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Progressing from Recurring Tissue Injury to Genomic Instability: A New Mechanism of Neutrophil Pathogenesis

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Aberrant neutrophil (PMN) infiltration of the intestinal mucosa is a hallmark of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis. While the genotoxic function of PMNs and its implications in carcinogenesis have been primarily associated with oxidative stress, recent work by Butin-Israeli and colleagues has defined a novel mechanism where PMN-derived microparticles through the delivery and activity of specific miRNAs promoted formation of double-strand breaks (DSBs), and in parallel, suppressed DSB repair through the downregulation of lamin B1 and Rad51. Respective downregulation of these two proteins compromised the nuclear envelope and high-fidelity repair by homologous recombination, increasing DSB accumulation and aneuploidy. This discovery defined a novel mode of action where PMN-mediated suppression of DSB repair leading to genomic instability in the injured mucosa may facilitate progression toward colorectal cancer.

Keywords: neutrophils, wound healing, microRNAs, inflammation, carcinogenesis, IBD

Introduction

Inflammatory bowel diseases (IBDs), encompassing ulcerative colitis and Crohn's disease, are characterized by recurring episodes of inflammation and tissue injury (Rieder *et al.*, 2007; Matricon *et al.*, 2010). Such relapsing inflammation is associated with dysregulated neutrophil (PMN) infiltration of the intestinal mucosa, and is regarded as a pathological feature and a hallmark of IBDs (Cho, 2008; Wera *et al.*, 2016). Aberrant immune response triggered by PMN accumulation in gut mucosa can adversely affect epithelial barrier integrity, enable translocation of microbes into the interstitium, perpetuate inflammatory response, and promote tissue damage which compromise gut function (Cho, 2008; Sekirov *et al.*, 2010; Weber *et al.*, 2014; Butin-Israeli *et al.*, 2016; Slater *et al.*, 2017). As such, high PMN blood count (Torun *et al.*, 2012; Nishida *et al.*, 2017) and accumulation of PMNs in stool of IBD patients (Silberer *et al.*, 2005; Langhorst *et al.*, 2008) are correlated with active flares and disease severity.

In addition, intestinal inflammation and recurring tissue injury have been shown to predispose IBD patients to gastrointestinal malignancies (Canavan *et al.*, 2006). Supporting this, many correlative studies have documented the increased risk of IBD patients to develop small bowel adenocarcinoma (Bojesen *et al.*, 2017), cholangiocarcinoma (Huai *et al.*, 2014), gastric cancer (Nissen *et al.*, 2016), intestine-associated non-Hodgkin's lymphoma (Farrell *et al.*, 2000), and colorectal cancer (CRC) (Ekbom *et al.*, 1990; Lakatos *et al.*, 2006; Choi *et al.*, 2016). Comparative analyses of different patient cohorts also reveal that the risk of CRC development is increased by sixfold in IBD patients (Herrinton *et al.*, 2012; Ording *et al.*, 2013). Indeed, CRC metastasis accounts for 10–15% of all IBD-related mortalities (Jensen *et al.*, 2006; Ording *et al.*, 2013; Althumairi *et al.*, 2016).

A particular subset of CRC arising on the background of IBD, known as colitis-associated cancer (CAC) (Grivennikov, 2013; Francescone *et al.*, 2015), is strictly associated with PMN accumulation in the gut and is likely driven by PMN-mediated exacerbated inflammation (Shang *et al.*, 2012). A high neutrophil-to-lymphocyte ratio in the systemic circulation of CRC or CAC patients is predictive of poor clinical outcomes and shorter progression-free survival (Shibutani *et al.*, 2013; Ozdemir *et al.*, 2014; Haram *et al.*, 2017; Kim *et al.*, 2019). As a result, there have been tremendous efforts to control aberrant immune activation through immunosuppression and the use of TNFα and interleukin monoclonal antibodies (Zenlea and Peppercorn, 2014; Feagan *et al.*, 2016; Adegbola *et al.*, 2018). Nonetheless, these treatments exert severe side-effects and suppress systemic immune response necessary for the elimination of invading pathogens (Stallmach *et al.*, 2010). To develop effective targeted therapy for IBDs and prevent CRC, it is important to gain a better understanding of mechanisms

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underlying PMN-driven tissue injury as well as the beneficial functions of PMNs in intestinal homeostasis.

