan et al., 1994). A series of von Frey filaments was applied perpendicular to the mid-planter region of the hind paw. The stiffnesses of the filaments were logarithmically incrementing from 0.0008g to 4g (Stoelting). The mean paw withdrawal threshold (in grams) was calculated based on Dixon's up-down method (Dixon, 1980).

Thermal hyperalgesia was measured by the duration of painful behavior (e.g. flinching, licking or biting) evoked by the application of one drop of acetone on the plantar surface of the hind paw. One minute was set as cut-off (Yoon et al., 1994). Increased duration of behavioral response to acetone stimuli indicated the development of cold hypersensitivity.

2.4. Serum injection

Serum was collected from both young and aging female mice. Blood was collected in a Microvette 200 serum collection tube (SARSTEDT, cat# 20.1291). After clotting at room temperature for 30min, blood was centrifuged to isolate serum. Serum was kept at -80ºC. 2-month old female C57BL/6 mice were given intravenous injection via a tail vein. A total amount of 100 µl serum from the same group of mice was pooled for intravenous injections. The vehicle mice received pooled serum from the young mice, and the treatment group received pooled serum from the aging mice.

2.5. Flow cytometry

Whole blood was collected from facial vein of the mice and was kept in Alsever's solution to prevent coagulation. ACK lysing buffer (gibco, cat#A10492-01) was used to lyze erythrocytes.

Tissue samples were diced into small pieces in DMEM containing collagenase IV for digestion. Tissue samples were kept in 37ºC incubator for 45mins with trituration every 8 mins. After filtration by 70 μm nylon filter and wash by FACS juice, 2.4-G2 blocking serum was added to avoid unspecific binding at 4 ºC for 30 min. Samples were then stained with specific fluorochrome-conjugated antibodies (listed in **Table 1**) for 30 min at 4 °C. Staining specificity was verified by correlation of spectral overlap was done by using negative and positive compensation beads (BD, cat# 552843, 552845). The absolute cell numbers of each sample were quantified by counting beads (eBioscience, cat#01123442). BD FACSCanto was used to acquire cellular events. Flow Jo software was used for analysis.

Antibody	dilution	Source	Catalog number	Clone
CD11b	1:50	BD	552850	M1/70
		Biolegend	101228	M1/70
CD115	1:50	Biolegend	135512	AFS98
CD45	1:50	Biolegend	103116	30-F11
CD3	1:50	Biolegend	100308	145-2C11
CD4	1:50	BD	550954	RM4-5
CD8	1:50	eBioscience	12-0081-83	53-6.7
CD19	1:50	Biolegend	115506	6D5
Ly6G	1:50	BD	560601	1A8
F4/80	1:50	eBioscience	17-4801-82	BM8
CX3CR1	1:50	Biolegend	149006	SA011F11
CCR2	1:50	Biolegend	150604	SA203G11
CD62L	1:50	BD	560513	MEL-14
CD44	1:50	Biolegend	103030	1M7
NK1.1	1:50	Biolegend	108718	PK136

Table 1. Antibodies used for FACS

2.6. Total RNA extraction and RT-PCR

Expression levels of target genes in the blood and skin were measured using real-time quantitative PCR. Whole blood was collected from facial vein of the mice and was kept in Alsever's solution to prevent coagulation. ACK lysing buffer was used to lyze erythrocytes.

Tissue samples were frozen at -80^{o}C for 15min or longer. The diced tissue was homogenized in TRIzol Reagent (Ambion Life Technologies, cat#10296010) with tissue homogenizer beads (Sigma-Aldrich, cat#1002167648) and a Precellys 24 tissue homogenizer (Bertin Technologies) using two 20s pulses at 6500 rpm.

Total RNA of the blood immune cell and skin were extracted with TRIzol. Removal of total protein and genomic DNA were performed by chloroform (Sigma-Aldrich, cat#1001557082) and isopropanol (Sigma-Aldrich, cat#1003316270), respectively. After washing by 75% ethanol, total RNA was resolved in DEPC treated RNase-free H_2O. Nanodrop 200 (Thermofisher Scientific) was used for RNA purity and concentration detection. Total RNA of 1 μg was used as template for reverse transcription with SuperScript IV Reverse Transcriptase kit (Invitrogen, cat#18090050), Oligo-dt (Invitrogen, 18418020), and dNTP (Invitrogen, cat#18427-088). Real-time quantitative PCR (qPCR) reactions (in duplicate) were processed by a Rotor-Gene Q real-time PCR cycler (Qiagen) using SYBR Green Supermix (Bio-Rad, cat#1725271). qPCR primers as listed in **Table 2**. The levels of target genes were normalized against the housekeeping gene Actin-beta and interpreted using the delta/delta Ct method.

Primers	Forward	Reverse
ACTB	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATG
CCL2	CTACTCATTCACCAGCAAGA	TCAGCACAGACCTCTCTC
CCR2	AGAAGAGGGCATTGGATT	CGTGGATGAACTGAGGTA
CXCL5	AGCTGCGTTGTGTTTGCTTA	CAGACAGACCTCCTTCTGGTTT
CXCL10	CCTATGGCCCTCATTCTCAC	AAGTGCTGCCGTCATTTTCT
IL1-β	CTATACCTGTCCTGTCTA	GCTCTTGACTTCTATCTTG
IL10	CTATGCTGCCTGCTCTTA	GCTGGTCCTTTGTTTGAAA
IL6	CTGAAACTTCCAGAGATA	TTCATGTACTCCAGGTAG
TNF-α	TTCTGTCTACTGAACTTC	CCATAGAACTGATGAG

Table 2. Sequences of qPCR primers

2.7. Data analysis

For FACS results: In **Fig**. **4A**, the absolute cell numbers of the young mice were normalized to 1, the fold change represented the relative cell abundance in the aging mice compared to the young mice. In **Fig**. **6C** and **7B**, the fold change ipsilateral over contralateral cell number indicated the level of immune cell infiltration in both young and aging groups.

For qPCR results: In **Fig**. **4B**, **6E** and **7D**, the relative gene expression levels of young mice were normalized to 1, the fold change represented the relative expression level in the aging mice compared to the young mice.

All data are presented as means ± SEM. Statistical analysis was based on the following:

1) two-way ANOVA followed by Bonferroni's multiple comparison test for von Frey and acetone tests of post-surgery young and aging mice (**Fig. 5**);

2) One-way ANOVA repeated measures followed by Bonferroni's multiple comparison test for acetone test of young mice received young serum injection, von Frey and acetone tests of young mice received aging serum injection (**Fig. 3A** right panel and **B**);

3) paired *t* test for young mice received young serum injection (**Fig. 3A** left panel);

4) two-tailed unpaired *t* test for FACS (**Fig. 1**, **4A**, **6B**&**C**, **7A**&**B**), qPCR analysis (**Fig. 2**, **4B**, **6D**&**E**, **7C**&**D**), baseline mechanical and thermal threshold comparison (**Fig. 4C**).

A value of $P < 0.05$ was accepted as statistically significant. All statistical analyses were performed using GraphPad 7.0 prism software.

RESULT

3. 1. Blood samples showed elevated systemic inflammation in the aging mice

We firstly used FACS to identify the circulating immune cells of female C57BL6 mice in young and aging groups. As the key contributor of innate immunity, monocyte was defined by its surface receptors CD115 and CD11b. A larger number of monocytes (CD115+CD11b+) was found in the blood of aging mice (**Fig. 1A**, upper panel: unpaired 2-tailed t-test, young vs. aging, $p=0.0096$, $t=3.19$, $df=10$). Coincidently, the numbers of inflamed monocytes (CD115+CD11b+CCR2+) and homeostatic monocytes (CD115+CD11b+CX3CR1+) were both higher in aging mice than in young mice (**Fig. 1A**, lower panel: $p=0.0085$, $t=3.262$, $df=10$ and $p=0.01$, $t=3.111$, $df=10$, respectively). Compared with young mice, an increased pattern was seen in Ly6G+ neutrophils of aging mice (**Fig. 1B**: $p=0.001$, $t=4.096$, $df=15$), and a decreased pattern was seen in NK1.1+ NK cells of aging mice (**Fig. 1C**: $p=0.0099$, $t=3.510$, $df=7$).

We also analyzed adaptive immune cells. CD3+ T cell number showed a decrease in the aging group (**Fig. 1D**, upper panel: $p<0.0001$, $t=14.34$, $df=6$) with reduction of CD4+ and CD8+ T cells (**Fig. 1D**, middle panel: $p=0.0017$, $t=4.917$, $df=7$ and $p<0.0001$, $t=7.899$, $df=15$, respectively), while the effector T cell (CD3+CD44+CD62L-) number of aging mice appeared to be higher than young mice (**Fig. 1D**, lower panel: $p=0.2042$, $t=1.424$, $df=6$). Moreover, the number of CD19+ B cells was lower in the blood of aging mice (**Fig. 1E**: $p=0.0028$, $t=4.497$, $df=7$). From the FACS results, we observed altered cell numbers and phenotypes in different types of immune cells, suggesting the prevailing of innate immunity with accumulated effector T cells and weakened adaptive immunity of aging mice.

