BRAIN
ORIGINAL ARTICLE

OnabotulinumtoxinA alters inflammatory gene expression and immune cells in chronic headache patients

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Occipital headache, the perception of pain in the back of the head, is commonly described by patients diagnosed with migraine, tension-type headache, and occipital neuralgia. The greater and lesser occipital nerves play central role in the pathophysiology of occipital headache. In the clinical setup, such headaches are often treated with onabotulinumtoxinA, a neurotoxin capable of disrupting ability of nociceptors to get activated and/or release proinflammatory neuropeptides. Attempting to understand better onabotulinumtoxinA mechanism of action in reducing headache frequency, we sought to determine its effects on expression of inflammatory genes in injected occipital tissues.

To achieve this goal, we injected 40 units of onabotulinumtoxinA into four muscle groups (occipitalis, splenius capitis, semispinalis capitis, and trapezius muscles—all located on one side of the occiput) of patients with chronic bilateral occipital headache scheduled for occipital nerve decompression surgery 1 month later. At the time of surgery, we collected discarded muscle, fascia and periosteum tissues from respective locations on both sides of the neck and occiput and performed targeted transcriptome analyses to determine expression level of inflammatory genes in onabotulinumtoxinA-injected and onabotulinumA-uninjected tissues.

We found that (i) onabotulinumtoxinA alters expression of inflammatory genes largely in periosteum, minimally in muscle and not at all in fascia; (ii) expression of inflammatory genes in uninjected periosteum and muscle is significantly higher in historical onabotulinumA responders than historical non-responders; (iii) in historical responders' periosteum, onabotulinumA decreases expression of nearly all significantly altered genes, gene sets that define well recognized inflammatory pathways (e.g. pathways involved in adaptive/innate immune response, lymphocyte activation, and cytokine, chemokine, NF-kB, TNF and interferon signalling), and abundance of 12 different immune cell classes (e.g. neutrophils, macrophages, cytotoxic T-, NK-, Th1-, B- and dendritic-cells), whereas in historical non-responders it increases gene expression but to a level that is nearly identical to the level observed in the uninjected periosteum and muscle of historical responders; and surprisingly (iv) that the anti-inflammatory effects of onabotulinumA are far less apparent in muscles and absent in fascia.

These findings suggest that in historical responders' periosteum—but not muscle or fascia—inflammation contributes to the pathophysiology of occipital headache, and that further consideration should be given to the possibility that onabotulinumA mechanism of action in migraine prevention could also be achieved through its ability to reduce pre-existing inflammation, likely through localized interaction that lead to reduction in abundance of immune cells in the calvarial periosteum.

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Abbreviations: GON = greater occipital nerve; onabotA = onabotulinumtoxinA

Introduction

Occipital headache, the perception of pain in the back of the head, is commonly described by patients diagnosed with migraine,[1] tension-type headache,[2] occipital neuralgia[3] and whiplash injury.[4] It is often accompanied by tenderness in posterior neck muscles, allodynia affecting the back of the head, and the perception of pain outside and inside the occipital bone.[5] Relevant to the current study, headache in the occipital region is frequently the first sign of a commencing migraine or migraine-like headache that over time (minutes or hours) can migrate frontally to involve the periorbital and temporal regions of the head.[6,7]

Recent anatomical findings suggest that axons of C2 and C3 dorsal root ganglion cells that make up the greater and lesser occipital nerves and carry sensory and nociceptive signals from muscles of neck, occipital periosteum, scalp and dura overlying the cerebellum and occipital cortex may play central role in the pathophysiology of occipital headache, occipital allodynia and neck muscle tenderness.[8]

Following a traditional neural path, this knowledge had paved the way to attempts to prevent/reduce occurrence of migraine and migraine-like headache using procedures such as occipital nerve blocks,[9] occipital nerve stimulation,[10] occipital nerve decompression surgeries,[11] radiofrequency lesions of C2-3 dorsal root ganglia,[12] and local injections of onabotulinumtoxinA (onabotA)[13] that disrupt the ability of sensory nerves in the occipital region to transmit nociceptive signals to the spinal trigeminal nucleus. Given the peripheral nature of these interventions and current understanding of onabotA ability to block release of presynaptic vesicles and attenuate receptors insertion into the synaptic membrane,[14,15] it is not surprising that until now, all effort to explain the mechanism by which onabotA reduces migraine frequency, has focused on its ability to interfere with proper synaptic detection of nociceptive stimuli and their transmission along sensory nerves of the calvaria, as well as its ability to relax cranial and pericranial muscles by blocking acetylcholine release at the neuromuscular synapse.[16]

While much progress has been made in understanding the neural mechanism by which extracranial injections of onabotA can interfere with the detection and transmission of nociceptive signals by trigeminal and cervical nerves endings whose role in migraine headache is widely recognized, it is also imperative to note that sensory neurons and nociceptors play a variety of roles in regulating immune and inflammatory responses.[17,18] Seeking to widen the scope of scientific thinking about the mechanism by which onabotA prevents migraine, we hypothesized that extracranial injections of this neurotoxin may affect inflammatory processes in injected tissues. The pursue of this novel and less traditional 'inflammatory' path, follows a recent study showing that expression of key proinflammatory genes is increased in the occipital periosteum of chronic migraine patients whose headaches begin in the back of the head, as compared to subjects with no history of headache.[5] Based on that study, and on the effectiveness of anti-inflammatory drugs in the acute treatment of migraine attacks[19,20] and in delaying progression from episodic to chronic state,[21–23] we sought to determine levels of expression of inflammatory genes, strength of inflammatory pathways, and relative density of immune/inflammatory cells in respective onabotA/injected and onabotA/uninjected periosteum, muscle and fascia tissues of the posterior neck and occiput of patients whose headache involves both sides of the occiput.

Materials and methods

This study was designed to evaluate whether treatment with onabotA was associated with modulation of inflammatory genes. It is worth noting that this was not a full genome analysis rather restricted to the genes on the nanostring inflammatory panel. We designed this study in patients with headache who were scheduled for a surgical procedure and thus provided an opportunity to pretreat with onabotA systematically, in a controlled setting. Consequently, the initial analyses plan was to evaluate inflammatory gene findings associated with treatment. A subsequent analyses was performed to integrogate whether gene findings were associated with clinical characteristiscs in this patient population.

All aspects of this study were carried out in compliance with the 1983 revision of the 1975 Helsinki Declaration, and according to the clinical ethical standards of Beth Israel Deaconess medical center (2016-C-00612) and Massachusetts General Hospital (2017-P-00183) Committees on Clinical Investigation on Human Experimentation.

Participants

Included in the study were (i) patients experiencing bilateral occipital headache with migrainosus features; (ii) who deemed to be appropriate candidates for an occipital nerve decompression surgery;

and (iii) fulfil protocol criteria for treatment with onabotA prior to surgery. Those deemed appropriate candidates for the surgery were presented with an option to hear about the study and sign the informed consent. Criteria for selecting patients for the nerve decompression surgery included: (i) diagnosis of chronic bilateral occipital headache with and without migraine symptoms; (ii) headache and pain that correspond to the anatomical distribution of the greater occipital nerve (GON); and (iii) refraining from taking medication that affect blood clotting processes [such as non-steroidal anti-inflammatory drugs (NSAIDs)] for 7–10 days before surgery. Excluded from the study were patients <18 years of age, patients with medical conditions that increase risk of anaesthesia and those who were pregnant or trying to become pregnant within the timeframe of the study, and patients unable or unwilling to give written informed consent.

Tissue collection and preparation

The tissue biopsies were taken by the study surgeon (W.G.A.). During GON decompression, a midline incision was made in the hair bearing scalp distal to the occipital protuberance. Tissues compressing the GON were removed including the trapezius fascia, semispinalis capitis muscle, and occipital periosteum (Fig. 1). The most superficial layer that was biopsied was the trapezius fascia directly overlying the GON nerve. The trapezius fascia is a distinct layer of white tissue that surrounds the trapezius muscle in its entirety. Fascia is usually a thin and pliable layer of white connective tissue. However, as shown by Gfrerer *et al.*,[24] in patients who present for surgery with a diagnosis of occipital neuralgia, and/or chronic migraine and/or chronic headache, the fascia is thickened and fibrotic. In patients with thickened trapezius fascia, the white colour still allows distinction of fascia from red muscle fibres. Based on histologic staining, small nerve fibres, vessels and muscle fibres can be present in fibrotic trapezius fascia. The next type of tissue that was biopsied was the semispinalis capitis muscle. The semispinalis capitis muscle lies deep to the trapezius muscle and trapezius fascia and has vertical muscle fibres as compared to the trapezius muscle that has diagonal muscle fibres. This allows for differentiation of both muscles. The semispinalis capitis muscle appeared normal in all patients, which is consistent with prior findings.[24] The semispinalis capitis muscle was harvested at the exit point of the GON as the nerve emerges from the muscle. The deepest tissue type that was harvested was the occipital periosteum that lies directly under the GON overlying the occipital bone at the nuchal ridge. The periosteum is a shiny white layer of tissue that surrounds the occipital bone and that is well vascularized. This tissue appeared macroscopically normal in all patients. All tissues were harvested sharply with a knife and immediately placed in Storage solution. Collected tissues were stored in RNAlater and snap frozen in liquid nitrogen immediately after removal from the body. Outside the operating room, tissues were stored at −80°C until processing. Frozen tissues were homogenized using automated cell homogenizer for extraction of RNA. RNA was extracted using a total RNA preparation kit from Qiagen Biotechnology Company. RNA quantity and quality (i.e. percentage of fragments longer than 200 bp) were determined using Agilent Bioanalyzer. Only high-quality RNA (distribution value 200 or higher) was used for the transcriptome profiling.

