Osteoarthritis-associated basic calcium phosphate crystals alter immune cell metabolism and promote M1 macrophage polarization

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SUMMARY

Objective: A number of studies have demonstrated that molecules called ‘alarmins’ or danger-associated molecular patterns (DAMPs), contribute to inflammatory processes in the OA joint. Metabolic reprogramming of immune cells, including macrophages, is emerging as a prominent player in determining immune cell phenotype and function. The aim of this study was to investigate if basic calcium phosphate (BCP) crystals which are OA-associated DAMPs, impact on macrophage phenotype and metabolism.

Methods: Human monocyte derived macrophages were treated with BCP crystals and expression of M1 (CXCL9, CXCL10) and M2 (MRC1, CCL13)-associated markers was assessed by real-time PCR while surface maturation marker (CD40, CD80 & CD86) expression was assessed by flow cytometry. BCP induced metabolic changes were assessed by Seahorse analysis and glycolytic marker expression (hexokinase 2(HK2), Glut1 and HIF1α) was examined using real-time PCR and immunoblotting.

Results: Treatment with BCP crystals upregulated mRNA levels of CXCL9 and CXCL10 while concomitantly downregulating expression of CCL13 and MRC1. Furthermore, BCP-treated macrophages enhanced surface expression of the maturation markers, CD40, CD80 and CD86. BCP-treated cells also exhibited a shift towards glycolysis as evidenced by an increased ECAR/OCR ratio and enhanced expression of the glycolytic markers, HK2, Glut1 and HIF1α. Finally, BCP-induced macrophage activation and alarmin expression was reduced in the presence of the glycolytic inhibitor, 2-DG.

Conclusions: This study not only provides further insight into how OA-associated DAMPs impact on immune cell function, but also highlights metabolic reprogramming as a potential therapeutic target for calcium crystal-related arthropathies.

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Introduction

Osteoarthritis (OA) is the most common form of chronic arthropathy and leading cause of disability worldwide. It affects 10% of males and 18% of females over the age of 45, a figure that is set to rise as life expectancy increases. In addition, the current obesity epidemic has resulted in an increased incidence of OA in younger individuals, many of whom will undergo multiple joint replacements due to the limited lifespan of artificial joints.

Extensive research has highlighted a complex interplay between chondrocytes, synovial fibroblasts and the innate immune network and while much progress has been made in elucidating the cellular and molecular events contributing to OA, the complex nature of the disease has hampered the development of a successful disease-modifying drug. Current therapies are focused on providing symptomatic relief rather than halting or reversing disease progression, hence, there is a clear need to identify specific orchestrators that initiate and/or contribute to eventual joint destruction.

Immunometabolism is an emerging field of research that focuses on changes in intracellular metabolic pathways in immune cells and how these alterations impact on cell fate and function. It is now recognised that a metabolic shift from oxidative phosphorylation to a highly metabolically active glycolytic state...
(designed to maintain energy homeostasis under conditions of low oxygen and acute stress) can lead to an accumulation of reactive oxygen species (ROS) and metabolic intermediates that promote the synthesis of degradative enzymes and inflammatory mediators. Increased glycolytic rates and glucose consumption are associated with enhanced expression of the glucose transporter, GLUT1, as well as elevated lactate production. Furthermore, activated immune cells exhibit Warburg metabolism and HIF-1α induction even under normoxic conditions. Recent studies have also demonstrated that macrophage activation and phenotype is intricately linked to cellular metabolic status. For example, lipopolysaccharide (LPS) activated M1 macrophages [M1(LPS)] exhibit an enhanced glycolytic profile characterised by increased expression of glucose transporters, glycolytic enzymes and rapid production of ATP.

While this metabolic switch is required to meet the energy demands of the cell during infection and provide biosynthetic components required to carry out effector functions, dysregulated immune-cell metabolism which results in sustained glycolytic flux can lead to alarmin production, contribute to disease pathology and regulate disease pathology.

Modulating metabolic reprogramming represents a novel approach to treating diseases and it has been strongly suggested that strategies to alter immune cell metabolic reprogramming and change immune cell phenotype and function can be developed by targeting metabolic pathways.

