Journal of Translational Autoimmunity 5 (2022) 100161

Contents lists available at ScienceDirect

Journal of Translational Autoimmunity

journal homepage: www.sciencedirect.com/journal/journal-of-translational-autoimmunity

Red blood cells in type 1 diabetes and multiple sclerosis and technologies to measure their emerging roles

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ARTICLE INFO

Keywords:
Red blood cells
Adenosine triphosphate
Nitric oxide
Autoimmune disease

ABSTRACT

Autoimmune diseases affect over 40 million people in the United States. The cause of most autoimmune diseases is unknown; therefore, most therapies focus on treating the symptoms. This review will focus on the autoimmune diseases type 1 diabetes (T1D) and multiple sclerosis (MS) and the emerging roles of red blood cells (RBCs) in the mechanisms and treatment of T1D and MS. An understanding of the role of the RBC in human health is increasing, especially with respect to its role in the regulation of vascular caliber and vessel dilation. The RBC is known to participate in the regulation of blood flow through the release of key signaling molecules, such as adenosine triphosphate (ATP) and the potent vasodilator nitric oxide (NO). However, while these RBC-derived molecules are known to be determinants of blood flow in vivo, disruptions in their concentrations in the circulation are often measured in common autoimmune diseases. Chemical and physical properties of the RBC may play a role in autoimmune disease onset, especially T1D and MS, and complications associated with downstream extracellular levels of ATP and NO. Finally, both ATP and NO are highly reactive molecules in the circulation. Coupled with the challenging matrix posed by the bloodstream, the measurement of these two species is difficult, thus prompting an appraisal of recent and novel methods to quantitatively determining these potential early indicators of immune response.

1. Introduction

Over the past few decades, much attention has been given to the roles of T-cells in immune function and autoimmune disease due to their function as a major determinant in immune response. The roles of other leukocytes in autoimmune disease have also been described, including B-cells [1–3] and macrophages [4–6]. Less discussed, with respect to autoimmune disease, is the role other circulatory cells may have in the onset of autoimmune disease. For example, many recent reports have discussed the potential role of the red blood cell (RBC) in autoimmune disease (Table 1). This review will focus on two specific autoimmune diseases, type 1 diabetes (T1D) and multiple sclerosis (MS), not necessarily for overall prevalence, but rather to exemplify the role of the RBC in each disease despite contrasting properties of the RBC. In the next few sections, we introduce the reader to important features of the RBC and how these specific features may play a potential role in T1D and MS.

2. A potential indirect role of the red blood cell in immune response

An adult human has, on average, ~5 L of whole blood in their body, yet less than 1% of this volume is comprised of white blood cells [62]. The remainder of the circulation is comprised of plasma (~55%) and RBCs, which account for nearly 40–45% of the total bloodstream volume. While prevalent in number, the RBC is not generally thought of as a major determinant in immune response, nor autoimmune disease. Perhaps one reason for a lack of attention given to the RBC with respect to autoimmune disease is its perception of being an unexciting cell with no nucleus or internal organelles. The lack of a nucleus and organelles provide additional space for hemoglobin, which is responsible for binding and releasing carbon dioxide and oxygen to demanding surrounding tissue [62,63]. Despite this key role in oxygen delivery to demanding tissues in vivo, the lack of a nucleus and organelles often render the RBC to be viewed as a “dead cell” even though the RBC membrane consists of a variety of receptors and transporters that are

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https://doi.org/10.1016/j.jtauto.2022.100161
Received 20 April 2022; Received in revised form 14 July 2022; Accepted 21 July 2022
Available online 7 August 2022

Table 1
Autoimmune Disease Characteristic Comparison. A table summarizing red blood cell, adenosine triphosphate (ATP), and nitric oxide (NO) characteristics in various autoimmune diseases.

Autoimmune Disease	Characteristics
Type 1 Diabetes	• Decreased RBC-derived ATP release [7–9] • Altered eNOS function and decreased NO release [8, 10–14] • Reduced RBC deformability [15–20]
Multiple Sclerosis	• Increased levels of NO [21–28] • Increased RBC-derived ATP release [26,29] • Debated RBC deformability [30–33]
Alzheimer's Disease	• Reduced RBC-derived ATP release [34–37] • eNOS deficiency and lower NO concentrations [38–43] • Decreased RBCs deformability [44–46]
Rheumatoid Arthritis	• Endothelial dysfunction and elevated NO levels [47–49] • Decreased RBC elasticity and deformability [50–53] • Increased ATP in plasma [54]
Systemic Lupus Erythematosus	• Decreased RBC deformability [55–57] • Increased NO levels [57–59] • Endothelial dysfunction and reduced endothelium-dependent vasodilation [60,61]

functionally active [64–69]. In fact, the RBC is known to release key molecules that are determinants in immune response, specifically, adenosine triphosphate (ATP) and nitric oxide (NO), and reports suggest that release levels of these molecules are often abnormal in autoimmune disease.

2.1. RBC-derived ATP

The RBC has been shown to release ATP in response to a variety of stimuli, such as shear stress, hypoxia, and hypercapnia [70,71]. Although ATP is a ubiquitous extracellular signaling molecule, the mechanism of its release from the RBC has not been fully resolved. However, the eventual release of ATP is thought to be channel-mediated because RBCs do not form vesicles [72]. RBCs express pannexin 1, a gap junction protein that forms a channel that is permeable to ATP and mechanosensitive in the non-junctional plasma membrane [73]. RBC-derived ATP release is reduced by the gap junction blocker carbenoxolone, which supports the role of pannexin 1 as a channel for ATP release [74].

