Brain and Spine 2 (2022) 101698

Contents lists available at ScienceDirect

Brain and Spine

journal homepage: www.journals.elsevier.com/brain-and-spine

Exploring macrophage differentiation and its relation to Modic changes in human herniated disc tissue

N. Djuric [a,b,*], G.C.M. Lafeber [a,b], W. Li [a,b], S.G. van Duinen [c], C.L.A. Vleggeert-Lankamp [a,b,d,e]

[a] University Neurosurgical Center Holland, the Netherlands
[b] Department of Neurosurgery, Leiden University Medical Center, the Netherlands
[c] Department of Pathology, Leiden University Medical Center, the Netherlands
[d] Department of Neurosurgery, The Hague Medical Center and HAGA Teaching Hospital, The Hague, the Netherlands
[e] Department of Neurosurgery, Spaarne Hospital Haarlem/Hoofddorp, the Netherlands

ARTICLE INFO

Keywords:
Intervertebral disc herniation
Lumbar
Cervical
Modic changes
Inflammation
Macrophage
M1
M2

ABSTRACT

Introduction: Cervical- and lumbosacral radiculopathy symptoms due to disc herniation are likely to be influenced by macrophage infiltration of the herniated disc. Vertebral endplate changes are hypothesized to, at least partially, correlate to the inflammatory condition of the disc and its environment.
Research question: The present study aims to evaluate several immunohistochemical M1-and M2-markers for their suitability to discern pro-inflammatory M1-and anti-inflammatory M2 macrophage differentiation patterns in herniated intervertebral disc tissue. In addition, their associations with Modic changes (MC) of the vertebral endplates will be evaluated.
Materials and methods: Herniated disc samples were collected from 45 patients undergoing surgery for cervical- or lumbosacral radiculopathy. Samples were processed for immunohistochemistry and stained for the presence of macrophages: CD68 (macrophage marker), CD40 (M1), iNOS (M1), CD192 (M1), CD163 (M2), Arg1 (M2) and CD209 (M2). T-cells (CD3) and neutrophil (CD15) expressions were studied additionally.
Results: CD68 positive cells were present with a median density of 50/cm^2, M2 markers CD163 and CD209 were expressed most dominantly, followed by M1 marker CD192. Other M1/M2 markers, T-cell and neutrophil expression was limited. Lumbar samples showed higher expression of iNOS and Arg1 compared to cervical samples. Presence of Modic changes was associated with higher levels of CD68$^+$ cells ($p = 0.046$), but no significant differences in M1/M2 markers were found.
Discussion and conclusion: For studying M1 macrophages, CD192 is the most suitable marker due to its high expression; whereas for M2 macrophages, this is CD163 due to its high expression and selectivity. Further, the relatively high expression of M2 markers indicates predominance of anti-inflammatory over pro-inflammatory macrophages in symptomatic lumbar and cervical disc herniations. No associations between M1/M2 markers and MC were seen in this limited number of samples. In order to further explore the role of macrophage differentiation and its relation with MC in radiculopathy, a large prospective trial with elaborate clinical follow-up is required.

1. Introduction

Herniation of the intervertebral disc is a common phenomenon, and when accompanied by radicular symptoms, it causes a major burden for society worldwide. These severe radicular symptoms are thought to be partially caused by the compression of the adjacent nerve root, but the nerve root can also be sensitized by inflammatory cells responding to local damage and/or disc material (Djuric et al., 2019, 2020b).

These abovementioned infiltrating inflammatory cells mostly consist of macrophages (Cunha et al., 2018; Kawaguchi et al., 2001), which have shown to considerably aid disc resorption through phagocytosis of herniated tissue (Kobayashi et al., 2009), thereby increasing the rate of recovery. In contrast, they may also excrete pro-inflammatory cytokines (Woods and Hilibrand, 2015), which may sensitize the nerve root and have been associated with a decreased rate of recovery (Djuric et al., 2020a). The discrepancy in these effects can potentially be explained by

* Corresponding author. Department of Neurosurgery of the LUMC, Albinusdreef 2, 2333ZA, Leiden, the Netherlands.
E-mail address: n.djuric@lumc.nl (N. Djuric).

https://doi.org/10.1016/j.bas.2022.101698
Received 1 March 2022; Received in revised form 9 October 2022; Accepted 23 November 2022
Available online 24 November 2022
2772-5294/© 2022 The Authors. Published by Elsevier B.V. on behalf of EUROSPINE, the Spine Society of Europe, EANS, the European Association of Neurosurgical Societies. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

the various differentiation profiles of macrophages. Each set of environmental cues will lead to distinct macrophage phenotypes, which show unique behaviors and expression profiles (Martinez and Gordon, 2014). Even though each differentiation profile produces a unique phenotype, they can be polarized to pro-inflammatory (M1) or anti-inflammatory (M2) macrophages (Vogel et al., 2014). M2 macrophages express markers such as CD163, CD206 and CD209, and may be responsible for the abovementioned beneficial effects on recovery through expression of anti-inflammatory factors like IL-4 or arginase-1 (Vogel et al., 2014; Pourcet and Pineda-Torra, 2013; Gerrick et al., 2018; Tarique et al., 2015) and phagocytosis factors such as IL-10 (Gerrick et al., 2018; Li et al., 2022). By contrast, M1 macrophages express markers such as CD40 and CD192 and may exacerbate pain symptoms through expression of pro-inflammatory cytokines such as, IL-1β, IL-6 and TNF- α (Djuric et al., 2020a; Vogel et al., 2014; Gerrick et al., 2018; Lee et al., 2016), aggravating the immune response.

