The role of inflammation in sciatica: the contradictory effect of macrophages

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Chapter 7

Exploring macrophage differentiation in herniated disc tissue in patients with radiculopathy; is this associated with Modic changes?

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Submitted

"Failure is an option here. If things are not failing, you are not innovating enough." (Elon Musk)

Abstract

Background & Objective

Cervical- and lumbosacral radiculopathy symptoms due to disc herniation are likely to be influenced by degenerative endplate changes and macrophage infiltration of the herniated disc. The present study was conducted to assess the efficacy of immunohistological methods to discern pro-inflammatory M1- and anti-inflammatory M2 macrophage differentiation patterns in herniated intervertebral disc tissue and to evaluate their associations with Modic changes (MC) of the vertebral endplates.

Methods

Herniated disc samples were collected from 38 patients undergoing surgery for cervical- or lumbosacral radiculopathy. Samples were processed for immunohistochemistry and stained for the presence macrophages: CD68 (macrophage marker), CD163 (M2), CD40 (M1), Arg1 (M2) and iNos (M1). In order to assess whether other immune cells also play a pivotal role, T-cells (CD3) and neutrophil (CD15) expressions were studied additionally.

Results

CD68 positive cells were present with a density of 10-100/cm2 in 10 samples and of >100/cm2 in 18 samples. In 12 of these samples 10-50% of the CD68+ cells were M2-type (CD163+) and in 6 of these samples this was even more than 50%. Whereas in 7 of the CD68+ samples, 10-50% of the CD68+ cells were M1-type (iNOS+), and in none of the samples more than 50%. Arg1 and CD40 were expressed in minimal quantities. Expression levels for CD68 were slightly higher in lumbar than cervical patients (OR=2.7, p=0.18). Presence of Modic changes was associated with higher levels of CD68+ cells (OR=6.0, p=0.023) and with lower relative expression of CD163 (OR=0.123, p=0.02). T-cells (CD3) and neutrophils (CD15) were present in a limited number of samples.

Discussion

The relative high expression of CD163 (M2 marker) indicates predominance of an anti-inflammatory over a pro-inflammatory macrophage presence in symptomatic disc herniations, both at lumbar and cervical level. The association of M2 marker positive cells with absence of Modic changes implies possibilities for prediction of rate of recovery in radiculopathy patients. However, the opposite, an association between presence of Modic changes and pro-inflammatory macrophages could not be established.

Introduction

Herniation of the intervertebral disc is common phenomenon that causes a major burden for society worldwide. With an incidence of 1-3% it occurs most often in lumbar discs [1], followed by cervical discs with an incidence of 0,018% [2]. Lumbar patients suffer from disabling leg pain that radiates down the dermatome, whereas for cervical patients this radiating pain is localized in the arm. These symptoms seem to be caused by a multiple factors and vary tremendously in intensity and duration between patients. Recent evidence has indicated that an important factor that may influence radicular symptoms is infiltration of the herniated disc with inflammatory cells [3, 4], which are mostly macrophages [5, 6].

Macrophages have shown to considerably aid disc resorption through phagocytosis of herniated tissue [7], thereby increasing the rate of recovery. In contrast, they may also excrete pro-inflammatory cytokines [8], which may sensitize the nerve root and have been associated with a decreased rate of recovery [9]. The discrepancy in these effects can potentially be explained by the various differentiation profiles of macrophages. Each set of environmental cues will lead to distinct macrophage phenotypes, which show unique behaviors and expression profiles [10]. Even though each differentiation profile produces a unique phenotype, they can be polarized to pro-inflammatory(M1) or anti-inflammatory (M2) macrophages[11]. M2 macrophages express markers such as CD163, and may be responsible for the abovementioned beneficial effects on recovery through expression of anti-inflammatory factors like IL-4 or arginase-1 [11-13] and phagocytosis factors such as IL-10 [13]. By contrast, M1 macrophages express markers such as CD40 and may exacerbate pain symptoms through expression of pro-inflammatory cytokines such as, IL-1β, IL-6 and TNF- α [9, 11, 13].

