Writing for **Portal, Professor Michael O Hottiger** of the Institute of Veterinary Biochemistry and Molecular Biology at the University of Zürich outlines his investigations into epigenetic regulation of inflammation and metaflammation

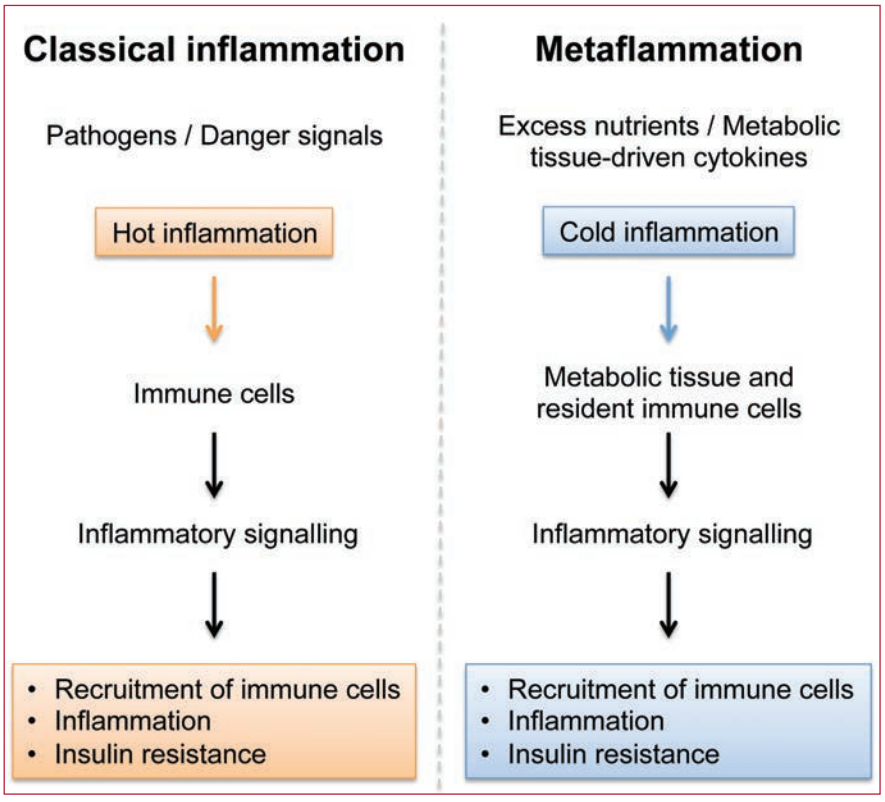
Inflammation/metaflammation

Inflammation is a general cell and tissue response elicited upon recognition of pathogens (e.g. viruses or bacteria) or danger-associated molecules (e.g released by damaged cells). Its principal function is to repair or regenerate cell and tissue damage. Innate immunity is the defensive frontline that co-ordinates the response of an organism to injury and infection to initiate the transition to adaptive immunity, eradicate pathogens and facilitate healing. Thus, the innate immune system plays a crucial role in the primary ('non-specific') inflammatory response and also for long term ('specific') immunity.

Professor Michael O Hottiger, DVM PhD

Chronic inflammation can result from dysregulation of the inflammatory response and is implicated in a plethora of medical conditions such as chronic infections, cardiovascular diseases, inflammatory neuropathies, neurodegenerative diseases and even cancer. Thus, while inflammation promotes and protects the integrity and healthy state of cells or tissues, it can also lead to serious complications if not properly controlled.

Interestingly, an inflammatory response can also be induced in metabolic tissues in the absence of pathogen or danger-associated signals by an excess of nutrients (e.g. in obesity or during excessive muscle training). Enhanced generation of reactive oxygen species (ROS) formed as byproducts of normal mitochondrial energy metabolism and by the subsequently recruited inflammatory cells have been implicated in the pathogenesis of many age-related diseases such as diabetes, cardiovascular disease and neurodegenerative disorders, such as Alzheimer's disease. This sterile (non-infectious) and low-grade, but chronic inflammatory response has been termed 'metaflammation'.