Since not all herniations are of the same origin, it is possible that macrophage differentiation may possibly vary depending on the characteristics of the disc lesion and condition of the vertebral endplates. Vertebral endplate changes are visible on MRI (Modic changes; MC) and are considered to be a sign of inflammatory fibrosis and/or edema (Dudli et al., 2017), possibly induced by decreased vascularization. In a previous study, we demonstrated that in patients without Modic changes, macrophage infiltration was associated with faster recovery after surgery, whereas in patients with Modic changes, macrophage infiltration was associated with a decreased recovery rate after surgery (Djuric et al., 2019). Based on these findings, a higher percentage of M2 macrophages is expected in patients without MC, whereas in those with MC, M1 macrophages are likely to be present more abundantly.

Another factor that could be of influence is the location of the herniation: cervical herniated discs have been proposed to contain a lower degree of neovascularization after herniation compared to lumbar ones (Chitkara, 1991), which likely results in a lower degree of macrophage infiltration in cervical as compared to lumbar patients.

A better understanding of macrophage differentiation in the herniated disc will help to understand its role in recovery and may open new doors for treatment possibilities. For example, shifting a macrophage profile towards M2 could fasten the resorption process of herniated discs and alleviate radiculopathy symptoms. Therefore, the aim of the present study is to immunohistochemically explore macrophage differentiation in both cervical and lumbar disc herniations and investigate the association with Modic changes. Additionally, in order to explore whether other immune cells may also play a pivotal role in radiculopathy, T-cells and neutrophils will be analyzed as well.

2. Methods

2.1. Study population

Discectomy patients from a single center were included between January 2018 and July 2022 if they suffered from radicular symptoms due to an intervertebral disc herniation, verified by MRI, for 8 or more weeks.

In cervical radiculopathy patients, an anterior approach was performed for discectomy and the bulk of the disc was removed. In lumbar radiculopathy patients, a posterior approach was performed using a standard unilateral transflaval approach. The herniated part and some of the intervertebral part of the disc was collected. Study was approved by the medical ethics committee.

2.2. Sample processing

All harvested discs were fixed in a 4% formaldehyde solution for 3–7 days. Tissue was subsequently embedded in paraffin blocks and a 5-μm thick slices were taken from the middle of the block for haematoxylin and eosin staining, which was performed according to the Leica ST 5020-mulitstainer standard protocol. Samples were evaluated under the microscope for presence of inflammatory cells. If tissue from one sample exceeded the capacity of 1 paraffin block, multiple blocks were formed and a slide of each block was evaluated.

2.3. Immunohistochemistry

From each disc containing inflammatory cells, the slide with the most inflammatory cells was submitted to further analysis using immunohistochemistry: Macrophages were characterized using CD68, M1 macrophages were identified using CD40, iNOS and CD192 M2 macrophages were identified using CD163, Arginase 1 (ARG1) and CD209, T-cells were identified using CD3 and neutrophils using CD15. 5-μm paraffin slices were rinsed in ethanol and methanol solutions and prepared for the expression of CD68 (DAKO, Denmark), CD40 (Sanbio, Netherlands), iNOS (Spring bioscience, USA), CD192 (Thermo Fisher Scientific, Netherlands), CD163 (Abcam, Netherlands), ARG1 (Spring bioscience, USA), CD209 (Thermo Fisher Scientific, Netherlands), CD3 (DAKO, Denmark). Immunohistochemistry was performed using a three-step indirect method. Antibodies CD68, Arg1, iNOS and CD3 were cooked in Citrate pH 6.0 buffer, CD163, CD192 and CD15 in EDTA pH 8,5 and CD40 in pronase as a pre-treatment, for CD209 no pretreatment was used. Subsequently, an avidin-biotin complex technique was performed with the Vectastain ABC-Elite Kit (Vector Lab. USA) and the appropriate biotinylated antibodies. Visualization of the peroxidase reaction was done with DAB solution (Sigma). Samples were counterstained with Harris haematoxylin. All samples were accompanied by a positive control, which was atherosclerosis tissue for all macrophage markers, and tonsil tissue for T-cells and neutrophils. For evaluation all samples were photographed using Philips ultra-fast scanner. Since previous studies have reported the expression of CD68, CD40 and iNOS by nucleus pulposus cells and chondrocytes (Yang et al., 2016; Bai et al., 2019; Gotoh et al., 2004), cells were analyzed based on morphological features and only macrophages were photographed and evaluated. The same approach was used for CD163, CD192, CD209 and Arg1. For CD3 and CD15 morphological features of T-cells and neutrophils respectively were taken into account as well.

2.4. MRI

For the evaluation of Modic Changes, a 3T MRI was used. Both sagittal T1-and T2-weighted images of the lumbar or cervical spine were obtained. Image evaluation of MC was according to the criteria of Modic et al., 1988a, 1988b. Image evaluation was done by two independent researchers in a blinded manner (ND & CVL). Inter agreement analysis was performed and kappa values were calculated. Upon disagreement, a third observer was consulted (GL).

2.5. Data analysis

Preceding cell counting, quality of all staining results was evaluated by a senior pathologist (SVD). Cell counts were performed using ImageJ and evaluation was executed by two independent researchers (ND & GL). Inter-observer correlation coefficients were calculated for each staining

separately. For each antibody 50 pictures were evaluated by hand in the above-mentioned manner. Subsequently an automated cell count algorithm in ImageJ was matched on the average count of the two observers with a correlation coefficient of >0.8, which was regarded as a strong correlation. This algorithm was used to evaluate the remaining bulk of pictures.

Positive macrophage/lymphocyte counts were divided by the surface of the evaluated herniated disc material in cm^2. M1 and M2 dominance could only be analyzed in samples where inflammation was present, hence it was only determined in if at least 10 positive $CD68^+$ cells/cm^2 were seen, Also, for relative M1/M2 dominance, M1 and M2 markers were expressed as percentages (ranging from 0% to 100%) of the number of CD68 positive cells.

