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**Gadolinium retention effect on macrophages - a potential cause of MRI contrast agent  
Dotarem toxicity**

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**Running Title:** Gadolinium retention in macrophages

## Abstract

Gadolinium is a component of the MRI contrast agent Dotarem. Although Dotarem is the least toxic among MRI contrasts used, gadolinium present in Dotarem accumulates for many years in various organs and tissues exerting toxic effects. We showed previously that gadolinium remains in macrophages for at least 7 days after exposure to Dotarem. However, very little is known about the effect of gadolinium retention on the immune cells such as macrophages. We studied the effect of 1-day and 7-day retention of gadolinium on various functions and molecular pathways of macrophages. Gadolinium retention for 7 days decreased macrophage adhesion and motility and dysregulated the expression of adhesion and fibrotic pathway-related proteins such as Notch 1 and its ligand Jagged1, adhesion/migration-related proteins PAK1 and Shp1, immune response-related transcription factors Smad3 and TCF19, and chemokines CXCL10 and CXCL13, and dysregulated the mRNA expression of fibrosis-related genes involved in extracellular matrix (ECM) synthesis, such as Col6a1, Fibronectin, MMP9, and MMP12. It also completely (below a level of detection) shuts down the transcription of anti-inflammatory M2 macrophage polarization marker the Arg.1. Such changes, if they occur in MRI patients, can be potentially detrimental to the patient's immune system and immune response-related processes.

**Key words:**, macrophage, fibrosis, adhesion, movement, gadolinium, Dotarem, MRI contrast

## Introduction

Gadolinium is a rare earth metal with paramagnetic properties used in MRI contrast agents to increase the contrast and quality of MRI-acquired images. Among many contrast agents, one commonly used is the gadolinium-based Dotarem (gadoterate meglumine, Artirem, Clariscan, Gd-

DOTA). Because gadolinium in solution forms very toxic  $Gd^{3+}$  ions, gadolinium in MRI contrast agents is chelated (conjugated with a sequestering ligand) to increase stability and limit or prevent toxicity. For example, studies showed that even 10 min exposure to gadolinium chloride ( $GdCl_3$ ) caused the apoptotic death of rat alveolar macrophages and that injection of  $GdCl_3$  to the newborn rats significantly reduced lung macrophage content (Mizgerd et al. 1996; Jankov et al. 2001). Dotarem is the most stable and least toxic among MRI contrast agents and, for many years, was believed to be promptly washed out from the body and completely harmless. However, over the years, studies showed that gadolinium could not only cross the blood-brain barrier (BBB) but remain in the body for many years, possibly exerting toxic effects on the brain, heart, vasculature, kidney, bone, skin, and other organs and tissues (Harvey et al 2020.; Iyad et al. 2023; McDonald et al., 2018; Semelka et al. 2023). Gadolinium retention toxicity is inculcated in nephrogenic systemic fibrosis (NSF), a debilitating disease involving thickening of the skin and subcutaneous tissues, and systemic inflammation and fibrosis of organs, reported in patients with renal insufficiency who received gadolinium-based contrast MRI tests. Studies showed that, in NSF patients, gadolinium accumulation in the heart and blood vessels associates with myocardial fibrosis, atherosclerosis, and shortened life span (Grobner, 2006). Findings like these identified gadolinium deposition disease (GDD) and led to new recommendations limiting the frequency of MRI contrast use in the clinical setting (Harvey et al. 2022; Semelka et al., 2023). So far, we do not know much about the effect of gadolinium accumulation on immune cell functions and molecular pathways and how they contribute to gadolinium organ toxicity. We showed previously that 24-hour exposure of mouse macrophages to Dotarem affects their morphotype and cell organelles and upregulates pro-inflammatory and downregulates anti-inflammatory markers characteristic for the ant-inflammatory M2 macrophages [Chanana et al., 2022]. Recently, we also

developed the 7T MRI-based method to analyze gadolinium accumulation in isolated immune cells and showed that macrophages retained gadolinium for at least seven days after exposure, which caused downregulation of RhoA, mTORC1/Raptor and mTORC2/Rictor proteins, and affected expression of organelle markers (Uosef et al., 2022). Macrophages are functionally versatile and not only participate in and regulate immune responses *per se* but also, by supporting fibrogenesis and fibrolysis, regulate tissue injury, regeneration, healing, and, dependent on these events, physiological and pathological fibrosis, including myocardial fibrosis (Chen et al., 2022; Lech and Anders, 2013; Ma et al., 2022). Thus, we focused our study on the effect of 7-day gadolinium retention on macrophage motility, adhesion, and the expression of fibrotic and immune-response pathway proteins and on the transcription of the anti-inflammatory M2 macrophage genes involved in the synthesis of extracellular matrix (ECM).

## **Material and Methods**

### **Cells and treatment**

Mouse macrophages RAW 264.7 (cat# ATCC TIB-71) were cultured in DMEM medium (Corning® 10-013-CV) supplemented with 10% FBS (HyClone, SH30071.03) and Penicillin-streptomycin (Thermo Fisher Scientific, 15140122) at a concentration of 100 units/mL. Cells were kept at 37° C and 5% CO<sub>2</sub>. At 70% confluency, cells were treated with gadolinium-based MRI contrast agent Dotarem (cat. NDC67684-2001-3, Guerbet, Raleigh, NC, USA) at 1.4mM concentration. Cells were incubated with Dotarem for 24 hours, washed twice in PBS, and immediately collected for Western blot processing. For checking the effects of gadolinium retention, cells were incubated with Dotarem for 24hr then washed and incubated in Dotarem-free medium for seven days before further processing.