To see the functions of the circulating immune cells, we performed qPCR to determine the mRNA levels of several inflammatory markers. The result showed increased expression levels of IL6, IL10, CCR2, CXCR2, CXCL5 (**Fig. 2A**: $p=0.0160$, $t=3.766$, $df=13$; $p=0.0475$, $t=2.258$, $df=10$; $p=0.0015$, $t=4.008$, $df=13$; $p=0.0263$, $t=2.604$, $df=10$ and $p=0.0254$, $t=2.584$, $df=11$ respectively) and decreased expression level of CCL2 ($p=0.0159$, $t=2.77$, $df=13$) in the blood of aging mice. However, no difference was observed in TNFa, IL1B, CXCL10 expressions. These results characterized the systemic immune profile of aging mice, including the immune cell number and inflammatory markers, is changed in the aging mice.

Figure 1

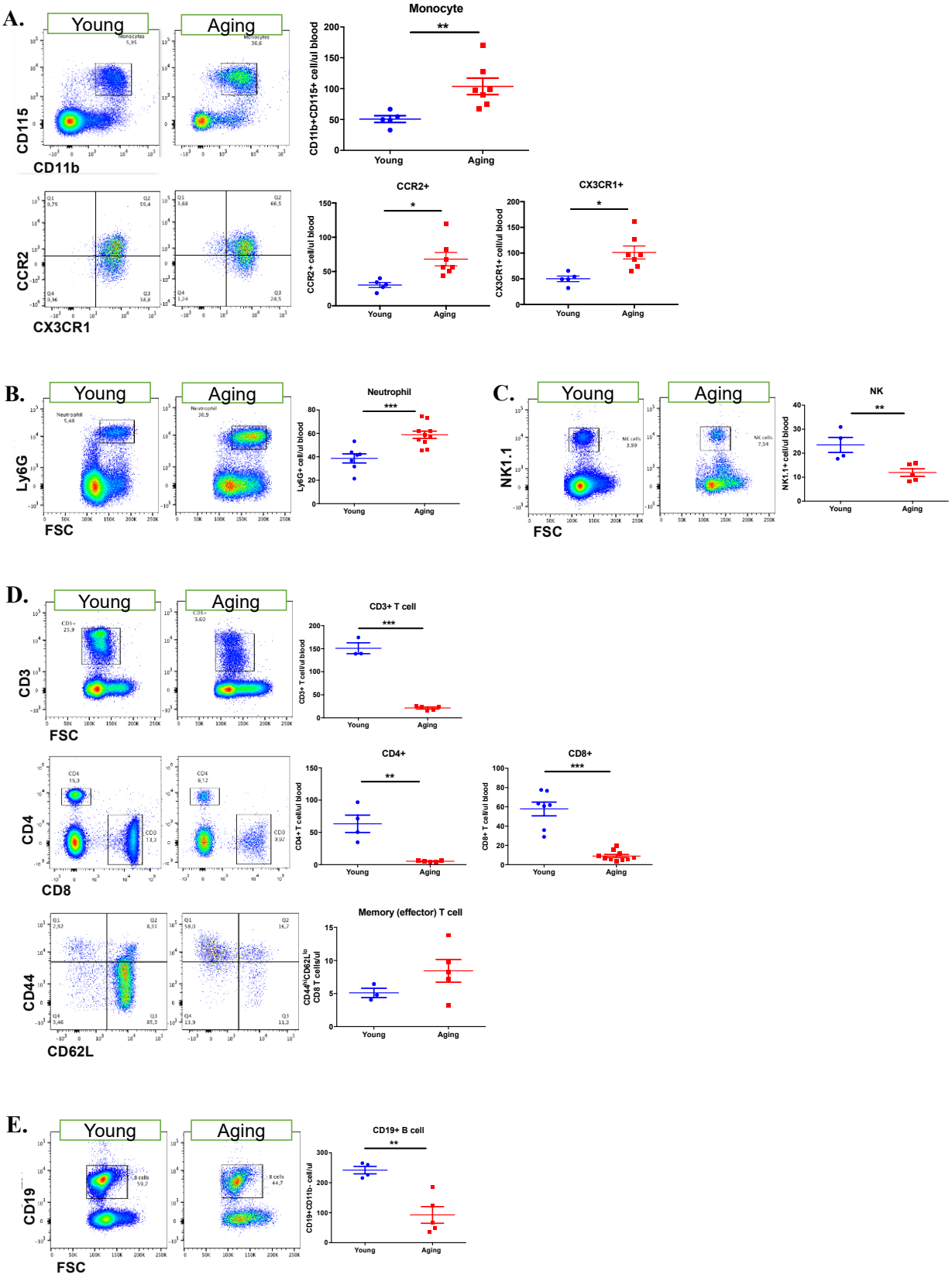

A.
Young
Aging
CD115
CD11b
Monocyte
CD11b+CD115+ cell/ul blood
**
Young
Aging
CCR2
CX3CR1
CCR2+
CCR2+ cell/ul blood
*
CX3CR1+
CX3CR1+ cell/ul blood
*
B.
Ly6G
FSC
Neutrophil
Ly6G+ cell/ul blood
***
C.
NK1.1
NK
NK1.1+ cell/ul blood
**
D.
CD3
CD3+ T cell
CD3+ T cell/ul blood
***
CD4
CD8
CD4+
CD4+ T cell/ul blood
**
CD8+
CD8+ T cell/ul blood
***
CD44
CD62L
Memory (effector) T cell
CD44hiCD62Llo CD8 T cells/ul
E.
CD19
CD19+ B cell
CD19+CD11b- cell/ul
**

Fig. 1. Altered abundance and phenotype of circulating immune cells in the blood of aging mice.

(A) $CD11b^+CD115^+$ monocytes increased in the blood of aging mice (18-19 months). Specifically, both $CCR2^+$ and $CX3CR1^+$ phenotypes showed higher absolute number in the aging mice compared with the young mice (2-3 months). **(B)** $Ly6G^+$ neutrophils increased in the blood of the aging mice. **(C)** $NK1.1^+$ cells decreased in the blood of the aging mice. **(D)** $CD3^+$ T cells decreased in the aging mice, with significant reduction of both $CD4^+$ and $CD8^+$ T cells. $CD44^+CD62L^-$ effector T cells showed an increasing trend in the aging mice, although not statistically significant. **(E)** $CD19^+$ B cells decreased in the aging mice. Data shown as mean ± SEM. n=5-10 per group. *P<0.05, **P<0.01, ***P<0.001.