For statistical analysis, expression level of each marker was analyzed using a multiple regression with MC and location of herniation (cervical/lumbar) as predictors. Additionally, since type of herniation (bulging/extrusion/sequester) is known to influence the extend of macrophage infiltration (Djuric et al., 2020b), it was included as covariate. Moreover, since epidural corticosteroid injections have been suggested to alter inflammation profiles, this variable was also considered as a covariate (Ehrchen et al., 2019).

Data was log transformed to achieve a normal distribution and linear regression assumptions were met. Furthermore, the correlations between duration of symptoms and expression of inflammatory markers were evaluated using Spearman's correlation. For all analyses, alpha was set at 5%.

3. Results

3.1. Study population

Herniated disc samples were retrieved from 45 patients who consecutively underwent discectomy for radiculopathy and fulfilled the inclusion criteria. Twenty-two patients underwent lumbar discectomy for sciatica, and twenty-three patients underwent anterior discectomy for cervical radiculopathy. No statistically significant difference was found in age, gender and symptom duration between lumbar and cervical patients, nor between patients with and without MC (Table 1).

3.2. Histopathology

The tissue that was removed, embedded and stained mostly consisted of nucleus pulposus (NP) material with varying degrees of annulus fibrosus (AF) and cartilage endplate (EP) present. 33/45 samples (73%) showed inflammatory cells which were localized in the edges of NP tissue (Fig. 1a). Only CD163 showed to be specific for macrophages. CD68, CD40, iNOS, CD192, Arg1 and CD209 also stained nucleus pulposus cells and chondrocytes (Fig. 1).

3.3. Inter observer agreement and algorithm efficacy

For all macrophage markers, an inter observer correlation coefficient of >0.8 was found between the two observers for 50 randomly chosen pictures from different samples. For CD68, CD40, CD192, CD163 and CD209 a correlation coefficient of >0.8 was also achieved between the average count of the observers and ImageJ automated cell count. For iNOS and Arg1 however, the required correlation coefficient could not be achieved and cells were counted manually. Regarding CD3 and CD15, the total number of pictures with positive cells was <50, hence no automated cell count could be validated and cell counts were performed manually. For all manual counts, average counts of the observers were used for data analysis (Table S1).

3.4. Quantification of inflammation markers

In 33 of the 45 samples, inflammatory cells were present. Number of macrophages ($CD68^+$) varied widely between samples ranging from 8/cm^2 till 19,341/cm^2 with a median of 50/cm^2 (median = 261/cm^2 within the 33 samples with inflammation). The M1 and M2 markers were only analyzed in the population with >10 macrophages ($CD68^+$)/cm^2. The median expression of M1 markers was 52/cm^2 for CD192, 5/cm^2 for iNOS and 3/cm^2 for CD40. Median expression of M2 markers was higher: CD209 was expressed with a median of 159/cm^2, CD163 with a median of 70/cm^2, Arg1 was barely expressed with a median of 0/cm^2.

CD3 and CD15 were mostly expressed in limited quantities: CD3 median<1/cm^2, CD15 median<1/cm^2. Further, high expression of CD68 correlated with higher levels of CD3 ($p < 0{,}001$) and CD15 ($p < 0{,}001$).

3.5. Overall suitability of M1 and M2 markers in disc tissue

Taken together, CD163 was shown to be a suitable marker for M2 macrophages in both lumbar and cervical radiculopathy patients due to its abundant presence and specific, intracellular macrophage staining. CD209 is also a useful M2 marker due to its abundance and staining intensity, but it was also found to stain NP cells, thereby making CD163 the preferential marker. Furthermore, Arg1 was deemed unsuitable as M2 marker due to its limited expression and extracellular staining.

Regarding M1 markers, CD192 was regarded as a suitable marker due to its relatively high abundance and staining intensity. Further, both iNOS and CD40 can be used as M1 markers, but with some limitations: iNOS showed the highest expression levels, but also illustrated extracellular reactivity and limited staining intensity, making it unsuitable for algorithm counting. CD40 was expressed in lower quantities, but staining was intracellular and the intensity was high, thereby making it suitable for algorithm counting. Further, Both CD3 and CD15 have shown to be proper markers for T-cells and neutrophils respectively. Nonetheless, as they are only expressed in limited numbers, their relevance in the inflammation response of the herniated disc is questionable.

3.6. Inter observer agreement MRI

For the presence of MC, inter observer agreement was moderate with an agreement percentage of 78% (Cohens Kappa = 0,58). Of the 45 patients, 22 patients did show MC, of which four type 1, seventeen type 2 and one type 3, 22 did not show MC and one MRI was lost (Table 1b).

3.7. Macrophage expression in subgroups

For the multiple regression analysis, only type of herniation was included as a covariate, as the usage of corticosteroid injections was not of any influence on the regression model.

Regarding the differences between cervical and lumbar samples, expression levels of most markers were comparable. Nevertheless, even though the expression levels of iNOS (M1) and Arg1 (M2) were very limited, the logistic regression showed significantly higher levels of these markers in lumbar discs (table 3A). Regarding differences between patients with and without MC, those with MC + had significantly higher levels of $CD68^+$ cells/cm^2 ($p = 0.046$), none of the M1 and M2 markers showed significant differences between MC+ and MC- groups, and neither did the T-cell (CD3) or neutrophil (CD15) markers (Table 3A).

In an additional multiple regression analysis, relative expression of M1 and M2 markers as percentage of CD68 positive cells was assessed. In

line with the absolute expression levels, the relative analysis showed that lumbar patients had significantly higher percentages of iNOS (M1) and Arg (M2) compared to cervical samples (Table 3B). Other M1 and M2 markers showed no significant differences. No significant differences in relative expression were seen between MC+ and MC- patients. Overview of all absolute expression levels can be found in Fig. 2 and relative expression levels in Fig. 3. Linear regression results are listed in Table 3.