Since not all herniations are of the same etiology, it is possible that macrophage differentiation may vary depending on the characteristics of the disc lesion and condition of the vertebral end plates. Vertebral endplate changes are visible on MRI (Modic changes; MC) and are considered to be a sign of inflammatory fibrosis and/or edema [14], possibly induced by decreased vascularization. In a previous study, we demonstrated that in patients without MC, macrophage infiltration was associated with faster recovery after surgery, whereas in patients with MC, macrophage infiltration was associated with a decreased recovery rate after surgery [4]. 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. In this study we aim to verify this by characterizing the macrophages present in symptomatic herniated discs of patients that were subjected to surgery for radiculopathy. Additionally, In order to explore whether other immune cells may also play a pivotal role in radiculopathy, T-cells and neutrophils will also be analyzed.

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 [15], which likely results in a lower degree of macrophage infiltration in cervical as compared to lumbar patients. Moreover, In cervical spine herniated disc pathology, MC are observed less frequently [16]. If true, this would imply predominance of M2 macrophages in cervical herniated discs.

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. Inducing a shift towards M2 macrophage presence in radiculopathy patients could fasten the resorption process of herniated discs and alleviate radiculopathy symptoms. Additionally, the presence of MC may be indicative of the dominant type of macrophage present and predict the natural course of clinical symptoms. Therefore, the aim of this study is to immunohistochemically explore macrophage differentiation in both cervical and lumbar disc herniations and investigate the association with Modic changes.

Methods

Study population

Discectomy patients were included if they suffered 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.

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 hematoxylin staining, which was performed according to the Leica ST 5020-mulitstainer standard protocol. Samples were evaluated under the microscope for clear signs 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.

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 and iNOS, M2 macrophages were identified using CD163 and Arginase 1(ARG1), 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) CD163 (Abcam, Netherlands), ARG1 (Spring bioscience, USA), 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 and CD15 in EDTA pH 8,5 and CD40 in pronase as a pre-treatment. 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 hematoxylin. All samples were accompanied by a positive control, which was atherosclerosis tissue for all macrophage markers, and tonsil tissue for T-cells and B-cells. In order to standardize the evaluation of the samples, all samples were photographed under a light microscope before they were evaluated, using Philips ultra fast scanner. Since previous studies have reported the expression of CD68, CD40 and iNOS by nucleus pulposus cells and chondrocytes [17-19], cells were analyzed based on morphological features and only macrophages were photographed and evaluated. The same approach was used for CD163 and Arg1. For CD3 and CD15 morphological features of T-cells and neutrophils respectively were taken into account.

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 [20, 21]. 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).

Data analysis

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. If an intercorrelation of >0.8 was found, a third observer was consulted (SVD). 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. This algorithm was used to evaluate the remaining bulk of pictures. Positive Macrophage / lymphocyte counts were divided by the surface of the herniated disc in cm^2. First the total amount of macrophages was counted using CD68, subsequently, M2 dominance was determined as the fraction of positive CD163 or ARG1 macrophages out of the CD68 positive macrophages. M1 dominance was determined as fraction of positive CD40 or iNOS macrophages out of the CD68 positive macrophages.

For statistical analysis CD68, CD3 and CD15 positive cells were each categorized as: No infiltration: (<10 cells/$cm^{2)}$), moderate infiltration (10-100 cells/cm^2) and considerable infiltration (>100 cells/cm^2). For all analyses that focus on the quantification of differences in M1 and M2 macrophage marker expression (CD163 Arg1, CD40, iNOS), only samples of patients with at least moderate inflammation were used (>10 CD68+ cells/cm^2) and the positive marker expression was presented as a percentage of the CD68+ cells present in that sample. These were subsequently categorized as: low expression (<10% of CD68+), medium expression (10-50% of CD68+) and high expression (>50% of CD68+). The effect of Modic changes and location of herniation (cervical/lumbar) on the absolute and relative expression categories were tested in ordinal logistic regressions, for which assumptions were met. In this model, type of herniation (bulging/extrusion/sequester) was included as a covariate, since this is known to influence macrophage infiltration. At last, the correlations between duration of symptoms and expression of inflammatory markers were evaluated using Spearman's correlation. For all analysis, alpha was set at 5%.