Human blood monocyte-derived macrophage isolation

Cells were pre-treated with PD98059 (20 μM), SB203580 (20 μM), for 45 min prior to stimulation with BCP crystals (50 μg/ml) for 24 h. Supernatants were harvested and cytokine concentrations of TNFα, IL-6 and IL-8 were quantified by ELISA (eBioscience, San Diego, CA) according to manufacturer’s protocol.

Real-time PCR

Macrophages were treated with BCP crystals (50 μg/ml) alone or in the presence of indicated inhibitors (PD98059 (20 μM), SB203580 (20 μM)), for 45 min prior to stimulation with BCP crystals (50 μg/ml) for 24 h. Supernatants were harvested and cytokine concentrations of TNFα, IL-6 and IL-8 were quantified by ELISA (eBioscience, San Diego, CA) according to manufacturer’s protocol.

Immunoblotting

Primary macrophages were stimulated BCP crystals (50 μg/ml) for 3, 6, or 24 h for the detection Hexokinase 2 (HK2), Glut1 or HIF1α protein. Cells were lysed by addition of RIPA buffer (Tris 50 mM; NaCl 150 mM; SDS 0.1%; sodium deoxycholate 0.5%; Triton X-100). Lysates were electrophoresed on SDS-PAGE gels (12% gel for Glut1, 10% gel for HK2 and 7.5% gel for HIF1α) and transferred to PVDF membranes (Millipore, Massachusetts) prior to detection with anti-HK2 or anti-Glut1 antibodies.
Flow cytometry

Primary human macrophages were plated at a concentration of 1 × 10^6 cells/ml and stimulated with BCP crystals (50 µg/ml) for 24 h in the absence or presence of the glycolytic inhibitor 2-DG (25mM). Cells were collected, washed, Fcγ blocked and stained extracellularly with amine-binding markers for dead cells (Fixable Viability Dye; eBioscience) for 10 min. Cells were washed in PBS and stained with fluorochrome-conjugated antibodies specific for CD14, CD11b, CD86, CD40 and CD80 (all ebiosciences) and fixed with 2% paraformaldehyde (PFA) washed and acquired. The model antigen DQ-Ova, which fluoresces upon processing by proteases inside the cell, was used to assess phagocytic capacity of the cells. Macrophages were cultured with complete RPMI containing DQ-Ovalbumin (500 ng/ml; Invitrogen) for 20 min at 37 °C, followed by incubation for 10 min at 4 °C. Cells were then washed in PBS and immediately acquired. Cells were acquired on a FACSWilliam TM II (BD Biosciences) and analysis was performed with FlowJo v.10 software (Tree Star Inc.). Gating strategies utilised in all experiments are detailed in Supplementary Fig. 1.

Seahorse analyser

Macrophages were cultured at 1 × 10^6 cells/ml for 6 days prior to re-seeding at 2 × 10^3 cells/well in a Seahorse 96-well microplate and allowed to rest for 5 h prior to BCP or LPS stimulation. The Seahorse cartridge plate was hydrated with XF calibrant fluid and incubated in a non-CO2 incubator at 37 °C for a minimum of 8 h prior to analysis. To determine the effect of BCP crystals, macrophages were incubated with BCP crystals (50 µg/ml) for 24 h, prior to analysis using a Seahorse XFe24 analyser. 30 min prior to placement into the XF/XFe analyser, cell culture medium was replaced with complete XF assay medium (Seahorse Biosciences; supplemented with 10mM glucose, 1mM sodium pyruvate, 2mM L-glutamine, and pH adjusted to 7.4) and incubated in a non-CO2 incubator at 37°C. Blank wells (XF assay medium only) were prepared without cells for subtracting the background oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) during analysis. Oligomycin (1 µM, Cayman Chemicals), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1 µM; Santa Cruz biotechnology), rotenone (500 nM), and antimycin A (500 nM) and 2-deoxy-D-glucose (2-DG) (25 mM; all Sigma–Aldrich) were prepared in XF assay medium and loaded into the appropriate injection ports on the cartridge plate and incubated for 10 min in a non-CO2 incubator at 37 °C. OCR and ECAR were measured over time with sequential injections of oligomycin, FCCP, rotenone and antimycin A and 2-DG. Analysis of results was performed using Wave software (Agilent Technologies). The rates of basal glycolysis, max glycolysis, glycolytic reserve etc. were calculated as detailed in the manufacturer’s protocol and supplied in Supplementary Table 3.