In this review, we summarize our recent discovery of the novel mechanism that links neutrophil infiltration and genomic instability, and will discuss the long-term implications of this biological process in tissue homeostasis and carcinogenesis. Insights into how neutrophil infiltration and genomic instability are mechanistically connected in the context of intestinal inflammation will pave the way for new therapeutic options to alleviate IBD symptoms and prevent progression toward CRC/CAC.

Neutrophils, Reactive Oxygen Species Production, and Induction of Oxidative Stress: A Classical View

PMN-induced exacerbated inflammation is a hallmark of many inflammatory conditions (Wright *et al.*, 2010; Delgado-Rizo *et al.*, 2017), including but not limited to acute lung injury (Zhou *et al.*, 2012), atherosclerosis (Baetta and Corsini, 2010), chronic obstructive pulmonary disease (COPD) (Hoenderdos and Condliffe, 2013), and IBD (Rieder *et al.*, 2007; Cho, 2008; Matricon *et al.*, 2010; Wera *et al.*, 2016). Upon tissue transmigration and detection of invading pathogens, PMNs undergo oxidative burst and degranulation (Nguyen *et al.*, 2017). The tissue-damaging effects of PMNs are primarily attributed to their capacity to generate high levels of reactive oxygen species (ROS). Accumulation of ROS in tissues gradually increases intracellular levels of hydrogen peroxide and superoxides, which oxidize and generate modified DNA bases (Cadet and Wagner, 2013). For example, activated PMNs have been shown to induce ROS-mediated DNA damage in lung and respiratory tract tissue in the context of acute lung injury (Kellner *et al.*, 2017) and COPD (Boukhenouna *et al.*, 2018).

One of the nucleotides that is primarily oxidized and modified by ROS is 8-oxoguanine (8-oxoG), whose presence markedly contributes to G/A nucleotide mismatch and DNA replication errors (Cadet and Wagner, 2013; Cadet *et al.*, 2017). In addition, ROS-mediated base modification or direct alkylation of the DNA sugar backbone generate DNA lesions that stall replication fork and form single-strand breaks (SSBs) (Eccles *et al.*, 2011; Cadet *et al.*, 2017), which are gradually converted to double-strand breaks (DSBs) if not repaired in a timely manner (Mehta and Haber, 2014; Cannan and Pederson, 2016). On this basis, these ROS-dependent genotoxic stressors can contribute to mutagenesis and malignancy transformation.

There is a long-standing doctrine that neutrophils contribute to DNA damage solely through ROS-dependent mechanisms. However, we recently expanded this dogma by identifying a novel, ROS-independent mechanism, whereby PMNs deliver microparticles (MPs) that transport regulatory miRNAs to promote the formation of DSBs in inflamed epithelial cells (Butin-Israeli *et al.*, 2019). We found that coculture of PMNs with cultured colon epithelial cells, HCT116 and Caco2, or with primary patient-derived colonoids induced the formation of γH2AX foci, indicating DSBs, as well as led to activation of downstream DNA damage response. Intriguingly, PMN-MPs did not increase 8-oxoG levels in epithelial cells and were proved to be non-responsive to ROS inhibition by several well-characterized ROS scavengers. These findings implicate PMNs in causing DSBs through mechanisms distinct from the known induction of ROS-mediated oxidative stress.

PMN-MP-Derived miRNAs Are Novel Mediators of Biological Activities

In addition to releasing soluble mediators, including cytokines and various metalloproteinases, activated PMNs have been recently shown to secrete microvesicles or microparticles (PMN-MPs) as an additional way to exert their effector functions (Dalli *et al.*, 2013; Butin-Israeli *et al.*, 2016; Bui *et al.*, 2018). PMN-MPs are vesicles that include exosomes and larger particles, ranging from 50 to 1000 nm that are generated during PMN activation and migration across endothelial and epithelial barriers (Bui *et al.*, 2018; Finkielsztein *et al.*, 2018). The PMN-MP cargo include proteins, miRNAs, and lipid mediators, all of which are encapsulated and protected by the vesicle lipid bilayer and are efficiently shuttled to surrounding target cells. As such, the exchange of extracellular vesicles or MPs has recently emerged as a novel way of intercellular communication (Hwang, 2013; Pitt *et al.*, 2016). Following uptake, PMN-MP cargo is discharged and functions to modulate many biological activities in recipient cells. A simplified list of PMN effector molecules and their respective biological functions is summarized in Table 1. Importantly, the biological content of PMN-MPs is stimulus dependent and is reflective of the condition, whereby the PMNs were activated. As such, the heterogeneity of MP composition has been shown to drastically change depending on the environmental milieu and tissue condition (Distler *et al.*, 2005; Shai *et al.*, 2012; Alexy *et al.*, 2014). The fact that we and other groups have confirmed rapid induction of miRNAs in activated PMNs, followed by their packaging into PMN-MPs, has redefined the notion that PMNs have low transcriptional activity and operate only through the release of granular stores.