NanoString gene expression analysis

NanoString technologies were used for the targeted transcriptome profiling. NanoString is a polymerase-free and amplification-free

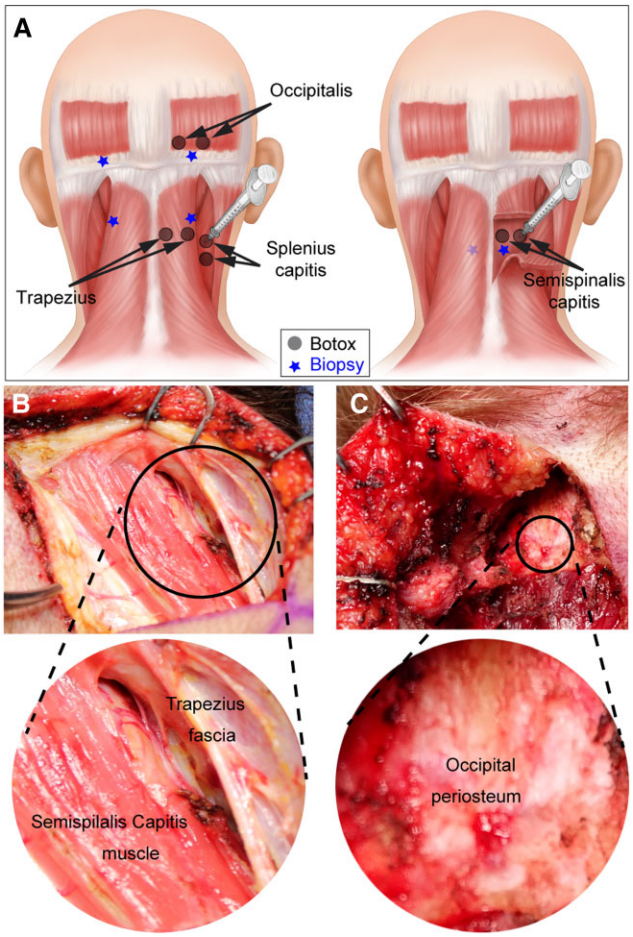


Figure 1 Locations of onabotA injections and biopsies collection. (**A**) Anatomical illustration of eight unilateral locations at which onabotA was injected (5 units/site), and six bilateral locations at which periosteum, muscle and facia biopsies were collected. (**B** and **C**) Photomicrographs depicting sites of biopsies collections in the trapezius facia, semispinalis muscle and occipital periosteum.

nucleic acid quantification platform based on hybridization chemistry. The method involves mixing RNA with pairs of capture and reporter probes tailored to each gene, hybridizing, washing away excess probes, immobilizing probe-bound genes on a surface, and scanning colour-coded bar tags on the reporter probes to calculate expression level or copies of target genes in solution. This solution-phase hybridization results in minimizing background signal and improving detection of low-abundance genes (<1 mRNA per cell).

Approximately 100 ng of total RNA extracted from frozen tissues were hybridized to the NanoString™ Human Inflammation panel (579 genes related to inflammation and immune responses) at 65°C overnight. Hybridized samples were processed on an nCounter prep station and data collected on an nCounter digital analyser (NanoString™), following manufacturer's instructions. Raw data were imported into nSolver4.0 (NanoString™) for data quality check, background thresholding and normalization. Background level was determined by mean counts of eight negative control probes plus 2 standard deviation (SD). Samples that contain <50% of probes above background, or that have imaging or positive control linearity flags, were excluded from further analysis. Probes that have raw counts below background in all sample groups were excluded from differential expression analysis to avoid false

positive results. Advanced Analysis package2.0 (NanoString™) was used for statistical analysis. Briefly, raw data were first normalized by geometric mean of housekeeping genes. Samples with normalization factor >3 or housekeeping gene mean square error >0.5 (indicating insufficient or degraded RNA input) were excluded from analysis. Qualified samples were grouped based on tissue types and treatments. Differential expression (DE), gene set analysis (GSA) and cell type profiling analyses were performed among groups with matched tissue types and/or treatments.[25,26] All statistical analyses on NanoString data were performed on log2 transformed normalized counts.

Statistical analyses

Prior to conducting the analyses, the assumptions underlying the statistical approaches were evaluated with histograms and descriptive statistics. Because the gene expression profiles exhibited substantial variability and positive skew, the log of the values were used in the statistical modelling. A linear mixed effects model was conducted to examine differences in gene expression while accommodating the two sources of repeated measures within individuals: side (i.e. injected versus uninjected side) and inflammatory genes (i.e. 579 genes for each side). To model the sources of variation, a random intercept was specified at the level of individual and gene. Fixed-effects were specified as main effects for responder (i.e. responder versus non-responder), injection-side (injected versus uninjected side), and the interaction between responder and injection-side (i.e. responder × side). To facilitate interpretation, descriptive statistics are presented in the original mRNA units using median (25th, 75th) while effect sizes are reported as the ratio of the geometric means using per cent change. The models were conducted using the 'lme4' package in R4.0 and R-Studio. Where appropriate, all analyses are two-tailed with $P < 0.05$ denoted for statistical significance.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Participants

Included in the study were patients with bilateral occipital headache with migrainous features, who were deemed to be appropriate candidates for an occipital nerve decompression surgery.

Experimental protocol

After signing the informed consent, participants filled a migraine surgery screening form and a headache questionnaire. Those deemed appropriate candidates, received eight injections of onabotA (40 units total) in anatomical areas involved in the nerve decompression surgeries. These areas include: occipitalis (two injections, five units each), splenius capitis (two injections, five units each), semispinalis capitis (two injections, five units each) and trapezius muscle groups (two injections, five units each) in only one side of the occiput (Fig. 1). In all but one case (Patient 18), injections were made on the right side. Thirty days later, participants were scheduled to undergo bilateral GON decompression surgery using a standard midline incision approach, as previously described.[27] Discarded tissues containing the semispinalis capitis muscle, trapezius fascia and occipital periosteum were collected from onabotA/injected and onabotA/uninjected sides of each participant (to ensure that each serves as her/his own control), processed and analysed for expression of inflammatory genes using the NanoString technologies.

Demographics and responses to onabotA

Demographics, headache history, frequency and characteristics of the 18 patients are shown in Table 1. Patients were 21 to 74 years old, with an average of 19 years of headache and about 25 headache days per month. Most (61%) had a family history of migraine and all but two fulfilled criteria of migraine or historical migraine based on International Classification of Headache Disorders (third edition). All 18 patients presented with bilateral occipital headache and 78% reported occipital allodynia. Based on participants' responses to questions regarding their experience with onabotA therapy in years leading to the surgery (provided in their Initial Visit Form—Headache History Questionnaire, Migraine Surgery Screening, review of clinical notes in their online medical record, or routine post-surgery follow up phone calls) they were divided into those who self-reported a clinically relevant reduction in frequency with onabotA treatment in the past (historical responders) and those self-reported never having had a clinically relevant reduction in frequency with onabotA treatment (historical non-responder); this group included one patient (Patient 11) whose headache severity (but not frequncey) decreased, but not to the extent that it was clinically relevant. As shown in Table 1, headache/migraine history and characteristics were similar between those who found onabotA helpful (marked historical responders in the absence of daily headache diaries) and those who found onabotA unhelpful (marked historical non-responder).

Exploratory gene expression analysis

One hundred and eight processed samples (18 periosteums, 18 muscles and 18 fascias obtained from injected sides and 18 periosteums,18 muscles and 18 fascias obtained from uninjected sides of the same patients) yielded sufficient RNA for multiplexed gene expression analysis with a panel of 579 inflammation related genes. Our initial high-level exploratory analyses [Fig. 2A (unsupervised clustering of all genes) and Fig. 2B (principal components analysis)] indicate that the gene expression profiles were predominantly segregated by tissue types (periosteum, muscle and fascia) rather than by treatment (injected versus uninjected). Differential expression analysis between the treatment groups using all 108 samples (Fig. 2C) revealed only one gene that was differentially expressed between the injected and uninjected samples. In contrast, tissue specific analysis of differential expression patterns induced by injection of onabotA (paired analyses of injected and uninjected periosteum, muscle and fascia) yielded significant treatment effect differences in the periosteum [where expression of 45/47 genes was significantly higher in the injected than uninjected side, i.e. had unadjusted P-value < 0.01 and log2 fold change (log2FC) > 0.5; Fig. 2D], and muscle (where expression of 4/6 genes was significantly lower, and expression of 2/6 genes was significantly higher in the injected than uninjected side, unadjusted P-value < 0.01 and log2FC > 0.5; Fig. 2E), and no significant differences in the fascia (where no genes have unadjusted P-value < 0.01 and log2FC > 0.5; Fig. 2F).

