DNA REPAIR AND IMMUNE RESPONSE

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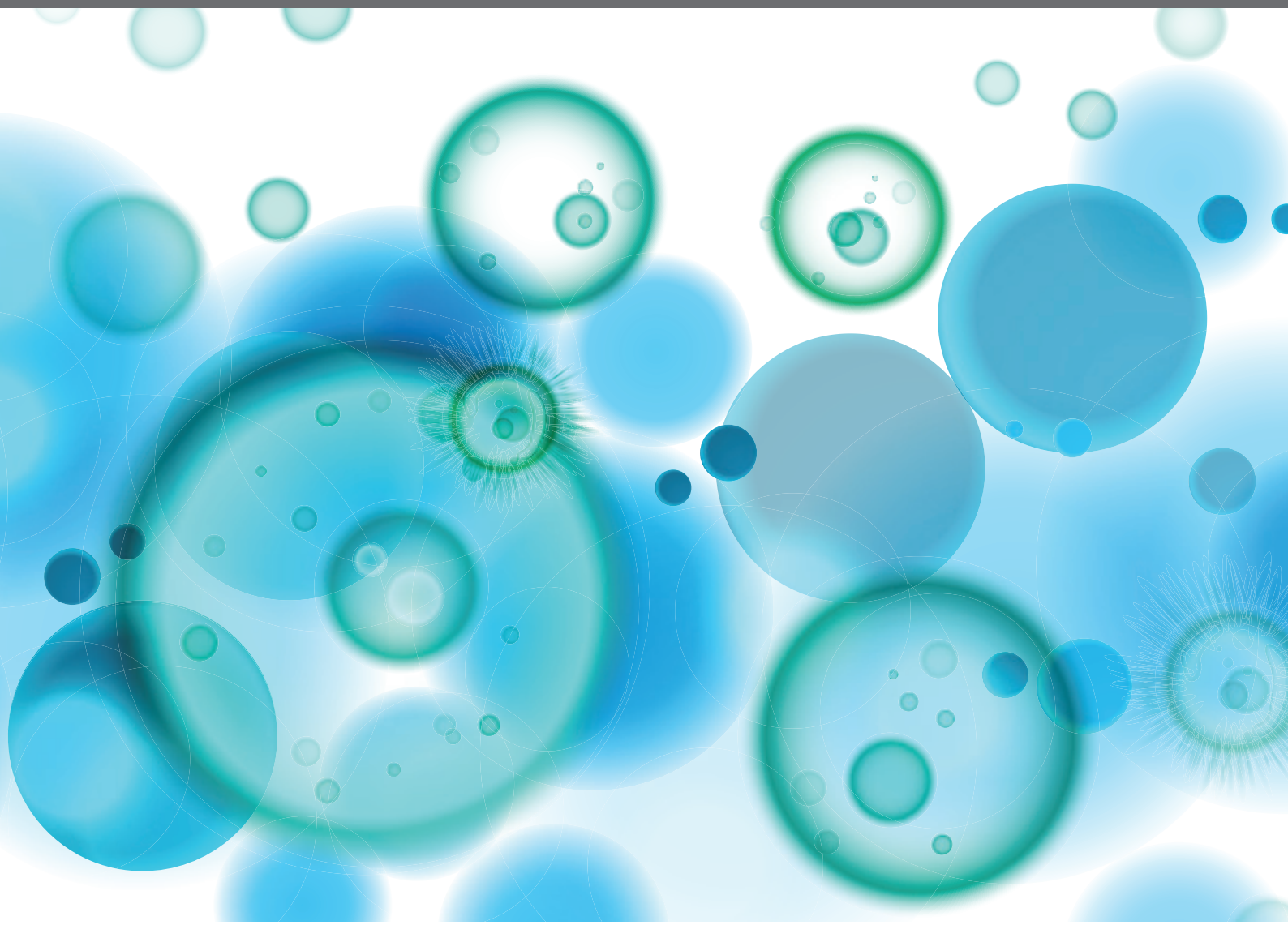

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DNA REPAIR AND IMMUNE RESPONSE

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Editorial: DNA repair and immune response

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Editorial on the Research Topic

DNA Repair and immune response

1 Introduction

1.1 Duality of the relationship between DNA damage responses and immunity: Health and disease

Genetic stability allows for the reliable transfer of genetic information to succeeding generations. In this regard, a complex and overlapping protein network operates to fix the DNA damage caused by internal or external stressors (1). This so-called "DNA Damage Response" (DDR) is a fine-tuning process and works actively to guarantee systemic homeostasis.

The imbalance between DNA damage and repair mechanisms accelerates the aging process and increases the risk of developing several age-related diseases such as cancer, cardiovascular diseases, and neurodegeneration. However, not all genetic modifications are harmful and some are essential for the correct functioning of the organism. Somatic mutations, for instance, guarantee the diversification and broad repertoire of immune receptors, ensuring an effective protective immunity against a wide variety of pathogens. Thus, the cooperation between DDR and the immune system has been discussed and offers a new field of investigation in which in-depth comprehension may provide new insights into the cellular and molecular mechanisms of inflammatory diseases.

1.1.1 An effective cooperation between DDR and immunity promotes health

When DDR is unable to deal with extended and irreparable DNA lesions, cellular alterations (e.g., misplaced cytosolic DNA fragments) will be promptly recognized by innate immune receptors and initiate or amplify inflammatory responses that will work to remove potentially malignant cells and, thus, preventing the perpetuation of DNA damages (Figure 1A).

Although there are DNA damage sensors that elicit an immune response (e.g., Ku70, DNA-dependent protein kinase, MRE11, Rad50, RNA polymerase III, and DExD/H-box helicase 41), there are also effector components that play multiple cellular roles, including in DDR and inflammation such as the poly(ADP-ribose) polymerase-1 (PARP-1), the enzyme mutY Homolog (MUTYH), and the 8-Oxoguanine DNA glycosylase-1 (OGG1) (2). In this edition, Oliveira et al. also discuss the role of apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1), a member of the base excision repair (BER) pathway, as another regulator of immunity through control of cellular signaling, redox status, senescence, and chromatin demethylation. Furthermore, Zhang and Li report on relevant observations regarding the structure and function of a versatile protein family belonging to the E3 ubiquitin ligase superfamily, called Pellino (Pellino-1, Pellino-2, and Pellino-3). The authors dissect Pellino's roles in the pattern recognition receptor, tumor, and microRNA signaling pathways. Ye et al., in turn, notably discuss current evidence on how DDR components communicate with both innate and adaptive immunity.

The adaptive arm of the immune system, composed essentially of lymphocytes and their subsets, requires random and purposeful DNA breaks to generate a vast repertoire of receptors that will recognize a broad range of antigens from infectious agents. Even after the receptors have been correctly produced during lymphocyte development, the DNA breaks may continue later in the lymphocyte's life. These processes are called class switch DNA recombination (CSR) and somatic hypermutation (SHM) and are essential for the generation of immunological memory and the production of highly specific antibodies. Any impairment of these mechanisms leads to critical DDR deficiency-driven immune system disorders and these are examined here by Gullickson et al.

CSR is a molecular mechanism that allows changing of antibody class from one to another (e.g., IgM to IgG or IgA). Previous studies showed that CSR requires DNA mismatch repair (MMR) and non-homologous end joining (NHEJ) pathways to replace the constant regions of immunoglobulins (Jhamnani et al.). On the other hand, SHM is a mechanism that introduces new mutations into antibody regions that recognize the antigens to increase antibody affinity (Pilzecker and Jacobs). This process is mediated and dependent on activation-induced cytidine deaminase (AID), which putatively distributes the

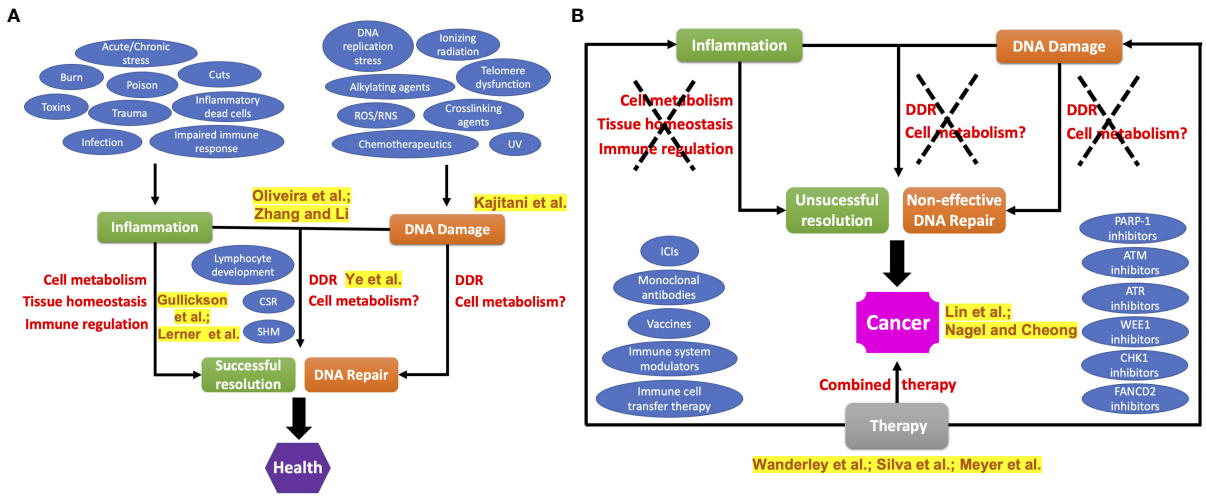

FIGURE 1
Relationship between inflammatory response and DNA damage in health and disease. **(A)** DNA damage response and immunity are closely related systems. They work together to deal with several negative stressors as well as to perform some physiological processes that require deliberate DNA breaks and rearrangement (e.g., lymphocyte receptor assembling, CSR, SHM), yielding a diverse set of immune receptors and antibodies capable of recognizing a broad range of antigens. **(B)** Chronic low-grade inflammation induces persistent DNA damage, and vice-versa, leading to cancer development. Current cancer treatment options envisage approaches targeting inflammatory or DNA-damaging agents separately. However, combined therapies have gained special attention as a potential strategy to improve the efficacy of cancer treatment. Here, we positioned the respective study from the manuscript collection closely to its main topic with the names of the authors highlighted in yellow, featuring the importance of the study's contribution to the field. CSR, class switch recombination; DDR, DNA damage response; ICIs, immune checkpoint inhibitors; SHM, somatic hypermutation; UV, ultraviolet (radiation).

mutations at G/C and A/T bases in similar ratios. However, even with the development of high throughput sequencing technologies, studying both CSR and SHM mechanisms remains challenging considering the limitations of current *in vitro* and *in vivo* approaches. Here, Lerner et al. describe that the Ramos cell line, a commonly G/C mutation-prone *in vitro* model used to evaluate SHM mechanisms, is capable of recapitulating the mutations at A/T bases by inhibiting ubiquitin-specific protease 1 (USP1) deubiquitinase activity and reestablishing the balance between proliferating cell nuclear antigen (PCNA) ubiquitination and deubiquitination.

Dissecting physiological and pathophysiological mechanisms is still the main path to the development of new therapeutic strategies against cancer and other illnesses. Here in this issue, Kajitani et al. show that the transgenic expression of cyclobutane pyrimidine dimers (CPDs) or 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs) photolyases in nucleotide excision repair (NER)-deficient mice exposed to UVB completely abrogated or reduced the inflammation, epidermal thickness, and cell proliferation in basal keratinocytes, indicating a central role of these cells in the control of responses to UVB-induced DNA lesions.

1.1.2 When the cooperation between DDR and immunity fails: the disease

When the organism is unable to counteract the high number of DNA damage through DDR and immune responses, a chronic low-grade inflammatory environment is established. This process is considered one of the strongest risk factors for cancer development (Figure 1B). In this issue, Cheong and Nagel not only review the cancer risk from dysfunctional DDR and immunity, but also discuss the influence of other factors such as genetics, aging, environment, lifestyle, circadian rhythm, and diet. The authors also emphasize the role of ongoing technologies in the advancement of knowledge in the DDR-immunity axis. In fact, the use of technology to determine both DDR and immune profiles is useful to improve clinical management since the intra- and inter-tumor heterogeneity among the patients remains a challenging concern. For this purpose, Lin et al. find two different profiles of patients with hepatocellular carcinoma based on their genomic landscape of DDR. The so-called "DDR-activated group" was categorized by patients with aggressive cancer and poor outcomes, while the "DDR-suppressed group" had a better prognosis.

Immune checkpoint inhibitors (ICIs) have revolutionized cancer therapy with their capacity for modulating the immune response (3). Because their use as single agents has shown unprecedented clinical benefits, the present state-of-the-art approach has focused on combining them with different anti-tumoral drugs to improve clinical outcomes. Wanderley et al. bring to light the potential of using ICIs with PARP1 inhibitors. As previously mentioned, PARP1 has multiple cellular functions, acting as a DDR agent and immune cell modulator. A limitation of ICIs relies on their effectiveness in less immunogenic tumors. In this regard, Silva et al. point out that ICIs also show better prognosis when used in MMR-deficient tumors and discuss ongoing approaches to increase ICI sensitivity in homologous recombination (HR)-deficient tumors. On the other hand, increased HR rates can also mitigate the immune response to cancer. Meyer et al. observe that ALDH1-positive breast cancer stem cells in phase S are resistant to radiation by increased HR activity, being a potential target to increase sensitization to radiotherapy.