Scheme 1 – Comparison between classical and metaflammation

Innate immunity-related healthcare costs are estimated to exceed $10bn (~€7.33bn) annually in the United States. The global pain management market was worth $46.4bn in 2007 and is expected to reach $57.2bn by the end of 2014. Understanding the global innate immunity signalling network is therefore of large economical and medical importance.

Inflammatory responses

Induction of an inflammatory or metaflammatory response alters gene expression and therefore drastically changes the level and nature of the proteins present in or secreted by a cell. An external inflammatory stimulus is translated into a response in the cell nucleus. One of the most important mediators of inflammatory signalling is the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). In response to an inflammatory stimulus, NF-κB translocates from the cytoplasm to the nucleus, where it binds to the regulatory sequences of target genes and activates their transcription. These genes encode for factors that can activate other cells, so-called 'cytokines' such as tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1) or interleukin 6 (IL-6), or adipokines in adipose tissue. Cytokines further stimulate NF-κB, causing a positive feedback loop that can lead to an excessive, deleterious inflammatory response when not properly controlled.

It is becoming clear that NF-κB-mediated expression of inflammatory genes in the nucleus is co-ordinated by the synergistic

interaction of various co-factors and signalling networks. These networks regulate the accessibility of the DNA-encoded information, which is wrapped in a three-dimensional structure around histone complexes, known as chromatin, and is thereby condensed and protected. Induction of the structural chromatin changes is mainly achieved by modifying the histones that interact with DNA, and thereby establish the complex of chromatin.

In general, highly condensed DNA stretches are not transcribed, while genes in relaxed DNA segments are actively transcribed. Intriguingly, inflammatory stimuli induce chemical modification of histones (post-translational modifications, or PTM), thereby altering the chromatin structure, which consequently leads to changes in gene expression. Chromatin modifications can be passed on to daughter cells and consequently also to offspring, thereby causing heritable changes in gene function that are independent of any change in DNA sequence (so-called 'epigenetics'). A more recent and broader definition of epigenetics is "a structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, A). Since histone modifications are critical players in the orchestration of NF-κB function, understanding inflammation and metaflammation-induced epigenetic changes are biologically as well as medically highly relevant.

ADP-ribosylation of proteins

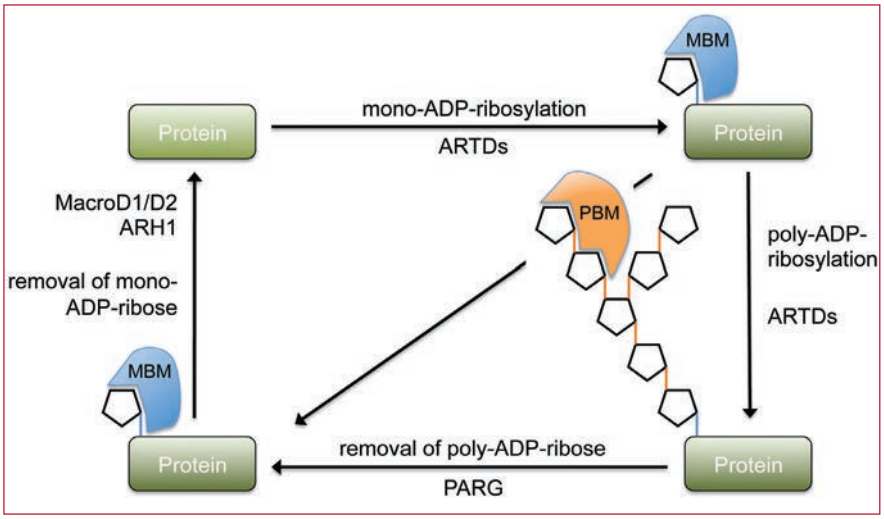
ADP-ribosylation is an important PTM of proteins such as histones and intensely studied by our group. A group of enzymes called ADP-ribosyltransferases (ARTs) mediates the reaction. They attach one or several ADP-ribose groups to specific sites marking them (i.e. 'writer' function) by using nicotinamide dinucleotide (NAD^+) as substrate. NAD^+ carries out essential functions as an electron acceptor in primary energy metabolism of cells (e.g. ATP generation in mitochondria). Due to this shared dependence on NAD^+, ADP-ribosylation is indirectly linked to the oxidative state of a cell.