Gene expression analysis based on direction and extent of their alteration by onabotA

Focusing on the periosteum, an initial analysis done by a biostatistician who was blinded to patient identity, to any of the data

Table 1 Characteristics of participants with bilateral occipital headache

Identification No.	Participants																		All	NR	R
	Historical[a] non-responders									Unknown		Historical[a] responders							*n* (%)		
	18	1	3	14	16	10	17	11	21	9	13	4	8	12	15	19	20	23			
Age, mean years ± SD	43	21	31	32	18	50	61	53	74	29	34	57	51	45	48	45	53	38	45 ± 14	43 ± 19	48 ± 6
Sex	M	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F			
Years of daily headache (mean ± SD)	4	6	3	15	2	7	51	43	9	20	17	43	8	15	13	40	12	31	19 ± 17	16 ± 18	23 ± 15
Monthly migraine days (mean ± SD)	25	23	30	20	5	30	2	20	18	20	20	20	15	10	20	20	27	15	19 ± 10	19 ± 8	18 ± 5
Monthly headache days (mean ± SD)	30	30	30	25	20	30	30	30	18	20	30	20	30	10	28	30	27	30	26 ± 6	27 ± 5	25 ± 7
Family history of migraine				Yes			Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes				
Headache locations																					
Bilateral headache	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	16 (100)	9 (100)	7 (100)
Occipital	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	16 (100)	9 (100)	7 (100)
Temporal	Yes			Yes			Yes	Yes		Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	10 (63)	4 (44)	6 (86)
Frontal	Yes			Yes	Yes		Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	12 (75)	5 (56)	7 (100)
Headache characteristics																					
Throbbing	Yes	Yes		Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14 (88)	7 (78)	6 (100)
Nausea/vomiting		Yes					Yes	Yes		Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	9 (56)	3 (33)	6 (86)
Photophobia	Yes	Yes		Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13 (81)	6 (67)	7 (100)
Phonophobia	Yes	Yes		Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	12 (75)	6 (67)	6 (86)
Osmophobia				Yes				Yes		Yes	Yes				Yes	Yes		Yes	5 (31)	2 (22)	3 (43)
Aura						Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes	7 (44)	3 (33)	4 (57)
Occipital allodynia	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	12 (75)	6 (67)	6 (86)
Headache severity																					
Mild																			0 (0)	0 (0)	0 (0)
Moderate	Yes	Yes	Yes	Yes									Yes	Yes	Yes				7 (44)	4 (44)	3 (43)
Severe					Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	9 (56)	5 (56)	4 (57)
Treatment tried (pre-surgery)																					
Response to botox	NR	NR	NR	2	NR	NR	NR	1	NR	nt	nt	R	R	R	R	R	R	R	7 (43)	0 (0)	7 (100)

1 = decreased pain severity but not migraine or headache days; 2 = worked in the past, did not work in last 2 years. F = female; M = male; NR = historical non-responders; nt = never tried (but included in the probable non-responder group as their gene expression was similar to the other nine subjects in this group); R = historical responders.

[a]Historical: in the absence of daily headache diaries (before and after treatment with onabotA), participants could only be classified as historical responders or non-responders based on answers they provided and hospital clinical notes taken during follow-up visit in years or month leading to the surgery.

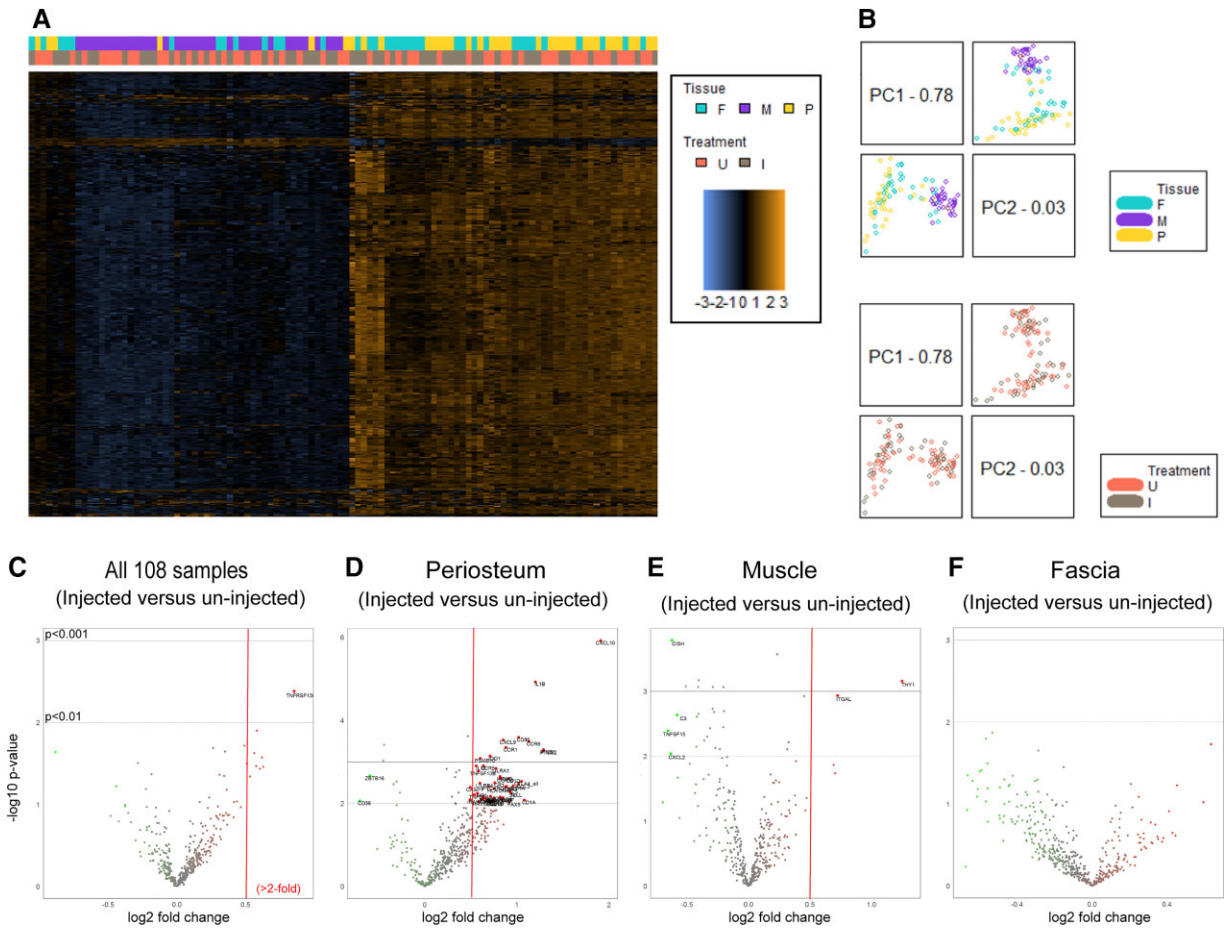


Figure 2 High-level exploratory gene expression analysis of injected and uninjected periosteum, muscle and fascia tissues. (**A**) Unsupervised clustering of all 579 genes found in injected (I) and uninjected (U) periosteum (yellow), muscle (purple) and fascia (blue) of all 18 patients. (**B**) Principal component analysis indicating that the gene expression profiles are predominantly segregated by tissue types (periosteum, muscle and fascia) rather than treatment (injected versus uninjected). (**C**) Volcano plots displaying differential gene expression analysis between onabotA injected and onabotA uninjected tissues using all 108 samples. (**D–F**) Tissue specific analysis of differential gene expression in injected and uninjected periosteum, muscle and fascia. Note significant treatment effect in periosteum and to a lesser extent in muscle (where expression of some genes had unadjusted *P*-value < 0.01 and log2FC > 0.5), but not in the fascia (where 0 genes have unadjusted *P*-value < 0.01 and log2FC > 0.5).

presented in Table 1, and most importantly to whether they were historical responders or historical non-responders, identified 37 genes whose expression levels were altered (log2FC > 0.5, excluding genes with low signals) by the onabotA treatment in at least 50% of the patients. An algorithm sorted patients based on the direction and extent of how these 37 genes were altered (Fig. 3). Of the nine patients whose gene expression was nearly universally upregulated (e.g. *NOD2*, *IRF4*, *TLR2*, *CXCL1*, *CCL5*, *IL2RB*), eight were historical non-responders and one (Patient 9) never tried onabotA therapys (Fig. 3, left nine columns). Of the eight patients whose gene expression was nearly universally downregulated (e.g. *CD45RA*, *CCL5*, *IL18RAP*, *NOD2*), seven were historical responders (they are presented in the seven right columns) and one (Patient 21) was historical non-responder. In only one case (Patient 13 who never tried onabotA therapy), some genes were upregulated and others downregulated.