Results

Study population

Herniated disc samples were retrieved from 38 patients that consecutively underwent discectomy for radiculopathy in 2018 and fulfilled the inclusion criteria. Twenty two patients underwent lumbar discectomy for sciatica, and 16 patients underwent anterior discectomy for a cervical disc herniation with 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).

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. 29/38 samples (76%) showed inflammatory cells; which were localized in the edges of NP tissue (Figure 1a). Only CD163 showed to be specific for macrophages. CD68, CD40, iNOS and Arg1 also stained nucleus pulposus cells and chondrocytes (Figure 1b). Most of the immune cells stained positive for macrophage marker CD68. A limited number of immune cells stained positive for the T-cell marker CD3 and neutrophil marker CD15. CD3 and CD15 exclusively stained the T-cells and neutrophils respectively. CD68, CD163, CD40 and CD3 stained cells with high intensity, whereas iNOS and Arg1 staining was of lower intensity and both intra and extracellular (Figure 1c-h).

Inter observer agreement and algorithm efficacy

The number of pictures taken from each sample varied from 0 (if no inflammatory cells were present) till >200. 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, CD163 and CD40, a correlation coefficient of >0.8 was also achieved between the average count of the observers and ImageJ automated cell count. Hence the automated cell count was used for the remaining pictures. For iNOS and Arg1 however, the

required correlation coefficient could not be achieved due to the low staining intensity and extracellular staining. The extracellular staining was namely often (falsely) counted as 'positive' by the algorithm; hence all pictures were evaluated 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 (Table S1). For all manual counts, average counts of the observers were used for data analysis.

Quantification of inflammation markers

Expression levels of CD68 varied widely between samples (median=48,3/cm^2).

Of the 38 samples, 10 showed mild (<10/cm^2), 10 moderate (10-100/cm^2), and 18 considerable infiltration (>100/cm^2). The distribution of M1/M2 markers (as% of CD68+) is as follows: highest levels were seen in CD163 (M2 marker): 11 showed low (<10%), 12 showed medium (10-50%) and 6 showed high expression (>50%). Whereas for Arg1 (M2 marker) 27 samples showed mild, 1 medium and 1 high expression. CD40 (M1 marker) also showed limited expression with 24 samples in the mild, 4 in the medium and 1 in the high expression group. Comparable results were seen for iNOS (M1 marker): 22 samples were scored as mild, 7 as medium and 0 as high expression (Table 2).

CD3 and CD15 were mostly expressed in limited quantities: CD3 median<1/cm^2 (32 mild, 6 moderate), CD15 median=1/cm^2 (25 mild, 10 moderate and 3 considerable). Further, high expression of CD68 correlated with higher levels of CD3 (p<0,001) and CD15 (p<0,001) (Table 2).

Inter observer agreement MRI

For the presence of MC, inter observer agreement was moderate with an agreement percentage of 78% (Cohens Kappa = 0,58). When results were separated for location of herniation, a strong inter observer agreement was seen in cervical patients with an agreement percentage of 94% (Cohens Kappa = 0,82), whereas in lumbar patients this was 71% (Cohens Kappa = 0,42).

Association of macrophage type and Modic changes

Cervical patients showed a median expression/cm^2 of 16 in patients without MC (MC-) and 40 in patients with MC (MC+). This was lower than in lumbar patients, where MC- patients showed a median cell count/cm^2 of 223 and MC+ a median of 231. Regarding the expression of M1 and M2 markers relative to CD68, cervical MC- patients showed the highest levels of CD163 (median of 59%), in cervical MC+ patients this percentage was 21%. In lumbar patients, MC- patients had 26% CD163, whereas in MC+ patients this was 8%. Arg1 and CD40 expression was very low in all subgroups. iNOS expression was also low: 5,6% for MC- patients and 3,7% for MC-. An overview of the medians per subgroup can be found in table 3 and an overview of all expression levels per sample in table S2.