Figure 2

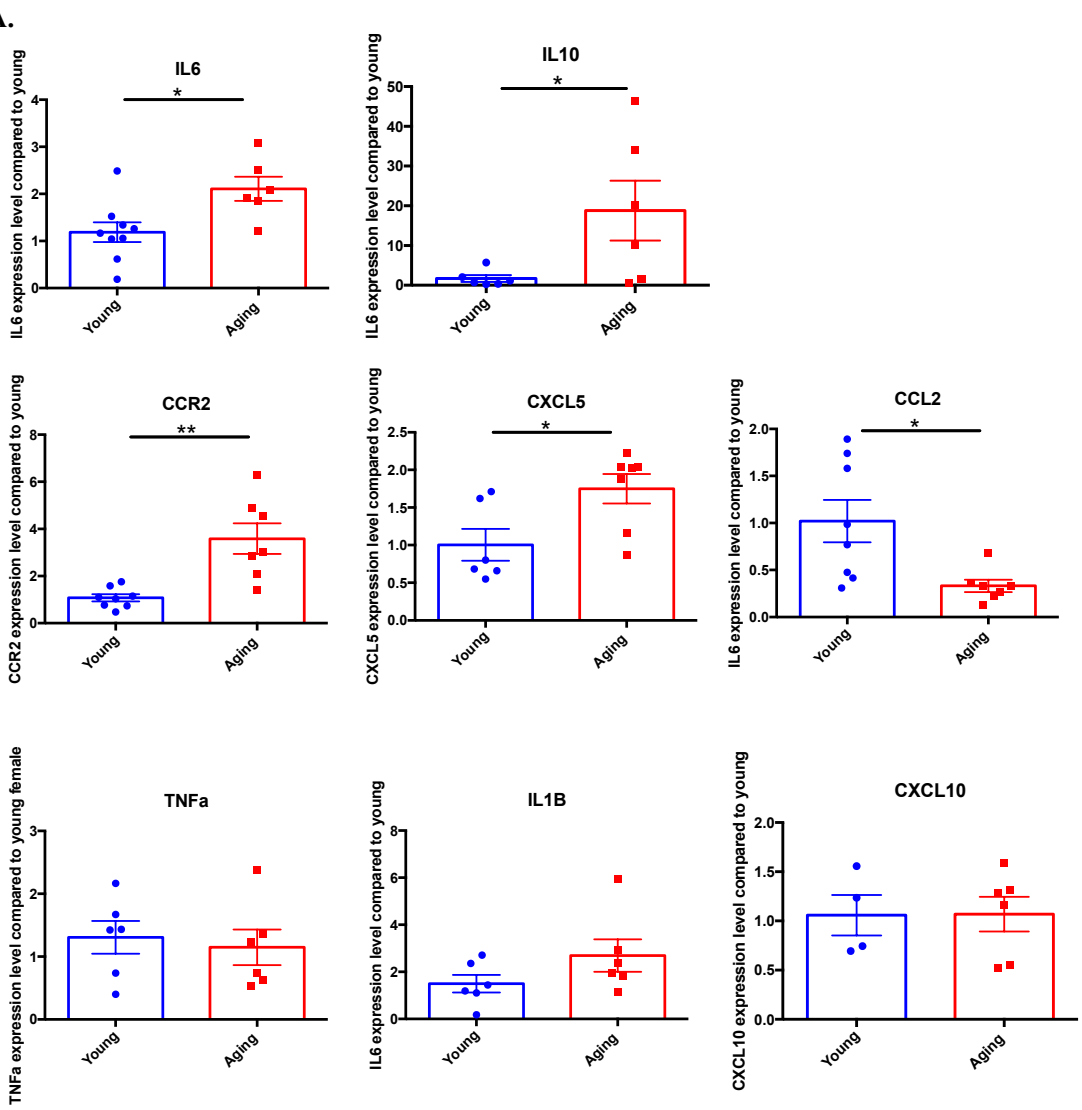


Fig. 2. Altered expression levels of inflammatory markers in the blood of aging mice.

qPCR results showed the relative expression level of inflammatory markers in the aging mice compared with the young mice. IL6, IL10, CCR2, CXCL5 had increased expression levels in the aging mice. CCL2 showed decreased expression level in aging mice. TNFα, IL1β and CXCL10 didn't show any significant difference between the two age groups. Data shown as mean ± SEM. n=4-9 per group. *P<0.05, **P<0.01, ***P<0.001.

3. 2. Serum of aging mice induced mechanical and thermal pain in the young mice

To investigate if the serum of aging mice could induce allodynic effect. We transferred the pooled serum of aging mice into young mice and monitor their behaviors. Young mice serum injecting into young mice was used as the control. Compared with the baseline, no difference was found in the behavior of the control group (**Fig. 3A**). However, in the group received serum from aging mice, von Frey and acetone tests showed significantly reduced mechanical thresholds and increased pain duration in response to acetone stimulation (**Fig. 3B**: repeated measurement one-way ANOVA), which lasted for five days. The development of hypersensitivity to mechanical and cold stimuli induced by the serum from aging mice indicated its allodynic effect.

Figure 3

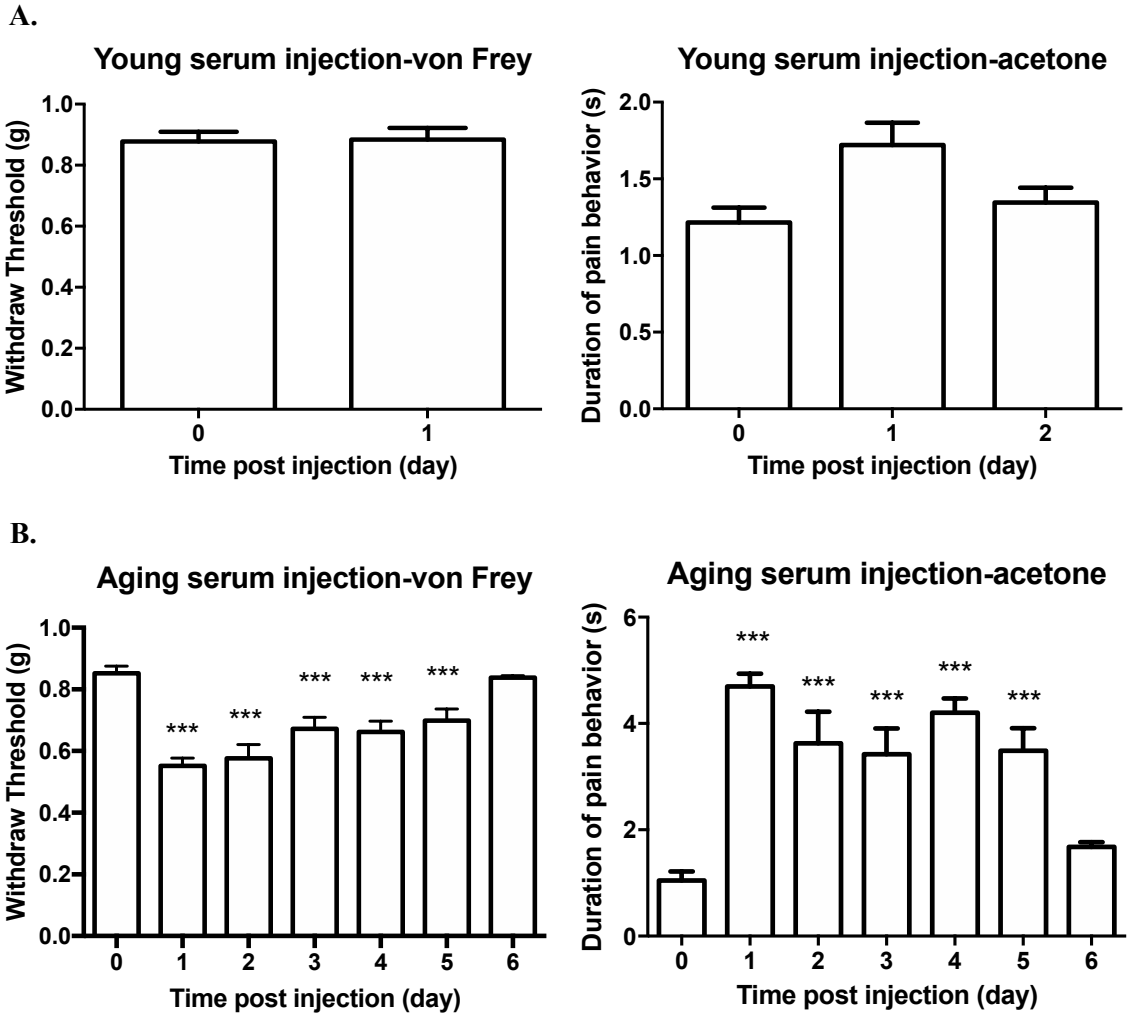


Fig. 3. Serum from the aging mice induced altered mechanical and thermal sensitivities in the young mice.

(A) Vehicle mice received serum from the young mice. No change was observed in von Frey and acetone tests post-injection. **(B)** Young mice received pooled serum from the aging mice, showed decreased mechanical threshold and increased sensitivity to cold stimuli, starting at day 1 and lasting for 5 days. Data shown as mean ± SEM. n=5-6 per group. ***P<0.001.

3. 3. Skin samples showed increased immune cells in the aging mice with altered nociceptive behaviors

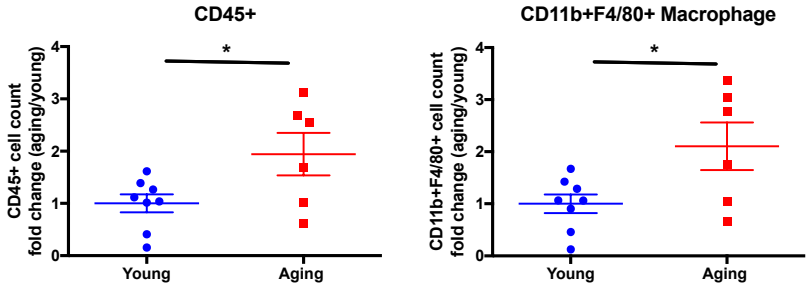
We firstly assessed the numbers of immune cell in normal skin by FACS. Significant larger numbers of CD45+ and CD11b+F4/80+ were found in aging group (**Fig. 4A**: $p=0.0372$, $t=2.342$, $df=12$ and $p=0.0283$, $t=2.493$, $df=12$, respectively).

We then analyzed the baseline expression levels of several inflammatory markers of the skin by qPCR. No difference was observed between young and aging groups for IL6, IL1B, TNFa, CCR2, and IL10 (**Fig. 4B**).