Sprague has provided multiple reports on the pathway between stimulation (e.g., mechanical deformation, hypoxia, etc.) and the subsequent channel-mediated release of ATP from the RBC (Fig. 1). In many of these pathways, heterotrimeric G protein (G_s) activation prompts adenylyl cyclase (AC) to generate cyclical adenosine monophosphate (cAMP). The phosphorylation of cAMP results in the activation of phosphokinase A (PKA), which opens the cystic fibrosis transmembrane conductance regulator (CFTR) to import anions [69]. The anion influx results in ionic balance when pannexin 1 releases ATP into the bloodstream [69].

Multiple reports describe the link between such external stimuli as shear stress or hypoxia and the intracellular signaling pathway(s) that result in the release of ATP from the RBC [75–78]. Interestingly, it also appears that some of these external stimuli may be intrinsically related. For example, Rifkind et al. reported that the conformational changes of hemoglobin during unloading of oxygen due to hypoxia results in a deformation of the cell membrane through membrane-bound hemoglobin [79]. In this construct, hypoxia is considered an external stimulus for ATP release, but at its core, it is a change in membrane properties that trigger the intracellular signaling pathway. If one considers flow-induced shear stress on the RBC as a type of deformation, then any changes to RBC rigidity or deformability would be considered a determinant in the RBCs ability to release ATP. This concept of cell deformability and the RBCs role in autoimmune disease will be examined further in subsequent sections on T1D and MS.

2.2. RBC-derived ATP stimulation of NO production by eNOS

RBC-derived ATP binds to the endothelial cell P2Y receptor, which increases Ca^{2+} uptake into the endothelium (Fig. 1). This Ca^{2+} influx binds calmodulin and increases eNOS activity that converts L-arginine to L-citrulline with the production of NO as a byproduct [69,80–85]. The gaseous NO diffuses into the vascular smooth muscle cell, resulting in cyclic guanosine monophosphate (cGMP) production and downstream vessel dilation [80–82]. Numerous reports have confirmed the relationship between RBC-derived ATP release and endothelium-derived NO stimulation/vasodilation [7,86–90]. From a beneficial standpoint, the

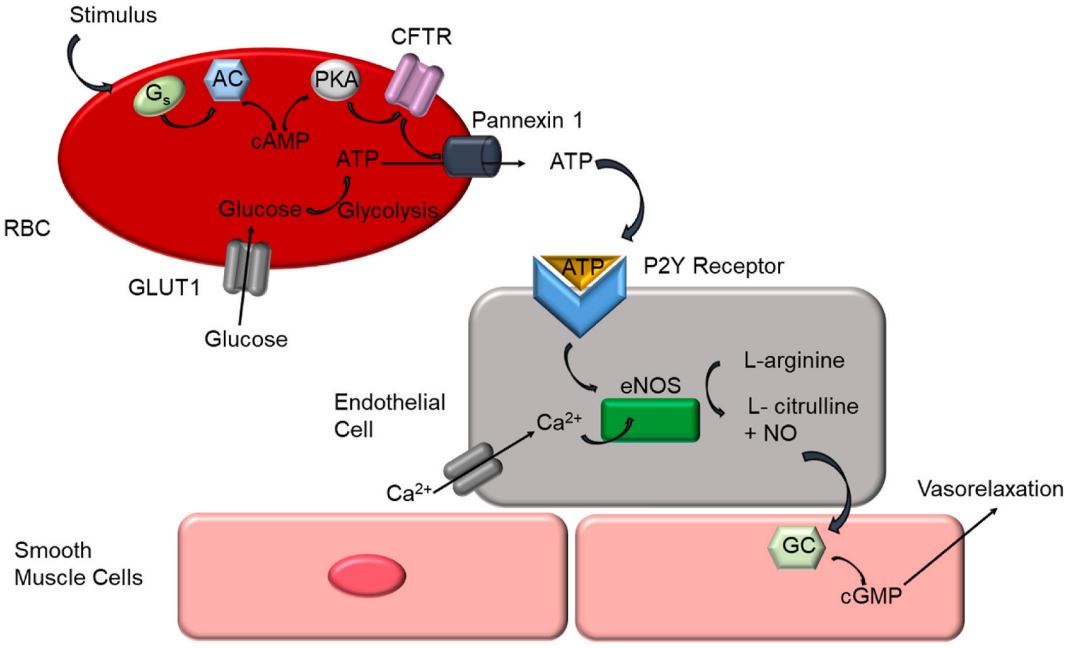

Fig. 1. Red Blood Cell-derived ATP Release. A proposed mechanism for the RBC release of ATP after stimulation. ATP binds to endothelial cells to increase NO production and subsequent vasorelaxation to improve blood flow.

NO produced by endothelial cells has vasoprotective and anti-atherosclerotic effects upon release from the cell and is a factor in blood pressure due to its effect on vessel relaxation [83]. Hemorheological abnormalities have been described for a variety of autoimmune diseases that may hinder microvascular blood flow [91,92].

As a stimulus of eNOS, ATP levels in vivo become important not only in the control of blood flow, but also in immune response. As eNOS plays a significant role in the vasculature, it is necessary to examine the role of eNOS in autoimmune disease, especially due to the increased risk of cardiovascular disease associated with a dysregulated inflammatory response [93]. An improved understanding of the role of RBC-derived ATP and eNOS in immune dysregulation will lead to a better understanding of autoimmune disease and its treatment. The remainder of this review will highlight studies that have examined the role of RBC-derived ATP and NO in T1D and MS. These two diseases were specifically chosen because in one case (T1D) the RBC-derived ATP levels (and downstream NO production) are reduced in comparison to healthy, non-T1D controls; in contrast, the release levels of ATP from the RBCs of people with MS are significantly increased in comparison to controls, as are the levels of NO and its metabolites.