Ordinal logistic regression illustrated that the presence of MC was associated with higher levels of CD68 (OR=6.0, p=0.023) and with lower relative levels of M2 marker CD163 (OR=0.123, p=0.02) compared to patients without MC. No significant differences between cervical and lumbar samples were seen with regard to expression levels of CD68, or the relative expression levels of CD163, Arg1, CD40 or iNOS (Table 4). No other significant results were seen.

T-cell count (CD3) was <1 in all subgroups. The median cell count/cm^2 of CD15+ neutrophils was 3.45 in MC- lumbar patients and 2.95 in MC+ lumbar patients, whereas it was <1 in MC- cervical patients and 2.15 in MC+ cervical patients. Moreover, the ordinal regression analysis showed no differences for CD3. However, it revealed that lumbar samples had higher levels of CD15 positive neutrophil infiltration as compared to cervical samples (OR=12.6, p=0.038).

Influence of time on macrophage differentiation

The median duration of symptoms was 6.5 months (IQR: 5 – 13). Neither the absolute expression of CD68, CD3 or CD15 nor the relative expression (% of CD68) of inflammatory markers was correlated to the duration of symptoms (Table 5).

Discussion

The present study indicates that M2 macrophages (CD163+) are the dominant type of inflammatory cells in herniated intervertebral disc tissue harvested from cervical and lumbar radiculopathy patients, and are more dominant in MC- as compared to MC+ patients. By contrast, the M1 phenotype (iNos+ and CD40+) only forms a small portion in all inflammatory cells identified, and we were unable to associate this with MC+ patients. Based on our findings, we conclude that CD163 is a suitable marker for M2 macrophages in both lumbar and cervical radiculopathy patients, whereas Arg1 was deemed unsuitable as M2 marker due to its limited expression and extracellular staining. 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 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.

Our results imply a higher degree of macrophage infiltration in lumbar herniated discs, compared to cervical discs. This difference among the locations are in line with results from Chitkara et al (1991), who reported a lower degree of neovascularization in cervical discs [15], and could be the result of morphological and pathological differences affecting vascular structure and growth, which have yet to be elucidated.

Additionally, the present study showed that inflamed cervical herniated discs contain a larger proportion M2 (CD163+) macrophages and a smaller proportion of M1 (iNOS+) macrophages when compared to lumbar ones, which was in line with our hypothesis. Since M2 macrophages tend to advance recovery by disc resorption through phagocytosis of the herniated tissue [12, 13] and M1 macrophages delay recovery by exacerbating pain symptoms [9], cervical patients might benefit from inflammation more than lumbar patients. This could be the result of structural differences between cervical and lumbar HNPs. That act on phenotypic differentiation of macrophages, but the exact mechanism yet has to be unraveled.

Moreover, present findings demonstrate a higher proportion of M2 (CD163+) macrophages in MC- patients. This is in accordance with previous findings [4], proposing that patients without MC benefit from macrophage infiltration as this was associated with faster recovery after surgery, whereas in MC+ patients, macrophage infiltration was associated with decreased recovery after surgery. Hence we expected MC+ to be associated with an increase in M1 macrophages. The fact that we were not able to find this association could be due to our limited sample size, as both iNOS and CD40 were rather insensitive marker and only few discs were found positive. Alternatively, it could be that the wrong M1 markers were used, and that other M1 markers such as CD64, CD80,CD86 or CD192 would provide better results [11, 13, 22].