2 Conclusion and perspectives

As evidenced by the latest cutting-edge research, DDR-related proteins are not only restricted to DNA repair processes, but also participate in other cellular circuits that regulate immune cell signaling and function. The crosstalk between DDR and immune response has only begun being dissected, opening new perspectives for understanding regulatory mechanisms controlling inflammation and providing new potential strategies to treat inflammatory (age-related) diseases by targeting the DDR-immunity axis.

Author contributions

PB wrote and drafted the whole editorial. CR, EB, IP, CM, and NC reviewed and approved the editorial.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DNA Damage Repair Profiles Alteration Characterize a Hepatocellular Carcinoma Subtype With Unique Molecular and Clinicopathologic Features

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Hepatocellular carcinoma (HCC) is one of the most common malignancies and displays high heterogeneity of molecular phenotypes. We investigated DNA damage repair (DDR) alterations in HCC by integrating multi-omics data. HCC patients were classified into two heterogeneous subtypes with distinct clinical and molecular features: the DDR-activated subtype and the DDR-suppressed subtype. The DDR-activated subgroup is characterized by inferior prognosis and clinicopathological features that result in aggressive clinical behavior. Tumors of the DDR-suppressed class, which have distinct clinical and molecular characteristics, tend to have superior survival. A DDR subtype signature was ultimately generated to enable HCC DDR classification, and the results were confirmed by using multi-layer date cohorts. Furthermore, immune profiles and immunotherapy responses are also different between the two DDR subtypes. Altogether, this study illustrates the DDR heterogeneity of HCCs and is helpful to the understanding of personalized clinicopathological and molecular mechanisms responsible for unique tumor DDR profiles.

Keywords: hepatocellular carcinoma, DNA damage repair, multi-omics, immune, survival

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INTRODUCTION

Liver cancer is the sixth most common cancer and the third most frequent cause of cancer-related death globally (1). Hepatocellular carcinoma (HCC), the most common form of liver cancer, accounts for about 90% of all cases and frequently develops in patients who are infected by hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, or metabolic syndrome (2). HCC commonly leads to inferior survival and requires molecules that help in refining prognosis and monitoring treatment response. Any attempt to improve the prognosis of HCC should involve clear recognition of HCC molecular characteristics. To date, several studies have proposed molecular and immune classifications of HCC based on genomic, transcriptomic, and proteomic data (3–5). These subtyping strategies broaden the knowledge into the molecular phenotype of HCC and provide effective targeted therapy options. However, the molecular mechanisms' response for the dismal prognosis of HCC are still unclear.

DNA damage repair (DDR) genes are the key to maintaining the stability of the human genome. Conversely, the loss of DDR function could lead to the onset and progression of cancer (6). Furthermore, treatment strategies focused on altered DDR function are becoming gradually realized. For example, Poly (ADP-ribose) polymerase (PARP), nuclear enzymes that recognize DNA damage, have been a therapeutic target for cancer treatment (7). DDR genes could be divided into some functional pathways based on their specific function in relation to DNA damage (8). Previously, The Cancer Genome Atlas (TCGA) work group comprehensively analyzed the influences of DDR pathway-related genes in cancers (8). The excellent study provides a rich resource for mechanistic and therapeutic analysis of cancer. However, transcriptomic and proteomic analysis of HCC from the perspective of DDR gene dysregulation and heterogeneity is still limited, especially in HCC. HCCs are complex ecosystems characterized by heterogeneity of molecular features and immune infiltrations. DDR actively participated in the processes of HCC carcinogenesis and immune characteristics. Recently, Yang et al. found that an important DDR gene TP53, its neoantigen may influence survival of HCC patients by regulating anti-tumor immunity thus could be an effective immunotherapy biomarker (9). Xu et al. also explored relationships between DDR gene RAD51 and immune infiltration in HCC (10). However, these studies mainly focused on role of single DDR gene in immune characteristics of HCC. Therefore, it is imperative to uncover the roles of DDR in HCC.

Here, we aim to comprehensively analyze transcriptional profile alteration of DDR genes in HCC. We have successfully identified two DDR gene-based subtypes based on 276 DDR genes. The two DDR-based subtypes have distinct clinical outcomes and molecular characteristics. Our data based on pan-cancer analysis also reveals heterogeneity among different cancer types and provides an alternative immune treatment response prediction approach. Our data shed light on the aspects of DDR alterations in HCC, which could be useful in guiding immunotherapy and prognosis monitoring.

METHODS

DNA Damage Repair Genes Curation

A total of 276 DDR genes were acquired from previous work by TCGA DDR-AWG (8, 11, 12). These genes were assembled based on MSigDB v5.0 and knowledge-based curation of DDR pathways. DDR genes mainly belong to ten DDR pathways: (1) base excision repair (BER); (2) nucleotide excision repair (NER); (3) mismatch repair (MMR); (4) the Fanconi anemia (FA) pathway; (5) homology-dependent recombination (HR); (6) non-homologous DNA end joining (NHEJ); (7) direct damage reversal/repair (DR); (8) translesion DNA synthesis (TLS); (9) nucleotide pool maintenance (NP); and (10) genes are either correlated with more than one DDR pathway, or coordinate cellular and molecular responses to DNA damage. This study of deidentified data was approved by the institutional review board of First affiliated hospital of Guangxi Medical University [2020(KY-E-119)].

DNA Damage Repair Genes-Based Clustering

First, we evaluated the global DDR alteration and proposed DDR gene-based subtypes based on two HCC cohorts included in the study. (1) Training cohort: Considering TCGA includes multi-omics resources for analysis, we characterized DDR characteristics based on TCGA. 371 primary HCC patients with RNA-seq date and corresponding survival information available from TCGA-Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset. The RNA-seq dataset and the corresponding clinical parameters were downloaded from UCSC-Xena (https://xenabrowser.net/datapages/). Gene expression value was transformed into log2 [Fragments Per Kilobase of transcript per Million mapped reads (FPKM) +1] for further analysis. (2) Validation cohort: 231 primary HCC RNA-seq and clinical information were downloaded from the International Cancer Genome Consortium (ICGC) dataset [accession ID: Liver Cancer RIKEN Japan (LIRI-JP)] dataset (13). Gene expression profiles were also converted into log2 (normalized read count + 1) for further analysis.

We performed K-means consensus clustering with transcriptomic profile of 276 DDR genes to identify subgroups. Consensus clustering was processed using the CancerSubtypes package in R software (14). The following details were set for subgrouping: number of repetitions = 1,000 bootstraps; pItem = 0.8 (resampling 80% of any sample); maxK=6 (k-means clustering with up to 6 clusters). An appropriate number of clusters was determined based on the clustering results and clinical ease of use. Similar clustering processes were performed in the training and validation cohorts. The Kaplan-Meier (K-M) method with log-rank test was performed to compare overall survival (OS) differences between the two subgroups.

Clinical and Molecular Characteristics Specific for the DDR Subtype

To observe clinicopathological and molecular characteristics between different DDR subtypes. We also compared clinicopathologic and molecular features between the two subgroups. Chi-square test was used to explore clinicopathological feature distribution between different DDR subtypes. The somatic mutation profile of HCC patients from TCGA was also downloaded from the TCGA database and ICGC, respectively. The somatic mutation data were further analyzed using the "maftools" R package (15).

We also compared transcriptomic alterations between the DDR-activated subtype and the DDR-suppressed subtype by using gene set enrichment analysis (GSEA). The GSEA procedures were performed based on the ClusterProfiler package in R software (16).

Here, we further conducted a metagene approach proposed previously for 28 immune cell subpopulations for HCC tumor microenvironment evaluation (17). Using the gene set variation

analysis (GSVA) algorithm, the relative infiltration score of 28 immune cell subpopulations was estimated (18). Metagenes for 28 immune cell subpopulations were obtained from a previous study (17). Then, immune profile differences between subtypes were estimated by Wilcoxon test.

DDR Subtype Signature Development and Validation

Considering too many genes, detection is hard for clinical application. We developed a gene signature for DDR subtype identification. Differentially expressed genes between DDR-activated and DDR-suppressed subtypes were identified by using Wilcoxon analysis. DDR genes with log2 (fold change)>1 and P-value <0.05 were considered as DDR subtype specific genes. In the era of precision medicine, proteogenomics could provide information about more direct executors and thus help in making a more precise diagnosis and prognosis monitoring of cancers. Considering that DDR-related proteins were major executors, we further compared the relationships between transcriptomic level and proteomic level. Proteomics data of 159 HCC patients were required from the clinical proteomic tumor analysis consortium (CPTAC) data portal (3). In the CPTAC cohort, 10,783 quantified protein expression levels were identified based on the Isobaric tandem mass tags (TMT) approach. Pairing transcriptomic and proteomic data were identified by Spearman correlation analysis. Genes that showed a significant correlation (spearman correlation coefficient >0.4) between protein levels and mRNA levels were submitted to DDR subtype signature construction. DDR genes that were significantly up-regulated in the DDR-activated subgroup and had high correlations between protein and mRNA levels were used for DDR subtype signature development. The DDR subtype signature score was calculated based on the average expression of the included DDR genes.

Prognostic Value of DDR Subtype Signature

To test the performance of the DDR subtype signature in survival prediction, five cohorts of HCC patients were included, including two RNA-seq datasets (TCGA, ICGC), two gene chips datasets acquired from Gene Expression Omnibus (GEO) [accession number: GSE14520 (19) and GSE54236 (20)] and proteomics dataset CPTAC (3). GSE14520 includes 242 HCC patients, while GSE54236 includes 78 HCC patients. Subsequently, we also explore whether the DDR-subtype signature could be a pan-cancer survival indicator. Therefore, RNA-seq data of 7779 cancer patients from 20 types of cancer were also downloaded from the TCGA database similar to the TCGA HCC download pipeline. Univariate Cox analyses were conducted in each cancer type to explore relationships between DDR subtype signature and OS. Hazard ratio (HR) and corresponding 95% corresponding interval (CI) were calculated. Then, Stata 14.0 software was used to integrate survival analysis results. Heterogeneity analyses used the I^2 and Q tests. When I^2>50% and the Q test P<0.1, it was considered that there was heterogeneity, and the random effect model was selected.

DDR Subtype Signature for Immunotherapy Response Prediction

To validate the value of the DDR subtype signature in immunotherapy prediction, we analyzed relationships between the DDR signature and immunotherapy response from the IMvigor210 cohort (21). The IMvigor210 cohort included 348 patients with locally advanced or metastatic urothelial cancer treated with an anti-PD-L1 agent (atezolizumab). The Kruskal-Wallis test was used to explore DDR signature score differences among different immunotherapy response groups [complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD)]. The area under curve (AUC) was used to estimate the DDR signature for immunotherapy response (CR/PR VS. SD/PD).

Single-Cell Analysis for DDR Heterogeneity Estimation

Single-cell data could provide higher resolution of gene alteration information. After filter out low low-quality cells, single-cell transcriptomic data of 12162 cells from 12 primary HCC samples was used for analysis from previous study (22). To explore DDR signature heterogeneity in different cell types, we calculated DDR signature in each cell and compared difference among different cell types. Seurat R package was used to generate t-SNE plot for cell types visualization.

RESULTS

DDR Gene Alteration Profiles in HCCs

To reveal the DDR gene heterogeneity of HCCs, all 371 HCC patients were divided into heterogeneous subtypes based on 276 DDR gene expression profiles (**Figure 1**). Considering the consensus clustering results and clinical significance, two DDR subgroups were identified. Cluster 1 (n=171, 46.1% of all HCCs) was designated as the DDR-activated subtype, owing to the relative upregulation of most DDR-related genes in this cluster. Cluster 2 (n=200, 53.9% of all HCCs), thereafter designated as the DDR-suppressed subtype based on the relative downregulation of DDR genes (**Figure 1A**). Furthermore, the two subtypes showed distinct clinical outcomes. K-M plots suggested that patients who were divided into DDR-activated subgroups suffered inferior OS (**Figure 1B**). We also compared clinical parameters between the two groups and found that advanced stage (chi-square value =5.757, P=0.016), high grade (chi-square value =18.013, P<0.001), and presence of vascular invasion (chi-square value = 4.135, P=0.042) were more frequently observed in the DDR-activated subgroup (**Figure 1C**).

In the validation ICGC cohort, all 231 HCCs were also divided into different subtypes based on the 276 DDR gene expressions. Similarly, K-means clustering indicated that patients who were also categorized into two subgroups had similar DDR pathway alterations with the training cohort (**Figure 1D**). Patients were also divided into DDR-activated and DDR-suppressed subgroups. A similar survival difference between two subgroups was also observed (**Figure 1E**).