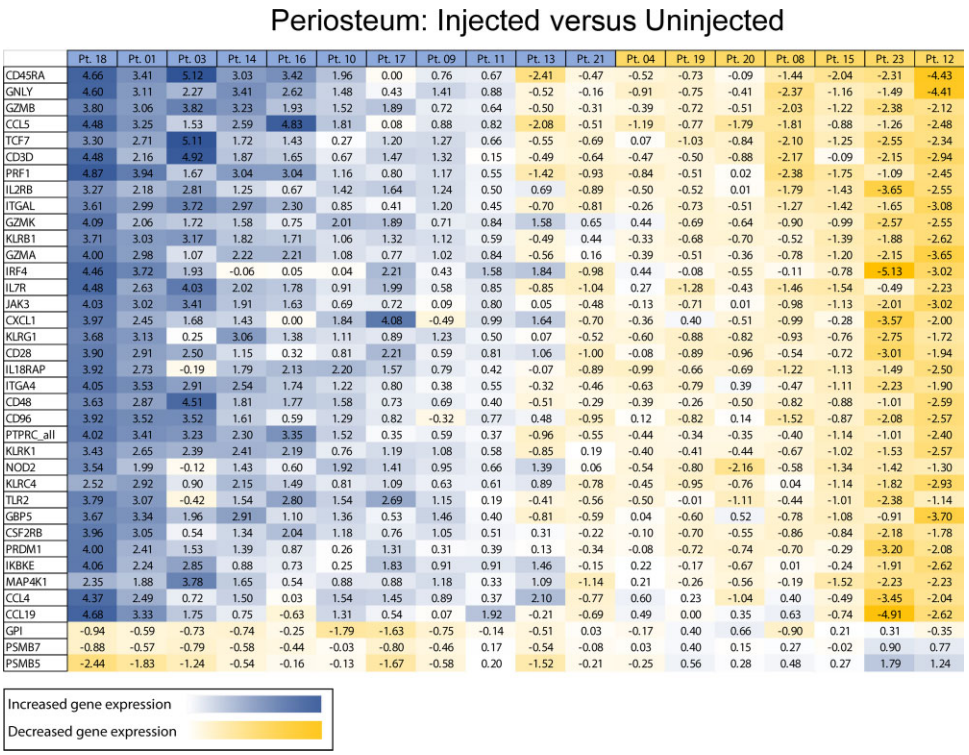
Baseline gene expression in historical responders and historical non-responders

We used the uninjected side samples to see if there is a baseline gene expression profile difference between the seven historical onabotA/responders and nine historical onabotA/non-responders (Fig. 4). Counting the total number of mRNA copies of all 579 pro-inflammatory genes of these participants showed that in the periosteum, their mean number in each historical responder is higher than in each historical non-responder by 95 279 copies, in the muscle by 9000 copies whereas in the fascia it is lower by 25 156 copies. Statistically (linear mixed-effect model), differences in baseline expression of inflammatory genes in the historical non-responders was significant in the periosteum [B = 0.58 (95% confidence interval, CI: 0.36 to 0.93), $P = 0.015$], and non-significant in the muscle [B = 0.88 (95%CI: 0.72 to 1.09), $P = 0.419$], and fascia [B = 1.44 (95%CI: 0.83 to 2.50), $P = 0.3$] (Fig. 4A–C). Genes whose expression differed by >2-fold ($P < 0.01$) are shown in Fig. 4D–F.

OnabotA effects on gene expression in historical responders and historical non-responders

Periosteum

Differential gene expression analyses of treatment effect showed that onabotA injections had large effect in the periosteum. Compared to uninjected periosteum, inflammatory genes are



	Pt. 18	Pt. 01	Pt. 03	Pt. 14	Pt. 16	Pt. 10	Pt. 17	Pt. 09	Pt. 11	Pt. 13	Pt. 21	Pt. 04	Pt. 19	Pt. 20	Pt. 08	Pt. 15	Pt. 23	Pt. 12
CD45RA	4.66	3.41	5.12	3.03	3.42	1.96	0.00	0.76	0.67	-2.41	-0.47	-0.52	-0.73	-0.09	-1.44	-2.04	-2.31	-4.43
GNLY	4.60	3.11	2.27	3.41	2.62	1.48	0.43	1.41	0.88	-0.52	-0.16	-0.91	-0.75	-0.41	-2.37	-1.16	-1.49	-4.41
GZMB	3.80	3.06	3.82	3.23	1.93	1.52	1.89	0.72	0.64	-0.50	-0.31	-0.39	-0.72	-0.51	-2.03	-1.22	-2.38	-2.12
CCL5	4.48	3.25	1.53	2.59	4.83	1.81	0.08	0.88	0.82	-2.08	-0.51	-1.19	-0.77	-1.79	-1.81	-0.88	-1.26	-2.48
TCF7	3.30	2.71	5.11	1.72	1.43	0.27	1.20	1.27	0.66	-0.55	-0.69	0.07	-1.03	-0.84	-2.10	-1.25	-2.55	-2.34
CD3D	4.48	2.16	4.92	1.87	1.65	0.67	1.47	1.32	0.15	-0.49	-0.64	-0.47	-0.50	-0.88	-2.17	-0.09	-2.15	-2.94
PRF1	4.87	3.94	1.67	3.04	3.04	1.16	0.80	1.17	0.55	-1.42	-0.93	-0.84	-0.51	0.02	-2.38	-1.75	-1.09	-2.45
IL2RB	3.27	2.18	2.81	1.25	0.67	1.42	1.64	1.24	0.50	0.69	-0.89	-0.50	-0.52	0.01	-1.79	-1.43	-3.65	-2.55
ITGAL	3.61	2.99	3.72	2.97	2.30	0.85	0.41	1.20	0.45	-0.70	-0.81	-0.26	-0.73	-0.51	-1.27	-1.42	-1.65	-3.08
GZMK	4.09	2.06	1.72	1.58	0.75	2.01	1.89	0.71	0.84	1.58	0.65	0.44	-0.69	-0.64	-0.90	-0.99	-2.57	-2.55
KLRB1	3.71	3.03	3.17	1.82	1.71	1.06	1.32	1.12	0.59	-0.49	0.44	-0.33	-0.68	-0.70	-0.52	-1.39	-1.88	-2.62
GZMA	4.00	2.98	1.07	2.22	2.21	1.08	0.77	1.02	0.84	-0.56	0.16	-0.39	-0.51	-0.36	-0.78	-1.20	-2.15	-3.65
IRF4	4.46	3.72	1.93	-0.06	0.05	0.04	2.21	0.43	1.58	1.84	-0.98	0.44	-0.08	-0.55	-0.11	-0.78	-5.13	-3.02
IL7R	4.48	2.63	4.03	2.02	1.78	0.91	1.99	0.58	0.85	-0.85	-1.04	0.27	-1.28	-0.43	-1.46	-1.54	-0.49	-2.23
JAK3	4.03	3.02	3.41	1.91	1.63	0.69	0.72	0.09	0.80	0.05	-0.48	-0.13	-0.71	0.01	-0.98	-1.13	-2.01	-3.02
CXCL1	3.97	2.45	1.68	1.43	0.00	1.84	4.08	-0.49	0.99	1.64	-0.70	-0.36	0.40	-0.51	-0.99	-0.28	-3.57	-2.00
KLRG1	3.68	3.13	0.25	3.06	1.38	1.11	0.89	1.23	0.50	0.07	-0.52	-0.60	-0.88	-0.82	-0.93	-0.76	-2.75	-1.72
CD28	3.90	2.91	2.50	1.15	0.32	0.81	2.21	0.59	0.81	1.06	-1.00	-0.08	-0.89	-0.96	-0.54	-0.72	-3.01	-1.94
IL18RAP	3.92	2.73	-0.19	1.79	2.13	2.20	1.57	0.79	0.42	-0.07	-0.89	-0.99	-0.66	-0.69	-1.22	-1.13	-1.49	-2.50
ITGA4	4.05	3.53	2.91	2.54	1.74	1.22	0.80	0.38	0.55	-0.32	-0.46	-0.63	-0.79	0.39	-0.47	-1.11	-2.23	-1.90
CD48	3.63	2.87	4.51	1.81	1.77	1.58	0.73	0.69	0.40	-0.51	-0.29	-0.39	-0.26	-0.50	-0.82	-0.88	-1.01	-2.59
CD96	3.92	3.52	3.52	1.61	0.59	1.29	0.82	-0.32	0.77	0.48	-0.95	0.12	-0.82	0.14	-1.52	-0.87	-2.08	-2.57
PTPRC_all	4.02	3.41	3.23	2.30	3.35	1.52	0.35	0.59	0.37	-0.96	-0.55	-0.44	-0.34	-0.35	-0.40	-1.14	-1.01	-2.40
KLRK1	3.43	2.65	2.39	2.41	2.19	0.76	1.19	1.08	0.58	-0.85	0.19	-0.40	-0.41	-0.44	-0.67	-1.02	-1.53	-2.57
NOD2	3.54	1.99	-0.12	1.43	0.60	1.92	1.41	0.95	0.66	1.39	0.06	-0.54	-0.80	-2.16	-0.58	-1.34	-1.42	-1.30
KLRC4	2.52	2.92	0.90	2.15	1.49	0.81	1.09	0.63	0.61	0.89	-0.78	-0.45	-0.95	-0.76	0.04	-1.14	-1.82	-2.93
TLR2	3.79	3.07	-0.42	1.54	2.80	1.54	2.69	1.15	0.19	-0.41	-0.56	-0.50	-0.01	-1.11	-0.44	-1.01	-2.38	-1.14
GBP5	3.67	3.34	1.96	2.91	1.10	1.36	0.53	1.46	0.40	-0.81	-0.59	0.04	-0.60	0.52	-0.78	-1.08	-0.91	-3.70
CSF2RB	3.96	3.05	0.54	1.34	2.04	1.18	0.76	1.05	0.51	0.31	-0.22	-0.10	-0.70	-0.55	-0.86	-0.84	-2.18	-1.78
PRDM1	4.00	2.41	1.53	1.39	0.87	0.26	1.31	0.31	0.39	0.13	-0.34	-0.08	-0.72	-0.74	-0.70	-0.29	-3.20	-2.08
IKBKE	4.06	2.24	2.85	0.88	0.73	0.25	1.83	0.91	0.91	1.46	-0.15	0.22	-0.17	-0.67	0.01	-0.24	-1.91	-2.62
MAP4K1	2.35	1.88	3.78	1.65	0.54	0.88	0.88	1.18	0.33	1.09	-1.14	0.21	-0.26	-0.56	-0.19	-1.52	-2.23	-2.23
CCL4	4.37	2.49	0.72	1.50	0.03	1.54	1.45	0.89	0.37	2.10	-0.77	0.60	0.23	-1.04	0.40	-0.49	-3.45	-2.04
CCL19	4.68	3.33	1.75	0.75	-0.63	1.31	0.54	0.07	1.92	-0.21	-0.69	0.49	0.00	0.35	0.63	-0.74	-4.91	-2.62
GPI	-0.94	-0.59	-0.73	-0.74	-0.25	-1.79	-1.63	-0.75	-0.14	-0.51	0.03	-0.17	0.40	0.66	-0.90	0.21	0.31	-0.35
PSMB7	-0.88	-0.57	-0.79	-0.58	-0.44	-0.03	-0.80	-0.46	0.17	-0.54	-0.08	0.03	0.40	0.15	0.27	-0.02	0.90	0.77
PSMB5	-2.44	-1.83	-1.24	-0.54	-0.16	-0.13	-1.67	-0.58	0.20	-1.52	-0.21	-0.25	0.56	0.28	0.48	0.27	1.79	1.24