Because the proportion of M2 macrophages is higher in patients without MC, It seems that MC status affects the macrophage differentiation process, possibly through the creation of a degenerative and pro inflammatory environment that could induce differentiation towards M1 [10, 14]. Vice versa, decreased M2 differentiation could also resemble a malfunction of the immune system that upon infiltration of the herniated disc material also creates endplate damage and thereby induces MC. Another scenario is that both observations are the result of a poor vascular status, which may have induced the degenerative changes of the endplate and alter immune function [23]. Another explanation, which is in line with a poor vascular status that leads to a degenerated endplate, would be the presence of anaerobic bacteria in the endplate, mostly Propionibacterium Acnes [24]. The presence of such bacteria has been associated with MC+ [24], and may induce a pro-inflammatory environment that could prevent macrophage differentiation towards M2. Unfortunately, these theories are still in concept, and in order to improve our understanding, more experimental research and knowledge of the vascular status of radiculopathy patients are required.

Moreover, the total number of macrophages was significantly higher in MC+ patients, whereas in our previous study we were not able to find this difference [4], and others have even reported lower numbers of macrophages in MC+ patients [1]. Due to this incongruency, we must conclude that more large studies are required in order to draw a conclusion on this association. 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[25], and switches to M2 in the following days [26, 27]. As patients in this study had symptoms >2 months, it is assumed that the initial switch from M1 to M2 is finalized, and that in this stage of chronic symptoms, a longer duration does not further influence macrophage differentiation. Nevertheless, even though our findings are in line with the literature, it should be noted 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 M1/2 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+. 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 is 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 by multiple testing, which we deemed unsuitable for the exploratory nature of this study.

Conclusion

M2 (CD163+) macrophages are abundantly present in intervertebral disc tissue that is herniating, compressing the nerve and associated with radiculopathy both at lumbar and cervical level. Moreover, M2 (CD163+) macrophages are more abundant in MC- patients which supports previous data and suggests patients without MC with macrophage infiltration have a quicker recovery rate after surgery. 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.

Acknowledgements
The authors 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.

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Appendix

Figure 1. Examples of immunohistochemistry staining results

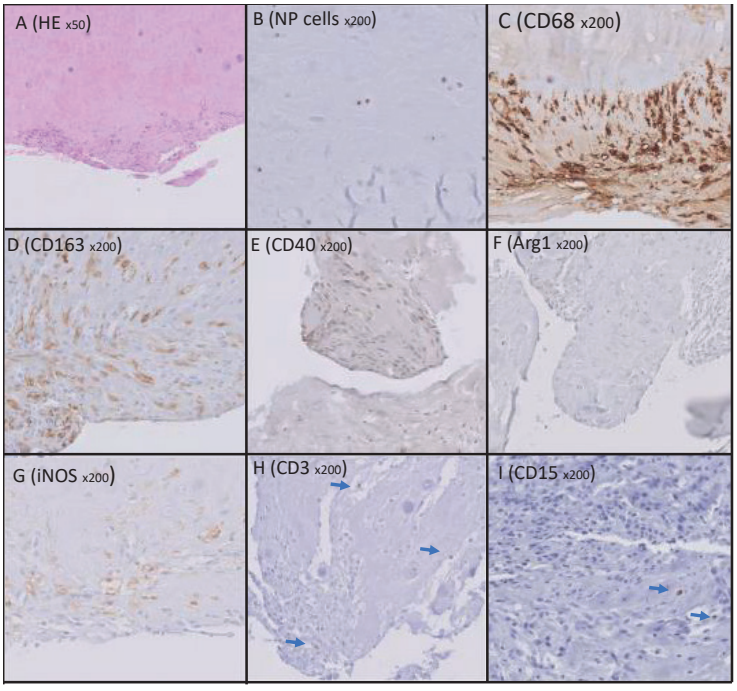

Figure 1 displays examples of the different antibody staining results. **1A** shows a HE coupe with infiltrating inflammatory cells in NP tissue. **1B** displays positively stained NP cells with CD68. **1C** shows infiltrating macrophages, intracellularly stained with CD68. **1D** shows infiltrating macrophages of which a large percentage is intracellularly stained with CD163. **1E** reveals infiltrating macrophages, some of which are stained intracellular with CD40. **1F** displays infiltrating macrophages of which a few are Arg1 positive, ECM surrounding Arg1 positive cells is also stained positively. **1G** illustrates infiltrating macrophages of which some are iNOS positive, ECM surrounding iNOS positive cells is also stained positively. **1H** reveals intracellular staining of infiltrating t-cells. **1I** shows intracellular staining of infiltrating neutrophils.