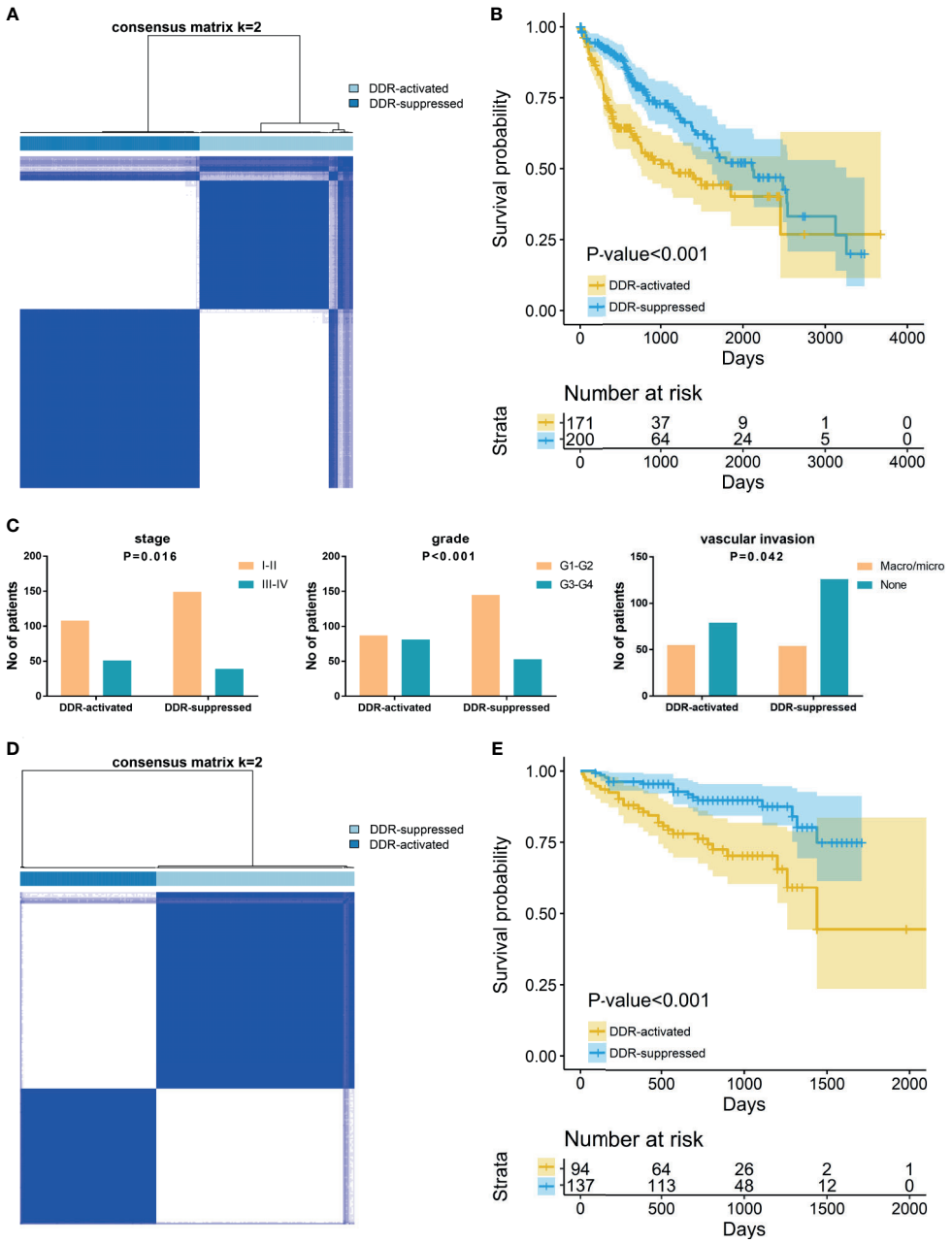

FIGURE 1 | Consensus clustering for DNA damage repair (DDR) related genes in HCC patients. **(A)** The consensus matrix shows patients with two distinct DDR statuses in the TCGA dataset. **(B)** Kaplan-Meier curves for overall survival based on DDR subgroups (Log-rank test) in TCGA dataset; **(C)** Tumor stage, grade, and vascular invasion distribution differences between DDR subgroups; **(D)** The consensus matrix shows patients with two distinct DDR statuses in the ICGC dataset; **(E)** Kaplan-Meier curves for overall survival based on DDR subgroups (Log-rank test) in ICGC dataset.

These findings further validate the inferior prognosis of patients in the DDR-activated group.

DDR Genes-Based Subtypes Show Distinct Clinical and Molecular Characteristics

When considering genomic alterations, we also compared gene mutation differences between two DDR subtypes. The most common mutational genes in patients from the training cohort were TP53 and CTNNB1 (**Figure 2A**). Considering the importance of these two genes, we compared and found that TP53 was more frequently mutated in the DDR-activated subgroup (78/165 Vs. 29/194, chi-square= 44.53, P<0.001) while CTNNB1 was more frequently mutated in the DDR-suppressed subgroup (32/165 Vs. 58/194, chi-square= 5.24, P=0.022, **Figure 2B**). In the validation cohort, we found TP53

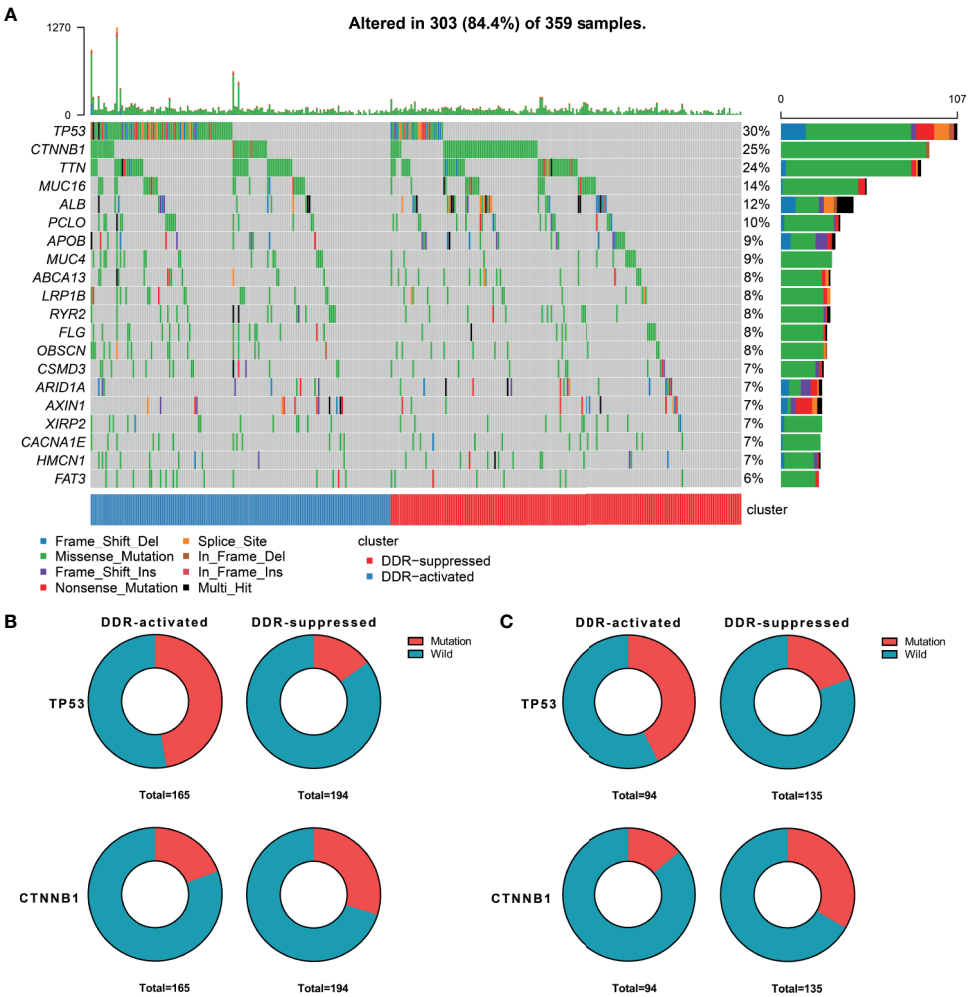

FIGURE 2 | Genomic alterations between DDR-activated and DDR-suppressed subgroups. **(A)** Landscape of mutation profiles in HCC samples. Mutation information of each gene in each sample is shown in the waterfall plot. Top panel shows individual tumor mutation burden. The data shown were analyzed based on the TCGA data portal. **(B)** The mutation rate of TP53 was higher in the DDR-activated subgroup, while CTNNB1 was higher in the DDR-suppressed subgroup in the TCGA dataset. **(C)** The mutation rate of TP53 was higher in the DDR-activated subgroup, while CTNNB1 was higher in the DDR-suppressed subgroup in the ICGC dataset.

also frequently mutated in the DDR-activated subgroup (40/94 Vs. 26/135, chi-square= 14.66, P<0.001) while CTNNB1 was more frequently mutated in the DDR-suppressed subgroup (13/94 Vs. 45/135, chi-square= 11.15, P=0.001, **Figure 2C**).

GSEA analysis revealed that DDR subtypes have distinct transcriptomic alterations. The top five most activated gene ontology terms in the DDR-activated subgroup were MCM complex, condensed chromosome outer kinetochore, mitotic chromosome condensation, single-stranded DNA-dependent ATPase activity, and entry of the bacterium into the host cell (**Figure 3A**). The top five most activated Kyoto Encyclopedia of Genes and Genomes terms in DDR-activated subgroup were DNA replication, mismatch repair, cell cycle, Fanconi anemia pathway, and homologous recombination (**Figure 3B**).

DDR Subtypes Characterized Different Immune Profiles

Immune cell infiltration markedly influenced tumor progression and immunotherapy treatment response. Therefore, we also explored differences in immune cell infiltrations between two DDR subtypes. Notably, activated CD4 T cells, central memory CD4 T cells, and effector memory CD4 T cells were significantly up-regulated in the DDR-activated subgroup regardless of the training (P=2.39E-17, 1.83E-06 and 7.01E-09 respectively, **Figure 4A**) and validation cohort (P=8.78E-07, 5.52E-04 and 1.70E-03 respectively, **Figure 4B**). Mast cell and neutrophil cell were significantly up-regulated in DDR-suppressed subgroup in the training (P=0.025 and 0.001 respectively, **Figure 4A**) and validation cohort (P=0.017 and 0.014 respectively, **Figure 4B**).

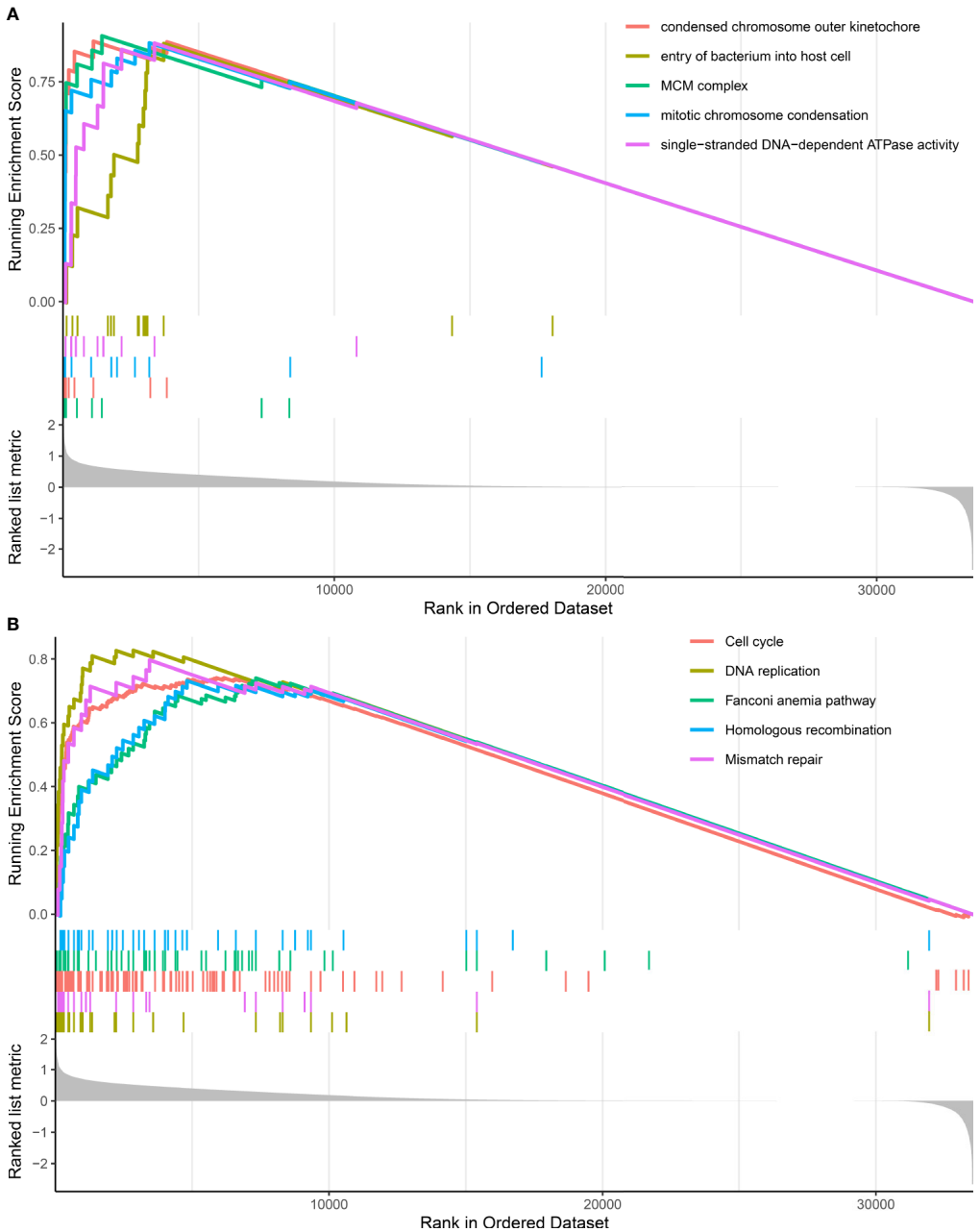

FIGURE 3 | Gene set enrichment analysis of DDR-subtype specific pathway analysis. **(A)** Top five most significant altered gene ontology terms in the DDR-activated subgroup when compared with the DDR-suppressed subgroup. **(B)** Top five most significant altered KEGG pathways in the DDR-activated subgroup when compared with the DDR-suppressed subgroup.

