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Autoreactivity to malondialdehyde-modifications in rheumatoid arthritis is linked to disease activity and synovial pathogenesis

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ABSTRACT

Oxidation-associated malondialdehyde (MDA) modification of proteins can generate immunogenic neo-epitopes that are recognized by autoantibodies. In health, IgM antibodies to MDA-adducts are part of the natural antibody pool, while elevated levels of IgG anti-MDA antibodies are associated with inflammatory and autoimmune conditions. Yet, in human autoimmune disease IgG anti-MDA responses have not been well characterized and their potential contribution to disease pathogenesis is not known. Here, we investigate MDA-modifications and anti-MDA-modified protein autoreactivity in rheumatoid arthritis (RA). While RA is primarily associated with autoreactivity to citrullinated antigens, we also observed increases in serum IgG anti-MDA in RA patients compared to controls. IgG anti-MDA levels significantly correlated with disease activity by DAS28-ESR and serum TNF-alpha, IL-6, and CRP. Mass spectrometry analysis of RA synovial tissue identified MDA-modified proteins and revealed shared peptides between MDA-modified and citrullinated actin and vimentin. Furthermore, anti-MDA autoreactivity among synovial B cells was discovered when investigating recombinant monoclonal antibodies (mAbs) cloned from single B cells, and 3.5% of memory B cells and 2.3% of plasma cells were found to be anti-MDA positive. Several clones were highly specific for MDA-modification with no cross-reactivity to other antigen modifications such as citrullination, carbamylation or 4-HNE-carbonylation. The mAbs recognized MDA-adducts in a variety of proteins including albumin, histone 2B, fibrinogen and vimentin. Interestingly, the most reactive clone, originated from an IgG1-bearing memory B cell, was encoded by near germline variable genes, and showed similarity to previously reported natural IgM. Other anti-MDA clones display somatic hypermutations and lower reactivity. Importantly, these anti-MDA antibodies had significant *in vitro* functional properties and induced enhanced osteoclastogenesis, while the natural antibody related high-reactivity clone did not. We postulate that these may represent distinctly different facets of anti-MDA autoreactive responses.

1. Introduction

Rheumatoid arthritis (RA) is a chronic and potentially disabling autoimmune disease affecting between 0.5 and 1% of the Western population [1]. RA is associated with synovial inflammation and progressive destruction of joints. There is also increased risk for morbidity and mortality from accelerated atherosclerotic cardiovascular disease [2]. Pathogenesis is associated with a characteristic autoimmune response to self-proteins post-translationally modified by citrullination, resulting in circulating anti-citrullinated protein antibodies (ACPA) that are detected in 65–80% of patients

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with established disease [3] (reviewed in Ref. [4]).

Seropositive RA is defined by clinical criteria that include the presence of IgG antibodies to synthetic peptides, termed cyclic citrullinated peptides (CCP), and/or IgM rheumatoid factors (RF) that bind aggregated IgG Fc regions [5]. Yet RA patients also commonly display other autoantibodies, including IgG binding to proteins post-translationally modified by carbamylation, that involves the generation of homocitrulline residues from lysines [6,7]. Antibodies to carbamylated antigens have been reported in both seropositive and seronegative RA patients. They can arise in parallel to ACPA responses and could at times reflect unique epitope recognition, but may also be partly explained by ACPA cross-reactivity [8].

The current report focuses on the contribution of immune responses to a distinctly different and less extensively studied self-protein modification, malondialdehyde (MDA) adducts, during RA pathogenesis.

MDA is a naturally occurring, highly reactive aldehyde, produced under oxidative stress states associated with excessive generation of reactive oxygen species (ROS). Elevated ROS catalyzes membrane lipid peroxidation and the formation of reactive MDA that can covalently modify proteins through carbonylation of amino acids carrying free amine groups (i.e. lysine, arginine, histidine), and to less extent amino acids with amide groups (i.e. asparagine, glutamine), which can generate structural changes and neo-epitopes [9,10]. A large number of self-proteins have been found to be modified by MDA under local inflammatory conditions, including vimentin, fibrinogen, α-enolase and albumin [11,12] (reviewed in Refs. [13,14]). Acetaldehyde (AcA) can further react with MDA adducts to form immunogenic malondiadehyde-acetaldehyde (MAA) modifications (Fig. 1) [15]. ROS levels sufficient to cause tissue injury can be generated by exogenous stimuli such as tobacco smoke, or be endogenously produced during inflammation [16]. Moreover, oxidized proteins and lipids are also formed as a consequence of programmed death pathways and the resulting adducts on apoptotic cells can be recognized by some anti-MDA antibodies [17–19].