Figure 3 Patient sorting based on the direction and extent of genes whose expression level was increased or decreased more than 1.5-fold (log2FC > 0.5) in at least 50% of the cases. Patient ID numbers correspond to their ID number in Table 1.

nearly unanimously downregulated in injected periosteums of historical responders, and nearly uniformly upregulated in the injected periosteums of historical non-responders [Fig. 5A(i–iv)]. Of the 80 significantly ($P < 0.01$) altered genes among the historical responders, 77 were downregulate and three were upregulated >2-fold [Fig. 5A(i)], whereas of all significantly ($P < 0.01$) altered genes among the historical non-responders, nearly 205 were upregulate and only 10 were downregulated >2-fold [Fig. 5A(ii)]. Attempting to understand better the biological significance of the increased gene expression in the injected side of the historical non-responders, we also examined differences in gene expression while accommodating the two sources of repeated measures (side and the 579 genes) within individuals. A linear mixed-effects model revealed a main effect for injected side [$F(1,20\,246) = 35.5$, $P < 0.0001$], no main effect for historical responder [$F(1,16) = 0.01$, $P = 0.917$], but an injected side × historical responder interaction [$F(1,20\,246) = 3494.4$, $P < 0.0001$] [Fig. 5D(i) and Suplementary Table 1]. The interaction was primarily driven by the increased gene expression in the injected side of the historical non-responders [Fig. 5A(iii)], which is 80% higher than the uninjected side [$B = 1.80$ (95%CI: 1.75 to 1.85), $P < 0.0001$] but is nearly identical (difference <3.4%) to the uninjected side of the historical responders [$B = 1.03$ (95%CI: 0.64 to 1.67), $P = 0.998$] [Fig. 5A(iv)], which is significantly higher (by 61%) than the injected side in this group [$B = 1.61$ (95%CI: 1.56 to 1.67), $P < 0.0001$].

Muscles

Differential gene expression analyses of treatment effect showed that onabotA injections had minimal effect in muscles. Of the six significantly ($P < 0.01$) altered genes among the historical responders, all were downregulate and none was upregulated >2-fold [Fig. 5B(i)], whereas of all significantly ($P < 0.01$) altered genes among the historical non-responders, 10 were upregulate and three were downregulated >2-fold [Fig. 5B(ii)]. A linear mixed-effects model yielded a main effect for injected side [$F(1,20\,246) = 90.3$, $P < 0.0001$], no main effect for responder [$F(1,16) = 0.15$, $P = 0.70$], but an injected side × responder interaction [$F(1,20\,246) = 211.6$, $P < 0.0001$] [Fig. 5D(ii) and Supplementary Table 1]. The interaction was primarily driven by the small gene expression increase in the injected side of the historical non-responders, which is only 3% higher than the uninjected side [$B = 1.03$ (95%CI: 1.01 to 1.05), $P < 0.0005$]; and nearly identical (difference <9.0%) to the uninjected side of the historical responders [$B = 0.91$ (95%CI: 0.74 to 1.12), $P = 0.66$], which is significantly higher (by 15%) than the injected side in this group [$B = 1.85$ (95%CI: 0.83 to 0.88), $P < 0.0001$] [Fig. 5B(iii–iv), D(ii) and Supplementary Table 1].

Fascia

Differential gene expression analyses of treatment effect showed that onabotA injections did not alter the expression of inflammatory genes significantly ($P < 0.01$) in historical responders [Fig. 5C(i)] or historical non-responders [Fig. 5C(ii)], and that this lack of treatment effect was also seen in the overall analysis of all 579 genes [Fig. 5C(iii–iv), D(iii) and Supplementary Table 1]. As onabotA injections altered gene expression in periosteum and muscle but not fascia, pathways analysis included periosteum and muscle only.

Gene set analysis and pathway scores

Gene set analysis

A gene set is defined as genes involved in the same cellular function or pathways. The inflammation panel include 32 predefined gene

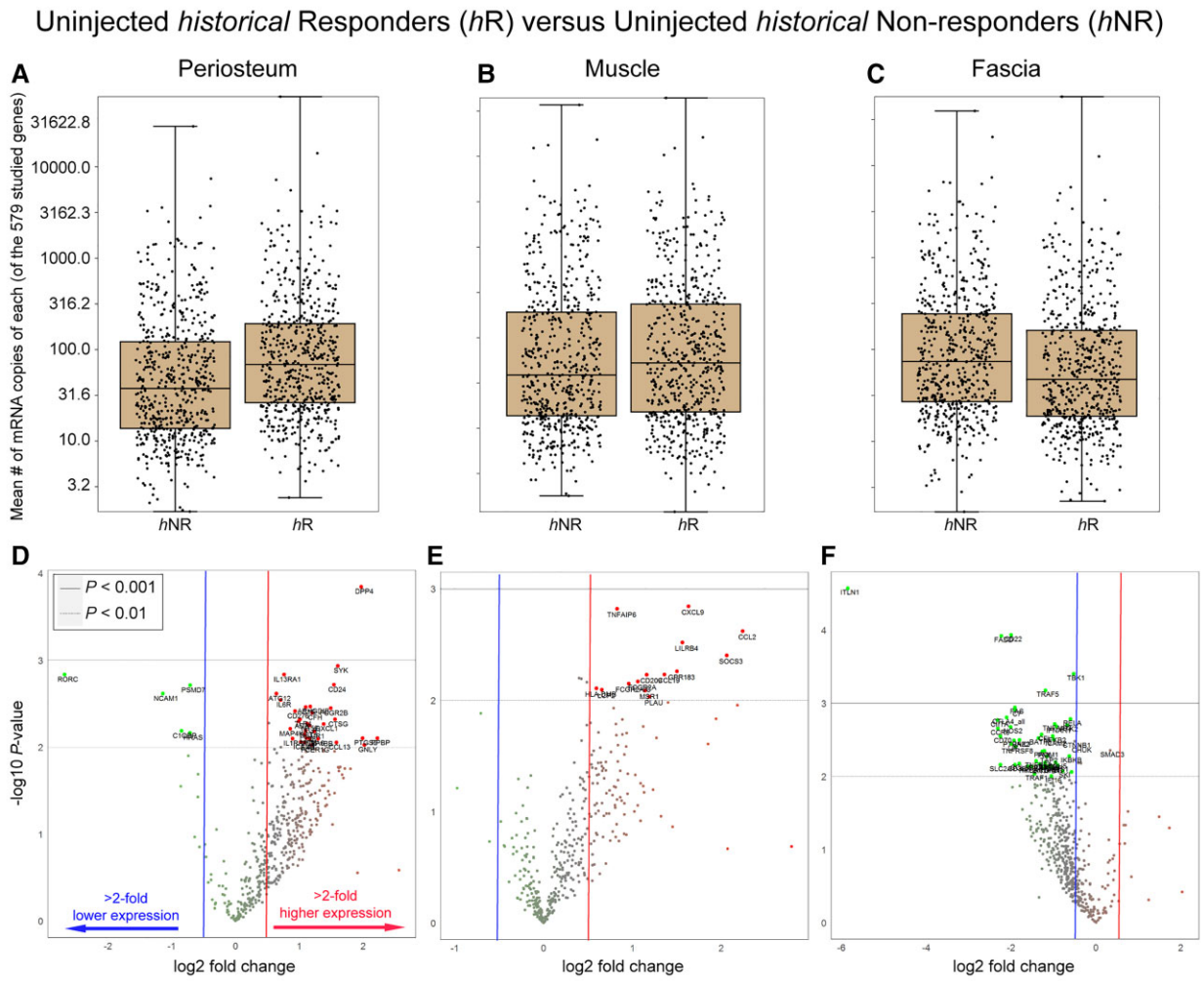


Figure 4 Baseline gene expression profile in historical onabotA/responders and historical onabotA/non-responders. (**A–C**) Box plot illustrating median, 95% CI, interquartile range (25th–75th percentile; lower and upper box boundaries) and observations below and above the 25th and 75th percentile of the mean number of mRNA copies of each of the 579 genes counted in the uninjected periosteum, muscle and fascia tissues of the historical responders and historical non-responders. (**D–F**) Volcano plot displaying each gene's −log10(*P*-value) and log2FC in uninjected responders versus uninjected non-responders. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side (red dots = higher expression; green dots = lower expression).