3. Type 1 diabetes

3.1. RBC dysfunction in diabetes

Bakhtiari et al. compared levels of ATP/adenosine diphosphate (ADP) to NO in healthy individuals and people with diabetes, finding significantly lower levels of ATP and NO in the patient group [8]. Satoh et al. studied in situ NO and reactive oxygen species in rat glomeruli tissues through confocal laser-scanning microscopy, which demonstrated decreased NO production [10]. Endothelial cells exposed to chronically high glucose environments similarly exhibited reduced levels of NO release [11]. Low measurements of cGMP levels in patients with T1D corresponded with decreased levels of NO and RBC-derived ATP release [12].

RBCs obtained from people with diabetes are known to exhibit abnormal properties, including decreased deformability, increased membrane microviscosity, and increased aggregation [15–20,94–96]. Although the molecular basis of the reduced deformability and increased membrane microviscosity is not known, it is hypothesized that protein non-enzymatic glycation and antioxidative imbalance play a role [97,98]. Regardless of the mechanism, a reduction in deformability (that is, a more rigid or stiffened cell) would likely lead to a reduction in ATP release and this has been demonstrated in multiple works [7,16,70]. Richard et al. focused on low oxygen induced ATP release [99–101]. Importantly, studies have demonstrated that basal levels of RBC-derived ATP release are lower in patients with T1D compared to healthy donors as shown in Fig. 2 [7–9].

In addition to its role in the regulation of blood flow and blood pressure, the reduction in ATP release from the RBC may have multiple downstream adverse effects with respect to early immune response, especially with respect to ATP-induced Ca^{2+} trafficking into the cell. For example, distribution of such immune cells requires blood flow and adherence of cells to sites of infection and/or injury [102–104]. As discussed in sections 2.1 and 2.2, it is well established that ATP release from the RBC results in endothelium-derived production of NO. The NO is catalyzed by eNOS, which is activated by Ca^{2+}-calmodulin binding; thus, an increase in Ca^{2+} into the endothelial cells is required for eNOS stimulation. A decrease in ATP-stimulated NO production would result in reduced NO-induced vessel dilation and blood flow, which may hinder distribution of immune cells in the circulation to infection sites.

Furthermore, a key determinant in migration of such cells as neutrophils and macrophages, once adhered to a surface, is cellular polarization [105,106]. The polarization often is dependent on Ca^{2+} trafficking through the cell membrane [107–109]. Much of the Ca^{2+} trafficking is dependent upon ATP binding to P2X purinergic receptors on the cell membrane. A reduction in RBC-derived ATP release may contribute to reduced migration of immune cells reported in people with T1D.

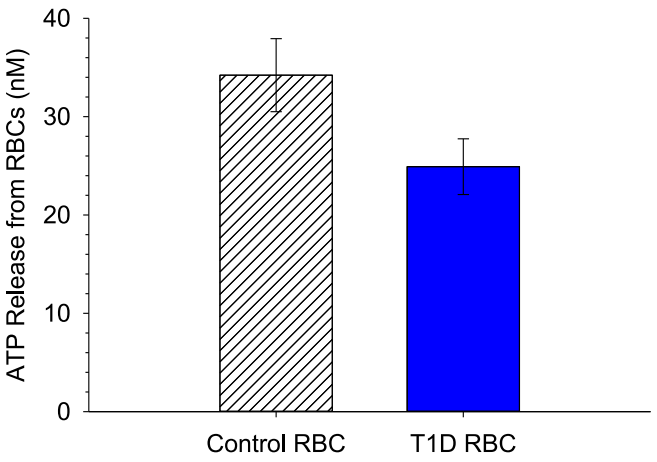

Fig. 2. RBC-derived ATP Release in Type 1 Diabetes. The ATP release (in nM) from RBCs collected from healthy donors and patients with type 1 diabetes (T1D) in albumin containing conditions (non-flow system). RBCs from patients with T1D release significantly less ATP than the healthy control at basal levels. ($n \geq 7$, error = SEM, $p < 0.05$). Figure adapted from Geiger et al. © Scientific Reports.

The reduced RBC-derived ATP measured in people with T1D may also be a determinant in pathogen killing post-migration. While many researchers acknowledge that eNOS is activated through shear stress, acetylcholine, adiponectin, and bradykinin, few have investigated ATP release from the RBC as a potential stimulus of eNOS activity [110–113]. If ATP is a key stimulus for NO production in other cell types besides the endothelium, then one would expect a reduction in pathogen killing in certain cell types after phagocytosis as it is established that NO and its metabolites are key in destruction of pathogens [114–116] once the pathogens are engulfed by a macrophage or neutrophil.

3.2. T1D clinical studies

Initial studies involving animal models of T1D have shown reduced levels of endothelium-dependent vasorelaxation, and upon returning vasorelaxation to normal levels, neuropathy was prevented [117–119]. Other studies focused on the ability of C-peptide to improve RBC deformability and increase RBC-derived ATP release [80,86,120]. C-peptide, the 31-amino acid peptide co-secreted with insulin from pancreatic β-cells, is lacking in patients with T1D, therefore, representing a potential therapeutic option alongside administration of exogenous insulin.

Small scale clinical studies have measured an increase in NO production by providing C-peptide to patients with T1D [121,122]. Johansson et al. demonstrated that forearm blood flow was improved by NO [122]. It has been noted that patients with T1D have lower forearm blood flow than healthy controls, both at rest and during exercise, prior to the appearance of late diabetic complications [123,124]. Measuring forearm blood flow while administering C-peptide with or without NOS blocked confirmed that C-peptide can increase NO levels [122]. This finding has a significant role in the potential for therapeutic development to improve vascular complications in patients with T1D.