DDR Subtype Signature Is a Prognostic Indicator for HCC's OS

Considering that many gene expression detections are difficult for clinical implication, it is imperative to have a signature that could be used for DDR subtype identification. Differential analysis indicated that 11 DDR-related genes, including TYMS, RRM2, UBE2T, HMGB2, SOX4, FEN1, RFC4, H2AFX, FANCI, PCNA, and RMI2, were most specifically upregulated in the DDR-activated subtype. Correlation analyses from the CPTAC cohort found that six genes (FEN1, H2AFX, HMGB2, PCNA, RFC4, and RRM2) were significant correlated between transcriptomic and proteomic data. Therefore, we used the average expression of six markers for the DDR-activated signature. AUC of ROC indicated that the gene signature

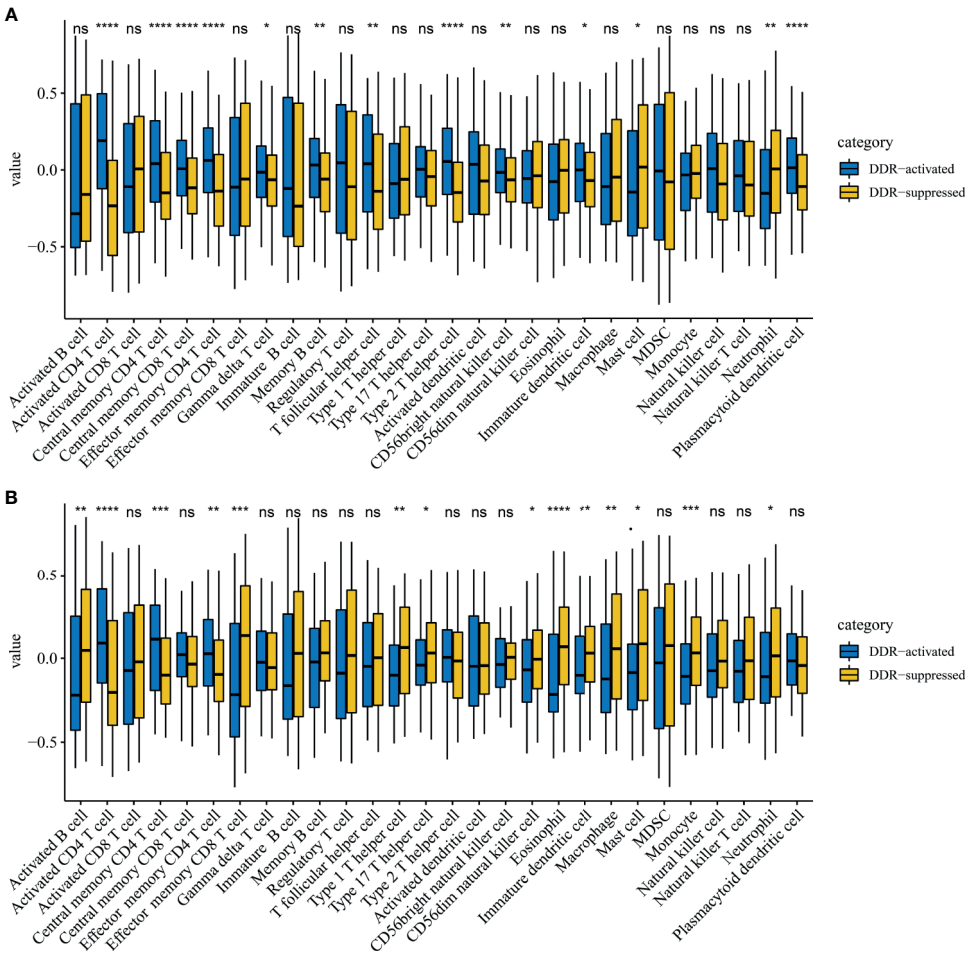

FIGURE 4 | Immune profile alterations between the DDR-activated and DDR-suppressed subgroups. **(A)** TCGA; **(B)** ICGC. * represents P < 0.05, ** represents P < 0.01,*** represents P < 0.01, **** represents P < 0.0001, ns represents no significant difference.

could be useful for stratification patients in different DDR subtypes (AUC= 0.909 in training cohort, **Figure 5A**; AUC= 0.932 in validation cohort, **Figure 5B**). Therefore, the six DDR gene signatures provided an alternative and clinically accessible method for DDR subtype identification.

To validate generalization performance of DDR subtype signature in different cohorts, a meta-analysis approach was utilized to integrate survival analysis results from five cohorts (TCGA, ICGC, GSE14520, GSE54236, and CPTAC). The DDR-subtype signature showed statistical significance in five cohorts. Meta-analysis revealed that a higher signature showed inferior OS (HR, 1.89; 95% CI, 1.49–2.38, **Figure 5C**). Time-dependent ROC was generated and showed that area under curves for 1, 3, 5 years were 0.71, 0.65 and 0.63 respectively (**Figure 6A**). K-M plot showed that patients could be divided into two groups with distinct prognosis based on median value of DDR signature (**Figure 6B**). To find optimal cut-off of DDR gene signature for risk stratification, we also evaluated the best significant cut-off value (**Figure 6C**). The optimal cut-off value was 4.51, which was also effective for risk stratification in ICGC cohort (**Figure 6D**).

Pan-cancer analysis that included 7779 patients from 20 types of cancer indicated that the DDR signature still remains a prognostic indicator. A higher DDR signature score suggested that patients had poor survival (HR, 1.26; 95% CI, 1.03, 1.54; **Figure 7A**). However, marked heterogeneity was observed among different cancer types (I-squared = 89.6%, p <0.001). For example, two pathological subtypes of lung cancer, lung adenocarcinoma and lung squamous cell cancer, showed distinct prognoses of the DDR-subtype signature.

DDR Signature Is a Promising Predictor for Immunotherapy

To explore the DDR subtype signature for immunotherapy response prediction, we explored 348 samples from the IMvigor210 cohort. For gene expression analyses with respect to response, 298 patients were used to estimate DDR subtype score for immunotherapy response prediction. We found that the DDR

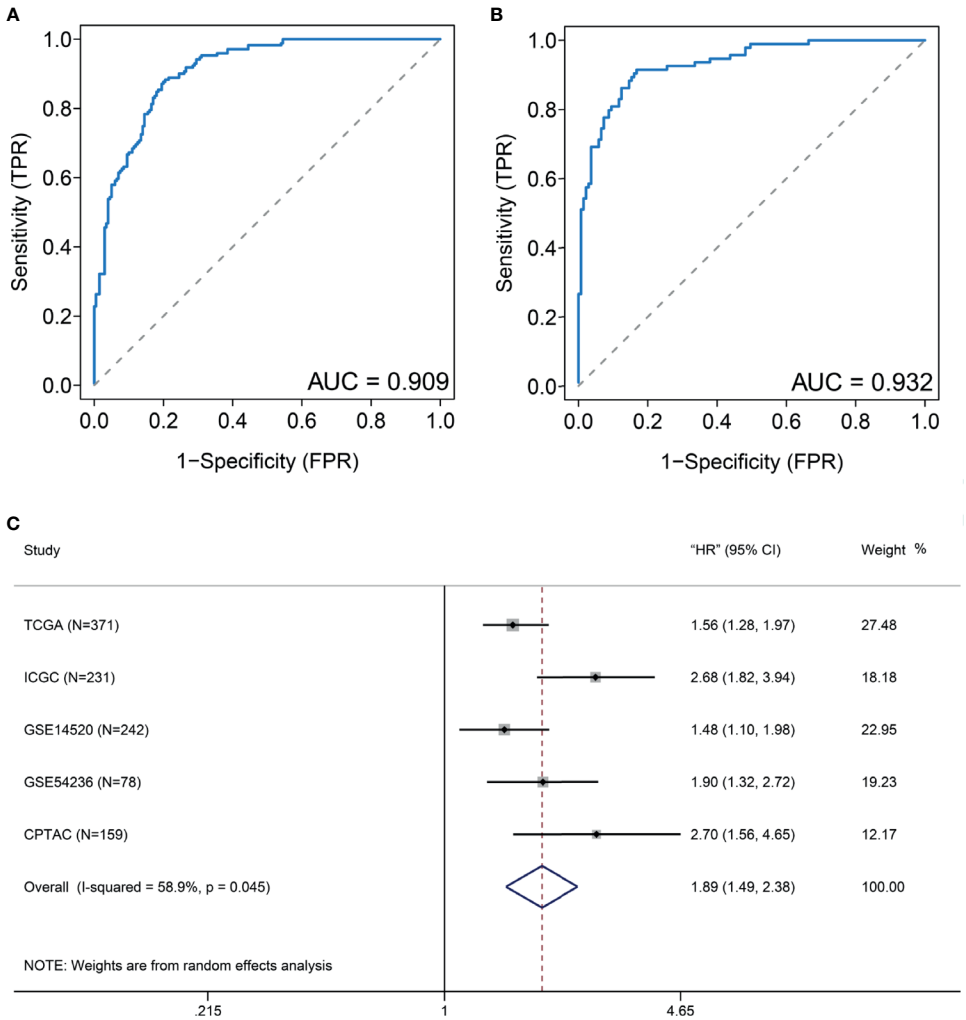

FIGURE 5 | DDR-subtype development and validation. Receiver operating characteristic curve (ROC) analyses of DDR signature to evaluate its performance in TCGA **(A)** and ICGC **(B)** datasets. **(C)** Forest plots show that a high DDR signature score is correlated with inferior overall survival based on five cohorts.

signature score was higher in the CR or PR group when compared with the SD and PD groups (Kruskal-Walls, P = 0.00014; **Figure 7B**). The results of the ROC curve indicated that the DDR signature could be used for immunotherapy response prediction (AUC=0.671, 95% CI, 0.598-0.743, P<0.001; **Figure 7C**).

DDR Signature Is Heterogeneous in Tumor Immune Microenvironment

In 12162 cells from 12 samples, cells were mainly divided into 10 types, including B cell, endothelial, epithelial, hepatic stellate cells (HSCs), myeloid, NK, pDC, plasma, T cell and tumor cell (**Figure 8A**). Results from single-cell analysis found that DDR signature score was significant different distribute in different clusters (**Figure 8B**). And DDR score was significant up-regulated in some particular clusters, especially for tumor and T cells. Kruskal-Wallis test also showed that DDR score was significant different among different cells (**Figure 8C**).

DISCUSSION

Despite the progress in the approaches to therapy, the prognosis of HCC remains poor owing to the high recurrence rate, even after surgical resection. Molecular heterogeneity often tends to limited treatment options and is a challenge for survival monitoring. Hence, some excellent previous studies that aimed at molecular-phenotypic subtype identification of HCC have provided novel insights into HCC precision medicine (3, 4). However, the roles of DDR in the ecosystems of HCC still need to be deciphered. In this study, we analyzed multi-omics data that included genomics, transcriptomics, and proteomics to characterize differences between DDR-based subtypes in HCC. Further study also explored immunotherapy response and immune profile differences between DDR-based subtypes.