sets/pathways centered around inflammatory responses. The global significance scores are an average of the significance measures across all genes in the pathways.[26] By using undirected and directed global significance score to respectively quantify the mean change of expression for each gene set (regardless of up- or down-regulation) and the direction of regulation, we noted that in historical responders' periosteum, onabotA injections resulted in robust and significant downregulation of gene sets that regulate well-defined inflammatory pathways such as cytokine signalling, lymphocyte activation, innate immune response, TNF family signalling, NF-kB signalling, and TLR signalling (Fig. 6A and Supplementary Figs 1 and 3A), whereas in historical non-responders, onabotA injections upregulated these gene sets (Supplementary Figs 1 and 3A). A similar analysis of onabotA effects on muscle tissue revealed much smaller, but nonetheless unidirectional down-regulatory effects in historical responders (Fig. 6D and Supplementary Figs 2 and 3B), and a mixed bi-directional (lack of) effect in historical non-responders (Supplementary Fig. 3B)

Baseline pathway scores in historical responders and historical non-responders

Pathway analysis of differentially expressed genes in corresponding uninjected tissues obtained from the seven onabotA historical responders and nine onabotA historical non-responders identified 29 inflammatory pathways whose scores were higher in the periosteum (Fig. 6B) and 27 such pathways in muscle (Fig. 6E) of historical responders than historical non-responders, and only two pathways whose scores were lower (all in muscle tissue).

Pathway scores impacted by onabotA treatment

All pathway scores were lower in the injected than uninjected periosteum of historical responders (Fig. 6C) and higher in the injected than uninjected periosteum of historical non-responders (Supplementary Figs 1 and 3A). In the muscle, treatment effects were minimal at most (Fig. 6F and Supplementary Figs 2 and 3B), suggesting negligible effect of onabotA in this tissue. As onabotA

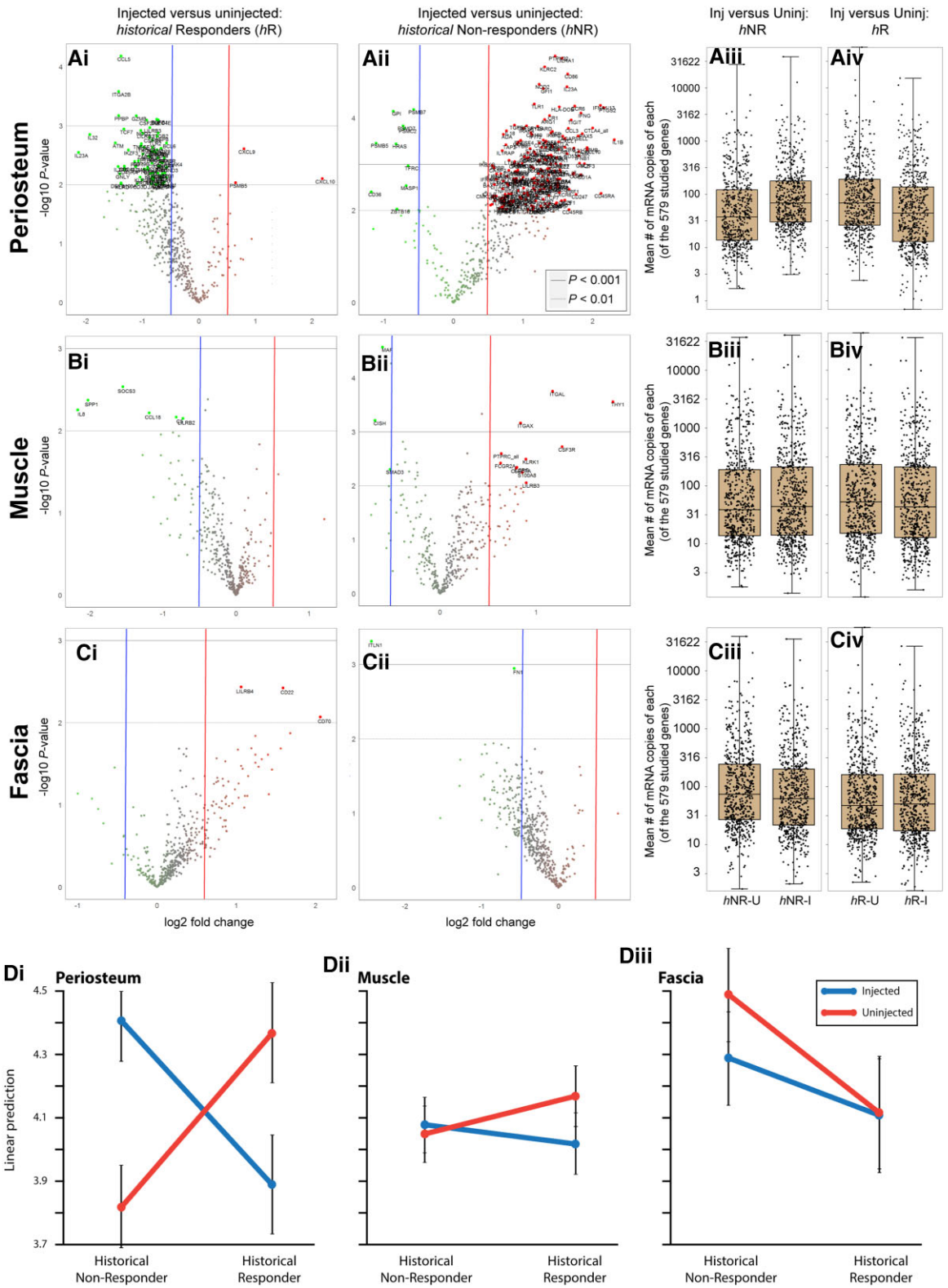


Figure 5 Treatment effect on gene expression in historical responders and historical non-responders. Volcano plots illustrate differential expression of genes in injected versus uninjected periosteum [**A(i–ii)**], muscle [**B(i–ii)**] and fascia [**C(i–ii)**] of historical responders [**A(i)**, **B(i)** and **C(i)**] and historical non-responders [**A(ii)**, **B(ii)** and **C(ii)**]. Highly statistically significant genes fall at the *top* of the plot above the horizontal lines, and highly differentially expressed genes fall to either side (red dots = higher expression, green dots = lower expression). Box plots illustrate median, 95% CI, interquartile range (25th–75th percentile; lower and upper box boundaries) and observations below and above the 25th and 75th percentile of the mean number of mRNA copies of each of the 579 genes counted in the uninjected and injected periosteum [**A(iii–iv)**], muscle [**B(iii–iv)**] and fascia [**C(iii–iv)**] of non-responders [**A(iii)**, **B(iii)** and **C(iii)**] and responders [**A(iv)**, **B(iv)** and **C(iv)**]. Linear mixed-effects models of source of variation in gene expression in historical

Continued

injections altered inflammatory pathways in periosteum but not muscle, analyses of cell type profiling included periosteum only.

Cell type profiling

We used a set of predefined cell type marker genes to calculate the immune cell type abundance scores for all samples as previously described.[26] As with gene expression and pathways activation, the baseline abundance (i.e. uninjected samples) of multiple inflammatory/immune cells was higher in the periosteum of historical responders than in the historical non-responders (Fig. 7A), and their abundance was reduced in the injected periosteum of the historical esponders (Fig. 7B) and elevated in the injected periosteum of the historical non-responders (Fig. 7C). The affected immune cells were T cells (exhausted CD8 cells, Th1 cells), NK cells, B cells, neutrophils, macrophages and dendritic cells.