When blood glucose is chronically elevated, as occurs in T1D, eNOS functions as a source of superoxide rather than NO, possibly due to reduced L-arginine and the cofactor tetrahydrobiopterin (BH4) levels [13]. Therefore, studies have aimed to modify the function of eNOS to shift the ratio from superoxide to NO production. One study tested the effectiveness of salsalate suppression on inhibitory kappa B kinase beta

(IKKβ), an upstream mediator in endothelial cell transcription nuclear factor kappa B signaling pathway, along with L-arginine/BH4 oral supplements (that were previously shown to increase NO and vasodilation in a mouse model) [13,125]. After a 16-week trial with the aforementioned combination, an improvement in vasodilation and eNOS expression was observed [13]. However, previous studies from other groups report conflicting effects of L-arginine, with some studies reporting an increase in NO production and others showing an abolishment of endothelial cell activation [126,127]. Due to the inconsistency of results, a larger sample set with a long-term diet set-up would be needed to determine the effect of L-arginine treatment [13]. However, it should also be noted that use of cardiovascular drugs and other pharmacological agents that are not specific to eNOS may result in off-target effects [14].

One goal of the research by Sharma et al. was to find a highly specific manner to increase NO production through eNOS [14]. Caveolin-1 (Cav-1) is an anchoring protein within the endothelial cell's plasma membrane caveolae [128]. Cav-1 anchors eNOS in the plasma membrane to reduce its ability to generate NO, while increased Ca^{2+} levels result in the translocation of eNOS to the cytosol, where it is fully activated [128–130]. CavNOxin, a Cav-1-derived peptide, has been shown to specifically increase eNOS activity and reduce diabetic atherosclerosis, a cardiovascular disease, by 84% [14]. A large benefit of CavNOxin is that it preserves Cav-1's natural biology, making it a potential candidate for pharmaceutical treatment [14]. Although the study of CavNOxin appeared promising, there were potential issues which need to be considered for the experiment. Specifically, the ability of CavNOxin to inhibit Cav-1 and upregulate eNOS activity efficiency, which is important to understand the cross-talk signaling, was not studied [131]. Also, the possibility that CavNOxin could result in hyperactivated eNOS and subsequent cardiovascular side effects was not considered [131].

4. Multiple sclerosis

4.1. RBC dysfunction in MS

Numerous groups have reported that patients with MS have increased levels of NO or NO metabolites in plasma or cerebral spinal fluid [21–25]. RBCs from patients with MS release higher concentrations of ATP than RBCs obtained from non-MS healthy donors (Fig. 3). This increased ATP release would subsequently increase the endothelial NO production [29]. An increase in NO concentration disrupts the blood brain barrier (BBB) and neuronal injury results [21,22,132]. More specifically, the high levels of NO impair oligodendrocyte DNA, leading to demyelination and axonal degeneration [133]. Increased NO concentrations in MS have a diagnostic biomarker potential that would reduce time for diagnosis [134].

In addition to ATP stimulation of NO, ATP itself has also been reported to be toxic to oligodendrocyte formation [135]. In this construct, a reduction in myelin-producing oligodendrocytes would be expected to lead to a compromised BBB layer. In support of this work and its association with myelin formation, it has been shown that in P2X7 knockout mice who are given reagents to induce autoimmune encephalomyelitis (EAE, an MS mimicking condition), the BBB is compromised, but the mice didn't become sick [135,136]. With the P2X7 being an ATP-binding purinergic receptor, these results suggest a role for ATP in the onset of MS.

There may be other roles for abnormal levels of RBC-derived ATP in MS. Recently, it was reported that increased levels of ATP affect NETosis, a type of regulated cell death involving neutrophil traps [137,138]. The NETosis pathways have been implicated in both inflammation and BBB integrity [139–141].

One of many questions remaining in the role of the RBC in MS involves the mechanism by which extracellular ATP levels are increased in comparison to controls. Though debated [30], one area of interest is the MS RBC overall deformability. While Pollock et al. did not find

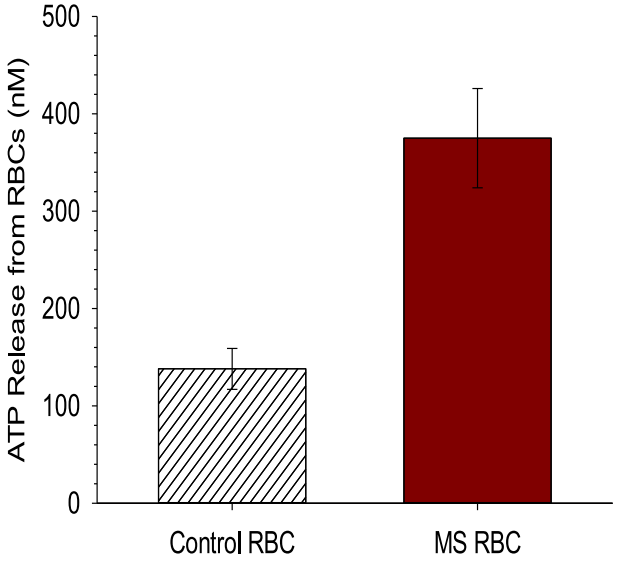

Fig. 3. RBC-derived ATP Release from RBCs in a Flow-based System. The average ATP release from RBCs was higher for RBCs collected from patients with multiple sclerosis than those collected from healthy donors. (n = 11 controls, n = 18 MS, error = SEM, $p < 0.001$). Figure adapted from Letourneau et al. © Analytical Bioanalytical Chemistry.

deformability abnormality in RBCs of patients with MS, others have reported reduced deformability [31–33]. Lower RBC deformability in MS patients compared to healthy controls was surprising because decreased deformability has been shown to decrease ATP and subsequent NO (in which MS patients demonstrate increased levels). Patients in the studies by Simpson et al. were receiving steroids, which decreases deformability [26,32]. The decrease in deformability in Janes' studies could be due to the variation in RBC diameter between MS and control RBCs. RBCs of patients with MS have been reported to have a larger diameter than those of healthy donors [142,143]. Since the author used a 5 μm membrane, the larger MS RBCs would have more difficulty passing through than the control RBCs [33]. The author stated that less cells would pass through the membrane in the experimental time frame, thus, skewing the data.