ADP-ribose consists of an adenine group (a nucleotide) that is linked via two phosphate groups to a particular sugar molecule (a ribose). We broadly distinguish two groups of ARTs in mammalian cells. Extracellular enzymes that resemble the mammalian C2/C3 toxins are called ARTCs, and ARTDs are intracellular ARTs with similarity to diphtheria toxins and have important functions during inflammation and stress responses, as well as in many diseases.

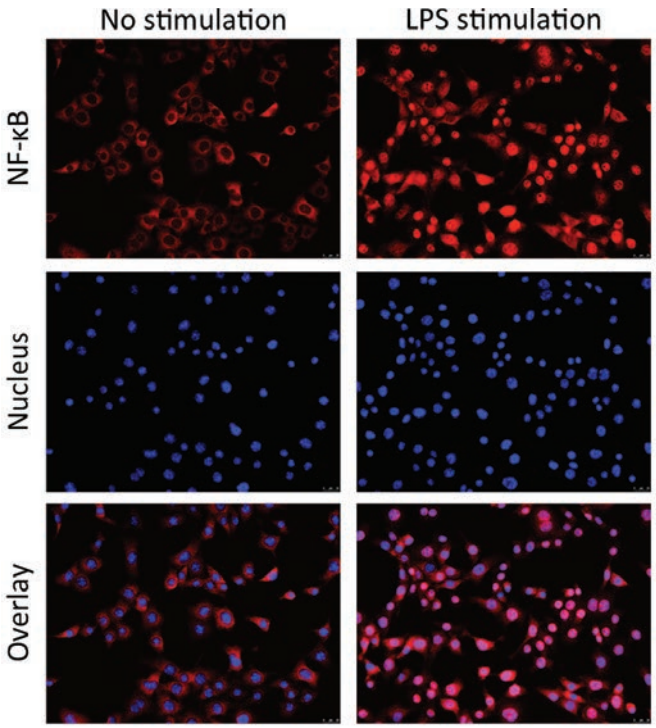
Of particular interest to our research is the stimulation of cells with an inflammatory mediator, which leads to a strong regulation of intracellular ADP-ribosylation. While certain ARTDs only mono-ADP-ribosylate their protein targets, other ARTDs can cause the formation of either linear or branched chains of poly-ADP-ribose (PAR). Mono or poly-ADP-ribose can in turn be recognised by specific domains of certain proteins (i.e. 'readers'). The ADP-ribose modification alters the physicochemical properties of the acceptor proteins, and thereby changes their functions, stability and interactions. For ADP-ribosylation to act as a regulator, the modification has not only to be synthesised and read in response to a specific stimulus, but must also be removed once the inflammatory signalling function needs to be terminated.

The proteins that remove ADP-ribose from modified proteins (i.e. 'erasers') are not as well characterised as the ARTs, but recent research from our group (Rosenthal *et al., Nat Struct Mol Biol.* 2013;20(4):502-7), as well as from other laboratories, has identified new, important players that carry out this function. The best-studied ADP-ribosylhydrolase activity so far has been ascribed to the enzyme poly-ADP-ribosylglycohydrolase (PARG). As the name implies, PARG degrades poly-ADP-ribose modifications, but is not able to release the primary ADP-ribose unit attached to the protein. Dependent on the modified amino acid, this activity is carried out by ADP-ribosylhydrolase (ARH1 for arginines) and the more recently described macrodomain-containing proteins (e.g. MacroD1 and MacroD2 for aspartic and glutamic acid). Interestingly, ARH1 or macrodomain-containing proteins are not able to release ADP-ribose from all modified proteins, suggesting that these enzymes are specific for distinct modification types and that possibly other erasers, for example for modified lysine residues, possibly exist.

The important finding that ADP-ribosylhydrolases (e.g. PARG, ARHs and MacroD proteins) can only remove certain ADP-ribose modifications highlights the importance of the nature of the modified protein and the specific ADP-ribose acceptor groups. The identification of all cellular ADP-ribosylated proteins and, most importantly, of the specific amino acid that is modified, is therefore essential to understand the molecular details



Scheme 2 – ADP-ribosylation cycle. Enzymes that attach and remove ADP-ribose molecules to and from proteins as discussed in text



Stimulation of cells with the inflammatory bacterial cell wall component lipopolysaccharide (LPS) leads to translocation of NF-κB (in red) from the cytoplasm to the nucleus (blue)

of the turnover, function and signalling of cellular ADP-ribosylation during inflammation. Unfortunately, specific antibodies that recognise ADP-ribose modifications do not exist and the specific detection of a particular ADP-ribosylated amino acid has been challenging. This specific field of research has therefore been dominated by the development of new affinity purification protocols and mass spectrometry techniques.