Statistical analysis

Each experiment was performed in at least four healthy donors (defined by N) with 3–4 technical replicates run for each experiment, (defined by n) depending on the assay type. Paired one-way ANOVA or Kruskal–Wallis tests were used for the comparison of more than two groups for parametric and non-parametric analysis respectively, with the Tukey or Dunn’s post-test (where applicable). A paired Student’s t-test was used when there were only two groups for analysis and the data was normally distributed and a Two-tailed paired Wilcoxon Signed rank test was used for non-parametric data. All statistical analysis was performed on GraphPad Prism 7.00 (GraphPad Software). P value < 0.05 were deemed significant.

Results

BCP crystals promote M1 macrophage polarization

We and others have previously reported that BCP-crystals drive inflammatory mediator and alarmin production in macrophages, fibroblasts and chondrocytes22,29,31. In order to determine if BCP crystals impact on macrophage polarization, primary human macrophages were treated with previously published doses of BCP crystals (50 µg/ml) for 24 h and expression of established genes associated with M(LPS) (i.e., prototypic M1-like MDMS) and M(IL-4) (i.e., prototypic M2-like MDMS) macrophage polarization was assessed by real-time PCR. While BCP stimulation of macrophages significantly enhanced the mRNA expression of the M(LPS) markers, CXCL9 and CXCL10, compared to control samples, treatment of macrophages with the crystals led to a significant decrease in basal levels of the M(IL-4) macrophage markers, MRC1 and CCL13 (Fig. 1A–B). Furthermore, treatment of macrophages with BCP crystals significantly enhanced secretion of the chemokine, IL-8 [Fig. 1(E)]. Treatment of macrophages with BCP-crystals did not induce secretion of the cytokines TNFα or IL-6 (Fig. S2A), which is in agreement with published results examining BCP-induced responses in macrophages30 and together with results from the HEK-Blue™ iBLR4 assay system (Invivogen) (Fig. S2B) signifies a lack of endotoxin contamination in our BCP crystal preparation.

We have previously demonstrated that BCP crystals activate MAPKs downstream of Syk in a receptor independent manner38,39. Pre-treatment of macrophages with either SB203580 or PD98059 to inhibit p38 and ERK, respectively, significantly inhibited BCP-induced IL-8, CXCL9 and CXCL10 expression [Fig. 1(F)–(H)], demonstrating that MAPK activation is also involved BCP-induced macrophage polarization. Of note, inhibition of MAPKs had no effect on MRC1 or CCL13 mRNA expression in BCP treated macrophages (Fig. S3). In addition to CXCL9/10 expression, we assessed surface expression of the M1-associated maturation markers, CD86 and CD40, and the pro-inflammatory mediator, IL-8 [Fig. 1(E)]. Treatment of macrophages with BCP crystals significantly enhanced the secretion of the cytokines TNFα, IL-8 and CXCL9 and M1 macrophage polarization coincided with a metabolic switch facilitating glycolysis, macrophages were treated with BCP and established markers of glycolysis were assessed by real-time PCR and immunoblotting. BCP crystals significantly upregulated mRNA and protein expression of the glucose transporter, GLUT 1 the glycolytic enzyme, hexokinase 2 (HK2) and the transcription factor, HIF1α. There was also a trend towards increased expression of BCP crystals alter macrophage metabolism and promote glycolysis