It is also interesting to note that RBCs from patients with MS have significantly more C-peptide and Zn^{2+} binding than RBCs from healthy controls [134,144]. This increase in C-peptide and Zn^{2+} binding by RBCs could explain the elevated RBC-derived ATP release and subsequent endothelial NO production in patients with MS based on prior studies showing C-peptide and Zn^{2+} increasing RBC-derived ATP release [86,99,100,145,146].

4.2. MS treatment

Therapeutic interventions targeting the CNS are often blocked by the BBB, making it difficult to find an effective treatment for neurological conditions such as MS [147]. Another difficulty with MS treatment is that NO effects depend upon the NOS isoform location and kinetics [148]. Stojanovic et al. demonstrated that patients treated with interferon beta (IFN-β-1b) had significantly lower NO plasma concentrations than controls [27]. A 3-year clinical trial, focusing solely on IFN-β, demonstrated that IFN-β-1a reduced serum nitrite levels by 77%, while IFN-β-1b reduced serum nitrite levels by 71% [149]. Another study, which focused on serum nitrogen species in patients with MS, compared NO levels between two treatment groups [150]. The first group received IFN-β-1a and IFN-β-1b, while the second group received natalizumab and fingolimod. After at least 6 months of treatment, subjects in the first group had significantly higher NO levels than those in group two and

controls, signifying that natalizumab and fingolimod were more successful in lowering NO in this study. These MS treatments focus on the increased NO levels present in a patient with MS, however, the mechanisms of action are not understood and additional studies are needed on why NO levels are increased in the first place.

While the pharmaceutical trials aimed to reduce NO levels as a potential MS treatment, complications can result if NO levels are reduced below normal concentrations. Previous research in autoimmune encephalomyelitis (mimics MS in an animal model) mice have found severe demyelination after 15 days in the eNOS deficient mice compared to the wild-type [148]. The wild-type mice remyelination was complete by day 40, whereas demyelination remained in large areas in the eNOS deficient mice [148]. This indicates that while excess NO may result in BBB damage, the molecule cannot be completely removed due to its neuroprotective actions [151].

5. Technologies used to measure ATP and NO

The discussion above makes it clear that the role and implications of RBC-derived small molecules in T1D and MS are significant areas of study. A key part of such studies is the measurement of ATP and NO in various matrices, with the detection event being utilized as a diagnostic tool or to help determine the mechanisms for onset of these autoimmune diseases. These measurements have a few characteristics that must be considered. First, both ATP and NO are highly reactive molecules. ATP, with its multiple phosphate groups, can undergo hydrolysis to form ADP or AMP [152]. NO is a relatively unstable molecule. As a free radical, it is readily oxidized into nitrate by oxygen and water, two essential components of any biological system. In blood, NO can also be oxidized by oxyhemoglobin [153]. Hunter et al. compared NO concentrations in various physiological matrices and showed that the measured amount of NO in blood (via amperometry) was significantly decreased compared to measuring the same concentration in physiological buffer or even plasma and serum [153]. RBCs are a challenging matrix, even when separated from whole blood. In common detection methods, RBCs can cause interference and signal attenuation [154]. Therefore, RBCs are often diluted to low hematocrits for analysis [76,155], or standard addition is used to account for the matrix effect [156,157]. When using standard addition, care must be exercised to maintain constant hematocrit values across standards since ATP concentrations from RBCs trend with hematocrit and the matrix can have an impact on the signal [158].

5.1. ATP detection

One of the earliest manuscripts discussing ATP levels in blood was published in 1951 by Harry Albaum et al. who quantified the ATP proportion of adenine nucleotides with several enzymatic reactions by measuring changes in density of each reaction [159]. In this publication, they noted hydrolysis of ATP can be an issue depending on the storage method. To avoid this issue, ATP can be extracted from RBCs, and then analyzed using high-performance liquid chromatography (HPLC) with absorbance detection [160]. In the 1960s, the luciferin/luciferase assay began being used to quantify ATP in RBCs [161]. In this commonly used assay, the luciferase enzyme catalyzes a bioluminescent reaction with its substrate luciferin and ATP to produce oxyluciferin at an excited state [152]. As oxyluciferin relaxes to its ground state, yellow-green light is produced with a maximum emission between 550 and 570 nm, which can easily be detected with a photomultiplier tube (PMT). The reaction is selective for ATP, highly sensitive, and requires a relatively simple experimental setup. Although the signal from the bioluminescent assay can be attenuated by high hematocrits, this method accommodates RBC suspensions, unlike chromatography, mass spectroscopy, or other extraction methods. A standard addition curve can be used to accounts for the matrix effects allowing direct measurement of extracellular ATP from RBCs [156,157,162].

Novel methods of ATP detection from blood are still being published, for example, by anion exchange chromatography [163], LC/MS [164], HPLC with absorbance detection [165], and a florescent probe for ATP using a fluorescein amidite (FAM)-DNA/nanoceria complex [166]. Nishiyama et al. recently published an electrochemical detection scheme based on enzymatic reactions [167]. ATP extracted from whole blood was cycled into pyruvate by adenylate kinase and pyruvate kinase. Pyruvate oxidase led to the production of hydrogen peroxide, which was electrochemically detected on a Prussian blue-modified screen-printed electrode.

While there have been some alternate detection methods for ATP, the luciferin/luciferase reaction is by far the most common technique for measuring ATP levels from RBCs. Traditional methods of mixing the luciferin-luciferase reagent with a blood sample and measuring the resulting light in a luminometer is still being used for routine measurements [8,155,168,169]. Pierce et al. adapted a commercially available ATP luminometer to be used as a point-of-care testing for blood ATP levels [170]. Montalbetti et al. used a luciferase fusion protein, proA-luc, to detect ATP changes in the “nanoenvironment” of the surface of RBCs [171]. The Spence lab created a microfluidic polydimethylsiloxane (PDMS) device that allowed extracellular ATP to diffuse through a polycarbonate membrane into a well as the RBCs flowed through a channel underneath [172]. Luciferin/luciferase reagent was added to each well and the device was added to a spectrophotometer for measuring the chemiluminescence.