Our integrated analysis revealed that HCC patients have two distinct DDR statuses: the DDR-activated subtype and the DDR-

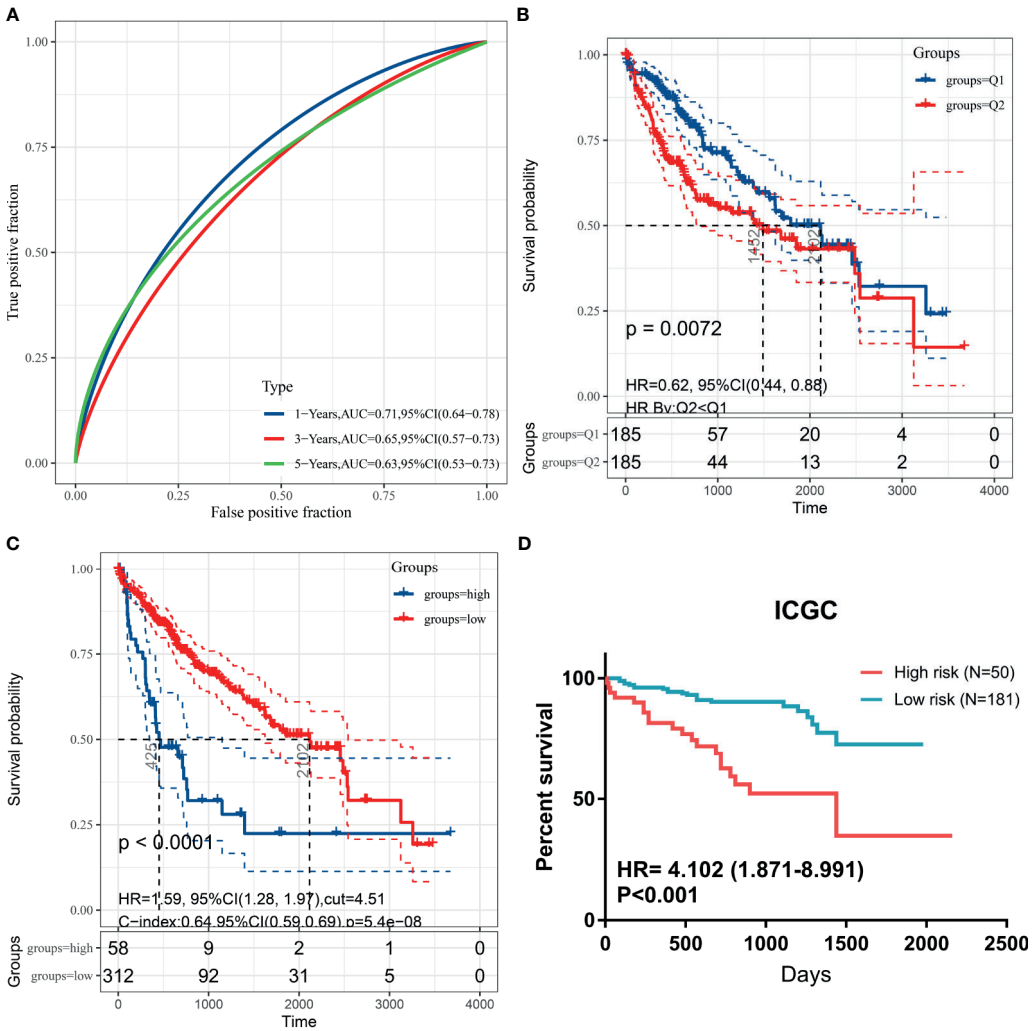

FIGURE 6 | Kaplan-Meier plots for DDR signature cut-off identification. **(A)** Time-dependent ROC for DDR signature survival prediction in TCGA database; **(B)** survival difference between high and low DDR signature based on median value; **(C)** survival difference between high and low DDR signature based on best separation in training cohort; **(D)** cut-off from TCGA cohort could be useful for risk stratification in ICGC dataset.

suppressed subtype. Patients in the DDR-activated subgroup are characterized by aggressive clinical behavior, including advanced stage, poor differentiation, and inferior prognosis. To identify the molecular characteristics of distinct DDR subtypes in HCC, we found that genomic alterations were significant between the two subtypes. TP53 mutation was more frequently observed in the DDR-activated subtype. The tumor suppressor p53 plays a key role in DNA repair and somatically mutated in many types of human cancers, including HCC (23). As the "guardian of the genome," TP53 mutations have been clinically recognized as an inferior survival indicator for HCC (24). Interestingly, in the DDR-suppressed subgroup, CTNNB1 was more frequently mutated when compared with the DDR-activated subgroup. CTNNB1 mutations activating ß-catenin and were mutually exclusive with TP53 (25). A previous excellent proteogenomics study revealed that the protein and phosphorylation differences between CTNNB1 mutant and wild-type HCC were mainly concentrated in metabolic pathways (3). In the era of immunotherapy, more studies have found that Wnt/CTNNB1 mutations are the characterization of immune-excluded class HCC (26, 27). Harding et al. showed that HCC patients with CTNNB1 mutations did not respond to PD-1 blocking therapy, which validated the hypothesis that HCC "cold tumors" defined by Wnt/CTNNB1 mutations are not responsive to immunotherapy (28).

GSEA analysis further indicated that our subgroup plan is credible. The DDR opens news perspectives for understanding the regulatory mechanisms of tumors. We also explore the immune microenvironment in HCCs. DDR subtypes have distinct immune profiles. Activated CD4 T cells, central memory CD4 T cells, and effector memory CD4 T cells were significantly up-regulated in the DDR-activated subgroup.

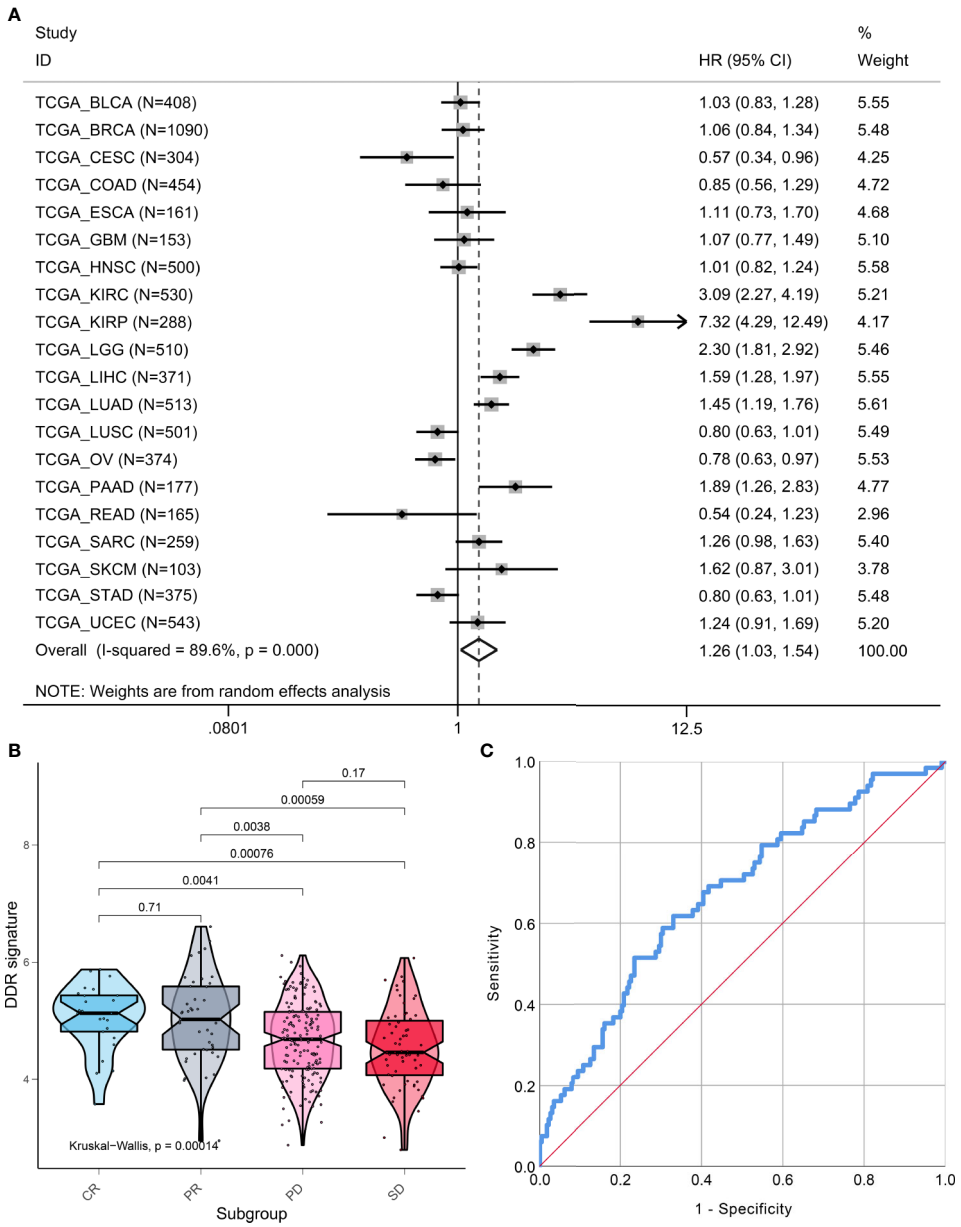

FIGURE 7 | Pan-cancer prognostic value and immunotherapy response prediction of DDR signature. **(A)** Pan-cancer analysis to evaluate prognostic value of DDR signature. **(B)** TME scores in groups with different anti-PD-L1 clinical response statuses. **(C)** A receiver-operating characteristic (ROC) curve was used to measure the performance of the DDR subtype signature in immunotherapy response prediction.

CD4+ T cells can target tumor cells in a variety of ways, either by eliminating tumor cells directly through cytolytic mechanisms or indirectly by regulating TME (29, 30). Mast cell and neutrophil cell were specifically enriched in the DDR-suppressed subgroup. Tumor-infiltrating mast cells have been identified as being associated with resistance to anti-PD-1 therapy (31). These findings have shown that DDR subtypes have distinct immune cell infiltration differences, which hints at different immunotherapy responses between subtypes. Therefore, we also found immunotherapy response differences between distinct DDR subtypes. By applying ROC curve analysis, we also identified that the DDR subtype signature is valuable for immunotherapy response in patients with metastatic urothelial cancer treated with the anti–PD-L1 agent. We found that the DDR subtype signature was significantly higher in responders than in non-responders undergoing checkpoint blockade therapy. However, the performance of this signature in HCC should be further tested through analysis of a large cohort of HCC patients who have received immunotherapy.

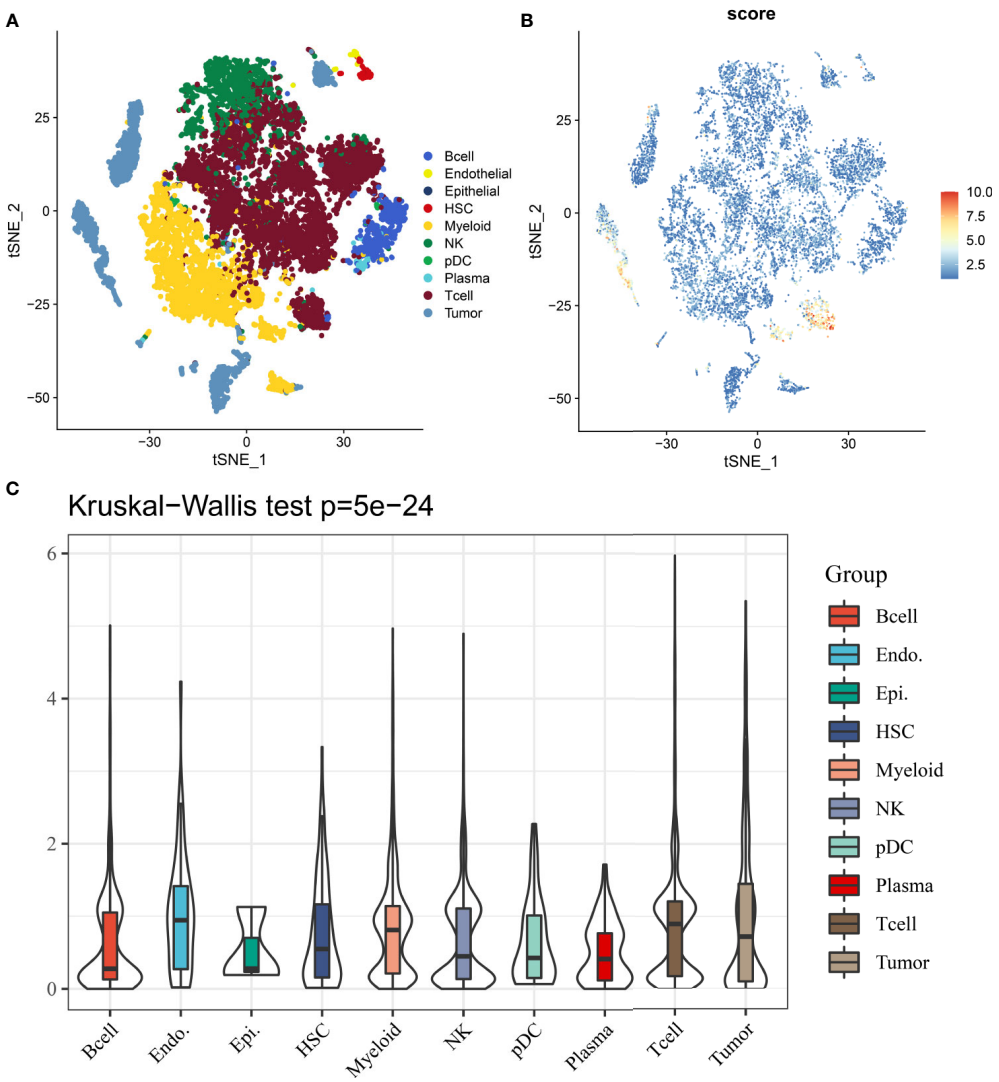

FIGURE 8 | The distribution and expression of DDR subtype signature in HCC. **(A)** The percentage of each type of cells in HCC. **(B)** The distribution of each type and DDR score expression in HCC. **(C)** DDR scores in different cells are various.