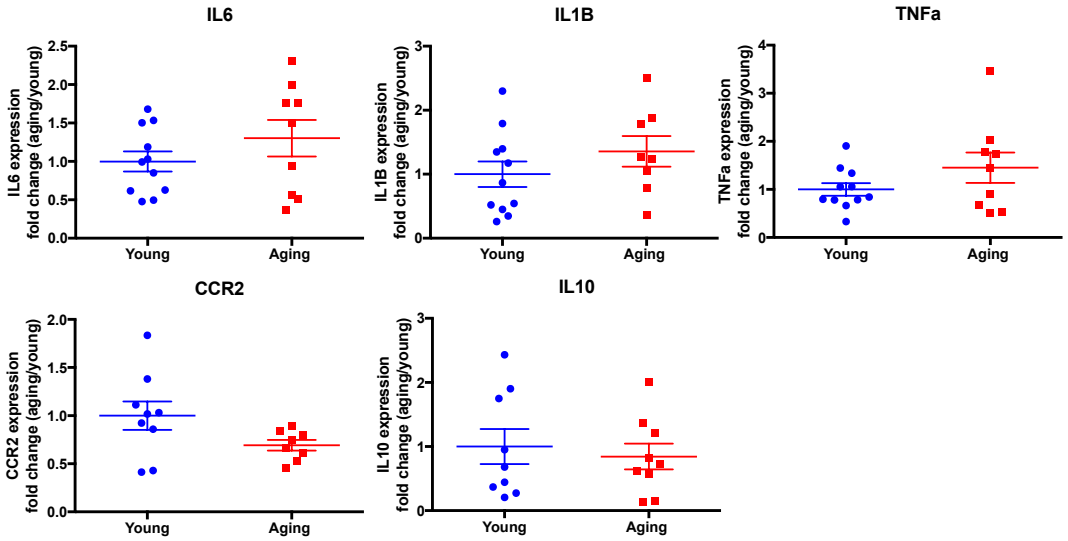
Last, we evaluated the impact of aging on the mouse nociceptive responses to mechanical and cold stimuli. Aging mice exhibited higher withdrawal thresholds on von Frey test and longer duration of painful behavior in acetone test (**Fig. 4C**: $p<0.0001$, $t=6.688$, $df=17$ and $p<0.0001$, $t=5.856$, $df=17$, respectively), suggesting that compared to the young mice, aging mice were less sensitive to mechanical stimuli but more sensitive to cold stimuli.

Figure 4

A.


CD45+
CD45+ cell count
fold change (aging/young)
*
Young
Aging
CD11b+F4/80+ Macrophage
CD11b+F4/80+ cell count
fold change (aging/young)
*
Young
Aging

B.


IL6
IL6 expression
fold change (aging/young)
Young
Aging
IL1B
IL1B expression
fold change (aging/young)
Young
Aging
TNFa
TNFa expression
fold change (aging/young)
Young
Aging
CCR2
CCR2 expression
fold change (aging/young)
Young
Aging
IL10
IL10 expression
fold change (aging/young)
Young
Aging

C.

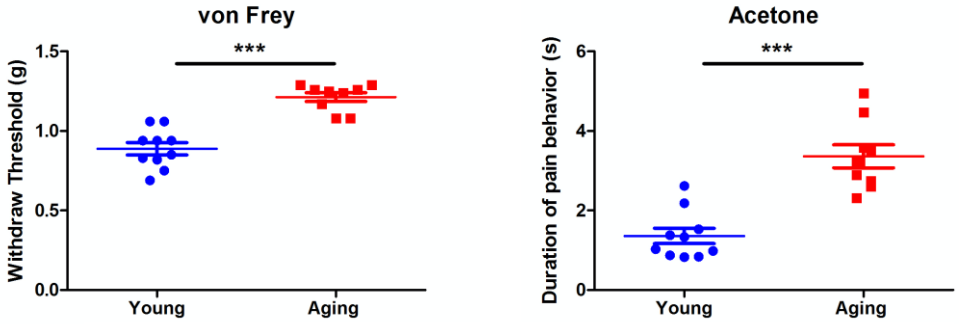

von Frey
Withdraw Threshold (g)
***
Young
Aging
Acetone
Duration of pain behavior (s)
***
Young
Aging

Fig. 4. Increased number of immune cells in the skin and altered pain behavior in normal aging mice.

(A) The fold change showed the relative abundance of immune cells in local skin in the aging group by normalizing the absolute numbers to the young group. Aging mice had increased abundances of $CD45^+$ and $CD11b^+F4/80^+$ immune cells at baseline level comparing with the young mice. n=6-8 per group. **(B)** The fold change showed the expression levels of the local inflammatory markers by normalizing to the young mice. No difference was observed in the expressions of IL6, IL1β, TNFα, CCR2 and IL10 in the local skin of aging mice comparing with young mice. n=9-11 per group. **(C)** Young and aging wild-type mice behavioral responses to mechanical and cold stimuli. Comparing with the young mice, the aging mice had increased mechanical threshold (less sensitive to mechanical stimuli) and longer duration of acetone-evoked behavior (more sensitive to cold stimuli). n=9-10 per group. Data shown as mean ± SEM. *P<0.05, ***P<0.001.

3. 4. Aging mice exhibited prolonged post-surgery pain

To monitor the pain behavior induced by incision on young and aging mice, we assessed their mechanical and thermal sensitivities by von Frey and acetone tests. The behavior testes performed from the first day post-incision till the end of the pain behavior. The left paw that received surgery was tested as the incision sample and the right paw was tested as the control sample.

Shortly after injury (at day 1), both young and aging mice presented significantly decreased mechanical thresholds and increased cold stimuli-induced response. In young mice, incision-induced pain peaked at day 1 and disappeared at day 6 in both von Frey and acetone tests (**Fig. 5A**). However, in aging mice, incision-induced pain lasted at least 13 days, completely disappeared at day 16 (**Fig. 5B**). This observation suggested that the duration of incision-induced pain was more than twice longer in aging mice than in young mice.

Figure 5

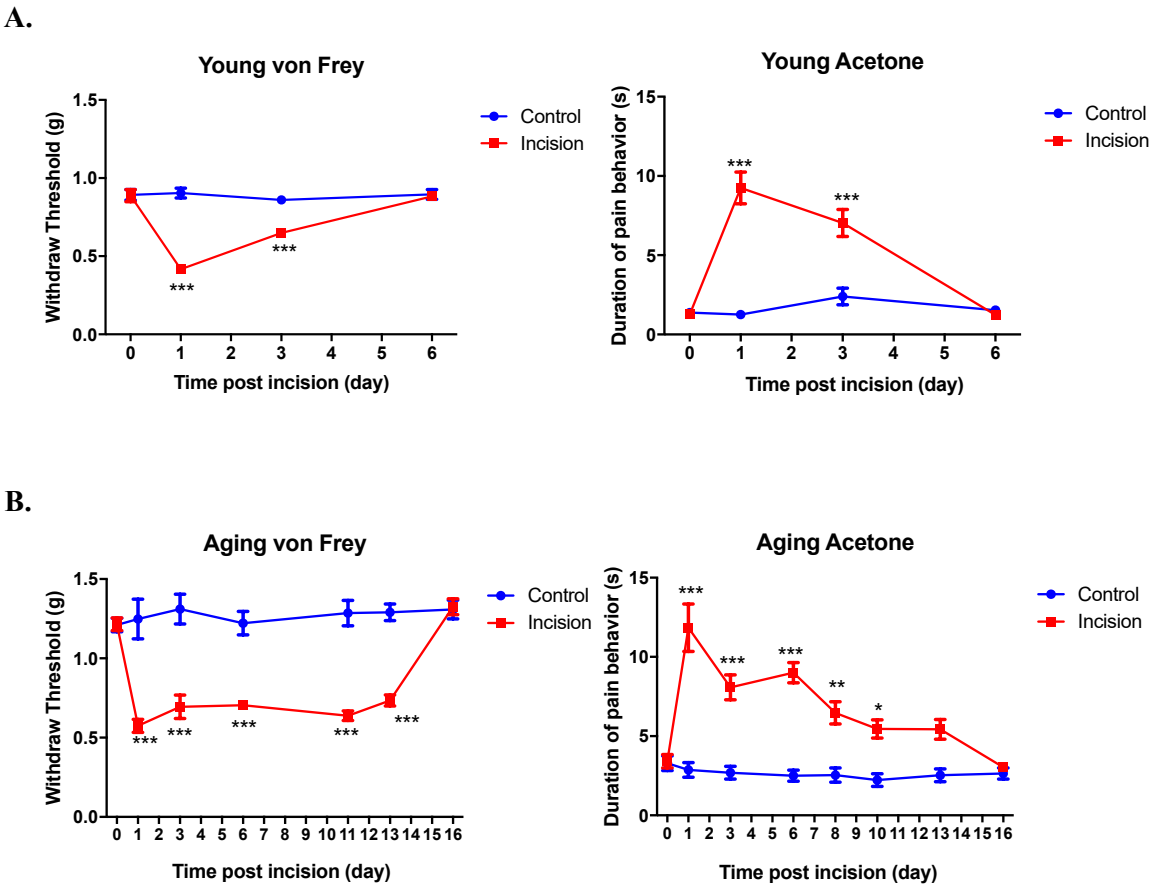


Fig. 5. Aging mice exhibited prolonged post-surgery pain assessed by von Frey and acetone tests.

(A) Surgery-induced mechanical and thermal hypersensitivities in young mice. The post-surgery pain of the young mice peaked at day 1 and ended at day 6. n=9-10 per group. **(B)** Surgery-induced mechanical and thermal hypersensitivities in aging mice. The post-surgery pain of aging mice peaked at day 1 and disappeared at day 16. n=4-5 per group. Data shown as mean ± SEM. Two-way ANOVA with Bonferroni's multiple comparison was applied. *P<0.05, **P<0.01, ***P<0.001.