3.8. Influence of time on macrophage differentiation

The median duration of symptoms was 7 months (IQR: 7,8). Neither the absolute nor relative expression of any of the inflammatory markers was correlated to the duration of symptoms (Table 4).

4. Discussion

For studying M1 macrophages in herniated disc tissue, CD192 was regarded as the most suitable marker due to its high expression For M2 macrophages however, CD163 was most suitable due to its high expression and selectivity. Furthermore, the present study indicates that M2 macrophages (CD163+, CD209+) are the dominant type of inflammatory cells in herniated intervertebral disc tissue harvested from cervical and lumbar radiculopathy patients. Moreover, for most inflammatory markers, no differences were seen between cervical and lumbar patients, only iNOS (M1) and Arg1 (M2), which were both expressed in limited quantities, were more abundant in lumbar patients. Further, a higher number of macrophages was seen in MC + patients, but no significant differences were seen in absolute or relative expression of the M1 or M2 markers between MC+ and MC- patients.

The dominance of M2 macrophages found in this study was not in line with a recent study by Yamagishi et al. (2021), where the authors looked at CD16 (M1) and CD206 (M2) expression in cervical herniated discs (Yamagishi et al., 2021). They found higher levels of CD16 in 61% of the inflamed samples, whereas in 39% CD206 was more dominantly expressed. As we have shown in the present study, not all M1 and M2 markers are expressed equally, making it difficult to draw conclusions on overall M1 or M2 expression based on a single marker. Moreover, only 3 random microscopy fields were evaluated in their study (Yamagishi et al., 2021), whereas in the present study all microscopy fields were evaluated.

Besides the differences in iNOS and Arg1, which were expressed in very limited quantities in all samples, our results imply a similar degree of macrophage infiltration in lumbar and cervical herniated discs. This seems to be in contrast with a previous study by Sadowska et al. (2017), who found higher mRNA levels of IL-15 in nucleus and higher levels of IL-6, TNF-α, TRPC6 in annulus of cervical discs (Sadowska et al., 2018). When interpreting our results, it should also be noted that a different surgical procedure was used for lumbar and cervical discs. During surgery for lumbar herniations, only the herniated part is removed, which is the part that is exposed to the epidural space and therefore more likely to undergo neovascularization, which may subsequently lead to a high macrophage cell density. By contrast, during surgery for cervical herniated discs, the whole disc is removed. This means that for cervical herniations, the investigated area under the microscope will contain more 'inner' nucleus pulposus material, which is relatively healthy disc material that is not exposed to epidural space and will thus contain no or less macrophages, leading to a lower macrophage density in the analysis. Thus, our cervical counts of inflammatory cells/cm2 could be underestimated.

Moreover, no significant differences in M1 or M2 markers were seen between MC+ and MC- patients. This is not in accordance with our hypothesis in which we, based on previous findings (Djuric et al., 2019), expected MC + to be associated with an increase in M1 macrophages. By contrast, we also expected a higher level of M2 in MC- patients. The fact that we were not able to find these associations could very well be due to our limited sample size. This speculation is supported by a non-significant trend towards higher relative expression of M1 (CD192) and lower relative expression of M2 (CD163) in MC + patients.

Alternatively, it could be that other M1 markers such as CD64, CD80 or CD86, or other M2 markers such as CD11b or CD206 would provide better results (Vogel et al., 2014; Gerrick et al., 2018; Tarique et al., 2015). Unfortunately, assessing all possible M1 and M2 markers was beyond the scope of this study.

In the present study, symptom duration did not affect results. In a normal wound healing response, the initial response is dominated by M1 macrophages on day 1 (Daley et al., 2010), and switches to M2 in the following days (Mahdavian Delavary et al., 2011; Lucas et al., 2010). In line with this process, a recent study by Nakawaki et al. (2019) showed that in an intervertebral disc injury model in mice, M1 related cytokine expression decreased from 1 to 7 days after the injury whereas M2 marker expression increased at 7 days and remained elevated after 28 days (Nakawaki et al., 2019). As patients in this study had symptoms >2 months, it is assumed that the initial switch from M1 to M2 was finalized prior to tissue harvesting, and that in this stage of chronic symptoms, a longer duration of symptoms could not affect macrophage differentiation. Alternatively, it could also be stated that since the disc is a degenerated environment, it cannot be compared with a normal wound healing response and no switch in macrophage type should be expected over time. In either case, is should not be forgotten that this analysis was conducted with a small number of samples and that data was obtained retrospectively during the intake visit, making it prone for recall bias.

A limitation of this study lies within the methodology of tissue processing and immunohistochemistry. For example, for each antibody a new slide of the same paraffin block was used, and sometimes the total number of macrophages was larger in the M1/M2 marker slide compared to the CD68 slide. Because the CD68 slide was used as a reference number to calculate the percentage of M1/M2, the positive fraction of M1/M2 could exceed 100% of $CD68^{+}$. In the relative analysis, these samples were scored as 100%. This difference in number of cells per slide was more frequently occurring in slides that were slit of the paraffin block at a relatively large distance from the location where the 'reference slide' was obtained, on which CD68 staining was performed. In this study the most distant slides were stained with CD209 and CD192, which may decrease the reliability of their relative analysis. Moreover, from some patients, more tissue could be collected than from others, resulting in multiple paraffin tissue blocks, of which only the one with the most inflammation was submitted for evaluation. This may have resulted in an overestimation of the number of inflammatory cells in patients with large amounts of tissue. Nonetheless, during surgery, not only the herniated tissue, but also some intervertebral disc tissue without any inflammatory cells was removed. Thus, the block with the most inflammatory cells logically resembles the sample with the most herniated tissue, thereby making it the most representable sample. Another limitation of this study is caused by the absence of correction for multiple testing, which we deemed unsuitable for the exploratory nature of this study. At last, this study did not look into resorption between MRI and what was seen intra operatively, which prevents translating the conclusions of this study to clinical practice.