To speed up clinical use, six DDR genes were composed as a signature for DDR-subtype identification. The signature showed high performance in dividing patients into distinct DDR subtypes in the training and validation cohort. The combination of RNA-seq data and mass spectrometry-based proteomics could provide a more comprehensive view globally. The DDR signature we proposed showed moderate prognostic value in HCC patients based on RNA-seq, microarray, and proteomics data. Their results also hinted that our results are robust and repeatable. However, pan-cancer analysis suggested that the prognostic value of the DDR signature is its heterogeneity. DDR alterations characteristics in different cancer types should be further analyzed.

Our study is not without its limitations. First, there is a lack of randomized trials of HCC patients who receive immunotherapy to validate the immunotherapy response prediction performance of the signature. Second, different expression detection platforms were used in our study, including RNA-seq, gene chip, and proteomics. Future studies are needed to validate the optimal cut-off for DDR subtype identification. Third, our study mainly focused on multi-cohort data for providing solid information for DDR-related survival information and molecular characteristics. Future *in vivo* and/or *in vitro* mechanism exploration may provide more information for DDR subtype alterations.

In conclusion, this study provides evidence of DDR heterogeneity and DDR categorized subtypes in HCC patients. Specific DDR subtype characteristics provide information for HCC clinical management and decision-making assistance. Our DDR subtype signature facilitates a deeper understanding of the mechanisms associated with HCC inferior prognosis and assists in developing more effective therapeutic targets and biomarkers for immunotherapies in HCC patients.

DATA AVAILABILITY STATEMENT

Data generated and analyzed during the current study are available from the UCSC TCGA data portal (http://xena.ucsc.edu/public/), ICGC (dataset ID: LIRI-JP, https://icgc.org/), CPTAC (https://proteomics.cancer.gov/programs/cptac) and GEO databases (dataset ID: GSE14520 and GSE54236, https://www.ncbi.nlm.nih.gov/).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by First Affiliated Hospital of Guangxi Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization: PL and R-zG. Methodology: PL, R-zG, and RW. Validation: YH and HY. Formal analysis: PL, R-zG, and RW. Data curation: YH and HY. Writing—original draft preparation: PL, R-zG, and RW. Writing—review and editing: YH and HY. Visualization: YH and HY. Supervision: YH and HY. All authors contributed to the article and approved the submitted version.

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REVIEW
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Function and Molecular Mechanism of the DNA Damage Response in Immunity and Cancer Immunotherapy

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The DNA damage response (DDR) is an organized network of multiple interwoven components evolved to repair damaged DNA and maintain genome fidelity. Conceptually the DDR includes damage sensors, transducer kinases, and effectors to maintain genomic stability and accurate transmission of genetic information. We have recently gained a substantially improved molecular and mechanistic understanding of how DDR components are interconnected to inflammatory and immune responses to stress. DDR shapes both innate and adaptive immune pathways: (i) in the context of innate immunity, DDR components mainly enhance cytosolic DNA sensing and its downstream STimulator of INterferon Genes (STING)-dependent signaling; (ii) in the context of adaptive immunity, the DDR is needed for the assembly and diversification of antigen receptor genes that is requisite for T and B lymphocyte development. Imbalances between DNA damage and repair impair tissue homeostasis and lead to replication and transcription stress, mutation accumulation, and even cell death. These impacts from DDR defects can then drive tumorigenesis, secretion of inflammatory cytokines, and aberrant immune responses. Yet, DDR deficiency or inhibition can also directly enhance innate immune responses. Furthermore, DDR defects plus the higher mutation load in tumor cells synergistically produce primarily tumor-specific neoantigens, which are powerfully targeted in cancer immunotherapy by employing immune checkpoint inhibitors to amplify immune responses. Thus, elucidating DDR-immune response interplay may provide critical connections for harnessing immunomodulatory effects plus targeted inhibition to improve efficacy of radiation and chemotherapies, of immune checkpoint blockade, and of combined therapeutic strategies.

Keywords: DNA repair, immune response, DNA damage, cGAS-STING, innate immunity, adaptive immunity, immunomodulatory, cancer therapy

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INTRODUCTION

Key cancer hallmarks critically include genomic instability, immune modulation, and altered DNA damage and other stress responses to favor overall cell survival (1, 2). Every day, tens of thousands of damaged DNA lesions occur in each human cell that could impact cell survival and genomic integrity (3). Importantly, the outcome of this DNA damage depends directly upon the nature and actions of the DNA damage response (DDR). Lesions become accurately or inaccurately repaired or left as unrepaired mutations depending upon the DDR. As a result, evolutionary selection ensures that the DDR is a carefully orchestrated response system consisting of multiple signaling pathways that largely maintain genomic stability and fidelity despite high levels of DNA damage (4, 5). Yet, comprehensive analyses of cancer genome databases reveal non-B DNA, mitochondrial dysfunction, and the activation of DNA repair/cell cycle pathways as major factors driving somatic mutation loads in cancer cells (2, 6). From a mechanistic standpoint, the positive correlations of these factors with mutations in cancer cells likely arise from increased reactive oxygen species (ROS), oncogenic replication and transcription stress, and the combination of resulting excessive DNA damage plus its escape from accurate repair.

In particular, DDR are activated by replication obstacles in proliferating cells that lead to replication stress: replication fork stalling, collapse or breakage, such as lesions from oxidation, deamination and alkylation, DNA breaks, protein-DNA cross-links, and non-B DNA structures including R-loops (RNA-DNA hybrids formed by replication-transcription conflicts) (7–9). DNA damage and activation of the DDR from endogenous replication stress are seen at pre- or early stages of oncogenesis, and adaptation to replication stress acts in tumor development (10). In breast-cancer susceptibility gene 2 (BRCA2)-deficient cancer cells, the inactivation of replicative stress response factors (e.g. poly (ADP-ribose) polymerase [PARP1] or ATM and Rad3-related [ATR] inhibition) triggers cyclic GMP-AMP synthase (cGAS)-STING-mediated innate immune responses (11, 12). Furthermore, inherent DNA repair defects in tumors may develop mutation-driven neoantigens that can cause the immune system to recognize the tumor cells as foreign while also increasing the amount of cytosolic DNA to trigger a cGAS-STING response. Thus, the DDR that largely protects against DNA damage in normal cells can often be defective or defeated in proliferating cancer cells with consequent impacts on immune responses. This finding implies a fundamental importance of DDR for cancer biology, for the elucidation of cancer vulnerabilities, and for optimal applications of immunotherapy.

The DDR machinery can conceptually be divided into at least six distinct DNA repair pathways responding to different types of DNA damage: (i) homologous recombination (HR), which repairs double-strand breaks (DSBs) using a homologous DNA template; (ii) non-homologous end joining (NHEJ), which repairs DSBs without a corresponding template; (iii) alternative end-joining (A-EJ), which repairs DSBs with insertion and deletion errors by employing micro-homology; (iv) nucleotide excision repair (NER), which repairs bulky DNA lesions globally or coupled to transcription; (v) mismatch repair (MMR), which repairs DNA single-strand breaks (SSBs) predominantly generated during DNA replication and recombination processes plus mismatches that escaped replication fidelity; and (vi) base excision repair (BER), which removes bases damaged by oxidation, alkylation, deamination, and methylation to avoid replication and transcription blocks and errors (4, 13–15).

The various DDR pathways share similarities in how they respond to the stress of damaged DNA, whereby a damage sensor that can also be a repair effector [e.g., RPA, MUTY, PARP1, Ku70/80, MRE11-RAD50-NBS1 (MRN) complex] recognizes specific DNA damage types (single-stranded DNA, base mismatches, SSBs, and DSBs) before recruiting and activating downstream transducer kinases (such as ATM, ATR, DNA-PKcs), which in turn transduce the signal to effector proteins (such as MRN, CHK1, EXO5, p53, RAD51, and BRCA1/2). The ensuing complexes ultimately orchestrate repair by employing damage removal and sequence replacement by handoffs or dynamic machinery that have evolved to avoid the release of toxic and mutagenic DNA intermediates (15, 16). Thus, the DDR is an ancient and evolutionarily conserved mechanism that is essential for genome stability and cell survival (17, 18).

As the major stress response system essential for surviving infection, the immune response is an evolved network of proteins and complexes that respond to invading pathogens and their associated toxins. Importantly, DDR defects can lead to imbalance between DNA damage and repair, impairing tissue homeostasis and leading to replication and transcription stress, mutation accumulation or outright cell death: this imbalance can drive tumorigenesis as well as secretion of inflammatory cytokines, and aberrant immune responses (19–23). All organisms possess mechanisms to detect and eliminate foreign pathogens *via* the innate immune system. Additionally, higher vertebrates employ a sophisticated adaptive immune system that includes antibodies as well as B and T lymphocytes with virtually limitless repertoires of receptors that mediate neutralization of foreign pathogens and removal malignant cells (24–26). To stimulate strong anti-tumor immune responses, cancer immunotherapy typically employs immune checkpoint inhibitors for the PD-1/PD-L1 and CTLA-4 pathways to amplify immune system responses and also to harnesses responses to neoantigens that are primarily tumor-specific antigens resulting from the higher mutation load in tumor cells (27–29). The validity of the PD-1/PD-L1 approach requires the functional MHC class I complex, which itself is often deleted during tumor evolution to escape immune regulation (30).

For clarity this review is divided into five overall sections: 1) Introduction, 2) DDR in innate immunity, 3) DDR in adaptive immunity, 4) DDR inhibition in antitumor immunity, and 5) Summary and prospects. Within these sections and their subsections, we furthermore consider how these critical and seemingly distinct DDR and immune stress responses are

intertwined and where defining their interconnections may enable novel insights into etiology and advanced molecular-based treatment of cancer and other human diseases.

DDR IN INNATE IMMUNITY

Innate immunity is the first immunological defense system against pathogens. Activation of the innate immune response relies on Pattern Recognition Receptors (PRRs). These PRRs detect Damage-Associated Molecular Patterns (DAMPs) or Pathogen-Associated Molecular Patterns (PAMPs) to initiate a signaling cascade resulting in production of interferons (IFNs), cytokines and chemokines (24, 26, 31). Importantly, non-self nucleic acids are the most well-characterized stimuli for the innate immune response (32, 33); furthermore, endogenous cytosolic DNA released from the nucleus or mitochondria stimulates the innate immune system.

DNA damage caused by genotoxic stresses or DNA damage stimulus (e.g., cytotoxic chemotherapy and radiation) can create cytosolic chromosomal fragments that may be recognized by cGAS, a cytosolic DNA sensor. Cytosolic exposure of chromosomal DNA by micronuclei rupture, breakage of chromatin bridges, or disintegration of micronuclei-like cytosolic chromatin fragments activates cGAS (34). Once bound to cytosolic DNA, activated cGAS can form dimers and multimer assemblies that undergo liquid–liquid phase separation to form biomolecular condensates that amplify cGAS activation (35). Activated cGAS produces 2′-3′cGAMP (cGAMP) as a second messenger to function in both the host cell and adjacent cells *via* secretion or by passage through gap junctions, which contributes to the bystander response to radiotherapy in non-irradiated neighboring cells (36–40). In the presence of cGAMP, STING is relocated from the ER to Golgi, where it recruits and activates TANK-binding kinase (TBK1), that activates interferon regulatory factor 3 (IRF3) and NF-κB signaling (41). Activated IRF3 and NF-κB then induce transcription of innate immune response genes, including IFNs and cytokines (36, 37, 42).

Interestingly, cGAS is also found in the nucleus. Nuclear cGAS is inactivated by its acidic patch binding to nucleosome core particles, which prevents DNA binding, thus preventing autoreactivity (34). Moreover, nuclear cGAS is recruited to DNA damage sites by γH2AX, which promotes its interaction with Poly (ADP-ribose) polymerase 1 (PARP1) and impedes formation of PARP1-Timeless complex to thereby suppress HR but not NHEJ (43, 44).

Another important cytosolic DNA sensor is γ-interferon-inducible protein-16 (IFI16). Like cGAS, IFI16 can detect both self and non-self dsDNA to promote IRF3 and NF-κB -dependent interferon production *via* STING (26, 45).

Emerging data reveal that DNA repair pathways and cytosolic pathological DNA sensing pathways have overlapping effectors that recognize and respond to damaged nuclear DNA, cytosolic endogenous DNA, or foreign DNA (46). These observations provide compelling evidence for inextricable links between the DDR and innate immune responses (**Figure 1**).

DDR Deficiency or Inhibition Enhances Innate Immune Responses

Interference in DDR signaling elicits innate immune responses. One of the most well-studied examples is PARP inhibition. PARP inhibition generates cytosolic chromatin fragments and significantly potentiates cGAS-STING-dependent immune responses (11, 47–54). Similarly, DNA damage as a result of cytotoxic chemotherapy, ionizing radiation (IR), metabolism, and deficiency of other DDR elements (including BRCA2, ATM, CHK1, RPA, RAD51, TREX1 and FANCD2), also leads to increased IFN signaling–mediated immune responses (11, 19, 55–61).