Our research group is at the forefront of these developments and has succeeded, for the first time, in identifying specific ADP-ribosylated lysine residues in histone proteins in cells. However, many other amino acids have also been suggested as ADP-ribose acceptor sites, and it is presently unknown which ARTD is responsible for generating which modification. One of our main goals is to resolve these questions and to establish reliable protocols to isolate, enrich and identify the entity of all ADP-ribosylated proteins in cells and tissues during inflammation.

ADP-ribosylation modulates inflammation

Our earliest observation that ADP-ribosylation is important for inflammation was that ADP-ribosyltransferase diphtheria toxin-like 1 (ARTD1, also known as PARP1) is absolutely essential for NF-κB-mediated gene expression. Not surprisingly, pharmacological inhibition of ADP-ribosylation also exhibits anti-inflammatory effects in various inflammatory disease models. For example, inhibition of ADP-ribosylation has been shown to reduce colonic inflammation (e.g. reduction in pro-inflammatory cytokines) and normalise cellular metabolic function and intestinal permeability in mice that spontaneously develop chronic, non-resolving colitis. Others have shown that mice deficient in ARTD1 are highly resistant to lipopolysaccharide (LPS)-induced endotoxic shock, suggesting that ARTD1 and ADP-ribosylation enhances the inflammatory response. Pharmacological ADP-ribosylation inhibition and/or ARTD1 deletion also reduce LPS-induced acute lung injury and cardiac or cerebral ischemia. ADP-ribosylation therefore regulates medically highly relevant inflammatory processes and their outcomes.

Laboratory research

Our previous work has led us to hypothesise that ADP-ribosylation affects NF-κB-gene expression either directly by modifying histones or indirectly by altering histone-modifying enzymes. The identification of nuclear protein ADP-ribosylation after inflammatory or metaflammatory stress signalling is therefore one of our main research focuses. We aim to identify the functional contribution of ADP-ribosylated proteins in the cell nucleus exposed to inflammatory stimuli and to characterise the already identified histones and histone-modifying enzymes in regard to their involvement in NF-κB-dependent gene expression. Importantly, we were the first to identify lysine residues in histone tails to be modified. To correlate distinct ARTD proteins with specific ADP-ribose modifications, we perform extensive analyses with cells lacking a particular ARTD member, as well as with purified ARTD proteins *in vitro*. Furthermore, we include clinically well tolerated PARP inhibitors in our studies, which have been developed as anti-cancer drugs and inhibit cellular ADP-ribosylation. We have shown that these inhibitors significantly reduce Helicobacter-induced neoplasia, ischemic damage to heart and brain, or the generation of atherosclerotic plaques. The molecular action of PARP inhibitors, however, is not well understood. We therefore also aim to correlate the presence and absence of specific ADP-ribose modifications to the activity of different PARP inhibitors.

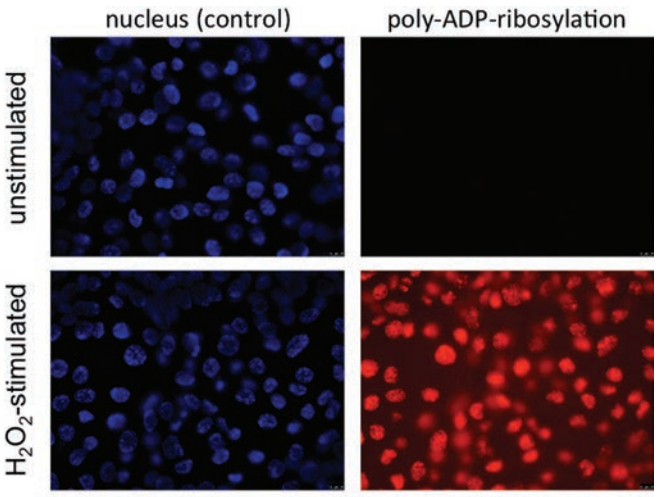
A further important aspect of our work is the development of new tools for analysing ADP-ribosylated proteins and the identification of genes regulated by ADP-ribosylation. In particular, we plan to use our technological innovations to define ADP-ribosyl modifications as specific biomarkers for stress conditions and employ our analytical tools to study the sensitivity of inflammatory and cancer cells to clinically used PARP inhibitors. The goal of these projects is to employ our newly developed tools to identify all ADP-ribosylated proteins in PARP inhibitor-sensitive and -insensitive cell lines, to identify important target proteins. This will help to further develop PARP inhibitors for cancer therapy.