Another issue to consider is the use of epidural corticosteroid injections in 18% of the patients. Corticosteroids have been shown to influence inflammatory cells through inhibiting the M1 response and stimulating an M2 related factors (Ehrchen et al., 2019). This could thus have increased the number of M2 macrophages found in certain study groups, thereby confounding the analysis. Nonetheless, when incorporating corticosteroid injections as a covariate in the regression analyses,

it was not of any significance for the outcome and was hence removed from the model.

5. Conclusion

Macrophages are abundantly present in herniating intervertebral disc tissue and our results demonstrate that M1 and M2 type macrophages can adequately be discerned by immunohistochemical staining. CD163 is the most suitable marker to indicate M2 macrophages, and CD192 is most appropriate to indicate M1 macrophages.

The abundant presence of M2 (CD163, CD209) macrophages in herniating intervertebral disc tissue, compromising the nerve, indicates the predominance of an anti-inflammatory over a pro-inflammatory macrophage response, both in lumbar and cervical patients. The predominance of the presence of macrophages in MC + patients suggests an association with vertebral endplate pathology. In order to further explore the role of inflammation and MC in recovery of surgical patients with lumbar and cervical radiculopathy, a large prospective trial with elaborate clinical follow-up is required.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Ingrid Hegeman-Kleinn for performing the immunohistochemistry experiments, Jesse van Oostrum for his help with the algorithm and Boyd Kenkhuis for assistance in the development of the antibody panel.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bas.2022.101698.

Appendix

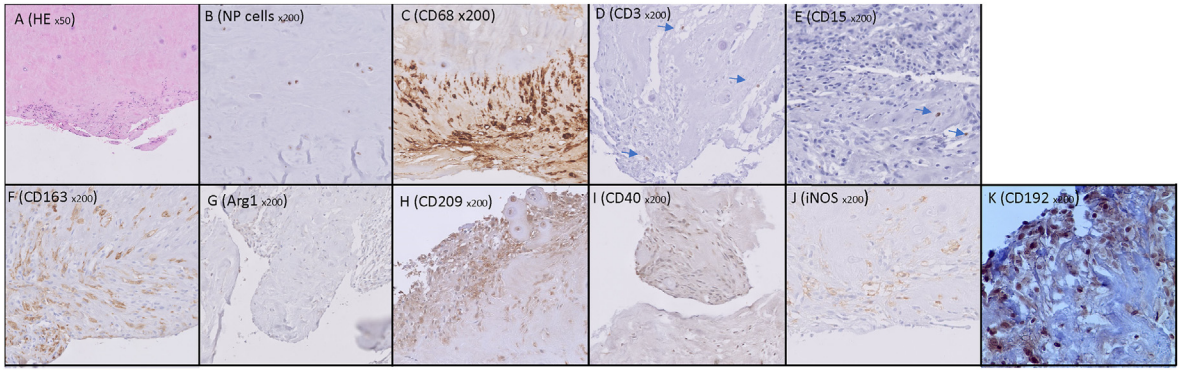

Fig. 1. examples of immunohistochemistry staining
Fig. 1 displays examples of the different antibody results. 1A shows a HE slide with infiltrating
inflammatory cells in NP tissue. 1B shows positively stained NP cells with CD68. 1C shows infiltrating macrophages intracellularly stained with CD68. 1D reveals intracellular staining of infiltrating T-cells with CD3. 1E shows intracellular staining of infiltrating neutrophils with CD15. 1F illustrates infiltrating macrophages of which a large percentage is intracellularly stained with CD163. 1G displays infiltrating macrophages of which a few are Arg1 positive, ECM surrounding Arg1 positive cells is also stained positively. 1H shows infiltrating macrophage intracellularly stained with CD209. 1I reveals infiltrating macrophages, some of which are stained intracellularly with CD40. 1J illustrates infiltrating macrophages of which some are positive for iNOS. The surrounding ECM flared up with iNOS staining as well. 1K displays infiltrating macrophages intracellularly stained with CD192..