3. 5. Strong inflammatory response observed at the period where both age groups exhibited post-surgical pain behavior

Based on the behavior results, we further explored the contribution of local inflammation to the post-surgery pain. The incision-induced pain could be divided into two periods, an early period when both groups were experiencing post-surgery pain (from day 1 to day 5) and a late period when only aging mice were painful (from day 6 to day 15).

As the schematic (**Fig. 6A**) description shows, behavior of the mice was monitored from day 1 post-incision till the complete recovery of their post-surgical pain. During the early period, both age groups exhibited enhanced pain (day 1). We collected the skin for immune cell analysis by FACS and inflammatory marker analysis by qPCR. During the late period (day 6 and day 12), when the young mice had already recovered from post-incision pain, while aging mice were still suffering pain from incision, skin samples from both groups were collected for FACS and qPCR.

On the first day after incision surgery, immune cell analysis showed increased CD45+ cells on the ipsilateral side of both age groups (**Fig. 6B** upper panel: $p=0.0213$, $t=3.675$, $df=4$ and $p<0.0001$, $t=25.34$, $df=4$, young and aging mice, respectively). Specifically, CD11b+F4/80+ macrophage was increased on the ipsilateral side (**Fig. 6B** lower panel: $p=0.0192$, $t=3.794$, $df=4$ and $p<0.0001$, $t=26.31$, $df=4$, young and aging mice, respectively). Considering the baseline of aging mice skin showed more immune cells. We analyzed the fold change of ipsilateral side over contralateral side within each age group. Both CD45+ and CD11b+F4/80+ cells of aging group showed higher ipsi over contra fold change than young group (**Fig. 6C**: $p=0.0486$, $t=2.804$, $df=4$ and $p=0.0666$, $t=2.502$, $df=4$, respectively).

In both young and aging groups, compared to the contra side, qPCR showed increased IL6, IL1B, and CCR2 expression levels on the injured side (**Fig. 6D**: IL6-young: p=0.0254, t=2.958, df=6; IL6-aging: p=0.0354, t=3.123, df=4; IL1B-young: p=0.0378, t=2.655, df=6, IL1B-aging: p=0.0110, t=4.473, df=4; CCR2-young: p=0.0206, t=3.119, df=6; CCR2-aging: p=0.0104, t=4.557, df=4). In aging mice, IL10 was also higher, although not significantly, on the injured side (p=0.1146, t=2.012, df=4). Comparing the expression levels of the ipsilateral paws, aging mice showed higher IL6, IL1B, and IL10 expression levels than young mice (**Fig. 6E**: p=0.0153, t=3.616, df=5; p=0.0025, t=5.622, df=5 and p=0.0085, t=4.203, df=5, respectively). In the early period post-incision, both young and aging mice showed elevated local inflammation with mechanical and thermal hypersensitivities. In general, incision triggered inflammatory responses in the skin, including the number of immune cells and the expression of inflammatory molecules, were much stronger in the aging mice than in the young mice.

Figure 6

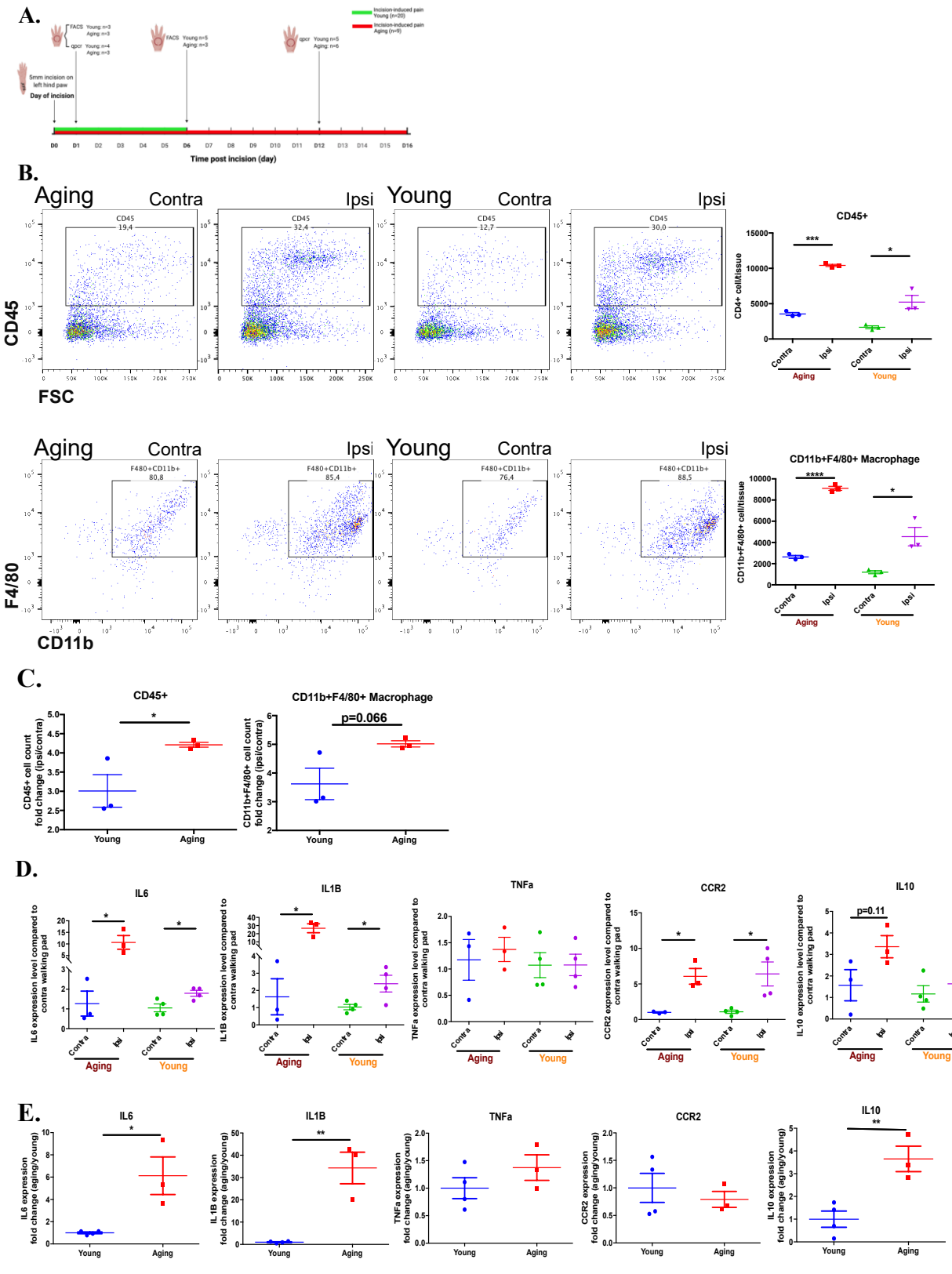

A.
FACS Young: n=3 Aging: n=3
qpcr Young: n=4 Aging: n=3
FACS Young n=5 Aging: n=3
qpcr Young n=5 Aging: n=6
Incision-induced pain Young (n=20)
Incision-induced pain Aging (n=9)
5mm incision on left hind paw
Day of incision
D0 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16
Time post incision (day)
B.
Aging
Contra
Ipsi
Young
Contra
Ipsi
CD45
FSC
CD45+
CD4+ cell/tissue
Aging
Young
F4/80
CD11b
CD11b+F4/80+ Macrophage
CD11b+F4/80+ cell/tissue
C.
CD45+
CD45+ cell count fold change (ipsi/contra)
CD11b+F4/80+ Macrophage
CD11b+F4/80+ cell count fold change (ipsi/contra)
p=0.066
Young
Aging
D.
IL6
IL1B
TNFa
CCR2
IL10
p=0.11
IL6 expression level compared to contra walking pad
IL1B expression level compared to contra walking pad
TNFa expression level compared to contra walking pad
CCR2 expression level compared to contra walking pad
IL10 expression level compared to contra walking pad
Contra
Ipsi
Aging
Young
E.
IL6
IL1B
TNFa
CCR2
IL10
IL6 expression fold change (aging/young)
IL1B expression fold change (aging/young)
TNFa expression fold change (aging/young)
CCR2 expression fold change (aging/young)
IL10 expression fold change (aging/young)
Young
Aging

Fig. 6. Young and aging mice showed elevated local inflammation at day 1 post-surgery.