### **Cell Viability Assay**

RAW264.7 cells were placed in a 96-well microplate at the density of 6000 cells/well. The cells were incubated with DOT (1.4 mM) for 24 hours. For the assessment of cell viability, the MTT Cell Proliferation Assay Kit (Ab211091) (Abcam, USA) was used according to the manufacturer's protocol. The absorbance was read at 590 nm using BioTek Synergy H1 Multimode Reader (Agilent Technologies, USA).

### **Wound Healing Migration Assay**

For 1 day experiment, RAW264.7 cells were placed in a 24-well plate at the density of  $4 \times 10^5$  cells/well. At 100% confluency cell monolayers were scratched with a sterile pipette tip, washed with PBS to remove loose cells, and treated with DOT for 24 hours in FBS-reduced culture media (2% FBS). The images of scratches were captured by the Olympus TH4-100 inverted microscope immediately after scratching and after 24 hours. For the 7-day experiment, cells were detached on day 6 after DOT treatment, centrifuged, and seeded to reach full confluency. The next day, cell monolayers were scratched with a sterile pipette tip and washed with PBS to remove loose cells. Cells were incubated in a reduced (2%) FBS medium for 24h and images were taken as described above. All images were analyzed with Fiji ImageJ software (NIH). Cell migration was determined at the edges of the wound, and the percentage of migration was determined as the ratio between the migrated distance and the initial distance from the wound.

### **Adhesion assay**

After DOT treatment, RAW264.7 cells were seeded in a 24-well plate ( $1 \times 10^5$  cells/well) and incubated for 2hrs in an FBS-free culture medium. Subsequently, the non-adherent (floating) cells and medium were removed. Adherent cells were rinsed with PBS, fixed with 4% paraformaldehyde (Cell Signaling, USA) stained with crystal violet (Sigma Aldrich, USA), and

visualized using the Olympus TH4-100 inverted microscope. Next, cells were lysed for 30 min, at room temperature in 2% SDS to dissolve crystal violet. 100  $\mu$ l of lysate was transferred to a 96-well microplate, and the crystal violet absorbance was read at 550  $\mu$ m.

### **TNF $\alpha$ secretion**

RAW264.7 cells were treated with DOT (1.4 mM) and 0.1  $\mu$ g/ml LPS (ThermoFisher, USA) in a reduced (2%) FBS culture medium for 24 hours. For 7 day experiment, cells were detached on the 6th day and treated with 0.1  $\mu$ g/ml LPS for 24 hrs. After incubation, the cell medium was collected, centrifuged, and stored at -80°C. The cells were lysed in RIPA buffer (Cell Signaling, USA, #9806), and a total protein concentration was determined by BCA Protein Assay Kit (Pierce, USA, #23227) according to the manufacturer's protocol. The concentration of secreted TNF $\alpha$  in cell medium was determined with TNF alpha Mouse ELISA Kit (BMS607-3) (ThermoFisher, USA) according to the manufacturer's protocol. The data were calculated in GraphPad Prism 10 and normalized to the total protein concentration.

### **Immunoblotting**

Dotarem-treated and untreated RAW 264.7 cells were harvested by 2000rpm centrifugation for 5 min at 4°C. Cells were washed once with cold PBS (1X) (Sigma, D8537) and centrifuged. Cell pellets were lysed with RIPA cell lysis buffer (Cell Signaling, #9806) supplemented with 1X protease inhibitor (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, #11836170001, Roche). Protein concentration was measured using the BCA protein estimation kit (Pierce™ BCA Protein Assay Kit, #23227). 1X SDS Dye (National Diagnostics) was added to the cell lysates and boiled at 95°C for 10 mins. After cooling, samples were electrophoresed on a 4-15% gradient SDS PAGE (Bio-Rad, Mini-PROTEAN® TGX™ Precast Protein Gels, #4561085) along with a protein marker (Bio-Rad Precision Plus Protein dual color standards marker, #161-0374). The gel was run



at 80V for around 2hrs. Subsequently, the proteins were transferred from SDS PAGE to the PVDF membrane (Bio-Rad, Trans-Blot Turbo Mini 0.2  $\mu$ m PVDF Transfer Packs #1704156) using the Trans-Blot Turbo Transfer System apparatus (Bio-Rad). The membrane was blocked with 5% nonfat dry milk (NFDM, #1706404, Bio-Rad) for 1hr and 30 mins. The primary antibody was added in 5% NFDM, and the membrane was incubated overnight at 4°C with shaking. The following day membrane was washed thrice (5 mins each) with 1X TBST (Tris Buffered Saline Tween). Subsequently, the membrane was incubated for 1hr and 30 mins at RT (with shaking) with a secondary antibody in 5% NFDM. After washing thrice, the membrane was developed using chemiluminescence reagents West Pico (Thermo Fisher Scientific, #34080), Immobilon (Millipore, #P90720), or super signal West Atto (Thermo Fisher Scientific, #A38554).

### **Antibodies**

We used the following antibodies: GAPDH (14C10) rabbit mAb (cat#2118), Jagged1 (D4Y1R) XP® rabbit mAb (cat #70109), PAK1 antibody (cat#2602), Smad3 (C67H9) rabbit mAb (cat#9523), Notch1 (D1E11) XP® rabbit mAb (cat #3608), SHP-1 (E1U6R) rabbit mAb (cat #26516), all from Cell Signalling, CXCL10 recombinant rabbit mAb (10H11L3) (Invitrogen, cat#701225), CXCL13 (BCA1) recombinant antibody (EPR19259-147) (Abcam, cat# ab199043), TCF-19 antibody (H-2) (Santa Cruz Biotechnology- cat# sc-390923). All these antibodies were used at 1:1000 dilution. The secondary antibodies: anti-rabbit IgG, HRP-linked antibody (cat #7074), and anti-mouse IgG, HRP-linked antibody (cat #7076) were from Cell Signalling and used at 1:3000 dilution.