A number of studies have demonstrated that activated macrophages exhibit an enhanced glycolytic profile characterised by increased expression of glucose transporters and glycolytic enzymes32. In order to determine whether BCP crystal-induced macrophage polarization coincided with a metabolic switch favoring glycolysis, macrophages were treated with BCP and established markers of glycolysis were assessed by real-time PCR and immunoblotting. BCP crystals significantly upregulated mRNA and protein expression of the glucose transporter, GLUT 1 the glycolytic enzyme, hexokinase 2 (HK2) and the transcription factor, HIF1α. There was also a trend towards increased expression of...
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) \( (p = 0.0634) \) [Fig. 2(A)]. No difference in mRNA expression of HIF2a was observed [Fig. 2(A)]. Furthermore, protein levels of both HK2 and GLUT1 and were found to be highly expressed at 24 h post BCP crystal treatment, while increased expression of HIF1a was observed at 3, 6 and 24 h post stimulation [Fig. 2(B)]. Densitometric analysis revealed a dose dependent upregulation of HK2 and Glut1 proteins at 3, 6 and 24 h with maximal and significant upregulation occurring at 24 h for both proteins [Fig. 2(C)]. To further investigate whether BCP treated cells were altering their energy metabolism to a more glycolytic state, we measured both glycolysis and oxidative phosphorylation using the Seahorse XF-Analyzer. Macrophages were seeded into a Seahorse microplate and stimulated with BCP (50 μg/ml) for 3, 6 or 24 h. The basal rate of ECAR of BCP-treated cells was higher than untreated controls with the highest ECAR observed at 3 h post-stimulation [Fig. 2(D)]. The basal rate of glycolysis and oxidative phosphorylation were determined by the measurement of ECAR (extracellular acidification rate) and OCR (oxygen consumption rate), respectively, before and after injections of oligomycin, FCCP, rotenone, antimycin A and 2-DG. Overall, the ECAR of BCP-treated cells was higher than untreated controls with the highest ECAR observed at 3 h post-stimulation [Fig. 2(D)].
glycolysis was increased in BCP-treated macrophages at all time points, compared to that of untreated control cells [Fig. 2(G)], while the maximum rate of glycolysis, was also enhanced in BCP stimulated cells, peaking at 3 h [Fig. 3(H)]. The respiratory profiles of macrophages appeared to mirror their observed glycolytic activity with 3 and 6 h BCP stimulation inducing the highest OCR, while 24 h stimulation appeared to marginally reduce OCR [Fig. 2(E)].

This was reflected in the calculated basal respiration and max respiration (Fig. 5S). Importantly, the ECAR:OCR ratio was increased in BCP treated cells at all time points tested [Fig. 2 (I)]. We also compared the metabolic profile of BCP-treated cells to that of a known PAMP, LPS which was marginally more potent than BCP at driving CXCL9 and CXCL10 gene expression (Figs. S6(I) and (J)). While both treatments enhanced glycolysis as well as oxidative

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phosphorylation (Figs. S5(A)–(H)), overall the ECAR/OCR ratio was higher for BCP-treated macrophages suggesting that it may be a more potent inducer of glycolysis [Fig. 2[J]]. Furthermore, when ATP production rate was assessed post BCP treatment, total cellular ATP was significantly enhanced at all time points tested, with BCP-treatment resulting in an increase in glycoATP production rates and a decrease in mitoATP production rates [Fig. 2(D)]. Taken together, these data indicate that human macrophages promote glycolytic metabolism upon BCP stimulation.

**Macrophage polarization and metabolic reprogramming by BCP crystals is dependent on particle uptake**

Having established that BCP crystals can induce a metabolic switch in human macrophages, it was of interest to determine if these responses are dependent on particle uptake. To test this, macrophages were pre-treated with the actin polymerization inhibitor, Latrunculin B (1 μM) (to inhibit phagocytosis) for 45 min prior to stimulation with BCP crystals. Results demonstrate that BCP induced CXCL9 and CXCL10 mRNA expression was significantly reduced in the presence of latrunculin B [Fig. 3(A) and (B)]. Inhibition of particle uptake also significantly reduced BCP crystal induced IL-8 production [Fig. 3(C)] as well as mRNA expression of the glycolytic markers, HK2, Glut1 and HIF1α [Fig. 3(D)–(F)]. To ensure that these observations were not due to loss of membrane integrity, which has been associated with Latrunculin B [26], cells were also pre-treated with the alternative actin polymerization inhibitors Cytochalasin D and B (5 μM) and the dynamin inhibitor, Dynasore (80 μM) prior to stimulation with BCP crystals. Similar trends were observed with all three inhibitors of phagocytosis (Fig. S7). Taken together, this indicates that particle uptake is essential for BCP crystal mediated activation of macrophages and glycolytic gene expression.