(A) Experimental design of the molecular experiments after the mice receiving surgery. The post-surgical time was divided into two periods. The early period: both young and aging mice experienced post-surgical pain, from day 1 to day 5. The late period: only the aging mice experienced post-surgical pain, young mice no longer exhibited hypersensitivity, from day 6 to day 15. Green and red bold lines indicated the duration of post-surgery pain of young and aging mice, respectively. Local immune cells and inflammatory markers in the early period were analyzed by FACS and qPCR at day 1. At day 6 and day 12, the late period, FACS and qPCR were performed to investigate the relationship of local inflammation and sustained post-surgery pain in aging mice. **(B)** FACS results at day 1 showed the absolute numbers of increased local infiltrated $CD45^+$ and $CD11b^+F4/80^+$ cells on the ipsilateral skins of both young and aging mice. **(C)** Fold change of ipsi over contra immune cells showed the level of increase in both young and aging groups. Higher ipsi/contra fold change was observed in the aging mice. **(D)** qPCR results at day 1 showed the expression levels of IL6, IL1β and CCR2 increased on the ipsilateral side in both young and aging mice. No difference was observed in TNFα expression level. **(E)** Expression levels of inflammatory markers on the ipsilateral side were normalized to the young mice. Aging mice showed higher expression level of IL6, IL1β, and IL10 on the ipsilateral side compared with the young mice. n=3-4 per group. Data shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3. 6. Persistent inflammatory response observed only in aging mice when post-surgical pain lasted

On day 6 post incision, the young mice had no longer exhibited mechanical and cold allodynia, while the aging mice were still painful. We collected the skin samples from both groups for FACS. CD45+ immune cell showed larger amount at the injured side of the aging mice (**Fig. 7A** upper panel: p=0.0293, t=3.324, df=4), while no difference between ipsilateral and contralateral sides was shown in the group of young mice. Similarly, an increased number of CD11b+F4/80+ macrophages at the ipsilateral side was found only in the aging mice (**Fig. 7A** lower panel: p=0.0310, t=3.264, df=4). Comparing the fold change of ipsi over contra within each age group, aging mice had higher fold changes than young mice for both cell populations (**Fig. 7B**: p=0.0375, t=2.661, df=6 and p=0.0241, t=2.996, df=6, for CD45+ and CD11b+F4/80+, respectively).

On day 12 post incision, we collected the skin samples for qPCR. Similar to day 1, IL6 and CCR2 were significantly higher on the injured side of aging mice (**Fig. 7C**: p=0.0267, t=2.594, df=10 and p=0.0114, t=3.09, df=10, respectively), IL1B also showed an increasing trend (p=0.0777, t=1.966, df=10). Different from day1, ipsilateral IL10 was decreased in aging mice. Comparing the expression levels of these markers between different age groups, the IL6, IL1B, TNFa and CCR2 were higher in the injured skin of the aging group (**Fig. 7D**: p=0.0432, t=2.313, df=10; p=0.0546, t=2.176, df=10 and p=0.0355, t=2.526, df=8, respectively), while IL10 expression was lower in aging mice (p=0.0034, t=4.117, df=8) compared to young mice at day 12.

In the late period post-incision, young mice had recovered from pain and local inflammation, while aging mice exhibited elevated inflammatory markers parallel with mechanical and thermal hypersensitivities.

Figure 7

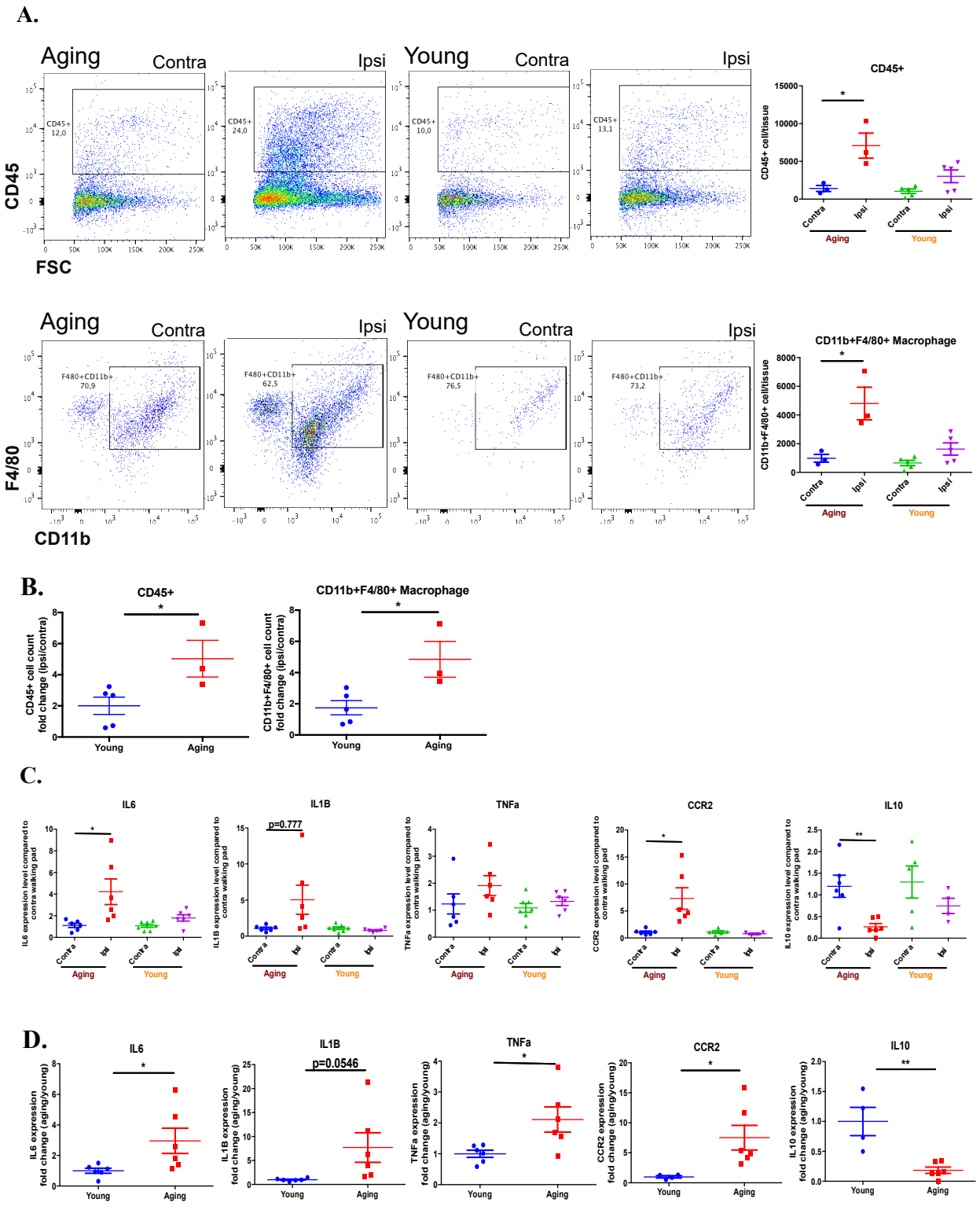

A.
Aging
Contra
Ipsi
Young
Contra
Ipsi
CD45
FSC
CD45+
CD45+ cell/tissue
Aging
Young
F4/80
CD11b
CD11b+F4/80+ Macrophage
CD11b+F4/80+ cell/tissue
B.
CD45+
CD45+ cell count fold change (ipsi/contra)
CD11b+F4/80+ Macrophage
CD11b+F4/80+ cell count fold change (ipsi/contra)
Young
Aging
C.
IL6
IL1B
TNFa
CCR2
IL10
IL6 expression level compared to contra walking pad
IL1B expression level compared to contra walking pad
TNFa expression level compared to contra walking pad
CCR2 expression level compared to contra walking pad
IL10 expression level compared to contra walking pad
p=0.777
D.
IL6
IL1B
TNFa
CCR2
IL10
IL6 expression fold change (aging/young)
IL1B expression fold change (aging/young)
TNFa expression fold change (aging/young)
CCR2 expression fold change (aging/young)
IL10 expression fold change (aging/young)
p=0.0546

Fig. 7. Aging mice showed elevated local inflammation at day 6 and day 12 post-surgery.

(A) FACS results at day 6 showed the absolute numbers of increased local infiltrated $CD45^+$ and $CD11b^+F4/80^+$ cells on the ipsilateral skins of the aging mice. No significant difference was observed in the young mice. **(B)** Fold change of ipsi over contra immune cells the level of increase in both young and aging groups. Higher ipsi/contra fold changed was observed in the aging mice. **(C)** qPCR results at day 12 showed the expression levels of IL6 and CCR2 were increased while IL10 level was significantly decreased on the ipsilateral side of the aging mice. No difference was observed in TNFα expression level compared with the contra side. No difference was observed in any of these markers in the young mice. **(D)** Expression levels of inflammatory markers on the ipsilateral side were normalized to the young mice. Aging mice showed higher expression levels of IL6, TNFα and CCR2 on the ipsi side compared with the young mice. IL10 expression level was decreased on the ipsi side of aging mice compared with the young mice. n=3-7 per group. Data shown as mean ± SEM. *P<0.05, **P<0.01.

DISCUSSION

In the present study, we investigated the contribution of inflammation to age-related altered pain behavior by characterizing the systemic inflammation in female young and aging mice. Secondly, we assessed their post-surgery local inflammation, which was accompanied by prolonged post-surgery pain. To establish the inflammaging profile, we analyzed the abundance, phenotype and functions of circulating immune cells. Administration of the serum from 18-month-old aging mice induced altered mechanical and thermal thresholds in the 2-month-old young mice. The aging mice receiving incision surgery exhibited longer-lasting post-surgery allodynia and local inflammation compared with the young mice. These data demonstrated that the inflamed serum of the aging mice had allodynic effects in the young mice. Local inflammation and hypersensitivity induced by surgery lasted longer in the aging mice compared to young mice.