A highly interesting translational aspect of our research is the role of ADP-ribosylation in obesity. To address this question, we studied the involvement of ARTD1 in adipocyte differentiation *in vitro* and *in vivo,* the latter by feeding mice with a high-fat diet. We found that ARTD1 is involved in the sustained expression of PPARγ2, the main driver of adipocyte differentiation and of PPARγ2 target genes at the later stages of adipogenesis, and promotes adipocyte function and differentiation *in vivo.* Similarly, treatment of mice with a pan-PARP inhibitor reduces the body weight gain, white adipose tissue content and cell size mediated by the high-fat diet, and reduces the increased expression of PPARγ2 in white adipose tissue of these mice. These studies uncovered yet another important (patho-) physiological function of ADP-ribosylation, which may be targeted in the future for controlling obesity and its associated complications.

Collaboration with the CABMM

In collaboration with the Competence Center for Applied Biology & Molecular Medicine (CABMM), hosted at our institute, we also address other translational aspects of our research. In our research investigating the molecular mechanisms by which ADP-ribosylation modulates inflammation, we often deplete cells of individual ARTD members by using non-viral delivery of silencing RNA using currently available transfection reagents. While this approach works well with most laboratory cell lines, primary cells isolated from tissues are more difficult to transfect, owing to their more complex extracellular makeup. This has also largely prevented the use of this strategy for potential therapeutic purposes. By using magnetic nanoparticles coated with the non-viral delivery agent polyethyleneimine combined with the application of a pulsed magnetic field, we were able to drastically reduce the time for efficient transfection, even in hard-to-transfect primary cells, making it possible to be potentially used *in vivo.*

The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) is another approach that has a high therapeutic potential for treating a variety of diseases. We found that ADP-ribosylation of the reprogramming factor Sox2 by ARTD1 plays an important role in the first days of the transduction with the reprogramming factors. Therefore, while inhibition of ADP-ribosylation is beneficial in inhibiting the growth of certain tumours, ADP-ribosylation promotes the generation of pluripotent stem cells, indicating that an exact understanding of the molecular pathways regulated by ADP-ribosylation is essential for the further development of pharmacotherapy targeting this PTM. Inflammatory signalling also contributes to low back pain caused by intervertebral disc (IVD) degeneration. Together with the CABMM, we found that break-down products of IVD extracellular matrix stimulate an inflammatory response in IVD cells similar to that in innate immune cells, which may therefore lead to further disc degeneration.



Stimulation of cells with the stress factor hydrogen peroxide leads to massive induction of poly-ADP-ribosylation (in red). Nuclei are stained blue

In conclusion, the transcription factor NF-κB is an important regulator of the innate immune response. It is becoming clear that NF-κB-mediated transcriptional activation is co-ordinated by the synergistic interaction of various co-factors and signalling networks. Histone modifications are being recognised as critical players in the orchestration of NF-κB functions. Therefore, an important goal of our research is to analyse the intricate, reciprocal regulation of histone modification and the NF-κB pathway. The long term goal of these studies is to understand the effect of nuclear and chromatin-associated ADP-ribosylation on the NF-κB function that is involved in acute or chronic inflammation and the subsequent progression to disease (such as cancer). These results will also consolidate the importance of NF-κB during inflammatory and metaflammatory stress signalling and may thereby generate new insights concerning the onset and development of diseases and disorders that will allow the development of better diagnostic, prognostic and therapeutic tools.

HORIZON 2020

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