### **RNA isolation**

For 1 day experiment, RAW264.7 cells were treated with DOT (1.4 mM) and TGF $\beta$  (10 ng/mL) for 24h. The purpose of the TGF $\beta$  treatment was to polarize macrophages into M2 phenotype,

which express fibrotic/ ECM synthesis-related genes Col6a1, Fibronectin, MMP9 and MMP12, and the M2 specific marker Arg-1. The next day, cells were washed twice with PBS, and RNA was isolated with RNeasy Mini Kit (Qiagen) according to manufacturer protocol. For a 7-day retention experiment, cells were treated with DOT (1.4 mM) for 24h. The next day, the culture medium was removed, and cells were cultured for 6 days in a DOT-free medium. On day 6 cells were treated with TGF $\beta$  (10 ng/mL) for 24h and then processed the same way as for 1-day experiment. The RNA yield and quality were assessed using a Nanodrop spectrophotometer NanoDrop-2000 (ThermoFisher).

### **Gene expression**

1  $\mu$ g of RNA was converted into cDNA using the SuperScript IV Reverse Transcriptase (ThermoFisher) according to the manufacturer's protocol. Obtained cDNA was used for qPCR (dilution 1:20). qPCR was performed using PowerTrack™ SYBR Green Master Mix (ThermoFisher) according to the manufacturer's protocol in the C1000 Touch™ Thermal Cycler machine (BioRad). Each sample was analyzed in duplicate. Gene expression was calculated using the 2-delta Ct method.

### **Forward and reverse primers:**

Arg-1 forward      ACACTCCCCTGACAACCAGC

Arg-1 reverse      AGGGTCTACGTCTCGCAAGC

Col6a1 forward      TCCTGCTGGTGAGAAAGGAT

Col6a1 reverse      TCCAGCAATACCCTGAGGTC

Fibronectin forward    GGTTACCCTTCCACACCCCA

Fibronectin reverse    GTGGCGTTGTCCGCCTAAAG

MMP9 forward      AAACCTCCAACCTCACGGACA

MMP9 reverse	ACAGCGTGGTGTTCGAATGG
MMP12 forward	GAGGGAACAGGTTGATGAGCTG
MMP12 reverse	GTCATTGGAATTCTGTCCTTTCCA
GAPDH forward	AGGTCGGTGTGAACGGATTTG
GAPDH revers	TGTAGACCATGTAGTTGAGGTCA

### **Statistical analysis and graphs**

The experiments were repeated three to five times, and the results were expressed as the mean  $\pm$ SEM. The graphs were prepared using GRAPH-PAD PRISM8. Statistical significance ( $p < 0.05$ ) was determined by paired T-test. The qPCR results were calculated using the 2-delta CT method, which is a relative quantification strategy for quantitative real-time polymerase chain reaction (qPCR) data. The 2-delta CT calculates 2-delta ct values for individual groups and compares these values between groups (independent samples).

### **Results**

We previously showed using the 7 T MRI method that gadolinium present in MRI contrast Dotarem is retained in mouse macrophages for at least 7 days (Uosef et al., 2022]. Here, we tested the effect of 1-day and 7-day retention of gadolinium on the functions of RAW264.7 mouse macrophages. Macrophages were incubated in the medium containing 1.4 mM Dotarem. After 1 day (24 hr ) of Dotarem exposure, macrophages were cultured in the Dotarem-free medium for 7 days. First, we established, by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which uses the cellular redox potential in metabolically active cells to convert a tetrazolium-based salt reagent—MTT to a brightly colored formazan product

that the 24hr exposure to Doraem did not affect cell viability (Fig. 1a). Next, we performed a wound healing motility assay to see if 1-day and 7-day retention of gadolinium affects cell motility. In this assay, the migration of cells into the scratch wound (wound closure) was monitored under the microscope. This assay showed that although both 1-day and 7 days gadolinium retention caused statistically significant (\*\*p = 0.056 and \*\*\*\*p <0.00, respectively) decreases in macrophage motility (Fig. 1 b-d’’), the effect of 7-day gadolinium retention was especially substantial causing > 50% decrease in motility in comparison to the control (Fig. 1c).

In the next set of experiments, we studied the effect of gadolinium retention on the expression of cell-cell interaction and fibrosis pathway proteins Notch1(Neurogenic locus notch homolog protein 1) and its ligand Jagged1 (Jagged canonical Notch ligand 1) and the focal adhesion-related proteins PAK1 (Serine/threonine-protein kinase PAK 1) and Shp1 (Src homology region 2 domain-containing phosphatase-1) in RAW264.7 macrophages (Fig.2). The Western blot analysis showed the statistically significant downregulation of Notch1 expression after 1 day and 7 days of gadolinium retention (Fig. 2a, a’), and, surprisingly, a very strong, especially after 7 days, upregulation of Nitch1 ligand Jagged 1 protein (Fig. 2a’’, a’’’). Such a disparity between the level of expression of the receptor (Notch 1) and its ligand (Jagged1) indicates that gadolinium retention dysregulates the Notch signaling pathway. Western blot analysis showed that gadolinium retention also downregulated the expression of adhesion- and migration-related proteins PAK1 and Shp1 (Fig. 2b-b’’’). A deleterious (and statistically highly significant) effect of gadolinium retention on macrophage adhesion was also confirmed by the cell adhesion assay (Fig. 2d-d’’’, e, e’’).