Table 1. Baseline characteristics

1A	Cervical	Lumbar	p-value
N	16	22	
Age	55	52	0.54
% male	31%	45%	0.51
Duration of symptoms (months)	7.7	13.2	0.63
% Modic Changes	27%	43%	0.48
1B	**MC**	**No MC**	**p-value**
N	13	21	
Age	49	55	0.45
% male	46%	41%	1.00
Duration of symptoms (months)	10.2	11.7	0,77
% Cervical discs	31%	48%	0.48

1A displays the baseline characteristics for Cervical and Lumbar patients separately. **1B** shows baseline characteristics for patients with and without Modic changes separately.

Table 2. Distribution of samples over the immune cell infiltration categories

A			
Marker	**<10/cm²**	**10-100/cm²**	**>100/cm²**
CD68	10	10	18
CD3	32	6	0
CD15	25	10	3
2B			
Marker as % of CD68	**<10%**	**10-50%**	**>50%**
CD163%	11	12	6
Arg1%	27	1	1
CD40%	24	4	1
iNOS%	22	7	0

Table **2A** displays for each immune cell marker the number of samples in each infiltration category. Table **2B** shows the relative expression of M2 markers (CD163 and Arg1) and M1 markers (CD40 and iNOS) relative to CD68 expression in all samples that showed at least moderate CD68 infiltration (>10 cells/cm²).

Table 3. Overview of inflammatory marker expression in subgroups for location of herniation and MC status

Subgroups	CD68/cm²	CD3/cm²	CD15/cm²	CD163 %	Arg1 %	CD40 %	iNOS %
Cerv_MC+	40	0	2	21%	0%	0%	0%
Cerv_MC-	16	0	0	59%	0%	2%	0%
Lumb_MC+	231	0	3	8%	2%	0%	4%
Lumb_MC-	223	0	3	26%	0%	0%	6%

The values are medians for CD68, CD3 and CD15, and percentages of CD163, Arg1, CD40 and iNOS are relative expressions compared to CD68+ cells.

Table 4. Results of ordinal logistic regression

	MC		Herniation location	
Marker	**p-value**	**Odds-ratio (95% CI)**	**p-value**	**Odds-ratio (95% CI)**
CD68	0,023*	6,0 (1,276 - 28.49)	0,18	2,7 (0,623 - 12,49)
CD3	0,172	5,0 (0.495 - 50.6)	0,999	1,3E9(0 - > 1E20)
CD15	0,124	4,08 (0,681 - 24,47)	0,038*	12,6 (1,145 - 139.08)
CD163%	0,020*	0,123 (0,021 - 0,721)	0,275	0,383 (0,068 - 2,145)
Arg1%	0,999	1,3E8 (0 - >1E20)	0,999	1,7E8 (0- > 1E20)
CD40%	0,748	0,702 (0,081 - 6,083)	0,509	2,3 (0,190 - 28,49)
iNOS%	0,998	2,1E-9 (0- > 1E20)	0,998	1,1E17 (0- > 1E30)

Table 4 lists the result of an ordinal logistic regression in which location of herniation 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 OR are provided. For the OR, 'no MC' and 'cervical samples' were used as reference category). % means relative to CD68 expression.

Table 5. Correlations between duration of symptoms and inflammatory markers

Absolute count	Spearman's rho	p-value
CD68	-0,016	0,924
CD163	-0,069	0,683
Arg1	0,119	0,497
CD40	0,143	0,397
iNOS	-0,124	0,465
CD3	-0,028	0,866
CD15	-0,158	0,343
Relative count		
CD163%	-0,198	0,323
Arg1%	0,042	0,834
CD40%	0,138	0,492
iNOS%	-0,178	0,364

Table 5 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 2-8 display the absolute cell counts whereas row 10-13 display expression as a percentage of CD68 positive cells.