In our study, the immune cell analysis by FACS suggested that aging affects cells in both innate immunity and adaptive immunity, which is consistent with the previous literatures describing immunosenescence (Franceschi, Bonafè, & Valensin, 2000; Ongrádi & Kövesdi, 2010). Innate immunity upregulated with age, while adaptive immunity is dampened (Franceschi, Bonafè, & Valensin, 2000). Our data showed that $CD11b^{+}CD115^{+}$ monocyte and its $CCR2^{+}$ and $CX3CR1^{+}$ proportions increased in the blood of the aging mice. Based on the expression of surface receptors, the monocytes of mice could be subdivided into $Ly6C^{high}CCR2^{high}CX3CR1^{low}$ classical monocytes and $Ly6C^{low}CCR2^{low}CX3CR1^{high}$ non-classical monocytes, with the functional equivalents $CD14^{++}CD16^{-/+}$ and $CD14^{+}CD16^{++}$ monocytes in humans, respectively (Cros et al., 2010; Geissmann et al., 2003). During infection,

the monocytes egress from the bone marrow to the periphery in the presence of CCR2 and preferentially homes to the sites of inflammation (Dunay et al., 2008). $CCR2^{+}$ inflammatory monocytes produce high levels of inflammatory cytokines (Kim et al., 2011). The increase of $Ly6C^{high}$ monocytes has been found in the aging mice with more pronounced CCR2 expression, which was demonstrated to be entirely dependent on the bone marrow microenvironment of the aging mice (Bodogai et al., 2018; Puchta et al., 2016; Strohacker et al., 2012). This increase in monocyte proportions has also been observed in human studies. Human monocytes from elderly participants showed increased levels of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ subsets (Hearps et al., 2012; Nyugen et al., 2010; Seidler et al., 2010). The classical monocytes produce more pro-inflammatory cytokines (e.g. IL6, TNF, IL1β) with higher expression levels of chemokine receptors (e.g. CCR2, CCR5, CX3CR1) (Álvarez-Rodríguez et al., 2012; Merino et al., 2011). Although monocytes are historically considered precursor cells for macrophages, they are also known as immune effector cells (Geissmann et al., 2010). The $CD14^{++}CD16^{+}$ monocytes derived from elderly individuals showed impaired phagocytosis capacity with senescent feature (i.e. have shortened telomeres) (Hearps et al., 2012). The defection of monocytes finally leads to an accumulation of cellular debris from apoptotic cells and mononuclear phagocytes in pro-inflammatory phenotypes, which ultimately contribute to the chronic inflammation in the aged population (De Maeyer et al., 2020).

Besides monocytes, we investigated increased absolute numbers of $CD45^{+}$ leukocytes and $F4/80^{+}CD11b^{+}$ macrophages in the skin, concordant with the increased blood macrophages in normal aging mice, as reported by the past study (Strohacker et al., 2012). Despite the enriched absolute number, aging macrophages have been previously reported to exhibit age-related functional and phenotype defects, including decreased Toll-like receptor expression and

reduced phagocytic capacity while increased production of inflammatory cytokines (Bouchlaka et al., 2013; Bruunsgaard et al., 2001; Koike et al., 1999; Renshaw et al., 2002).

In our study, we observed up-regulated number of neutrophils and downregulated number of NK cells in the aging mice. Dysfunction of NK cells is commonly seen in aging due to the maturation defect (Chiu et al., 2013; Hazeldine & Lord, 2013; Nair et al., 2015). The production rate of NKs is nearly halved in elderly people, which contributes to three times higher mortality risk (Gounder et al., 2018; Ongrádi & Kövesdi, 2010; Remarque & Pawelec, 1998). The reciprocal interaction between dendritic cells (DCs) and NKs is well-known (Cooper et al., 2004; Fernandez et al., 2002; Guo et al., 2014). DCs enhance NKs functions by direct cell-cell contact and cytokines release, such as IL12 and IL15 (Ferlazzo et al., 2004). Aging mice have significantly decreased and defective DCs, which lead to failed activation of NKs from either young or old mice in vivo or in vitro (Guo et al., 2014). NKs also play a role in the cross-talk between innate and adaptive immune responses (Vivier et al., 2011) by producing cytokines and expressing co-stimulatory molecules, resulting in the promotion of T cell priming and differentiation (Vivier et al., 2008; Zingoni et al., 2004).

Immunosenescence has a large impact on adaptive cell numbers and their repertoires. The exhaustion of the adaptive immunity in aging is characterized as a shift from naïve lymphocytes to memory lymphocytes (Cossarizza et al., 1996; Fagnoni et al., 2000; Johnson et al., 2002), which leads to a less functional adaptive compartment in the aging populations. This is also supported by our results, described as decreased $CD3^+$, $CD4^+$ and $CD8^+$ T cell with increased $CD44^-CD62L^+$ T cell, and decreased $CD19^+$ B cell. Adaptive immune incompetence seen in the aging population is mainly caused by the decline in the production of naïve lymphocytes and the increased survival of memory lymphocytes (Keren et al., 2011). Past middle age, the thymic

space for T cell development continuously declines (Steinmann et al., 1985). Early studies indicated that high CD8-positive T cell and low CD4-positive T cell percentages are associated with higher mortality, which was observed in elderly people (Ferguson et al., 1995; Goronzy et al., 2001; Wikby et al., 2002). The decline in T/B-lymphopoiesis is also associated with increasing prevalence and severity of infections, and impaired response to vaccination in the aging population (Ciabattini et al., 2018; Crooke et al., 2019; Lord, 2013; Riley, 2013). Old mice exhibited a significant decrease in B cell production and the frequency of circulating B cells (Gibson et al., 2009; Miller & Allman, 2003). In the last decade, the removal of "aging" senescent lymphocytes or tissue has emerged as a promising rejuvenation strategy in the last decade (Keren et al., 2011). B cell depletion in both elderly mice and humans induces de novo B lymphopoiesis, which rejuvenates the peripheral B cell compartments (Avivi et al., 2019). However, the result also showed by B cell rejuvenation alone is not sufficient to completely reconstitute the vaccine responsiveness in the aging mice. Therefore, it is warranted to look at strategies by rejuvenating other immunity components. Administrating IL7, IL12, IL15, and other cytokines have been demonstrated as emerging approaches in preclinical mouse models to restore thymic function and T cell regeneration (Bailey et al., 2017; Li et al., 2004; Rosenberg et al., 2006). IL21 is an important cytokine for thymic function. Administration of recombinant IL21 was proved to improve thymic regeneration and to restore the peripheral T cell compartment in the aging mice (Al-Chami et al., 2016).

Despite the declined functions of innate and adaptive immune cells with age, a chronic low level of systemic inflammation has been observed in the elderly, which is attributed to the increased basal inflammatory molecules production by the immune cells such as monocytes and neutrophils (Franceschi et al., 2007). Our study showed increased expression levels of IL6, IL10,

CCR2, CXCL5 and decreased CCL2 in the blood immune cells of the aging mice. Although studies have reported increased serum levels of TNFα and IL1β, we didn't observe any change of TNFα and IL1β at the transcriptional level (Álvarez-Rodríguez et al., 2012; Bruunsgaard et al., 2001; Franceschi, Bonafè, Valensin, et al., 2000; Puchta et al., 2016; Varadhan et al., 2014). Previous studies hypothesized that as the precursor of IL6 and CRP secretions, the production of TNFα could be inhibited by IL6, which maintained the level of TNFα (Mizuhara et al., 1994; Schindler et al., 1990; Starkie et al., 2003). We did observe high levels of IL6, which is well-known to be associated with increased morbidity and mortality among older individuals (Harris et al., 1999; Maggio et al., 2006; Puzianowska-Kuźnicka et al., 2016). Interestingly, as a potent anti-inflammatory mediator, IL10 mRNA and serum levels are also increased in aged individuals (Almanan et al., 2020; Garg et al., 2014). IL10 level appears to counter-act inflammation by balancing pro-inflammatory cytokines to promote healthy aging. High IL10 production is more prevalent in centenarians than in younger individuals (Lio et al., 2004). Moreover, the highest age-related risk of frailty-associated pathologies has been observed in the elderly men with the highest serum levels of inflammatory cytokines or with the lowest levels of IL10 (Cauley et al., 2016). Using a multidimensional approach on 19 inflammatory markers, increase in pro-/anti-inflammatory responses were identified, both of which were strongly correlated with age. The axis included IL6, hsCRP, TNFα, IL18 and IL1RA, indicating a more activated but not necessarily more inflamed immune system (Morrisette-Thomas et al., 2014). ELISA results elicited a profound age-dependent MCP-1 (CCL2) increase, which is related to cognitive impairments and mild Alzheimer's disease (Bettcher et al., 2019; Conley et al., 2016; Galimberti et al., 2006; Inadera et al., 1999). Discordantly, we detected a significantly lower level of CCL2 expression in blood immune cells. A possible explanation could be the difference between

protein and mRNA measurements. The higher serum levels of CCL2 could be released by other blood vascular cells (Khyzha et al., 2019).