Because macrophages are the first responders and effectors of the immune/inflammatory processes, in the next set of experiments, we studied the effect of gadolinium retention on the expression of immune response-related proteins: transcriptional modulator activated by TGF-beta

(transforming growth factor) and activin type 1 receptor kinase Smad3 (Mothers against decapentaplegic homolog 3), TCF19 (Transcription factor 19), CXCL13 (Chemokine (C-X-C motif) ligand 13), and CXCL10 (C-X-C motif chemokine ligand 10 also known as Interferon gamma-induced protein 10) (Fig. 3). Western blot analysis showed that gadolinium retention upregulated the expression of Smad3 and CXCL10 (Fig.3a, a'', b'', b''', c, c'), and downregulated the expression of TCF19 and CXCL13 (Fig.3a'', a''', b, b', c, c' ). However, interestingly, the secretion of TNF $\alpha$  (Tumor necrosis factor alpha), a major regulator of inflammation, in response to LPS (Lipopolysaccharide) stimulation was not significantly affected by gadolinium retention (Fig.3d). We also studied the effect of gadolinium retention on the transcription of the extracellular matrix (ECM)- and wound healing-related genes in the anti-inflammatory M2 macrophages. We induced M2 polarization by incubation of unpolarized macrophages in the TGF $\beta$ -containing medium and performed qPCR to assess the level of Arginase 1(Arg-1), type VI collagen alpha1 chain (Col6a1), fibronectin, Matrix metalloproteinase (MMP9), and matrix metalloproteinase 12 (MMP12) mRNAs after 1-day and 7-day gadolinium retention. All these gene products are involved in ECM synthesis/degradation and, thus, in the wound-healing processes [Etich et al. 2019; Pesce et al., 2009]. The results of qPCR showed that 1-day gadolinium retention upregulated the expression of Arg-1, fibronectin, and MMP9 (Fig.4a) while 7-day retention upregulated collagen (Col6a1) and fibronectin, and completely (below a level of detection) shut down the expression of the anti-inflammatory M2 marker Arg-1 (Fig. 4b). The changes in the expression of other studied genes were not statistically significant.

## Discussion

It is well established that gadolinium present in MRI contrast agents can be retained for many years in various organs, including the brain, causing organ fibrosis, systemic inflammation, atherosclerosis, and shortening lifespan. Although gadolinium retention toxicity has been well studied at tissue and organ levels, little is known about its cellular and molecular effects on the immune cells. To fill this knowledge gap, we studied the effect of 1- and 7-day gadolinium retention on fibrotic and inflammatory pathway components and cellular functions of macrophages. We showed that gadolinium retention inhibited macrophage movement and adhesion and dysregulated the expression of adhesion and fibrotic pathway components and inflammation/immune response-related factors.

Especially striking was a dysregulation of a Notch1/Jagged pathway, where gadolinium retention caused a downregulation of the Notch 1 receptor but an upregulation of its ligand Jagged 1. Ligand-induced Notch signaling regulates cell differentiation, proliferation, and apoptosis during organogenesis and is involved in the development of pulmonary, cardiac, and kidney fibrosis and cancer (Bielez et al. 2010; Fiúza and Arias, 2007; Ma et al. 2022; Maillard et al., 2003; Milano et al., 2004; Plantier et al., 2011; Russell et al., 2011; Wang et al., 2017). In mammals, there are four Notch receptors and five respective ligands: Jagged 1 and 2, and delta-like 1, 3, and 4. Ligand binding to the Notch receptor results in the Notch cleavage and release of its intracellular domain (NICD) that enters the nucleus acting as a transcriptional activator of the target genes (Fiúza and Arias, 2007; Kuintzle et al., 2023; Lee et al. 2015; Li et al. 2014). Studies showed that the excessive activation of Notch signaling in macrophages promotes kidney fibrosis and inflammation (Ma et al., 2022), while the overexpression of Jagged1 enhances cell proliferation (Simon et al., 2012) and correlates with tumor aggressiveness and poor clinical outcome (Silva et al. 2023).

Considering such pleiotropy of Notch/Jagged signaling effects, its dysregulation by gadolinium retention in macrophages is extremely concerning and requires further detailed studies.

Another effect of gadolinium retention we observed in macrophages was the downregulation of adhesion/migration-related molecules PAK1 and Shp1. PAK1 belongs to the family serine/threonine kinases activated by the Rho GTPases, which regulate cell polarity, adhesion, migration, and lamellipodia dynamics in macrophages (Smith et al. 2008) through the regulation of actin cytoskeleton dynamics (Somanath et al., 2023). Additionally, recent studies showed that downregulation/silencing of PAK1 resulted in the induction of genes expressed by M2 (antiinflammatory) macrophages and repressed the phenotypic markers of M1 (proinflammatory) macrophage polarization (Cheng et al. 2021). The Shp1 is a tyrosine phosphatase that regulates a variety of immune cell functions, including integrin-mediated cell adhesion in macrophages (Roach et al. 1998). Shp1 also interacts with the Hox domain proteins regulating transcription in the myeloid precursor cells. Downregulation of Shp1 activates transcription factor Stat5 resulting in overproliferation of hematopoietic stem cells (Abram and Lowell, 2017).

In our study, the negative effect of gadolinium retention was evident not only at the protein expression but also at the cellular function level. Migration and adhesion assays showed that gadolinium retention slows down macrophage movement and impairs their attachment to the substrate.