Discussion

Using targeted transcriptome analyses to determine levels of expression of inflammatory genes in respective onabotA/injected and onabotA/uninjected periosteum, muscle and fascia in the posterior neck and occiput of patients with bilateral occipital headache, we found that (i) onabotA alters expression of inflammatory genes largely in periosteum, minimally in muscle and not at all in fascia; (ii) expression of inflammatory genes in uninjected periosteum and muscle is significantly higher in historical onabotA responders than historical non-responders, and significantly lower in historical responders fascia; (iii) in historical responders' periosteum and muscle, onabotA decreases expression of nearly all significantly altered inflammatory genes evaluated whereas in historical non-responders it increases expression of these same genes but to a level that is nearly identical to the level observed in the uninjected periosteum and muscle of the historical responders; and (iv) in historical responders' periosteum (and to a far lesser extent muscles), onabotA treatment leads to robust and significant downregulation of gene sets that define well recognized inflammatory pathways (e.g. pathways involved in adaptive and innate immune response, lymphocyte activation, and cytokine, chemokine, NF-kB, TNF, TLR and interferon signalling) and 12 types of immune cells (e.g. neutrophils, macrophages, cytotoxic T-, NK-, Th1, B- and dendritic cells) whereas in historical non-responders, treatment appears to increase activation level of these pathways and abundance of cells—and as noted above, to a level that is similar and in many cases, just below the level seen in the uninjected tissues of responders. We interpret the significantly higher level of expression of inflammatory genes, activation level of the inflammatory pathways and abundance of immune cells in the historical responders (compared to the historical non-responders) as suggesting that inflammation of the occipital periosteum, and to a lesser extent occipital neck muscles, is likely to play a role in the pathophysiology of their occipital headache. Conversely, we suggest that inflammation is less likely to be involved in the headache pathophysiology of the historical non-responders. We interpret the lower expression of inflammatory genes, lower activation level of the inflammatory pathways and lower abundance of immune cells in the injected (compared to the uninjected) tissues of the historical responders, as suggesting that if inflammation exists (as a pre-existing condition), onabotA is capable of reducing it, possibly through yet-unknown direct or indirect interactions with periosteal immune cells. In the absence of more knowledge, we cannot explain the higher expression of inflammatory genes in the injected (compared to the uninjected) tissues of the historical non-responders—especially because it has not been reported in the literature that in those who fail to benefit from this preventive therapy, onabotA injections exacerbate the headache or make muscles hurt more 1 month after treatment. Along this line, it is possible that such reaction may occur after some onabotA injection but goes unnoticeable for lack of association with clinical pain.

Evidence for high level of expression of inflammatory genes in the occipital periosteum and to a far lesser degree in the muscle of the historical responders group and low level of gene expression in the periosteum and muscle of historical non-responders group, suggests that periosteal inflammation may play a pivotal role in the pathophysiology of some but not all migraine patients whose headaches involve bilateral occipital headache. These findings also bring attention to the possibility that a culprit of these headaches lays in the densly innervated periosteum rather than the muscle, as previously suggested. Evidence for increased expression of pro-inflammatory genes and decreased expression of anti-inflammatory genes in the periosteum of such patients,[5] lack of evidence for muscle pathophysiology,[28–31] and the fact that NSAIDs help some but not all migraine patients[32–35] support this view.

By far the most novel finding of this study is onabotA's ability to reduce expression of inflammatory genes, activation of inflammatory pathways, and abundance of classical immune cells in the historical responders group. Mechanistically, it suggests that the prevention of migraine by onabotA may, in part, be achieved through its ability to reduce inflammation via mechanisms that are distinctly different than NSAIDs. This is consistent with the observation that onabotA decreases exocytosis of inflammatory and excitatory neurotransmitters and peptides (i.e. substance P, CGRP) from primary afferent nociceptors.[36,37] In addition, in *in vivo* studies, onabotA inhibited 48/80-induced degranulation of both human and murine mast cells, LL-37-induced skin erythema in mice, and mRNA expression of rosacea biomarkers.[38] In a rat model of CFA-induced arthritis, intra-articular administration of onabotA decreased expression of proinflammatory cytokines IL-1β and TNF-α in synovial fluid. Furthermore, onabotA injection also led to

Figure 5 Continued
responders versus historical non-responders showing: [**D(i)**] Main effect in periosteum for injected side, no main effect for historical responders, but an injected side × historical responder interaction [$F(1,20\,246) = 3494.4$, $P < 0.0001$] driven mainly by the increased gene expression in the injected side of the historical non-responders which is 80% higher than the uninjected side, but is nearly identical (difference <3.4%) to the uninjected side of the historical responders [$B = 1.03$ (95%CI: 0.64 to 1.67), $P = 0.998$]. [**D(ii)**] Main effect in muscle for injected side, no main effect for historical responders, but an injected side × historical responder interaction [$F(1,20\,246) = 211.6$, $P < 0.0001$] driven by the small gene expression increase in the injected side of the historical non-responders, which is only 3% higher than the uninjected side and nearly identical (difference <9.0%%) to the uninjected side of the historical responders [$B = 0.91$ (95%CI: 0.74 to 1.12), $P = 0.66$]. [**D(iii)**] No main effect in fascia for injected side or historical responder, and no interaction between them. Values for each tissue type are presented in the original mRNA units using least square means (SE) while effect sizes are reported from the mixed model as the ratio of the geometric means. Note robust onabotA effects on periosteal gene expression, minimal effect on muscles and no effect on fascia. Also note that onabotA decreased gene expression in historical responders' and increased it in historical non-responders to a level that was similar to the level of expression seen in the uninjected tissues of the historical responders. *P*-values and difference probability for the level of response are shown in Supplementary Table 1.

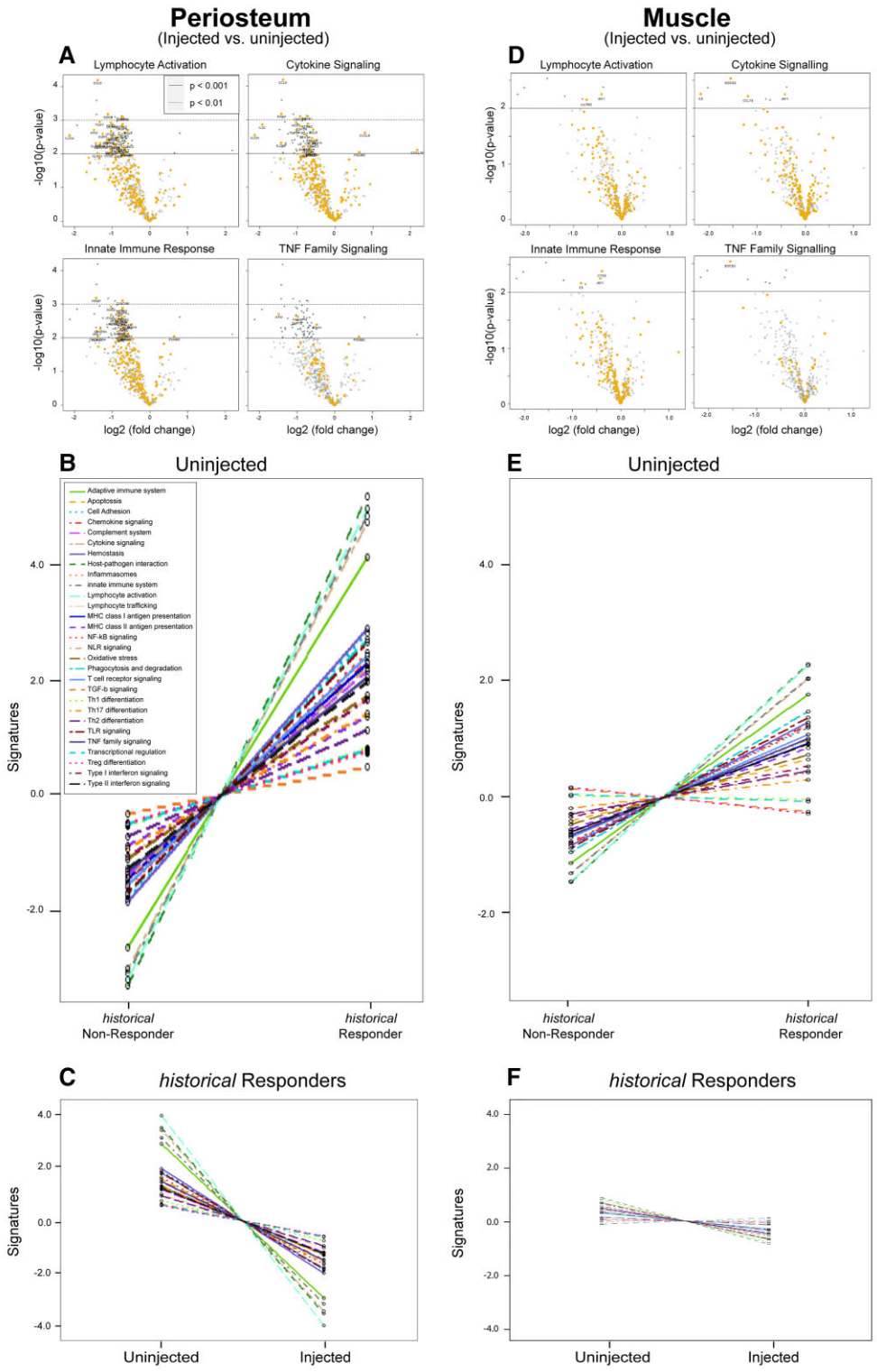


Figure 6 Gene set analysis and pathway scores in historical responders (A–C) and historical non-responders (D–F). (**A** and **D**) Volcano plots displaying onabotA effects on 4/32 representative predefined gene sets involved in inflammatory responses in historical onabotA/responders. Highly statistically significant genes fall at the *top* of the plot above the horizontal lines, and highly differentially expressed genes fall to either side (left = downregulation, right = upregulation). Horizontal lines indicate *P*-value thresholds. Genes are coloured if they belong to the selected gene set. The 40 most statistically significant genes are labelled in the plot. (**B** and **E**) Baseline pathway scores in historical responders and historical non-responders. Pathway are oriented such that increasing scores correspond to increasing expression of at least half the genes that define a pathway. Pathways colour codes are depicted in the *inset* in **B**. Note that in the uninjected periosteum (i.e. baseline), score values of all pathways are higher in the historical responders than historical non-responders and the similar trend in the muscle. (**C** and **F**) Pathway scores impacted by onabotA injections. Note that in historical responders' periosteum, onabotA decreases score values of all pathways whereas in the muscle, treatment effect is marginal.