**Inhibition of glycolysis abrogates BCP crystal induced macrophage polarization and alarmin expression**

To elucidate if direct inhibition of glycolysis abrogates BCP crystal induced macrophage polarization, cells were stimulated with BCP crystals as described, in the presence or absence of the glycolytic inhibitor, 2-DG. Pre-treatment of cells with 2-DG resulted in a significant decrease of BCP induced CXCL9 and CXCL10 mRNA expression [Fig. 4(A) and (B)]. We have previously demonstrated that BCP crystals strongly upregulate expression of the alarmin S100A8 [29,30] which together with S100A9, is heavily implicated in driving catabolic processes in OA [27–30]. mRNA expression of S100A8 was significantly inhibited in the presence of 2-DG ([Fig. 4(C)]) suggesting that inhibition of macrophage metabolic reprogramming impacts on the ability of BCP crystals to induce alarmin expression. Finally, inhibition of glycolysis with 2-DG decreased surface marker expression of CD40, CD80 and CD86 in BCP treated macrophages [Fig. 4(D) and (E)]. Taken together, the results suggest that glycolytic blockade inhibits BCP-induced pro-inflammatory effects in macrophages.

**Discussion**

While several studies have demonstrated that impaired mitochondrial dysfunction contributes to pro-catabolic processes in OA chondrocytes, the factors contributing to this phenomenon have not been fully characterized [21]. Furthermore, there have been few studies examining metabolic reprogramming in OA synovial fibroblasts or macrophages. During infection, stimulation of pattern recognition receptors (PRRs) leads to enhanced glycolysis which enables immune cells to generate sufficient ATP and pro-inflammatory mediators required to carry out effector functions [2]. It is now well accepted that sterile tissue injury can provoke an immune response analogous to that seen during infection and that...
endogenous molecules generated upon tissue damage are capable of exerting similar effects on cellular processes. Hence, while the switch to a highly active metabolic state is necessary for immune cell activation during infection, metabolic reprogramming by DAMPs/alarmins in the absence of infection has the potential to exacerbate existing inflammation. We now demonstrate that BCP crystals which are OA-associated DAMPs, polarize primary human macrophages towards an M1-like phenotype, enhancing expression of M1-macrophage associated markers and elevating expression of surface maturation markers. Furthermore, BCP crystal stimulation promotes a bioenergetic switch favouring glycolysis and this is accompanied by increased expression of HIF1α, GLUT1 and hexokinase 2, all of which are surrogate markers of glycolysis. Finally, BCP-induced expression of M1 markers and the alarmin, S100A8, was inhibited in the presence of glycolytic inhibitor, 2DG, suggesting that BCP-induced macrophage polarization and inflammation may be dependent on metabolic reprogramming.

Particulates such as calcium oxalate crystals have previously been reported to promote M1 macrophage polarization and, while a number of studies have investigated metabolic reprogramming in human immune cells in response to exogenous pathogen associated molecules (i.e., PAMPs), to our knowledge this is the first report of an endogenous particulate impacting on immune cell metabolism. We demonstrate that human macrophages stimulated with BCP crystals upregulate both glycolysis and oxidative phosphorylation within hours of activation however, the overall glycolytic and ATP production rate is higher. In our hands, LPS stimulation also resulted in enhanced glycolysis and oxidative phosphorylation in human macrophages however, BCP crystals promoted a higher ECAR:OCR ratio than LPS which is considered a potent PAMP. The observed effects of LPS on ECAR and OCR rates differs from reports in murine DC demonstrating that LPS strongly upregulates aerobic glycolysis whilst simultaneously down-regulating oxidative phosphorylation via the action of iNOS-derived NO\textsuperscript{41,42}. There is, however, controversy regarding the expression/activity of iNOS in human immune cells\textsuperscript{43,44} and our finding is in agreement with a recent study by Malinarich et al. who reported that, while mature human DC are more glycolytic than immature DC, they do not entirely downregulate oxidative phosphorylation, and instead display a more “balanced” switch to