Supplementary appendix

Table S1. Inter observer correlation and algorithm validation for cell counts

Marker	ND count	GL count	R (ND-GL count)	algorithm count	R (algorithm - average ND&GL)
CD68	3287	3563	0,95	3499	0,95
CD163	1716	1722	0,85	1.399	0,86
CD40	606	694	0,95	885	0,83
iNOS	615	678	0,81	634	0,61
Arg1	562	706	0,90	701	0,63
CD3	237	121	0,84	/	/
CD15	707	667	0,87	743	0,92

Table 1 displays the inter-observer correlation between the two independent researchers and the automated cell count from image J. The first column lists the antibody used, the second and third list the total positive cell count from 50 pictures by the two observers. The fourth column lists the correlation coefficient between the counts of the two observers (ND and GL). The fifth column shows the total cell count by the automated algorithm. In the last column, the correlation coefficient is listed for the correlation between the automated cell count and the averaged count of both observers. '/' illustrates that less than 50 pictures were available and no algorithm validation could be performed.

Table S2. Raw data file

CaseID	location	MC	CD68/cm²	CD3/cm²	CD15/cm²	CD163 %	Arg1 %	CD40 %	iNOS %
1	cervical	No	172	1	1	44%	1%	2%	0%
2	cervical	no	0	0	0				
3	cervical	no	145	0	5	71%	1%	8%	3%
4	cervical	No	0	0	0				
5	cervical	No	0	0	0				
6	cervical	No	324	0	43	101%	0%	13%	27%
7	cervical	no	0	0	0				
8	cervical	No	8	0	0	80%	0%	0%	0%
9	cervical	No	15	0	33	46%	0%	22%	0%
10	cervical	No	168	0	0	11%	0%	3%	2%
11	cervical	No	17	1	29	100%	0%	0%	0%
12	cervical	No	36	0	0	0%	0%	0%	0%
13	cervical	Yes	47	0	0	18%	0%	0%	0%
14	cervical	Yes	404	0	427	46%	0%	6%	0%
15	cervical	Yes	34	0	0	9%	0%	0%	0%
16	cervical	yes	25	0	4	25%	0%	0%	0%
17	lumbar	Missing	9183	0	3	1%	0%	0%	2%
18	lumbar	No	0	0	0				

CaseID	location	MC	CD68/cm²	CD3/cm²	CD15/cm²	CD163 %	Arg1 %	CD40 %	iNOS %
19	lumbar	No	396	14	15	46%	0%	0%	4%
20	lumbar	no	4708	0	190	5%	4%	0%	1%
21	lumbar	no	35	0	4	464%	0%	0%	20%
22	lumbar	No	1166	0	46	6%	0%	0%	7%
23	lumbar	No	19341	17	61	42%	0%	0%	3%
24	lumbar	no	0	0	0				
25	lumbar	No	1747	60	77	49%	0%	11%	22%
26	lumbar	no	0	0	0				
27	lumbar	No	0	0	0				
28	lumbar	No	50	1	1	10%	0%	0%	44%
29	lumbar	Yes	536	0	0	8%	0%	0%	10%
30	lumbar	Yes	0	0	0				
31	lumbar	Yes	148	0	5	2%	21%	10%	0%
32	lumbar	Yes	14	0	0	0%	100%	0%	37%
33	lumbar	Yes	4868	17	41	25%	4%	2%	4%
34	lumbar	Yes	983	1	1	38%	3%	0%	15%
35	lumbar	Yes	45	19	12	62%	0%	8%	12%
36	lumbar	Yes	225	0	0	9%	0%	0%	0%
37	lumbar	yes	14868	33	1097	4%	1%	0%	2%
38	lumbar	Yes	237	3	19	1%	2%	145%	1%

This table displays the location of the disc sample, MC status and absolute or relative (% of CD6+ cells) expression levels of all immune cell markers for all samples separately. If a percentage exceed 100%, slide with the M1/M2 marker had a higher total number of macrophages than the slide that was stained with CD68.