The microfluidic community has published many approaches for directly detecting ATP in flowing RBCs since the small channels or capillary tubing can be used to mimic the size and shape of small vessels and capillaries in vivo [158,173,174]. Mixing can occur in microbore/capillary tubing (with mixing Ts) [158,175] and “on-chip” in devices fabricated from PDMS/glass [70,176] or 3D-printed material [157]. In either case, light produced from the bioluminescent reaction is detected with a PMT that the tubing or chip is placed over. This allows online detection of RBCs as they flow through the biomimetic capillary/channel. Early work by Sprague et al. showed ATP release in response to mechanical deformation as RBCs were passed through a polycarbonate filter [177,178]. Similarly, Edwards et al. showed the shear stress-induced ATP release from RBCs flowing through capillaries with decreasing internal diameters, with Price et al. transferring this work to microchip-based PDMS channels that uniformly narrowed [158,173]. As shown in Fig. 4A, a microchip-based hydrodynamic approach was used by Moehlenbrock and co-workers to deform a stream of RBCs and integrate on-chip mixing with a luciferin/luciferase solution for chemiluminescence detection [78]. The amount of deformation could be changed by varying the focusing flow rate and the system was validated with inhibition studies involving diamide (stiffens RBC cell membrane) and glibenclamide (inhibits CFTR). Wan et al. investigated the mechanisms of the release by showing ATP release downstream from a constricting channel was dependent on the length and width of the channel [75]. ATP release by RBCs continues to be investigated by determining the impact of drugs on RBC deformability or diseased RBCs [179,180]. The Stone lab has even gone so far as imaging single RBCs as they move through capillary-sized channels [75,176]. In these experiments, a solution of RBCs in a luciferin/luciferase solution were pumped through a constricted channel. A microscope was used to image the deformation of RBCs flowing through the constriction, and a downstream detector was used to count photons (from the reaction of ATP with luciferin/luciferase) to determine the dynamics of ATP release from RBCs [75].

5.2. NO detection

Measuring NO in the blood is a relatively recent endeavor. As previously stated, 1987 was a revolutionary year for NO research, as nitric oxide was defined as the vasodilator released by endothelium [181,182]. A decade later, publications began discussing NO release by RBCs. This finding, along with studies to determine the actual source of NO, caused controversies that would last many more years. It was a “radical