The Synergistic Action of miR-23a and miR-155 Promotes DSB Accumulation and Paves the Road to Genomic Instability

By examining IBD patient biopsies, we observed that lamin B1 (LB1), a critical nuclear lamin that constitutes the nuclear envelope and protects replication forks (Butin-Israeli *et al.*, 2012, 2015), was significantly reduced. We further found that treatment of cultured colon epithelial cells or patient-derived colonoids with PMN-MPs downregulated LB1 through the action of miRNAs transported by these MPs. We then identified that miR-23a, which has been previously shown to regulate LB1 expression (Lin and Fu, 2009; Dreesen *et al.*, 2013), was highly upregulated in activated PMNs and was enriched in PMN-MPs. The lack of miR-23a primary and precursor transcripts (pri-/pre-miR-23a) in IBD colonic tissues or epithelial cells indicated that the increased level of miR-23a and its inhibitory effect on LB1 was due to PMN-MP deposition, and was not due to endogenous induction during inflammation. LB1 inhibition through miRNA activity compromised nuclear envelope integrity, resulting in replication fork stalling and S-phase arrest (Butin-Israeli *et al.*, 2015). Failure to restart replication precedes replication fork collapse and DSB induction at replication sites, perpetuating cell cycle arrest and affecting

Table 1. Neutrophil Effector Function in Inflammatory Bowel Disease

PMN effector function	*Contribution to IBD pathology*	*Association with MPs*	*References*
ROS	Contribute to oxidative stress, base damage/modification, replication errors, and single-strand breaks.	No	Nguyen *et al.* (2017), Cadet and Wagner (2013), Kellner *et al.* (2017), Cadet *et al.* (2017)
Myeloperoxidase	Generate ROS, promote cells death, and impede wound healing.	Yes	Slater *et al.* (2017)
Metalloproteinases	Disrupt tissue integrity, increase epithelial and vascular permeability, and immune cell recruitment.	Yes	Butin-Israeli *et al.* (2016)
Cytokines	Immune cells recruitment and inflammatory polarization, impede wound healing.	TBD	Wera *et al.* (2016), Wright *et al.* (2010), Wang *et al.* (2018)
miRNAs (miR-23a/-155, miR-9)	Posttranscriptionally regulate signaling pathways.	Yes	Butin-Israeli *et al.* (2019), Distler *et al.* (2005)

The table summarizes the pathological effector functions of PMNs and their association with MPs as it relates to IBD.
IBD, inflammatory bowel disease; MP, microparticles; PMN, neutrophil; ROS, reactive oxygen species; TBD, to be determined.

cellular fitness (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Zeman and Cimprich, 2014).

Intriguingly, inhibition of miR-23a activity by antagomir, although rescued LB1 expression, only partially reversed DSB accumulation, suggesting contribution of an additional DSB-inducing mechanism. We thus investigated two major DSB repair pathways, including homologous recombination (HR) and nonhomologous end joining (NHEJ) (Mao *et al.*, 2008; Brandsma and Gent, 2012), in the epithelial cells treated with PMN-MPs. While NHEJ activity was not affected by PMN-MP treatment during this short-term coculture experiment (24–48 h), HR was rapidly downregulated. Mechanistically, Rad51, a key HR regulator (Baumann and West, 1998), was found to be significantly downregulated by the specific action of miR-155 (Gasparini *et al.*, 2014), an additional miRNA that was found to be enriched in PMN-MPs (Butin-Israeli *et al.*, 2019). By suppressing Rad51 expression, miR-155 inhibits strand invasion and exchange between the homologous DNA strands on the sister chromatids, a rate-determining step of HR-mediated repair (Baumann and West, 1998; Anand *et al.*, 2017). Disruption of HR machinery and loss of HR activity result in ineffective resolution of DSBs incurred due to replication fork collapse (Costes and Lambert, 2012; Mijic *et al.*, 2017).