We also showed that gadolinium retention in macrophages impacted the expression of immune response-related proteins such as Smad3, TCF19, and chemokines CXCL10 and CXCL13. The expression of Smad3 and CXCL10 was upregulated, and TCF19 and CXCL13 were downregulated after 7-day gadolinium retention. Smad3, the main regulator of the transcriptional effects of TGF- $\beta$  signaling, mediates the acquisition of a phagocytic and anti-inflammatory

macrophage phenotype (Chen et al 2019). The transcription factor TFC19 regulates the inflammatory and DNA damage response pathways and regulates the polarity of tumor-associated macrophages (Cheng et al., 2022). Chemokines CXCL10 and CXCL13 act as chemoattractants, contribute to the inflammatory microenvironment, and promote macrophage chemotaxis (Iwanowski et al., 2017). We also showed that 1-day gadolinium retention increased the expression of anti-inflammatory M2 macrophage marker Arg-1, fibronectin, and matrix metalloproteinase 9, while the 7-day gadolinium retention completely shut down the expression of Arg-1, and increased the expression of fibronectin and collagen type VI alpha 1 chain (Col6a1). We previously showed that exposure to a high concentration (20mM) of gadolinium also decreases the expression of another M2-specific anti-inflammatory marker FcεR1 (Chanana et al., 2022). The dysregulation of these genes, all involved in the synthesis of ECM and the anti-inflammatory and wound-healing activities of M2 macrophages [11, 12], may, potentially, have a deleterious effect on the healing processes in MRI patients.

A question remains why the gadolinium affects all these pathways and what is the mechanism involved? One of the commonalities between all these affected pathways is their involvement in calcium signaling (Chen et al., 2018; Jeon et al. 2018; Rand et al., 2000; Sáez de Guinoa et al. 2011; Schareberg et al. 2007; Sui et al, 2006). In our previous studies on the effect of gadolinium on macrophages, we hypothesized that because the crystallographic radius of  $Gd^{3+}$  and  $Ca^{2+}$  is very similar,  $Gd^{3+}$  can compete with  $Ca^{2+}$  in the signaling pathways, inducing changes at the transcriptional and translational level, and affecting cell motility, adhesion, and other cellular processes (Uosef et al., 2022).

In summary, all data presented here indicate that gadolinium retention for 7 days profoundly affects immune-response signaling pathways and macrophage functions. Because MRI patients



are often immunocompromised or experience inflammation, infection, or cancer, such effects on the immune cells can be detrimental to the patient's health and the disease outcome. Further studies of the long-term (weeks, years) effect of gadolinium retention on the immune cells are also necessary. As a note of caution, because our studies consisted of mouse RAW 264.7 macrophage cell line, one must be very careful when translating these results into the *in vivo* situation in animals and humans. Further studies with different gadolinium doses, human primary monocytes/macrophages isolated from the blood, human macrophage cell lines, and *in vivo* animal studies are necessary to conclude how relevant our results are to human MRI patients.