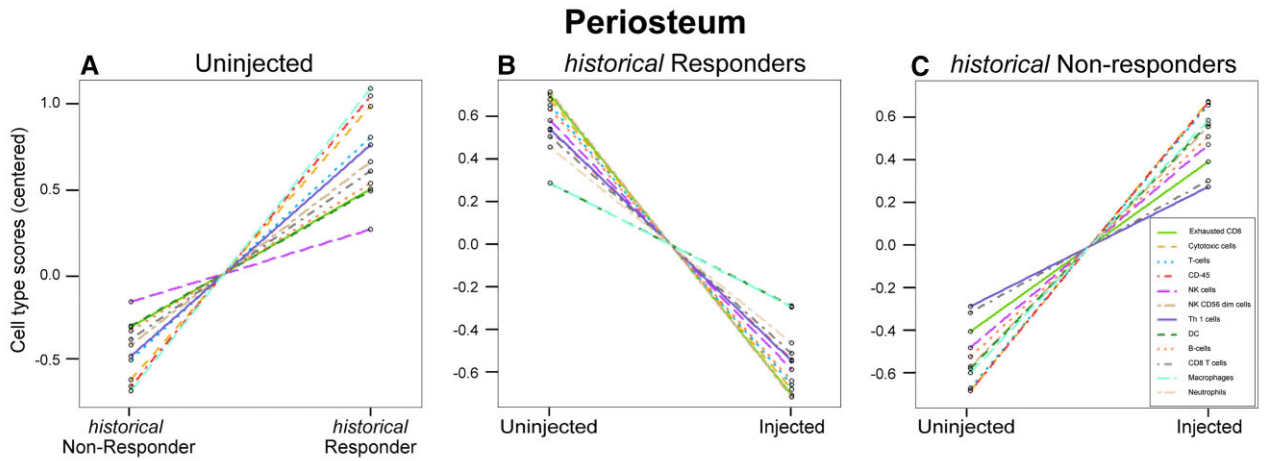


Figure 7 Cell type profiling in responders and non-responders. (**A**) Baseline scores showing higher abundance of 12 different classes of inflammatory/immune cells in uninjected periosteum of historical responders than historical non-responders. (**B**) Cell type abundance scores impacted by onabotA treatment in historical responders. (**C**) Cell type abundance scores impacted by onabotA treatment in historical non-responders. *Inset* in **C** depicts cell type colour codes. Note robust decrease in abundance of all 12 cells in the injected (compared to uninjected) periosteum of historical responders and opposite tendency in historical non-responders. Cell type marker genes: Exhausted CD8 cells: *LAG3*, *CD244*, *EOMES*, *PTGER4*; Cytotoxic cells: *RF1*, *GZMA*, *GZMB*, *NKG7*, *GZMH*, *KLRK1*, *KLRB1*, *KLRD1*, *CTSW*, *GNLY*; T cells: *CD6*, *CD3D*, *CD3E*, *SH2D1A*, *TRAT1*, *CD3G*; CD45 cells: *PTRPC*; NK cells: *XCL1*, *XCL2*, *NCR1*; NK CD56dim cells: *KIR2DL3*, *KIR3DL1*, *KIR3DL2*, *IL21R*; Th1 cells: *TBX21*; DC: *CCL13*, *CD209*, *HSD11B1*; B cells: *BLK*, *CD19*, *FCRL2*, *MS4A1*, *KIAA0125*, *TNFRSF17*, *TCL1A*, *SPIB*, *PNOC*, CD45: *PTRPC*; CD8 T cells: *CD8A*, *CD8B*; Macrophages: *CD68*, *CD84*, *CD163*, *MS4A4A*; Neutrophils: *FPR1*, *SIGLEC5*, *CSF3R*, *FCAR*, *FCGR3B*, *CEACAM3*, *S100A12*.

reduced cartilage degeneration and inflammatory cell infiltration.[39] The demonstration of onabotA anti-inflammatory effects in animals in which inflammation was first induced, support our clinical proposal that periosteal inflammation may be a pre-existing condition in patients with occipital headache, and that reducing the inflammation with onabotA may contribute to successful prevention of occipital headache by onabotA—a conclusion supported by the high expression of inflammatory genes in the historical responders group and low expression in the historical non-responders.

As this is the first report of onabotA's ability to reduce expression of inflammatory genes, activation of inflammatory pathways and abundance of immune cells in humans, no knowledge exists to allow evidence-based discussion on the mechanism by which onabotA may insert these anti-inflammatory effects. Given current understanding of onabotA's ability to cleave SNAP-25 and prevent docking, priming and fusion of synaptic vesicles with the cell membrane,[40] we can only speculate that the down regulation of gene expression and inhibition of pathway activation are secondary to the reduced abundance of the immune cells that express the inflammatory genes (e.g. the >2-fold decrese in expression of the Pro-Platelet Basic Protein coding gene, and activation level of the chemokine signalling pathway it relates to, could be secondary to the decreased population of neutrophils[41]). In the absence of evidence for onabotA ability to enter and eliminate immune cells, it may be reasonable to suggest that the local reduction in abundance of immune cells may be driven by the neurotoxin's ability to modulate nociceptors ability to release neuropeptides and chemokines[16] that attract immune cells[42,43] in tissues that are heavily innervated by nociceptors and contain large numbers of immune cells (e.g. periosteum) but not in tissues that are poorly innervated (e.g. fascia) or contain relatively small number of immune cells (e.g. tendons, ligaments, muscles). In raising this option, it must be noted that many cell types secrete multiple chemoattractants with distinctly different cellular signalling pathways that govern recruitment of immune cells (such as neutrophils) to site of inflammation, and that the 'decision' made by these cells on which chemoattractant pathway to follow to reach their end-target (and which chemoattractant to ignore) is organ-specific, tissue-speciifc, and largely influenced by the type of inflammation (e.g. bacterial, viral, sterile).[44] For example, whereas neutrophil recruitment to skin infected by *S. pyogenes* is suppressed by CGRP and facilitated by onabotA (presumably by blocking CGRP release from cutaneous nociceptors),[45] recruitment of neutrophils to the periosteum, where the inflammatory condition is unlikely to involve bacteria, appears to be regulated by other chemoattractants.

In summary, while onabotA mechanisms of action in migraine prevention is known to involve its ability to block activation of unmyelinated meningeal nociceptors by cortical spreading depression,[46] inflammatory mediators,[47] capsaicin and mustard oil[48]—effects that are secondary to onabotA ability to inhibit SNARE-dependent regulated exocytosis of proinflammatory and excitatory neurotransmitters and neuropeptides—the current study raises the novel possibility that onabotA may also reduce an elevated number of immune cells in the periosteum of patients with occipital headache. Beyond this study, onabotA's ability to reduce abundance of immune cells such as neutrophils, macrophages, cytotoxic (CD8 T cells), Th1 (CD4+T cells), NK cells and the TLR-regulating CD45 cells—all capable of releasing cytokines such as IL1, IL6, TNFα, IFNγ, CXCL2, CXCL10, CXCL8, IL13, IL12 and IL23[41,49–57]—may have far-reaching implications to its use in the treatment of other conditions associated with inflammation.

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Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author). R.B. is the John Hedley-Whyte Professor of Anesthesia and Neuroscience at the Beth Israel Deaconess Medical Center and Harvard Medical School. He has received research support from the NIH: R01 NS094198-01A1, R37 NS079678, R01NS095655, R01 NS104296, R21 NS106345, Allergan, Teva, Dr. Ready, Eli Lilly, Trigemina and the Migraine Research Foundation. He is a reviewer for NINDS, holds stock options in AllayLamp, Theranica and Percept; serves as consultant, advisory board member, or has received honoraria from: Alder, Allergan, Amgen, Autonomic Technologies, Avanir, Biohaven, Dr. Reddy's Laboratory, electroCore, Eli Lilly, GlaxoSmithKline, Merck, Pernix, Theranica, Teva, and Trigemina. CME fees from Healthlogix, Medlogix, WebMD/Medscape, and Patent 57546771 (Title: Method and apparatus for managing photophobia and migraine photophobia). A.A. and M.F.B. are employees of Allergan, an AbbVie Company. Other authors declare that they have no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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