Although DSBs are formed at much lower frequency than SSBs, they are the most lethal DNA lesions and can rapidly induce cell death (Ceccaldi *et al.*, 2016). Moreover, DSBs are repaired at a significantly lower rate compared with SSBs, and as a result, impairment of any one of the DSB repair pathways have severe implications to cellular survival and tissue homeostasis (Ceccaldi *et al.*, 2016). In the context of mucosal injury as seen in IBD, DSB accumulation in the inflamed epithelium due to synergistic activity of PMN-MP-derived miRNAs resulted in cell cycle arrest and apoptosis, thus delaying wound resolution. In the mouse model of acute colonic injury, using endoscopy-guided, biopsy-based injury, we showed that administration of antisense oligonucleotides (ASOs) that specifically target miR-23a and miR-155 successfully rescued the expression of LB1 and Rad51, reduced DSB levels, and substantially improved wound healing. These observations affirm the therapeutic potential of miRNA therapy in IBD treatment. Indeed, active flare regions of IBD patients, which are characterized by high PMN infiltration, also have elevated levels of miR-23/miR-155 (Butin-Israeli *et al.*, 2019). Further studies with human cell lines by our group mechanistically showed that enriched miR-23a/miR-155 in epithelial cells can induce DSB accumulation, delay wound healing, and increase aneuploidy, an established marker for onset of carcinoma. Thus, increased miR-23a/miR-155 level coupled with reduced LB1/Rad51 expression have a powerful diagnostic value and can serve as early markers for IBD progression toward CRC/CAC. Patient biopsies can be easily obtained during routine endoscopy, and evaluation of miR-23a/miR-155 enrichment can be effectively performed using commercially available qPCR kits.

Therapeutic Outlook: An Immediate Response for IBD Management and a Long-Term Approach to Prevent Progression Toward CRC/CAC

Although wound healing and durable clinical remission are considered as important clinical endpoints for IBD therapy (Rogler *et al.*, 2013), most current treatments rely on general immunosuppression and provide only temporary respite from the symptomatic disease. Standard therapies include systemic corticosteroids, immunosuppressives (mesalamine, azathioprine, 6-mercaptopurine), or biologics such as TNFα blockers (infliximab, adalimumab, golimumab), and in severe cases, surgical intervention (Hvas *et al.*, 2018). These therapies, although effective in inducing initial remission, have harsh side effects and lose efficacy with disease progression. As has been seen with TNFα blockers, while 30% of patients do not respond to such treatment (Kopylov and Seidman, 2016), others develop antibodies to the biologics and overtime fail to achieve remission (Yanai *et al.*, 2015). Similarly, prolonged use of corticosteroid-based immunosuppression or leukocyte adhesion inhibitors (natalizumab, vedolizumab) leads to leukopenia, bacterial infections, and the life-threatening condition of progressive multifocal leukoencephalopathy (Yousry *et al.*, 2006).

For these reasons, more selective treatments for IBD that specifically target detrimental functions of immune cells need

to be developed. Our findings identified one such potential therapeutic approach. As per our observations, targeting miRNAs transported by PMN-MPs can reduce tissue-damaging effects of PMNs without altering their recruitment or antibacterial/proresolving effector functions, highlighting the potential of miR-23a/miR-155 as novel molecular targets for miRNA-based therapy for IBD. In fact, multiple miRNA-targeted therapeutics have reached clinical development (Soroosh *et al.*, 2018), including an RNA mimic of the tumor suppressor miRNA, miR-34, which has reached phase I clinical trials for cancer treatment (Beg *et al.*, 2017), and an antagonist for miR-122, which has reached phase II trials for treating hepatitis (Zeisel and Baumert, 2017). The major challenges of miRNA-based therapy involve the multitude of targets for each miRNA as well as the delivery route of miRNA biologics (Chen *et al.*, 2015). Oral or intravenous delivery will increase off-target effects of therapeutic miRNA mimics/antagonists (Jackson and Linsley, 2010). In an attempt to minimize these issues, a number of studies have attempted to couple drug delivery with routine colonoscopic screening in IBD patients (Philip and Philip, 2010). In fact, colon-targeted drug delivery offers a number of desirable features. Intrarectal administration of ASOs can avoid unnecessary degradation of the biologics in the stomach, intestine, or liver (Philip and Philip, 2010; Ramalingam *et al.*, 2015). In addition, a combination of ASO delivery and colonoscopic screening can facilitate the localized distribution of ASOs at injured tissues and the specific inhibition of PMN-MP-derived miR-23a/miR-155 in these regions. As a result, this strategy enables the efficient combination of routine IBD surveillance and drug administration to increase wound healing and maintain a durable remission.