### **Conflict of interest**

The authors declare that they do not have any financial or conflict of interest

### **Ethical Approval**

Not applicable

### **Informed Consent**

Not applicable

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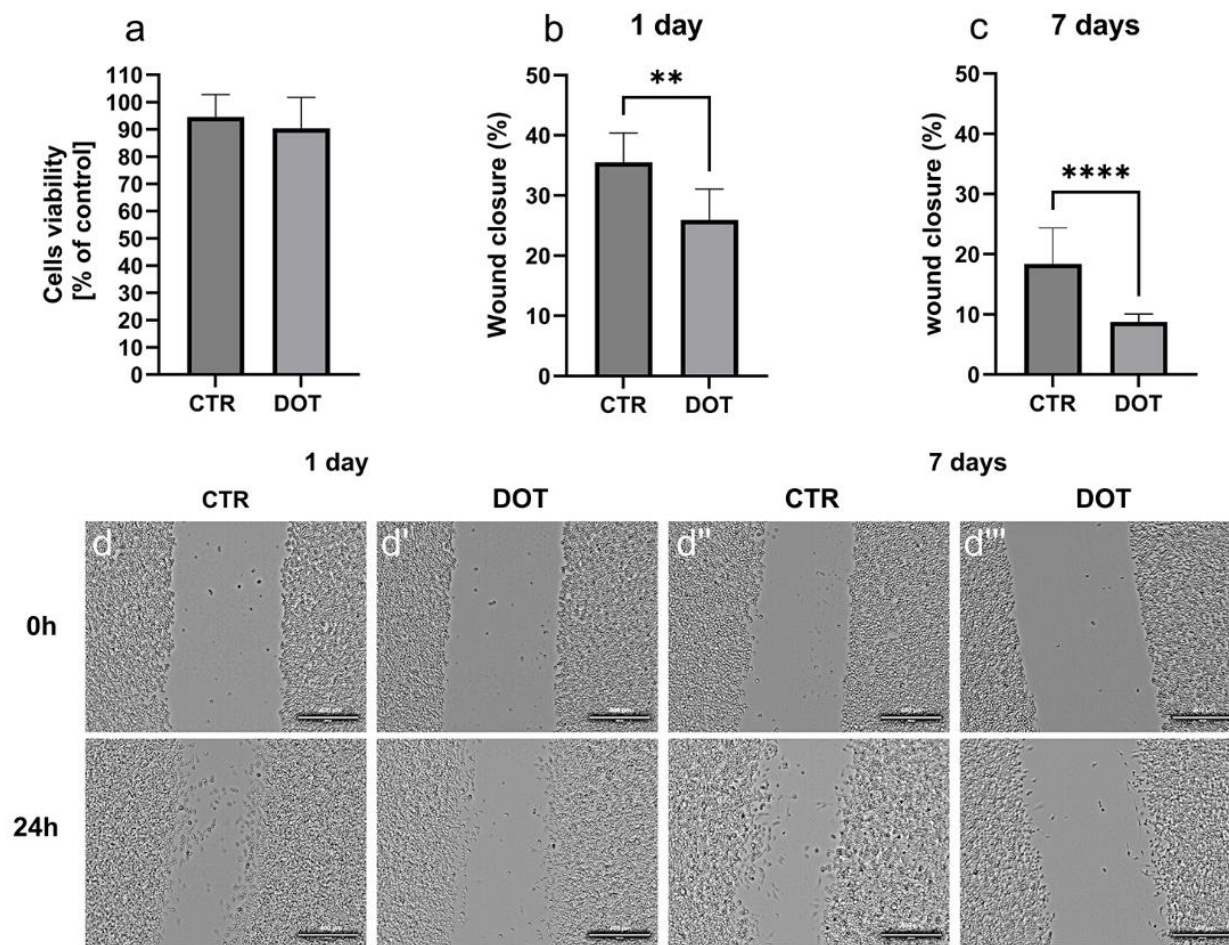
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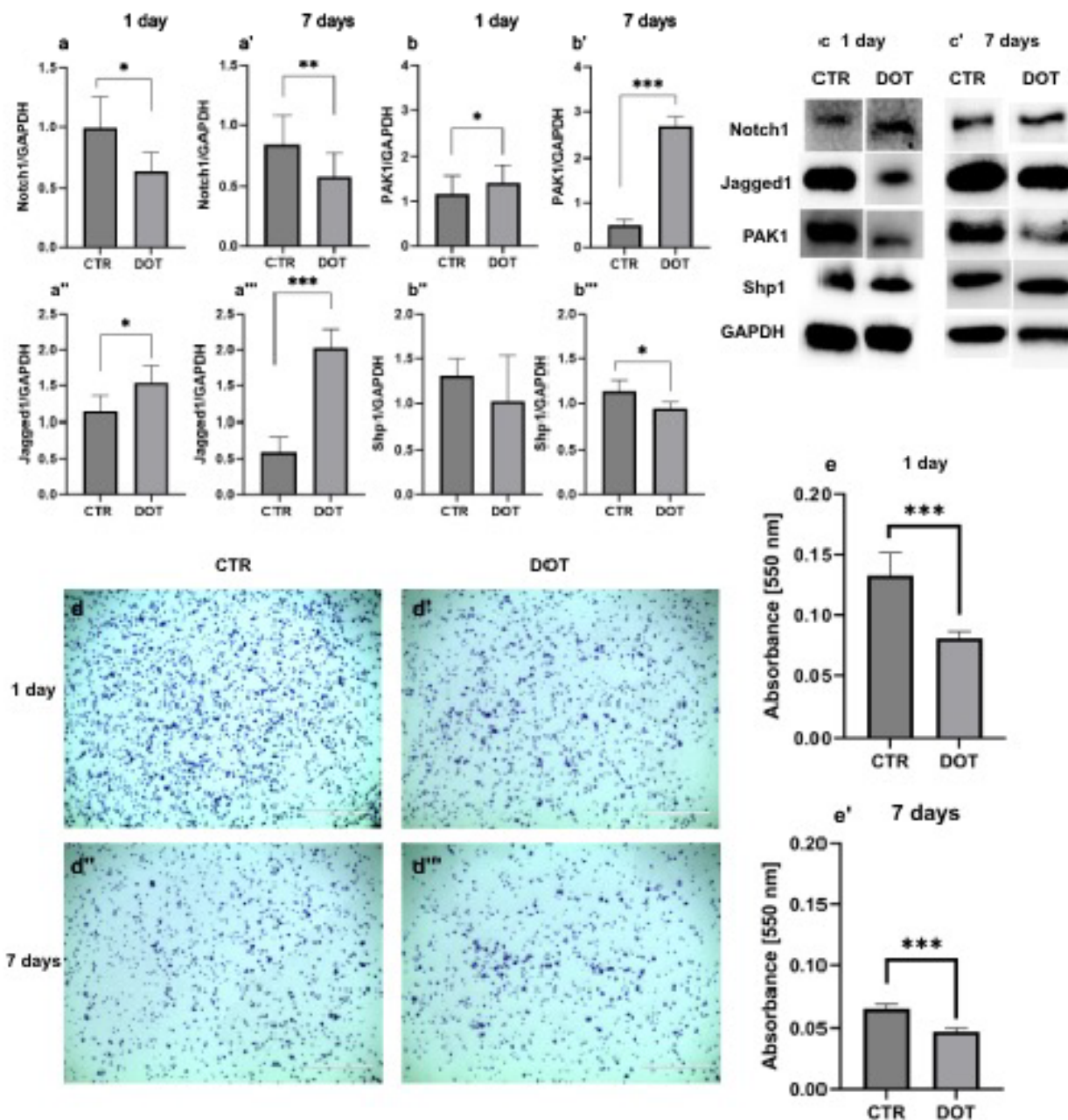
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## Figure legends



**Figure 1. The effect of gadolinium/Dotarem retention on cell viability and migration**

(a) The 1.4 mM Dotarem (DOT) used in the study was not toxic to RAW264.7 cells as assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell viability is expressed as a percentage of metabolically active cells after 24 hr DOT (1.4 mM) treatment compared to untreated control cells (CTR), (ns- statistically nonsignificant). (b, c) Effect of DOT 1 day and 7 days DOT retention on RAW264.7 cells motility assessed by the scratch wound healing assay. (b) Graphs illustrate the percentage of wound closure after 1 day and 7 days from the time of scratch (1 day DOT retention, \*\* $p = 0.056$ , 7 days DOT retention, \*\*\*\* $p < 0.001$ ). (d-d''') Representative images from the scratch wound healing assays demonstrated a decrease in macrophage motility after 1 and 7 days of DOT retention compared to untreated control (CTR) cells (scale bar is equal to 400  $\mu\text{m}$ ).