The RecQ–like BLM helicase partners with EXO5 and EEPD1 nucleases for stalled DNA replication restart and maintenance of genome integrity (62, 63). BLM deficiency in Bloom syndrome (BS) causes increased expression of inflammatory genes through the cGAS–STING–IRF3 pathway, suggesting it prevents unchecked inflammatory gene responses (64). ROS from radiation therapy or cell stress lead to cGAS-STING-mediated immune responses to cancer from DSBs as well as oxidative adducts that must be removed by DNA glycosylases, such as endonuclease VIII (Nei)-like proteins (NEIL) and oxoguanine DNA glycosylase (OGG1) (65–67). Furthermore, high levels of ROS that are not efficiently reduced by superoxide dismutases and catalase can leave unrepaired 8-hydroxyguanosine (8-OHG) (68, 69). 8-OHG stabilizes DNA against degradation by the cytosolic DNA exonuclease TREX1, leading to accumulated cytosolic DNA and increased cGAS activation (70). This ROS effect can be amplified by vicious cycles of oxidative damage and iron release from ROS-sensitive 4Fe-4S co-factors in multiple replication and repair proteins (62, 71–73).

Metabolism and innate immunity converge at the mitochondria, which can orchestrate innate immune signaling pathways in different cancer-relevant metabolic scenarios including a link to PARylation and cell death (74, 75). Metabolic cues including nucleotide imbalance can stimulate the release of mtDNA from mitochondria that drives an interferon response with MRE11 playing a leading role (76). The fundamental importance of DNA breaks in promoting such immune responses is evidenced by the observation that mtDNA breaks synergize with nuclear DNA damage to mount a robust cellular immune response (77).

In general, unresolved DNA damage can act as a mediator linking the DDR and immune recognition, and this can involve the formation of micronuclei as an initiating event in a cascade promoting genomic instability and innate immune responses (78, 79). Moreover, genome instability and imperfect cell cycle checkpoints in tumor cells enhance formation of micronuclei, making them more susceptible to targeting of the innate immune response (5, 22, 79). DNA damage responses occur in minutes to hours. Yet, there is a delayed onset of days for inflammatory cytokines that modify tumor microenvironment by immune cell recruitment as critical for local and systemic (abscopal) tumor responses to radiotherapy.

DNA-PK in Innate Immune Response

DNA-dependent protein kinase (DNA-PK) is a trimeric nuclear complex that functions as a central integrator of the DSB repair

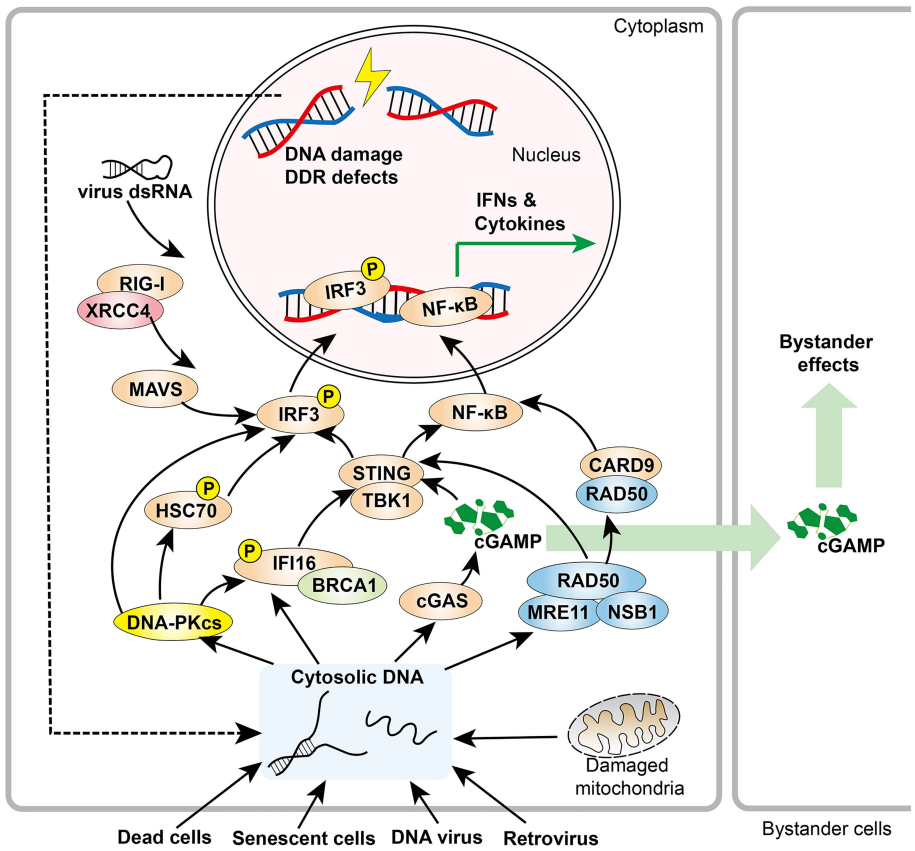

FIGURE 1 | Overview of DDR components in innate immune responses. DDR factors, including DNA-PK and MRE11, promote cytosolic DNA sensing signaling pathways. When activated by cytosolic DNA, cGAS produces cGAMP, a soluble second messenger that initiates STING-IRF3 signaling both within the host cell and adjacent cells. In addition, RAD50 associates with CARD9, leading to NF-κB activation and downstream cytokine production. XRCC4 interacts with RIG-I, which promotes the RIG-I-MAVS-IRF3 pathway.

system. The protein complex consists of a large catalytic subunit, DNA-PKcs, and the Ku70/80 heterodimer (Ku70/80) which recognizes DSB ends (80). DNA-PKcs is a Ser/Thr protein kinase and the largest member of the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family (81). Once activated by Ku70/80, DNA-PK undergoes autophosphorylation and is then positioned to phosphorylate other repair effectors and promote a synaptic complex for ligation of two dsDNA ends (80, 82–86). In recent decades, emerging evidence revealed that DNA-PK is a critical component of innate immunity against multiple viruses, including human immunodeficiency virus (HIV), Herpes Simplex Virus 1 (HSV-1), alphavirus M1, and vaccinia virus (87–92). As such, DNA-PK is a key DNA sensor that modulates innate immunity through several critical components of innate immune pathways.

In STING-dependent DNA sensing pathways, cGAS, IFI16, and IRF3 are substrates for DNA-PK (89, 93, 94). However, the role of DNA-PK within the cGAS-STING pathway remains controversial. One recent study reported that DNA-PK directly phosphorylates cGAS to suppress its enzymatic activity and thus attenuate innate immune responses (93). To this end, DNA-PKcs deficiency caused by missense mutations in its coding gene, *PRKDC*, leads to an increased inflammatory response in both human and mouse cells (93). In contrast, a pioneering study showed that DNA-PK interacts with and phosphorylates IRF-3, thus promoting its nuclear translocation (94). In a systematic profiling study, DNA-PKcs directly phosphorylated the DNA sensor IFI16 and promoted IFI16-driven cytokine responses (89). Furthermore, regardless of its partner cGAS, STING can localize to the inner nuclear membrane in breast cancer tumor samples and promote cancer cell survival by resistance to DNA-damaging agents through interacting with DNA-PK (95). Therefore, further studies are warranted to better understand mechanisms governing DNA-PK substrate selection within the context of the innate immune response.

As described above, although a potentially suppressive role of DNA-PK on cGAS was reported which may be context dependent, most studies suggest that DNA-PK promotes a STING-dependent innate immune response (96–100). Mechanistically, the HEXIM1-DNA-PK-paraspeckle components-ribonucleoprotein complex (HDP-RNP), containing DNA-PK subunits and paraspeckle proteins, is required for foreign DNA sensing through the cGAS-STING pathway. The HDP-RNP interacts with cGAS, and when

stimulated by cytosolic DNA, the paraspeckle proteins from the complex are released to recruit STING and activate DNA-PK and IRF-3. Knockdown of HDP-RNP subunits including Ku70, the DNA binding subunit in DNA-PK, resulted in loss of IFN stimulatory DNA–mediated immune response (97). In addition, Ku70 was identified as a cytosolic DNA sensor that translocates to the cytoplasm to form a complex with STING and induce production of IFN-λ1 (98, 99).

Besides STING-dependent DNA sensing mechanisms, DNA-PK also acts as a DNA sensor to trigger a robust and broad antiviral response in a STING-independent DNA sensing pathway (SIDSP) in human cells, but not in laboratory mice (101), perhaps a reflection that DNA-PK levels in human cells are much higher than in mouse cells (102–104). A recently characterized DNA-PK partner is LINP1, a lncRNA that can recruit multiple DNA-PK assemblies and promote formation of phase condensates (105). As LINP1 is present in both cytoplasm and nucleus, it will be important to test its potential role in cytosolic immune activation.

Overall, DNA-PK is considered a cytosolic DNA sensor for both STING-dependent and -independent DNA sensing pathways. The extent to which the role of DNA-PKcs in the innate immune response is distinct from its well-characterized nuclear functions in NHEJ is under active investigation.

MRN Complex in Innate Immunity

MRN, a core orchestrator that senses DSB damage and activates DNA repair cascades, is required to maintain genome integrity (13). In recent years, the MRN complex, which acts in DSB sensing, stabilization, signaling, and effector scaffolding (106), has furthermore been found to localize to viral replication sites and trigger innate immune responses (107–109).

An exemplary MRN role in regulating innate immunity comes from the meiotic recombination 11 homolog A (MRE11) nuclease subunit, which recognizes and processes DSB DNA ends as a part of HR repair, replication fork processing, and telomere length maintenance (110, 111). MRE11 has both endonuclease and exonuclease activities that, together, initiate HR repair (112). Furthermore, MRE11 functions as a key cytosolic DNA sensor in recognition of a broad spectrum of dsDNA and activates STING trafficking and type I IFN production in various cell types (108).

An intriguing observation is that nuclease activity is not required for the cytosolic DNA-sensing function of MRE11, which reinforces the notion that besides their nucleotide processing activity DDR nucleases also function to recognize and sculpt specific DNA structures (113–116). In fact, the nuclease-inactive mutant form of MRE11 triggers an even higher immune response than the wild-type form. Therefore, MRE11 may act as a regulatory switch within the STING-dependent immune response, initially functioning as a DNA sensor to activate STING-mediated signaling, then subsequently working as a nuclease to suppress excessive immune responses (108). Obviously, further studies are required to better elucidate the pro- and anti-immune–modulating mechanisms of MRE11 in STING-dependent signaling. Nevertheless, these data suggest that STING-mediated signaling may be activated by one of the existing MRE11 inhibitors (112, 117). It will also be interesting to see if the adaptor regulator GRB2 complex with MRE11, which promotes HR and suppresses A-EJ in the nucleus, plays a role in STING-mediated signaling (118). Intriguingly, multiple GRB2 molecules can also bind to Linker of Activation of T cells (LAT) to mediate its oligomerization, which is important for T-cell signaling under limiting stimulating conditions. Furthermore, GRB2 promotes metabolic reprogramming to support T cell activation (119–121). These and other data support the notion that tight protein and DNA binding plus conformational sculpting can regulate activities and switch DNA repair pathways (122).

MRE11 mutations that result in loss of binding ability to Nijmegen breakage syndrome protein 1 (NBS1) induce type I IFN comparable to wild-type MRE11 (108). This finding suggests that NBS1 is not instrumental for sensing cytosolic DNA and provoking an immune response. This concept is consistent with the mechanistic role implied by the NBS1 structure and its MRE11 interface, to flexibly restrict DNA end processing and homologous recombination activities to the vicinity of DSBs (123). On the basis of prior data showing that NBS1 loss promotes cytosolic MRE11 distribution (124), we propose that a deficiency of NBS1 may enhance cytosolic DNA sensing by MRE11.

The third component of the MRN complex is the ATP-binding cassette-ATPase (RAD50). MRE11 nuclease activity is regulated by ATP-dependent RAD50 helical coiled-coil conformations that switch the MRE11-RAD50 complex between DNA tethering, ATM signaling, and strand resection (125, 126). RAD50 plays an important role in innate immunity *via* a STING-independent signaling pathway (109). RAD50 binds a proinflammatory signaling adaptor amino-terminal caspase-recruitment domain (CARD9) through its structurally defined zinc-hook region (127). Together with MRE11, RAD50 recognizes cytosolic DNA and interacts with CARD9, which leads to the recruitment of Bcl-10 to induce NF-κB activation and pro-inflammatory cytokine IL-1β generation (109).

Other DDR Factors in Innate Immunity

BRCA1, which together with the MRN complex plays a central role in HR DNA repair, interacts with IFI16 (128, 129). In herpesvirus-infected cells, BRCA1 is required for IFI16-mediated recognition of foreign DNAs, association with STING, and subsequent IFN-β production (128). Aside from DNA virus sensing, X-ray repair cross-complementing group 4 (XRCC4), a DNA ligase IV (LIG4)-associated protein essential for NHEJ (130–132), acts in an RNA-sensing pathway through interaction with retinoic acid-inducible gene I (RIG-I) (133). XRCC4 promotes oligomerization and ubiquitination of RIG-I, which results in enhancement of the RIG-I-MAVS-IRF3-type I IFN signaling cascade and subsequent suppression of RNA virus replication in host cells. Reciprocally, RIG-I competes with LIG4 to interact with XRCC4, and therefore it impedes XRCC4-dependent NHEJ cascades and hinders retrovirus integration into the host genome by suppressing the NHEJ pathway (133). This finding highlights the critical role of XRCC4 in defense against RNA viruses and in potentiating innate immune response.