Genomic instability is an emerging hallmark of cancer that increases mutagenesis and chromosomal abnormalities (Negrini *et al.*, 2010). Both features serve as the driving forces for tumorigenesis and tumor progression (Campbell *et al.*, 2017; Levine and Holland, 2018). Genomic instability can be induced due to cell-intrinsic abnormalities (Levine and Holland, 2018) or extrinsically due to inflammatory activity, as seen in CRC (Colotta *et al.*, 2009; Kidane *et al.*, 2014; Li and Chen, 2018). Based on our findings, infiltrating PMNs in IBD patients may promote genomic instability through the induction of DSBs and suppression of HR repair. Genomic instability increases the rate of genetic mutations, accelerates adaptation of precancerous cells, and ensues carcinogenesis (Moon *et al.*, 2019; Raynes and Weinreich, 2019). A diagram schematic of how PMNs contribute to the progression of IBD to CAC/CRC is shown in Figure 1. On this basis, preventing DSB accumulation in the inflamed mucosa of IBD patients by therapeutically targeting miR-23a/miR-155 can preserve the genomic integrity of colon tissues.

Finally, PMNs have been shown to mediate both beneficial and detrimental effects in wound healing and cancer (Galdiero *et al.*, 2018; Wang *et al.*, 2018). Although we are still far from completely understanding the mechanisms that underlie these seemingly opposing functions of PMNs, recent insights into the temporal changes of PMN phenotypes and increased survival during disease progression may explain the ''good'' and the ''bad'' actions of tissue PMNs (Ng *et al.*, 2019; Yang *et al.*, 2019). As such, one may speculate that during recurring, inflammatory episodes with subsequent waves of recruited PMNs, distinct PMN subsets with specializing activity/function may evolve at injury sites or precancerous lesions. As per our observations, one important feature that may differ in PMN subsets, probably dictating detrimental versus beneficial PMN function, is the content and the ability to generate MPs. This of course has to be investigated in the future. Thus, continued exploration of the PMN biology can offer new therapeutic avenues for IBD therapy and CRC/CAC prevention.

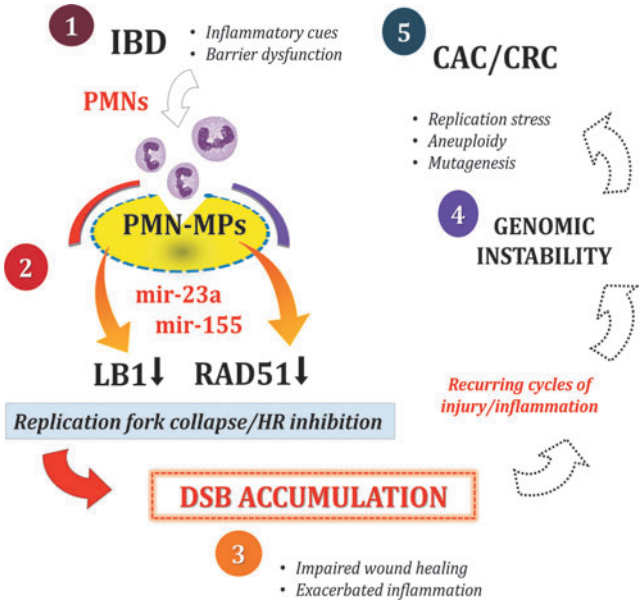

FIG. 1. Schematic diagram showing contribution of PMNs to genomic instability and progression toward CAC/CRC. (1) Recurring disease episodes in IBD patients elicit inflammatory cues and barrier dysfunction that facilitate PMN recruitment to the injured tissues. (2) PMN infiltration precedes deposition of PMN-MPs and delivery of miR-23a/miR-155 onto the intestinal mucosa. miR-23a and miR-155 downregulate LB1 and Rad51, respectively, leading to replication fork collapse and HR inhibition. (3) The synergistic compromise of nuclear envelope integrity and HR-mediated DSB repair results in DSB accumulation, which in turn increases apoptosis, impairs wound healing, and exacerbates inflammation. (4) Recurring cycles of tissue injury further perpetuate unresolved DSB accumulation and likely promote the induction of genomic instability. Genomic instability encompasses a multitude of genotoxic events, including replication stress, mutagenesis, and aneuploidy. (5) As an emerging hallmark of cancer, genomic instability, driven by PMN-mediated inflammation, thereby can drive the progression from IBD to CRC. CAC, colitis-associated cancer; CRC, colorectal cancer; DSB, double-strand break; HR, homologous recombination; IBD, inflammatory bowel disease; LB1, lamin B1; MP, microparticles; PMN, neutrophil.

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Disclosure Statement

No competing financial interests exist.

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