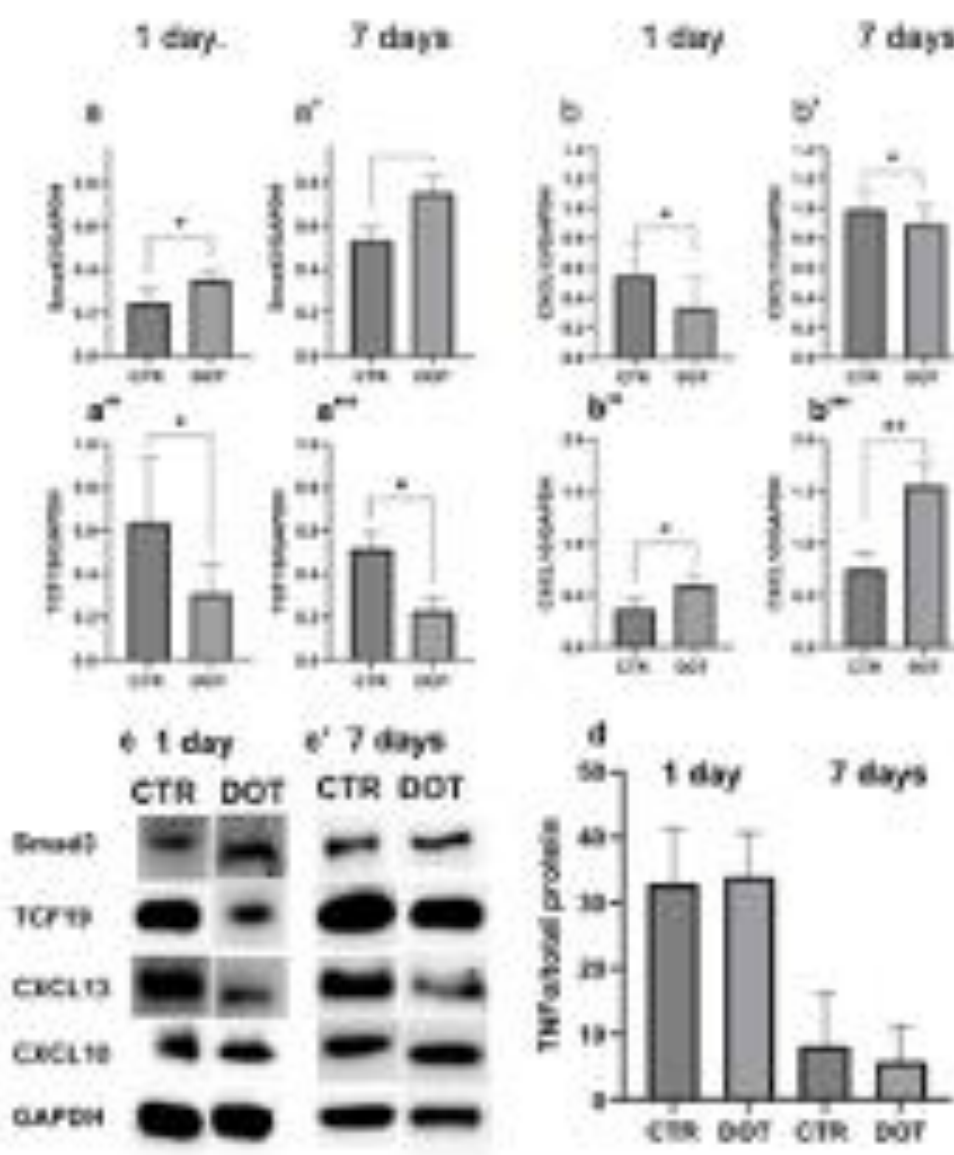


**Figure 2. Gadolinium/Dotarem retention affects the expression of cell-cell interaction, fibrosis, and focal adhesion-related proteins.**

(a-c') Western blot analysis of the expression of cell-cell interaction- and fibrosis-related proteins: Notch1 and its ligand Jagged1, the focal adhesion-related proteins: PAK1 and Shp1, and GAPDH (control) after 1 day and 7 days of DOT (1.4 mM) retention, and in the control untreated (CTR) RAW264.7 cells. (a-a'') Graphs illustrating Notch1 and Jagged1 signal intensity on the Western blots relative to GAPDH after 1 day (Notch1 \*p < 0.05, Jagged1 \*p < 0.01) and 7 days (Notch1



\*\*\* $p < 0.001$ , Jagged1 \*\*\* $p < 0.001$ ) of DOT retention. (b-b'') Graphs illustrating PAK1 and Shp1 signal intensity on the Western blots relative to GAPDH after 1 day (PAK1 \* $p < 0.05$ , Shp1 ns, statistically nonsignificant) and 7 days (PAK1 \*\*\* $p < 0.001$ , Shp1 \* $p < 0.01$ ) of DOT retention. (d-d'') Representative images of the effects of DOT retention on the adhesion of RAW246.7 cells. The adherent cells were stained with crystal violet. (e, e') Graphs illustrating changes in RAW246.7 cells adhesion after 1 day and 7 days of DOT retention assayed by the crystal violet absorption values.