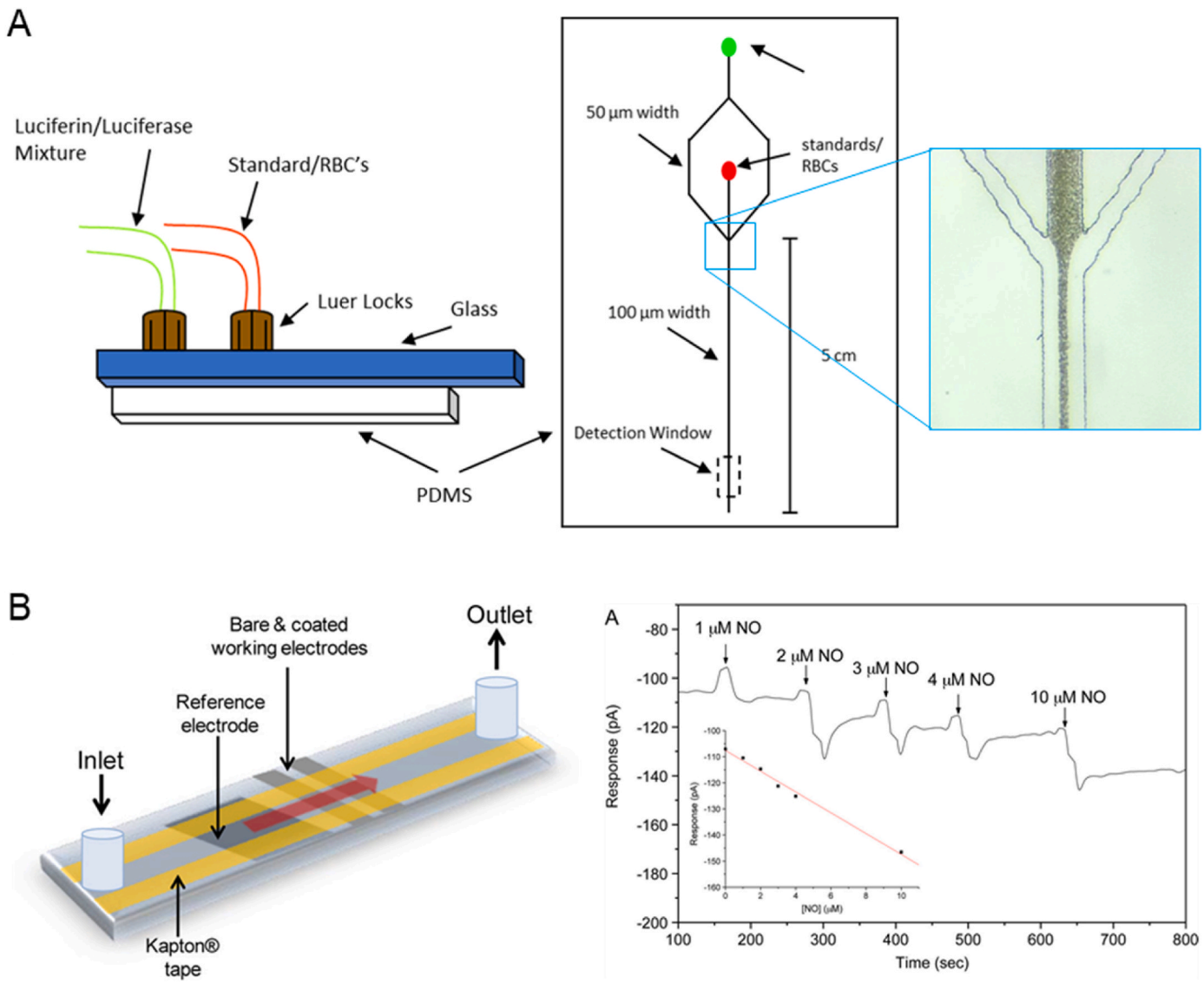

Fig. 4. RBC-derived ATP and NO Detection. A) Use of hydrodynamic focusing to deform RBCs, with resulting ATP mixing with a luciferin/luciferase solution, and the subsequent light being detected from a PMT that the device is placed over. The flow rate of the luciferin/luciferase flow streams could be varied to change the amount of deformation. Taken from Moehlenbrock et al. © Analyst with permission. B) Microfluidic amperometric sensor for NO detection is fabricated with a platinum working electrode coated with a xerogel membrane and Ag/AgCl reference electrode on glass substrate. Taken from Hunter et al. © Analytical Chemistry with permission.

theory" (in the words of Wallis in his 2005 review on NO and blood [183]) that RBCs could produce or release NO, and yet, nitric oxide synthases were found to be present and active in RBCs [184–186]. NO is a difficult analyte to measure in blood. The molecule has a fast diffusion rate, high reactivity, short half-life, and exists in low basal quantities [154]. Because of the difficulty in measuring NO in isolated RBCs, many labs have opted to study NO content in blood plasma, serum, or lysed RBCs.

Spectroscopic detection is one of the most common ways to measure nitric oxide levels. The Griess assay is a colorimetric reaction that has been used as an indirect method to quantify NO by reacting with its oxidative product, nitrite, with sulfanilamide and N-(1-naphthyl)ethylenediamine, to form an azo dye that absorbs light around 540 nm [154]. Detection limits (~0.5 µM) are relatively high compared to other methods [154]. RBCs can make optical detection difficult, as they can scatter and absorb light. One way to circumvent this issue is to analyze plasma or lysed RBCs. An automatic centrifuge-pneumatic disk was designed to centrifuge blood and analyze plasma levels of NO via the Griess assay [187]. Others have tried adapting the chemiluminescent reaction between NO and ozone for quantitation. With this method, NO gas reacts with ozone to form an excited state of nitrogen dioxide, which emits light above 600 nm as it relaxes to the ground state. The intensity of light is proportional to the concentration of gaseous NO in the sample [188]. This method is highly selective but requires the NO to be in gaseous form (an inert gas, such as nitrogen or argon, is often used to purge NO from the sample and carry the analyte to the reaction chamber) [153]. Some high protein biological solutions (i.e. media, blood) may present an issue to this detection method as the carrier gas can cause foaming of the solution. Rogers used triiodide to reduce NO metabolites in lysed RBCs back into NO, while Nagababu used ascorbic acid to convert nitrite in blood plasma back to NO [189,190].

Fluorescent probes are a popular way to measure NO. Probes have been used to label extracellular and intracellular NO to form a fluorescent product. In the first case, the probe can be mixed directly with the blood sample but to decrease the interference of the RBC, the blood is typically centrifuged before analysis. Fluorescently tagged NO can be separated by HPLC [191], microchip capillary electrophoresis [192], or detected directly [193]. Intracellular NO content of intact RBCs can be

imaged using the DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) dye [194,195]. The use of DAF-FM for intracellular measurements makes standardization difficult and relative values of NO are usually reported. In 2010, the Spence lab designed a microfluidic device to quantify NO with DAF-FM in a non-traditional way. They flowed a 7% RBC solution through a channel and extracellular NO diffused into a well filled with DAF-FM [196]. They were able to quantify NO release from both normoxic and hypoxic RBCs by placing the device in a conventional spectrophotometer for readout, showing the stimulated NO release by RBCs in response to low oxygen conditions. A limitation of fluorescent probes is the reaction time is typically on the tens of minutes time scale and, therefore, not capable of real-time analysis.

Amperometric detection of NO is well-established, with advantages including real-time detection, the use of coatings to impart selectivity, and quantitation being possible with low limits of detection (can be nM to pM) [154]. The redox active NO can be detected by oxidation or reduction, but oxygen is an interference at the potentials needed for reduction [154]. For this reason, many sensors employ electrochemical oxidation at potentials between +0.7 and 0.9 V (vs. Ag/AgCl), with NO undergoing a 3-electron oxidation to nitrate [197]. Electrodes are commercially available or can be fabricated in-house. Working electrodes are often coated with a membrane for selectivity, with gaseous NO diffusing through the membrane (and interferences not being transported) and oxidation of NO occurring at the electrode surface. This eliminates other potential interferences that can be oxidized at these potentials including nitrite. Another helpful electrode modification is applying a catalyst to the electrode surface. For NO detection, addition of a platinum black layer to an electrode can be used to increase the surface area of the working electrode, improve the electrode stability, enable faster electron-transfer kinetics, and decrease the oxidation potential [197].