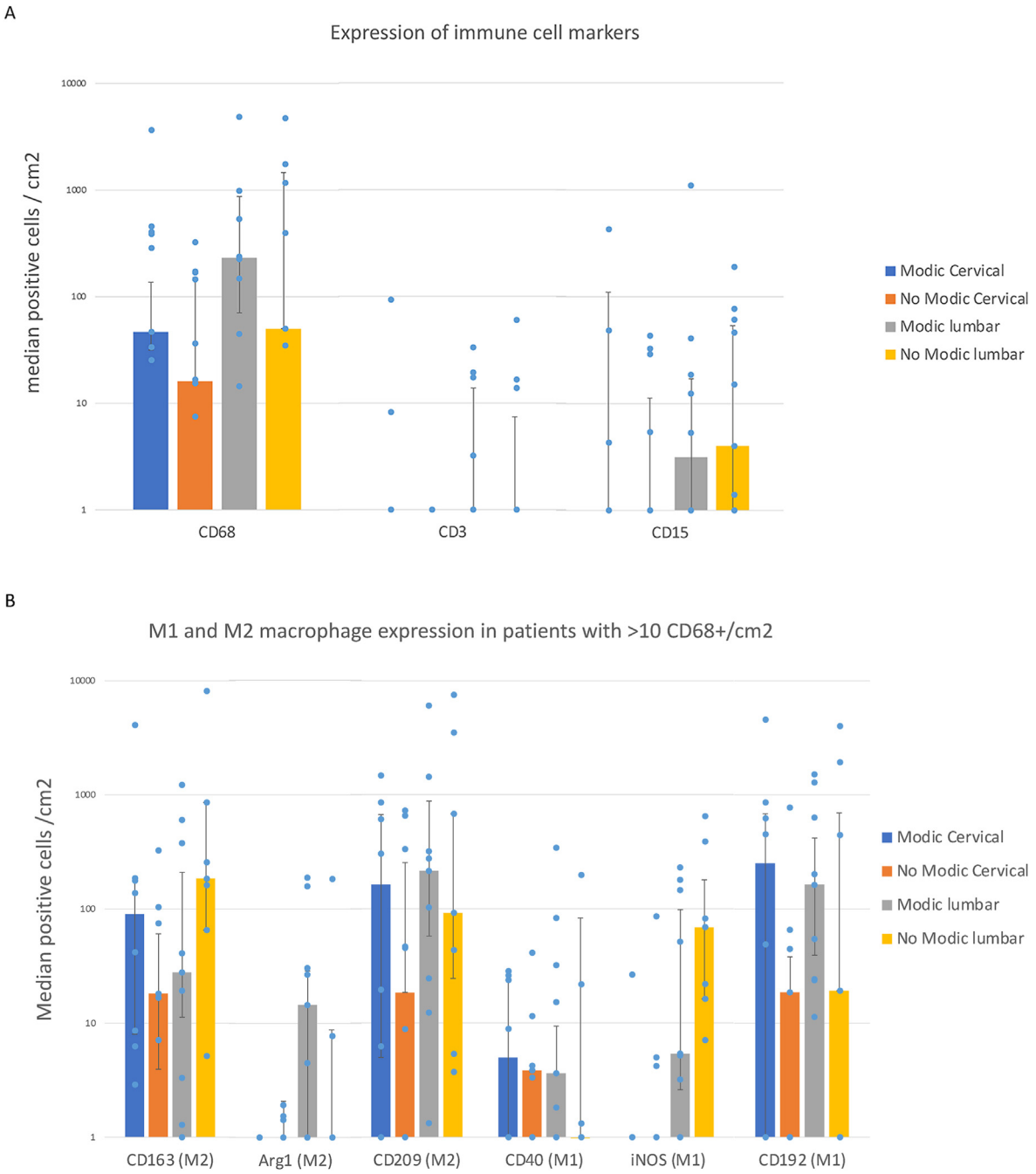

Fig. 2. Fig. 2A shows expression of immune cell markers for macrophages (CD68), T-cells (CD3) and neutrophils (CD15) for each of the 4 subgroups. Fig. 2B shows expression of M1 (CD40, iNOS, CD192) and M2 (CD163, Arg1, CD209) macrophage markers in the population with >10 $CD68^{+}$ cells per cm2. Values are expressed as median number of positive cells per cm2, medians and individual data points are shown, error bars are IQR values. For the Y axis, a logarithmic scale was used..

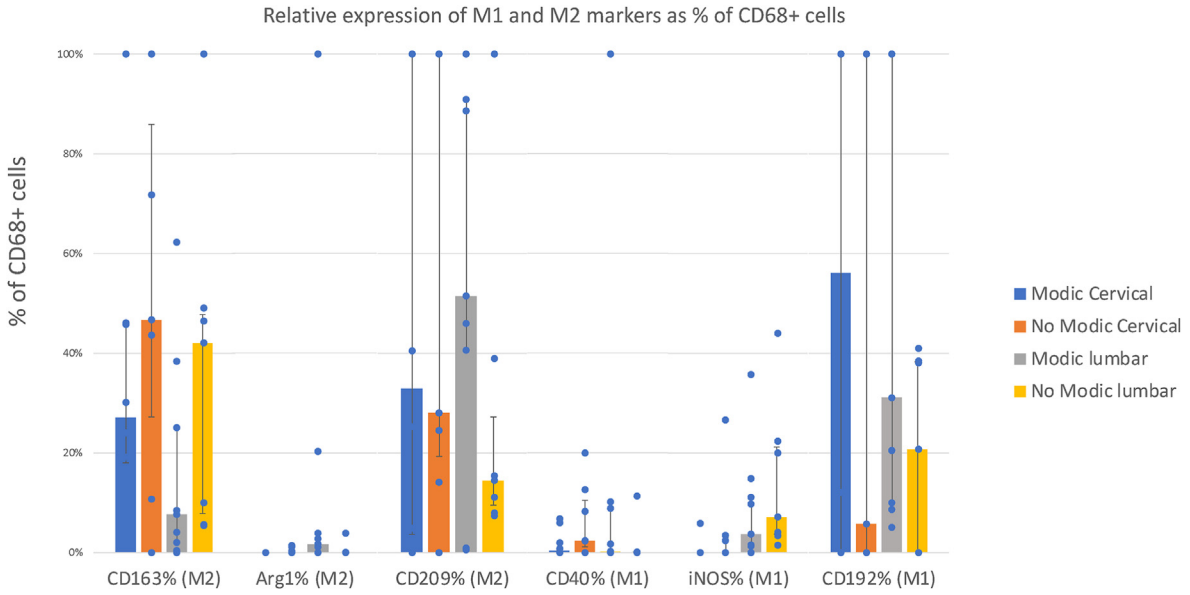

Fig. 3. Fig. 3 shows relative expression of M1 (CD40, iNOS, CD192) and M2 (CD163, Arg1, CD209) macrophage markers as a percentage of the CD68 positive cells. Only samples with >10 $CD68^+$ cells per cm2 were included. Values are percentages of $CD68^+$ cells, medians and individual data points are shown, error bars are IQR values..