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Fig. 4. BCP crystal-induced macrophage polarization is reduced in the presence of the glycolytic inhibitor 2-DG. Primary human macrophages (1 × 10\textsuperscript{6}) were stimulated with BCP (50 μg/ml) in the presence or absence of 2-DG (25mM) for 24 h. (A–C) Graphs demonstrating mRNA levels of CXCL9, CXCL10 and S100A8 (N = 4, n = 3). (D) Representative histograms from one experiment demonstrating CD40, CD80 and CD86 surface marker expression. (E) Pooled data (N = 4, n = 2) depicts mean Fluorescence Intensity (MFI) of surface marker expression. Data is represented as Mean ± SEM Data and was analyzed using one-way ANOVA with Tukey post-test for real-time PCR assays and Kruskal Wallis for Flow cytometry assays.
glycolysis whereby glycolysis and oxidative phosphorylation are both upregulated\(^\text{45}\).

BCP crystals have previously been reported to upregulate the expression of inflammatory and catabolic mediators in a number of cell types implicated in OA\(^\text{24,46–48}\). Our results show that, in addition to cytokines, chemokines and MMPs, BCP crystals can also upregulate expression of the key glycolytic enzymes, hexokinase 2, GLUT1 and HIF1α, an effect which was attenuated in the presence of phagocytosis inhibitors. Furthermore, when glycolysis was inhibited with the glucose analog, 2-DG, we observed a notable decrease in M1-macrophage marker expression, in addition to S100A8, which is a key OA-associated DAMP. Further analysis will determine if metabolic targeting impacts on downstream targets of S100A8 which include cytokines such as IL-6 and IL-8, as well as MMPs 1, 9 and 13\(^\text{29,62}\). Interestingly, BCP crystals themselves were potent drivers of IL-8 expression in our hands. This may represent a feed forward mechanism whereby BCP-induced S100A8 drives expression of IL-8, albeit it should be noted that, while S100A8 is known to stimulate the synovial membrane to generate pro-inflammatory mediators, including IL-8\(^\text{-17}\) in OA, this chemokine is generally not implicated in Milwaukee Shoulder Syndrome which is also characterized by BCP crystal deposition\(^\text{25}\). Further study is required to fully elucidate the relationship between BCP crystals, IL-8 and the downstream effects of S100A8 production. It will also be necessary to determine whether BCP crystals drive metabolic reprogramming in synoviocytes from OA patient samples and whether glycolytic inhibitors impact on inflammatory/catabolic processes. Indeed, upregulation of glucose transport and an increase in glycolytic metabolism has been demonstrated in OA and particularly in chondrocytes were accumulation of lactic acid contributes to matrix acidification and impaired ECM synthetic function\(^\text{51–54}\). Less is known regarding metabolic disturbances in OA-synoviocytes, however an increase in the glycolysis/OXPHOS ratio has been observed in OA-FLS\(^\text{11}\). Furthermore, silencing of PHD2, a global negative regulator of HIF-1α expression decreased expression of angiogenic genes, while high glucose levels were shown to induce ROS and VEGF in OA-FLS further implicating a metabolic switch and increased glucose transport in synovial joint cells in OA\(^\text{15,60}\).

While the field of immunometabolism is still in its infancy and much remains to be learned regarding the impact of metabolic reprogramming on disease pathogenesis, ultimately it may be possible to modulate metabolic changes in polarized macrophages by specifically targeting key glycolytic enzymes. However, extreme caution is required given the strong potential for off target effects. This is of particular importance in OA given that chondrocytes, in particular, exist in a hypoxic environment and rely on HIF1α and glycolysis to maintain homeostasis. Currently, there are no drugs available to target BCP crystal deposition in the OA joint and subsequent inflammatory responses, however, based on the above findings, we propose that targeting BCP-and DAMP-induced metabolic reprogramming represents a novel avenue that requires further consideration and exploration.

**Author contributions**

OM and AD contributed to conception and design of the study, analysis and interpretation of the data, drafting of the article. DK and GM contributed to the interpretation of data and provision of study materials.

**Competing interest statement**

The authors declare no conflicts of interest.

**Funding source**

This study was supported by a Trinity College Dublin Postgraduate Research Studentship.

**Acknowledgements**

We would like to thank Dr Eoin O’Brien and Eva Desmond for technical assistance.

**Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2019.10.010.

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