Amperometric detection is often the method of choice for in vivo detection of NO [198,199], with the sensors being robust enough to measure NO in the complex physiological environment of the blood stream and surrounding endothelium [200]. In 1993, in vitro amperometric detection was used to quantify NO in whole blood and in platelets [201]. The Schoenfisch lab has developed a microfluidic device with integrated electrodes that can be used to measure the amount of NO in 250 μL of whole blood, which they use to study upregulated NO levels in infected burns (see Fig. 4B) [202,203]. Carvalho et al. used a platinum electrode with a gas permeable membrane as a NO sensor to study RBC suspensions [204]. Basal levels of NO were below their limit of detection, but they were able to measure increased levels after stimulation with L-arginine. Selimovic et al. developed an amperometric detector for measuring NO from flowing RBCs, with a device made of PDMS and polystyrene with integrated gold and carbon electrodes [205]. Two parallel channels allowed RBC-derived NO to diffuse from the cell channel to the collector channel where the Nafion coated electrode was isolated from the flowing RBCs. They used the microfluidic device to show an increase of NO release in hypoxic conditions and that the NO levels from hypoxic cells could be reduced using the nitric oxide synthase inhibitor, L-N-nitroarginine methyl ester (L-NAME).

While there have been reports of electrochemically measuring NO release from RBCs, much more work has been done measuring NO that is released from the endothelium upon stimulation by RBC-derived ATP. In an effort to mimic blood vessels, in 2004, Spence et al. cultured endothelial cells directly on a microchip with an integrated electrode to measure endothelial cell-derived NO [206]. Plugs of ATP stimulated endothelial release of NO, which was detected on-chip by a Nafion-coated carbon ink electrode. Building upon work that showed the sensitivity of NO detection in PDMS/polystyrene microchip devices could be increased by using array electrodes modified with platinum black [207], Townsend et al. utilized a parallel channel design that cultured endothelial cells in one part of the device, with RBCs being pumped over the endothelial monolayer to truly mimic blood flowing through a vessel [89]. They were able to show that RBC-derived ATP could be used to stimulate NO release from the endothelial cells and that upregulation of ATP release from RBCs likewise increased endothelial NO production.

5.3. Simultaneous ATP and NO detection

The biological interdependence of ATP and NO and their importance in the autoimmune diseases described above leads to an opportunity for the development of methods that can measure both analytes in RBCs in close-to-real-time. We feel that future work in this field can utilize a 3D-printed multi-modal detector that simultaneously detects NO via amperometry and ATP with the luciferin/luciferase bioluminescent assay. Building on previously published work that was used for ATP and norepinephrine detection [208], standard addition can be used to identify the levels of both ATP and NO released by RBCs close to real-time. Fig. 5 shows preliminary data from our labs where this type of device can be used to measure both analytes, with successive injections of 7% RBCs yielding peaks for both nitric oxide and ATP that can be correlated to concentration in the flowing RBC stream. Future work will involve using this device to further understand the interplay of ATP and NO in autoimmune diseases such as MS.

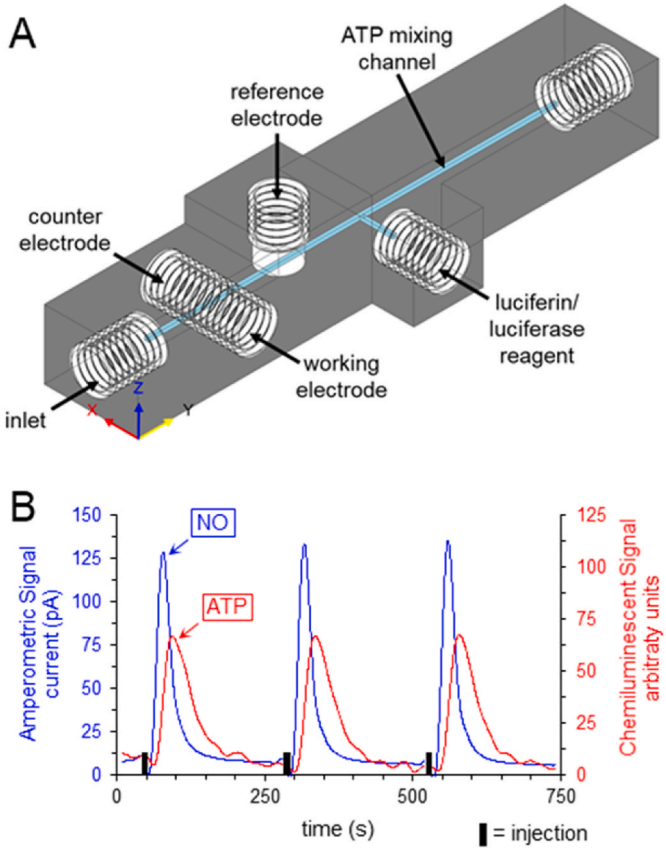

Fig. 5. A 3D-printed Multi-modal Detector Capable of Simultaneously Detecting NO and ATP. A) CAD rendering of the device (taken from Hayter et al. © Analytical Methods with permission). NO is detected via amperometry at a Pt-black/gold microelectrode that is threaded into the device (as are the counter and reference electrodes). Downstream, any ATP mixes with a luciferin-luciferase solution (in the ATP mixing channel) and the resulting light is detected by a PMT underneath the transparent device. B) Sample data of injections of 7% hematocrit RBCs using the device, with NO traces in blue and ATP in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

6. Conclusion

Although this review aimed to provide a comprehensive synopsis of research examining the role of RBC-derived ATP and NO in T1D and MS, the studies of ATP and NO dysfunction extend beyond what has been discussed in this review [209–211]. Moreover, RBC-derived ATP release and eNOS/NO dysfunction should be considered in the pathophysiology of diseases beyond those affecting the immune system. Several studies have analyzed eNOS in non-autoimmune diseases such as sepsis, migraines, and cerebrovascular diseases [212–220]. In conclusion, while detecting and maintaining RBC-derived ATP release may not be an end-all solution for autoimmune diseases, studies further examining the role of RBC-derived ATP and NO in autoimmune diseases could lead to more, and better, therapeutic choices for the growing number of affected individuals.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dana Spence reports a relationship with LifeBlood that includes: equity or stocks.

Acknowledgments

Financial support for this project is from the National Institutes of Health (R01 NS105888).

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