Table 1
Baseline characteristics

Table 1	Cervical no MC	Cervical MC	Lumbar no MC	Lumbar MC	p-value
N	12	11	10	11	
Age	52	57	53	50	0,587
% male	33%	64%	50%	45%	0,541
Duration of symptoms (months)	11.6	15.5	14.7	11.6	0,900
steroid treatment %	0%	36%	30%	10%	0,165

Table 1 illustrates baseline characteristics separately for cervical and lumbar samples and for patients with and without Modic Changes (MC). In de lumbar group, MRI data was missing for one patient.

Table 2
overview of immune cell marker expression in total population

2A			
Marker	N	median/cm2	IQR
CD68	45	50	431
CD3	45	<1	1
CD15	45	<1	24
2B			
marker	N	median/cm2	IQR
CD163	32	70	231
Arg1	32	0	7
CD209	32	159	706
CD40	32	3	23
iNOS	32	5	79
CD192	32	52	631

Table 2A displays the median number of positive cells per cm^2 for macrophages, T-cells and neutrophils. Table 2B shows the expression of M1 (CD40, iNOS and CD192) and M2 (CD163, CD3, CD15) markers in samples with >10CD68+ cells. Sample size, median cells per cm^2 and their corresponding inter quartile ranges are provided.

Table 3
Results of linear regression

3A	MC			
Marker	Beta	P-value	Beta	P-value
CD68	0.311	**0,046***	0,242	0097
CD3	0,29	0,065	0275	0,064
CD15	0,075	0639	0,237	0124
CD163	−0,091	0659	0,142	0465
Arg1	0,143	0423	0,494	**0006***
CD209	−0,009	0966	0,185	0333
CD40	0	0,554	0065	0.737
iNOS	−0,205	0187	0,636	**<0,001***
CD192	0.167	0.834	0,168	**0378**
3B	MC			
Marker	Beta	P-value	Beta	P-value
CD163%	−0,252	**0186**	−0,321	0077
Arg1%	0,124	0495	0,348	0048*
CD209%	0,068	0744	0,025	0898
CD40%	0.014	0,948	−0,03	0,877
iNOS%	−0,324	0061	0,494	0004*
CD192%	0,019	0941	0,047	0855

Table 3A lists the result of linear regressions in which the absolute cell counts per cm^2 of each marker were used as dependent variables. For CD68, CD3 and CD15 all samples were included in the analysis. For all M1 (CD40, iNOS, CD192) and M2 (CD163, Arg1, CD209) markers, only samples with >10 $CD68^+$ cells/cm^2 were included. In order to provide an overview of the relative distribution of M1 and M2 markers, table 3B displays the results of a linear regression with M1 and M2 markers as a percentage of $CD68^+$ cells. Only samples with >10 $CD68^+$ cells/cm^2 were included. In all regression analyses, location of herniation (cervical or lumbar) and MC were used as independent predictors, and the type of hernia (bulging/extrusion/sequester) was taken into account as a covariate. For both MC and location of herniation, estimator p-values and standardized beta are provided, 'no MC' and 'cervical samples' were used as reference category.

Table 4
correlations between duration of symptoms and inflammatory markers

Absolute count	Spearman's rho	p-value
CD68	−0,143	0349
CD163	0,034	0852
Arg1	−0,184	0313
CD209	0,252	0164
CD40	0,056	0761
iNOS	−0,163	0374
CD192	−0,043	0817
CD3	−0,006	0969
CD15	−0,195	0,2
Relative count		
CD163%	0,113	0536
Arg1%	−0,12	0,514
CD209%	0,331	0069
CD40%	0,169	0354
iNOS%	−0,244	0178
CD192%	0,054	0767

Table 4 displays the correlation coefficients and p-values for the Spearman correlation tests between the duration of symptoms and macrophage, T-cell and neutrophil markers. Row 2–10 display the absolute cell counts whereas row 12–17 display expression as a percentage of CD68 positive cells. For all M1 and M2 markers, only samples with >10 $CD68^+$ cells/cm^2 were included.

References

Bai, X., Ding, W., Yang, S., Guo, X., 2019. Higenamine inhibits IL-1beta-induced inflammation in human nucleus pulposus cells. Biosci. Rep. 39 (6).

Chitkara, Y.K., 1991. Clinicopathologic study of changes in prolapsed intervertebral disks. Arch. Pathol. Lab Med. 115 (5), 481–483.

Cunha, C., Silva, A.J., Pereira, P., Vaz, R., Goncalves, R.M., Barbosa, M.A., 2018. The inflammatory response in the regression of lumbar disc herniation. Arthritis Res. Ther. 20 (1), 251.

Daley, J.M., Brancato, S.K., Thomay, A.A., Reichner, J.S., Albina, J.E., 2010. The phenotype of murine wound macrophages. J. Leukoc. Biol. 87 (1), 59–67.

Djuric, N., Yang, X., Ostelo, R., van Duinen, S.G., Lycklama, A.N.G.J., van der Kallen, B.F.W., Peul, W.C., Vleggeert-Lankamp, C.L.A., 2019. Disc inflammation and Modic changes show an interaction effect on recovery after surgery for lumbar disc herniation. Eur. Spine J. 28 (11), 2579–2587.

Djuric, N., Lafeber, G.C.M., Vleggeert-Lankamp, C.L.A., 2020a. The contradictory effect of macrophage-related cytokine expression in lumbar disc herniations: a systematic review. Eur. Spine J. 29 (7), 1649–1659.

Djuric, N., Yang, X., El Barzouhi, A., Ostelo, R., van Duinen, S.G., Lycklama, A.N.G.J., van der Kallen, B.F.W., Peul, W.C., Vleggeert-Lankamp, C.L.A., 2020b. Lumbar disc extrusions reduce faster than bulging discs due to an active role of macrophages in sciatica. Acta Neurochir. 162 (1), 79–85.