Recent evidence implicates that autoimmune diseases are associated with an altered redox-state and elevated levels of oxidative species, which may contribute to disease pathogenesis [20–25]. Increased levels of MDA and MDA-modified proteins, observed both in systemic lupus erythematosus (SLE) and RA, may reflect disturbances in oxidation balance occurring during systemic inflammation [20–25]. Strikingly, at birth the human natural IgM repertoire has a strong bias towards antibody-recognition of oxidation-associated epitopes, especially MDA-modifications [18,19,26]. Hence, the immune system is primed from birth to recognize modified self-antigens, and it has been postulated that these antibodies play important roles in clearance of apoptotic cells, neutralization of harmful molecules, and maintenance of immune homeostasis [27]. However, whilst IgM may be protective, the constant regions of IgG autoantibodies with the same specificity may instead trigger inflammatory responses. IgG autoantibodies that bind MDA can be elevated in SLE, and levels are directly related to increased disease activity [25,28].

Herein, we have investigated the representation of MDA-modifications of self-proteins in the rheumatoid joint and their potential as immunogens for eliciting immune responses. In a cross-sectional RA cohort, we also evaluated serological levels of autoantibodies to MDA-modified self-protein epitopes in relation to disease activity. Furthermore, we recovered human mAbs isolated from individual synovial B cells and plasma cells from RA patients, and evaluated their binding specificity for MDA-modified self-proteins and their associated *in vitro* functional properties.

2. Material and methods

2.1. Patients and sample procedures

All RA patients fulfilled the 2010 ACR/EULAR criteria for diagnosis [5], and informed consent was obtained for all patients and controls according to protocols approved by the Human Subjects Institutional Review Board of NYU School of Medicine, the Ethics Review Committee at the University of Birmingham, and the Ethics Review Committee North at the Karolinska University Hospital. Patients were classified as seropositive RA and seronegative RA based on the clinical CCP2 assay. Synovial fluid samples for cell isolation were collected in connection to when the patients required arthrocentesis due to local disease activity, and the patients were given subsequent local steroid injections. Synovial tissues of RA patients or disease controls were obtained at the time of joint-replacement surgery.

2.2. Mass spectrometry analysis

In vitro MDA-modified BSA was analyzed in pilot studies for MDA-detection and for comparison between different MDA-modification times. Ten μg protein was reduced and alkylated by DTT and iodoacetamide (Sigma Aldrich), precipitated and digested by trypsin (sequencing grade, Promega) in 50 mM ammonium bicarbonate and 30% DMSO (Sigma Aldrich) at protease to protein concentration of 1:20, as previously described [29]. After desalting the peptides using C18 StageTips (Thermo Fisher Scientific), the peptides were analyzed by nanoLC-MS/MS a NanoUltimate 3000 coupled on-line to an LTQ Orbitrap Velos mass spectrometer, were the Velos Orbitrap had been upgraded to an Elite (Thermo Fisher Scientific, Germany). The peptides were separated using an Acclaim® PepMap100 precolumn (C18, 3 μm, 100 Å; Thermo Scientific) together with a 15 cm EASY-Spray PepMap® analytical column (C18, 3 μm, 100 Å; Thermo Scientific). The separation was achieved using ACN/water gradients (buffer A: 2% ACN, 0.1% FA; buffer B: 98% ACN, 0.1% FA) of 5–26% B over 55 min, followed by a 26–95% ACN gradient over 5 min and 95% ACN for 8 min, all at a flow rate of 300 nl/min. The instruments were operated in a data-dependent mode with a top 5 method. The mass spectra were acquired at a resolution of 60,000 followed by either CID only or HCD only MS/MS fragmentation. A normalized collision energy of 35 was used for CID and 30 for HCD. The HCD MS/MS spectra were acquired at a resolution of 15,000. One pmol of the three samples were analyzed by both MS analysis using CID only and HCD only, each in two technical replicates (i.e. four analyses per samples).

Data from an earlier reported study of post-translational citrullination in rheumatoid synovial tissue [30], were re-searched for the detection of MDA-modified proteins. As primary analyses sought to detect citrullinated peptides the samples were enzymatically processed with Lys-C to avoid digestion at arginine sites. Note that this will reduce the detection of lysine modified residues. As previously described, each synovial tissue sample was analyzed by four different MS methods: top5 using CID/ETD fragmentation, top5 using ETD only, top4 using HCD only, and top4 using an inclusion list together HCD only (the inclusion list contained citrullinated peptides identified in the same study). The 28 raw files [30] were re-processed by Raw2MGF v2.1.3 and re-searched against the human complete proteome database (downloaded from www.uniprot.org April, 2013; 71434 sequences; 24507501 residues). At least one spectrum from each peptide reported in the Supplementary Tables 1 and 2 was validated manually.

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