The aberrant circulating factors in the aging blood are the critical contributors to age-associated inflammation. Rejuvenation strategies in mice models have been studied to attenuate inflammaging and other age-related comorbidities. Administration of young blood plasma counteracts aging at the functional and cognitive levels in the aged brain by improving synaptic plasticity in the hippocampus (Villeda et al., 2014). By applying a shared circulatory system (i.e. heterochronic parabiotic pairing) between young and old mice, studies restored the Notch ligand expression and enhanced tissue-specific stem cell activity (Conboy et al., 2005), restored CNS remyelination by recruitment of monocytes to the repairing lesions (Ruckh et al., 2012), and improved neurogenesis and cognitive function (Villeda & Wyss-Coray, 2013), although it failed to restore the immune system of the old partner (Pishel et al., 2012). The extracted extracellular vesicles (EVs) or exosomes from the young serum pool was shown to decline systemic pro-inflammatory IL6 in the old mice, which was associated with the rejuvenation of thymic aging (Wang et al., 2018). However, the relationship between the aging systemic environment and the altered allodynic sensitivities remains a gap of knowledge.

In our study, we observed altered baseline responses to mechanical and cold stimuli in the aging mice. The mechanical threshold of normal aging mice varies between different laboratories (Sheldon R. Garrison & Cheryl L. Stucky, 2014; Muralidharan et al., 2020; Sadler et al., 2017; Weyer et al., 2016). Discordant changes in age-related pain perception have been reported in past studies, where there are mainly two schools of thought: 1) there is increased pain sensitivity, or 2) there is unaltered or decreased pain sensitivity in the aging population (Yezierski, 2012). Partially contradictory to our study, Millecamps et al. demonstrated that the

aging mice have a more pronounced response to tonic stimuli (acetone and capsaicin) while unchanged mechanical thresholds, compared with the young mice (Millecamps et al., 2020). Besides the differences in baseline pain sensitivities, formalin-induced acute pain and nerve injury-induced chronic pain have also been observed to be altered in the aging mice. Following formalin injection, aging mice developed more prolonged and intense mechanical allodynia which was maintained for 180 minutes (Sadler et al., 2017). Following spinal cord injury, aging mice were more susceptible to thermal hypersensitivity and reduced survival (Gaudet et al., 2021).

An interesting observation in this thesis is that we detected a decrease of both thermal and mechanical thresholds in the young mice after receiving serum from the aging mice, for which we assume that the increase of inflammatory molecules in the aging serum could player an important role. However, we also observed that in aging mice, their mechanical thresholds are indeed higher than young mice. Although we don't have a clear explanation for this discordant phenomenon now, we assume that, in addition to inflammation, mechanical sensitivity in aging mice might be mediated by other mechanisms as well. For examples, some mechanical sensitivity related neuronal function could be inhibited in aging mice, and such abnormalities might overcome the effect of inflammation. This hypothesis will need further investigation in the future. At least, our data support the concept that different pain modalities could be mediated by different mechanism.

The clinical and social burdens of chronic post-surgery pain have been largely recognized in the past 20 years. However, the specific impact of CPSP in the aging population is yet to be elucidated. To our knowledge, we are the first study that sought to compare post-surgery pain in young and aging mice. Our results showed prolonged post-surgery pain in female aging mice

compared to female young mice, without significant difference in their pain sensitivity. Besides, the local inflammation of the aging mice also lasted longer than in the young mice, which is parallel with the duration of post-surgical pain. Despite the increased number of local macrophages in the aging mice, the age-related aberration in their function is one of the contributing factors to the prolonged wound healing by delaying angiogenesis, re-epithelialization, and collagen remodeling (Krzyszczyk et al., 2018). The lack of matrix molecule production and the presence of inflammation lead to inferior extracellular matrix (ECM) production in the aging individuals, whereas the skin of younger individuals responds to the injury by a robust ECM production (Gould et al., 2015). The circulating pro-inflammatory cytokine, TGF-β, which has increased expression and secretion in the aging rodents (Tazawa et al., 2021), promotes the transformation of wounds from the acute phase into the chronic phase by attenuating re-epithelialization (Pohlers et al., 2009). Thus, even in the healthy elderly, the presence of increased basal inflammation could lead to the slow would healing process. Following a nerve crush injury, aging mice were found to exhibit damaged terminal Schwann cells (TSCs) extensions, which interrupted the regeneration and reinnervation process (Wang et al., 2007). Study on chronic complete Freund's adjuvant (CFA)-induced pain revealed the difference of nociceptor sensitization between young and aging mice, where transient receptor potential ankyrin 1 (TRPA1) played a key role in transition from acute pain to chronic inflammatory pain (Sheldon R Garrison & Cheryl L Stucky, 2014). During chronic inflammation, the mechanical sensitization in C fibers and responsiveness of A-mechanonociceptor (AM) fibers were reduced in the young mice. These reductions limited the pain afferent information carried to the CNS, where central sensitization plays a crucial role in chronic pain development (Woolf, 2011).

Two potential limitations of the current study are discussed here. Firstly, the post-surgical local inflammation assessments in the present study had a minimum experimental sample size of aging animals, which was due to the limited access to aging animals. Further study could include a larger number to solidify the results. Secondly, the present study has not addressed systemic inflammation or altered pain sensitivity in male mice due to limited time and access to the aging animals.

As our study focuses on female young and aging mice, future investigations with male mice should be considered. Despite the well-characterized sex differences in chronic pain prevalence, pain mechanism, immune regulation and longevity, studies have also investigated that aging differently affects peripheral blood cells and inflammatory makers in males and females. The genomic differences between sexes on peripheral blood mononuclear cells increase after age 65, which leads to higher innate and pro-inflammatory activity with lower adaptive activity in males than in females. Elderly females showed a bias towards adaptive immunity (Márquez et al., 2020). Model on older adults showed women have a higher level of inflammatory markers than males, with an overall rate of increase in inflammation with age (Yang & Kozloski, 2011). In the finding of positive correlations between blood hsCRP and IL6, female subjects presented stronger correlations with the highest values in the 51-60 age group (Milan-Mattos et al., 2019). The female sex hormone estrogen provides anti-inflammatory effects which improve host resistance to pathogens and reduce the risk of cardiovascular disease (Iorga et al., 2017; Villa et al., 2015). As a result, the age-related exhaust of ovarian oocytes and the decline of estrogen contribute to the more inflamed environment in females (Khera et al., 2005; McCarthy & Raval, 2020). Our pilot experiments using multiplex systemically screened the inflammatory markers in the serum of both male and female mice. A more inflamed profile

was detected in the female aging mice, including more elevated levels of TNFα, IL6, CCL2, CCL4, CCL5, and IL10, compared to male aging mice (data not shown). A detailed characterization of immune profile in male, as well as its relationship with pain sensitivity are needed in future investigation.

Lastly, although we characterized systemic and local inflammation, we could not appoint them as the causative factors of the altered post-surgical pain thresholds in the aging mice. We hypothesized that the systemic inflammation provided an inflamed environment and contributed to the local inflammation, which would make the aging mice more susceptible to prolonged allodynia stimuli. Modulation of systemic inflammation could be considered a valid strategy for pain improvement in the aging population. Recent human and rodent studies have confirmed the intake of anti-inflammatory diet and early-onset, lifelong physical exercise attenuated the progression of inflammaging (de Lemos Muller et al., 2019; Nilsson et al., 2019; Wawrzyniak-Gramacka et al., 2021). Moreover, bone marrow stromal stem cells (BMSCs) have been demonstrated to elicit immunomodulatory signals and attenuate the inflammatory responses (Fan et al., 2020; Hofer & Tuan, 2016). Besides, BMSCs in animal models showed analgesic effects by regulation of neuroinflammation in the peripheral and central nerve systems (Fischer et al., 2017; Li et al., 2017; Yousefifard et al., 2016). The contributions of anti-inflammatory diet, physical exercise or BMSCs in age-related pain modulation are remained to be elucidated. Complementary studies could consider applying BMSCs or physical exercise, as multi-target modulators, to delay the overall inflammatory process, and to improve the altered pain experienced in the aging mice.

CONCLUSION

In summary, our study characterized the systemic inflammation profile and described the prolonged post-surgical pain parallel with elevated local inflammation in the aging mice. The results described in this thesis suggested that (1) the female aging C57BL/6 mice possess systemic inflammation, characterized by prevailing innate immunity, decreased adaptive immunity and elevated expression levels of pro- and anti-inflammatory markers, (2) the serum from the aging mice exerts an allodynic effect and impacts both thermal and mechanical sensitivities of the young mice, (3) the post-surgical pain lasts longer in the aging mice, accompanied with local inflammation.

To our knowledge, this is the first study about post-surgical pain in the aging population. The applications of different pain models in aging mice warrants further elucidations of chronic pain conditions in humans. The present study provides an important advance in the current knowledge of post-surgical pain and inflammation in young and aging mice, which is valuable translational data for human studies.

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