**Figure 3. Gadolinium/Dotarem retention affects the expression of immune response-related proteins.**

(a-c') Western blot analysis of the expression of immune response-related proteins Smad3, TCF19, CXCL13, and CXCL10 in comparison to the GAPDH (control) after 1 and 7 days of DOT retention in comparison to the untreated control. (a, a'') Graphs illustrating Western blot band intensity of Smad3 and TCF19 after 1 day (Smad3 \* $p < 0.05$ , TCF19 \* $p < 0.05$ ) and 7 days (Smad3 \*\* $p < 0.01$ , TCF19 \* $p < 0.05$ ) of DOT (1.4 mM) retention in relation to GAPDH and in comparison to the untreated control. (a', a''') Graphs illustrating CXCL13 and CXCL10 signal intensity on the Western blots relative to GAPDH after 1 day (CXCL13 \* $p < 0.05$ , CXCL10 \* $p < 0.05$ ) and 7 days (CXCL13 \* $p < 0.05$ , CXCL10 \*\* $p < 0.01$ ) of DOT (1.4 mM) retention in comparison to untreated control. (d) TNF $\alpha$  secretion in response to LPS (0.1  $\mu\text{g/ml}$ ) stimulation after 1 day (ns, statistically nonsignificant) and 7 days (ns) of DOT (1.4 mM) retention in comparison to the untreated control.

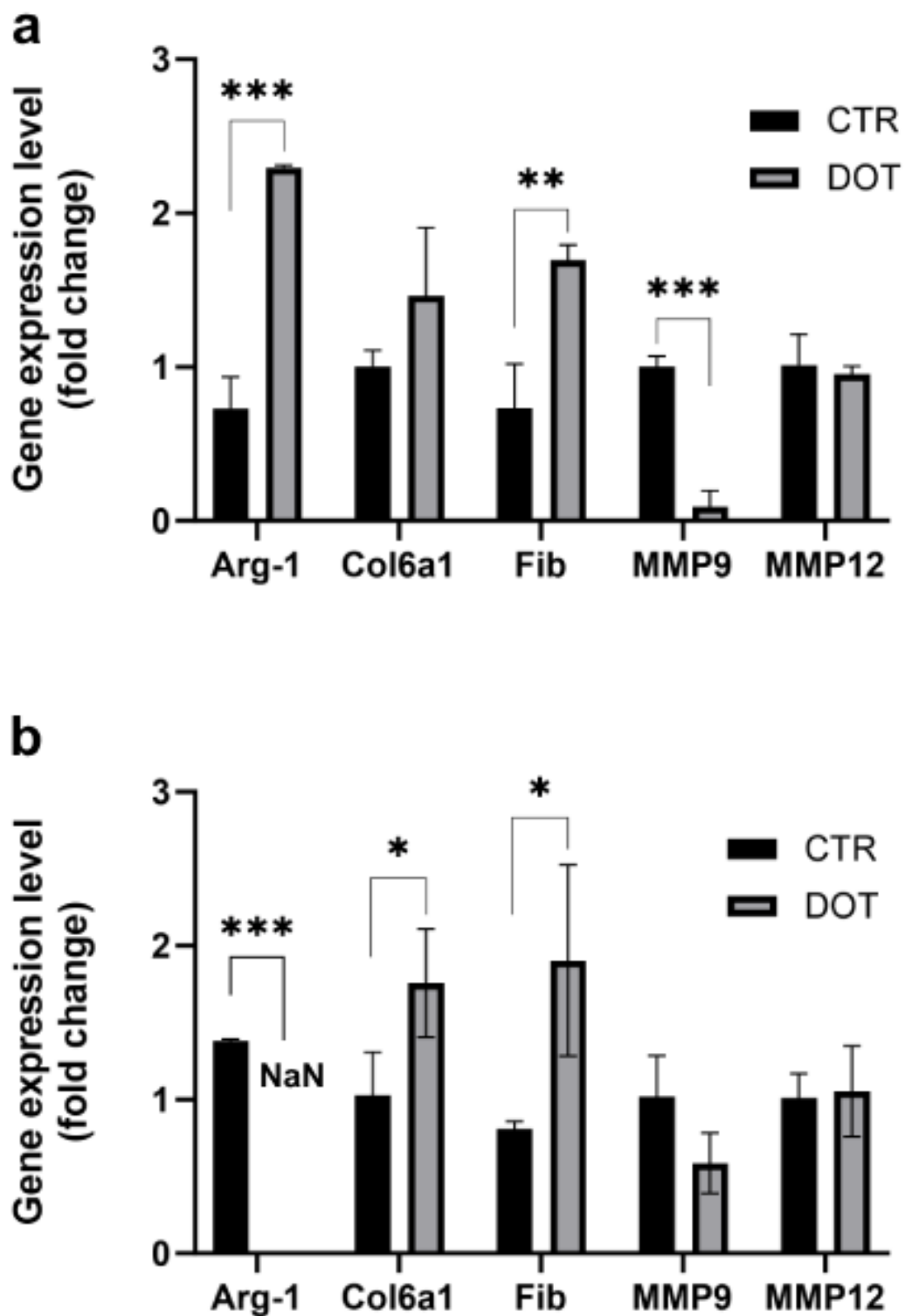


Figure 4. Expression of extracellular matrix (ECM)-related genes after 1 day and 7 days of gadolinium/DOT retention. Bar graphs depict mean relative gene expression levels and error

bars representing the standard deviations. Relative expression values were obtained by the delta Ct method, where GAPDH was used as the housekeeping gene and all results were normalized to the control average. Relative expression values were plotted on the y-axis. **(a)** Level of the expression of ECM-related genes: Arg-1, Col6a1, Fibronectin, MMP9, and MMP12 after simultaneous treatment with TGF- $\beta$  (10 ng/mL) and DOT (1.4 mM) for 24 hrs. TGF- $\beta$  was used to stimulate macrophage differentiation toward the M2 phenotype. **(b)** Level of ECM-related gene expression after 7 days of gadolinium /DOT retention. Interestingly, 7-day gadolinium retention completely shuts down (below a detection level, with the value NaN, where NaN- stands for Not a Number. It is a particular value of a numeric data type which is undefined or unrepresentable, such as the result of zero divided by zero) the expression of the M2 macrophage marker Arg-1.