Dudli, S., Sing, D.C., Hu, S.S., Berven, S.H., Burch, S., Deviren, V., Cheng, I., Tay, B.K.B., Alamin, T.F., Ith, M.A.M., Pietras, E.M., Lotz, J.C., 2017. Issls prize in basic science 2017: intervertebral disc/bone marrow cross-talk with Modic changes. Eur. Spine J. 26 (5), 1362–1373.

Ehrchen, J.M., Roth, J., Barczyk-Kahlert, K., 2019. More than suppression: glucocorticoid action on monocytes and macrophages. Front. Immunol. 10, 2028.
Gerrick, K.Y., Gerrick, E.R., Gupta, A., Wheelan, S.J., Yegnasubramanian, S., Jaffee, E.M., 2018. Transcriptional profiling identifies novel regulators of macrophage polarization. PLoS One 13 (12), e0208602.
Gotoh, H., Kawaguchi, Y., Harigai, M., Hara, M., Saito, S., Yamaguchi, T., Shimada, K., Kawamoto, M., Tomatsu, T., Kamatani, N., 2004. Increased CD40 expression on articular chondrocytes from patients with rheumatoid arthritis: contribution to production of cytokines and matrix metalloproteinases. J. Rheumatol. 31 (8), 1506–1512.
Kawaguchi, S., Yamashita, T., Yokogushi, K., Murakami, T., Ohwada, O., Sato, N., 2001. Immunophenotypic analysis of the inflammatory infiltrates in herniated intervertebral discs. Spine 26 (11), 1209–1214.
Kobayashi, S., Meir, A., Kokubo, Y., Uchida, K., Takeno, K., Miyazaki, T., Yayama, T., Kubota, M., Nomura, E., Mwaka, E., Baba, H., 2009. Ultrastructural analysis on lumbar disc herniation using surgical specimens: role of neovascularization and macrophages in hernias. Spine 34 (7), 655–662.
Lee, M.K., Moore, X.L., Fu, Y., Al-Sharea, A., Dragoljevic, D., Fernandez-Rojo, M.A., Parton, R., Sviridov, D., Murphy, A.J., Chin-Dusting, J.P., 2016. High-density lipoprotein inhibits human M1 macrophage polarization through redistribution of caveolin-1. Br. J. Pharmacol. 173 (4), 741–751.
Li, X.C., Luo, S.J., Fan, W., Zhou, T.L., Huang, C.M., Wang, M.S., 2022. M2 macrophage-conditioned medium inhibits intervertebral disc degeneration in a tumor necrosis factor-alpha-rich environment. J. Orthop. Res.
Lucas, T., Waisman, A., Ranjan, R., Roes, J., Krieg, T., Muller, W., Roers, A., Eming, S.A., 2010. Differential roles of macrophages in diverse phases of skin repair. J. Immunol. 184 (7), 3964–3977.
Mahdavian Delavary, B., van der Veer, W.M., van Egmond, M., Niessen, F.B., Beelen, R.H., 2011. Macrophages in skin injury and repair. Immunobiology 216 (7), 753–762.
Martinez, F.O., Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep 6, 13.
Modic, M.T., Masaryk, T.J., Ross, J.S., Carter, J.R., 1988a. Imaging of degenerative disk disease. Radiology 168 (1), 177–186.
Modic, M.T., Steinberg, P.M., Ross, J.S., Masaryk, T.J., Carter, J.R., 1988b. Degenerative disk disease: assessment of changes in vertebral body marrow with MR imaging. Radiology 166 (1 Pt 1), 193–199.
Nakawaki, M., Uchida, K., Miyagi, M., Inoue, G., Kawakubo, A., Satoh, M., Takaso, M., 2019. Changes in nerve growth factor expression and macrophage phenotype following intervertebral disc injury in mice. J. Orthop. Res. 37 (8), 1798–1804.
Pourcet, B., Pineda-Torra, I., 2013. Transcriptional regulation of macrophage arginase 1 expression and its role in atherosclerosis. Trends Cardiovasc. Med. 23 (5), 143–152.
Sadowska, A., Touli, E., Hitzl, W., Greutert, H., Ferguson, S.J., Wuertz-Kozak, K., Hausmann, O.N., 2018. Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression. Eur. Spine J. 27 (3), 564–577.
Tarique, A.A., Logan, J., Thomas, E., Holt, P.G., Sly, P.D., Fantino, E., 2015. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. Am. J. Respir. Cell Mol. Biol. 53 (5), 676–688.
Vogel, D.Y., Glim, J.E., Stavenuiter, A.W., Breur, M., Heijnen, P., Amor, S., Dijkstra, C.D., Beelen, R.H., 2014. Human macrophage polarization in vitro: maturation and activation methods compared. Immunobiology 219 (9), 695–703.
Woods, B.I., Hilibrand, A.S., 2015. Cervical radiculopathy: epidemiology, etiology, diagnosis, and treatment. J. Spinal Disord. Tech. 28 (5), E251–E259.
Yamagishi, A., Nakajima, H., Kokubo, Y., Yamamoto, Y., Matsumine, A., 2021. Polarization of infiltrating macrophages in the outer annulus fibrosus layer associated with the process of intervertebral disc degeneration and neural ingrowth in the human cervical spine. Spine J.
Yang, C., Cao, P., Gao, Y., Wu, M., Lin, Y., Tian, Y., Yuan, W., 2016. Differential expression of p38 MAPK alpha, beta, gamma, delta isoforms in nucleus pulposus modulates macrophage polarization in intervertebral disc degeneration. Sci. Rep. 6, 22182.