DDR IN ADAPTIVE IMMUNITY

Unlike the innate immune system, characterized by rapid sensing and elimination of pathogens as first line of defense, the adaptive immune system provides broader and more accurate discrimination between self and non-self immunogens based on the process of positive and negative selection during lymphocyte development (25). A robust adaptive immune response to any pathogen or biological macromolecule seen for the first time takes weeks to mount. However, subsequent exposure to the same pathogen promotes a rapid "memory" response that is often magnitudes stronger than the response following the first exposure. In adaptive immunity, the DDR is essential for lymphocyte development by facilitating the assembly and diversification of antigen receptor genes (134, 135). Thus, DDR deficiencies are linked with immunological disorders, including autoimmune diseases, such as systemic sclerosis, pediatric systemic lupus erythematosus, and severe sepsis (136–139).

Ataxia-telangiectasia (A-T), a disorder arising from *ATM* germline mutations, was one of the first-identified disorders whereby immunodeficiency was associated with an aberrant DDR (140–142). Missense mutations of *PRKDC*, which encodes the catalytic subunit in DNA-PK, were also found in patients with the organ-specific autoimmunity phenotype (93, 143). In addition, autoantibodies directed against Ku 70/80 were detected in autoimmune patient sera (144, 145). Indeed, Ku was first identified *via* autoantibodies in sera from patients with the autoimmune disease polymyositis-scleroderma overlap syndrome (146).

Adaptive maturation of T and B lymphocytes is guided by the "blueprint" of different cell surface receptors. During the process of lymphocyte maturation, three highly regulated processes, including variable, diversity, and joining [V(D)J] recombination, class-switch recombination (CSR), and somatic hypermutation (SHM) together with negative and positive selection (147), lead to generation of a functional, genetically diverse, and non-autoreactive antigen receptor repertoire. Interestingly, these processes naturally generate DSBs and/or trigger a DDR in adaptive immunity (**Figure 2**) (136, 148). In this section, we review these pathways highlighting roles of important DNA repair factors.

DDR in V(D)J Recombination

V(D)J recombination occurs in G1 phase of naive, progenitor T and B lymphocytes, and enables rearrangement of gene segments at both immunoglobulin and T-cell receptor loci in a lineage specific and developmental stage specific manner (148, 149). V(D)J recombination is initiated by the recombinase activating gene (RAG) endonucleases RAG1 and RAG2, which is directed by RAG recognition sequences (recombination signal sequences [RSS]) (**Figure 2A**). The RAG complex creates a nick between the coding segment and the flanking RSS which leads to a DNA hairpin at the ends of the gene segment containing the coding regions (coding-ends) and a blunt-ended DSB at the end of the RSS, so called signal-ends. Alignment of coding regions, excision, and formation of hairpin-ended coding-ends and blunt-ended signal-ends takes place within the RAG1/2 complex, aided by HMGB1 (148, 149). RAG-mediated DSBs are processed by the NHEJ machinery to assemble genes encoding immunoglobulin, and heterodimeric B- and T-cell receptors (150–152). The rapid repair of RAG-mediated DSBs by NHEJ is essential for normal lymphocyte development. Failure to repair RAG-mediated DSBs in immature B cells leads to a DDR including ATM-mediated upregulation of NF-κB signaling (134, 153–157).

DNA-PKcs in complex with Artemis, a member of the metallo-β-lactamase protein family, is required for successful V(D)J recombination and lymphocyte development. DNA-PKcs interaction is required for Artemis endonuclease and exonuclease activities for the RAG-mediated hairpin-opening step in V(D)J recombination and for 5' and 3' overhang processing in NHEJ (158). The two coding-ends, each terminating with a DNA hairpin, are released from the RAG1/2 complex first. Prior to rejoining, the DNA hairpins are opened by the Artemis-DNA-PKcs complex, which cleaves 3' to the apex of the DNA hairpin. Artemis requires DNA-PKcs for its hairpin opening activity but how this occurs is still an open question (159, 160). Nevertheless, both the interaction of DNA-PKcs with Artemis, and DNA-PKcs phosphorylation are important for Artemis activation (161, 162).

Irrespective of the mechanism, DNA-PKcs protein and Artemis are both required for opening the coding-end hairpins, as the unopened hairpins accumulate in cells lacking either Artemis or DNA-PKcs (158). Indeed, mice, dogs and horses with mutations that compromise DNA-PKcs protein levels are characterized by radiation sensitivity (due to defects in NHEJ and DSB repair) as well as severe loss of T and B cells resulting in severe combined immunodeficiency (SCID) (163–165). Kinase-dead (KD) point mutation in the catalytic domain of DNA-PKcs blocks end-ligation without abolishing hairpin opening in knock-in mouse models (166). However, hairpin opening in the DNA-PKcs-KD mice requires ATM kinase activity (166). While pathogenic *PRKDC* mutation in humans is rare, six patients with SCID and DNA-PKcs mutation have been identified, five of whom share mutation of L3062R in the C-terminal FAT domain (85). Interestingly, DNA-PKcs with the L3062R mutation maintains full catalytic activity, but the mutation appears to hinder activation of the Artemis nuclease (167). In addition, one patient with two DNA-PKcs mutations that severely impair (but do not completely ablate) catalytic activity presented both with SCID and a severe neurologic deficit incompatible with life (168). Description of this patient has led to speculation that complete loss of DNA-PK in humans is not compatible with life, and may have a unique function in neuronal development. Deficiencies in Artemis are also associated with SCID with radiation sensitivity (RS-SCID) (169–171).

Once the coding end hairpins are opened, they can be acted upon by nucleases, and extended by error prone polymerases such as V(D)J specific terminal deoxynucleotidyl transferase (TdT) and/or the more general NHEJ polymerases mu and lambda (148, 149, 172, 173). This processing of the coding-end creates additional diversity for antigen selectivity. Finally, the

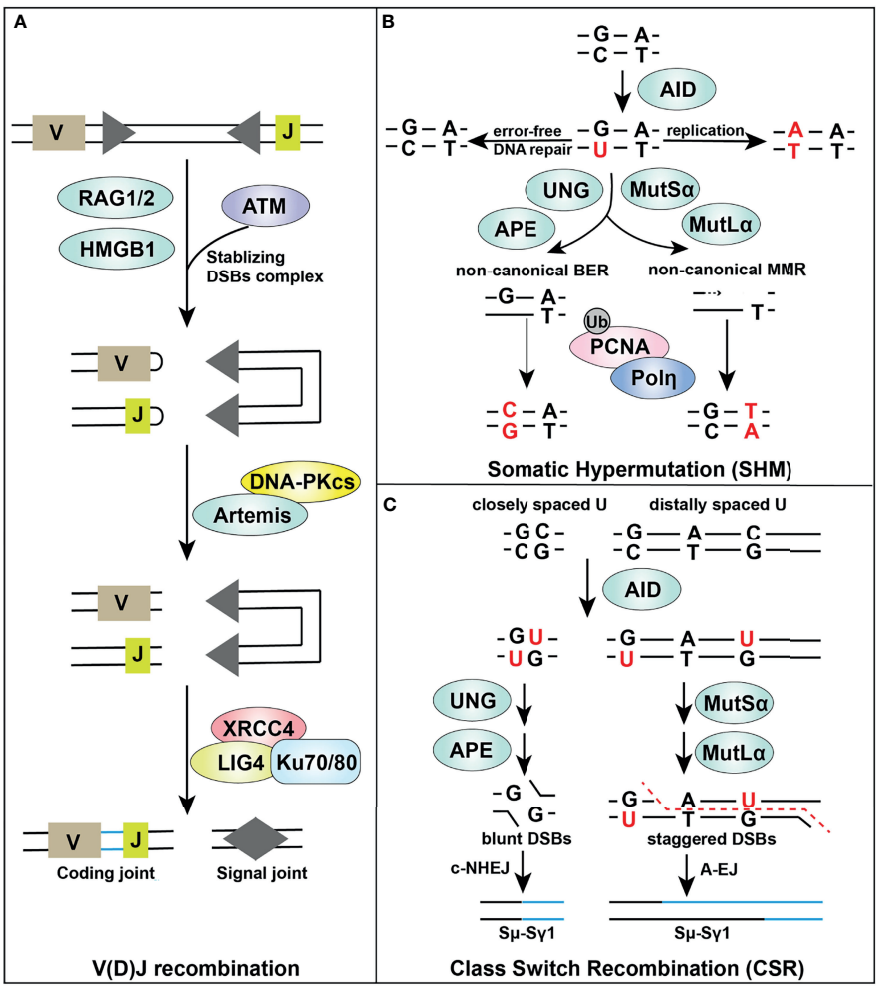

FIGURE 2 | Overview of DDR components in adaptive immune responses. Certain DDR signaling pathways, such as MMR, BER, NHEJ, and A-EJ, are required in V(D)J recombination **(A)**, SHM **(B)** and CSR **(C)** processes, supporting successful lymphocyte development.

processed coding-ends are ligated by the XLF-XRCC4-LIG4 complex in conjunction with Ku (174). The RSS signal ends are released after the coding-ends and directly ligated by the Ku-XLF-XRCC4-LIG4 complex (175–177). DNA-PKcs, but not Artemis, also plays a role in rejoining of signal ends (166, 178, 179).

Although NHEJ is required for both repairing DSBs produced by IR and those produced by the RAG endonuclease in V(D)J recombination, there are both similarities and differences between the two processes. IR introduces complex forms of DNA damage resulting in DSB ends with diverse sequences and overhanging ends, some of which will contain non-ligatable ends (180). Thus, after IR, NHEJ must be able to 1) respond to DSBs wherever they occur in the genome and 2) hold and tether the ends while they are processed before ligating them. The recently determined structures of NHEJ synaptic complexes reveal how NHEJ proteins can both tether and secure DSB ends while DNA-PKcs autophosphorylation provides a mechanism for handover to end processing enzymes and subsequent ligation by the XLF-XRCC4-LIG4 complex (82, 130, 181–184). In V(D)J recombination, defined DSBs with discrete coding-ends and signal-ends are generated and held within the RAG1/2 heterotetrameric complex (185, 186) before being released and opened by DNA-PKcs-Artemis (coding-ends) and ligated by Ku-XRCC4-LIG4 (coding-ends and signal-ends) (177, 185–187). After hairpin opening, coding ends are processed to include both additional antibody diversity (e.g. TdT) and generate ligatable ends. It will be interesting to determine how the NHEJ machinery interfaces with the RAG1/2 complex and the DNA-PK/Artemis hairpin opening complex.

While the role of Artemis in V(D)J recombination is clear, its role in NHEJ after IR is enigmatic (160). It may act to remove overhanging DNA ends, acting at ds-to-ssDNA transitions as a flap-endonuclease or by direct exonuclease activity and/or it may be required to open secondary structure elements formed by looping of ssDNA at the ends of DSBs. It is likely that Artemis is required for repairing only a subset of DSBs after IR, as Artemis-null cells are not as radiation sensitive as those lacking Ku, XRCC4, LIG4 or DNA-PKcs (148, 188–191).

Animals lacking DNA-PKcs, Artemis, Ku70 or Ku80 are viable but radiosensitive due to defects in NHEJ and immune-deficient due to defects in V(D)J recombination (192–195). For V(D)J recombination in mice lacking functional DNA-PKcs or Artemis, unopened coding-end DNA hairpins accumulate, producing a profound defect in coding joint formation (192, 193, 196). Signal joints are unaffected by loss of Artemis whereas mutation of DNA-PKcs has variable effects on signal joints (157, 175). In SCID horses signal ends are profoundly affected by DNA-PKcs mutation, while SCID dogs and mice have intermediate signal end rejoining, indicating species differences in V(D)J recombination at signal ends, possibly due to relative levels of DNA-PKcs and ATM (165). In contrast, in animals lacking Ku70 or Ku80, both coding and signal joins are affected (194, 195). Mice lacking XRCC4 or LIG4 are non-viable, with embryos undergoing neuronal apoptosis, while cells lacking XRCC4 or LIG4 are radiation sensitive and defective in coding and signal joints, consistent with a more severe V(D)J recombination defect (148, 188–190). Notably, deletion of Ku rescued the embryonic lethality, but not the V(D)J recombination defects in LIG4-null mice, likely through aberrant end-resection and the repair by the Alt-EJ pathway (197, 198).

Besides the DNA-PKcs-Artemis/Ku-XRCC4-LIG4 axis, the MRN-associated kinase ATM plays a critical role in lymphocyte development *via* direct or indirect involvement at various stages of development. Although many details are still unclear, ATM is required for stabilization of the RAG post-cleavage complex that releases the DNA ends to the NHEJ pathway (157, 199, 200). Inactivating somatic *ATM* mutations are associated with T- and B-cell lymphoma (201, 202); dysregulated V(D)J recombination results in translocations in ATM-deficient lymphocytes, potentially promoting tumorigenesis (203, 204). While XLF-deficient cells have significant V(D)J recombination, ATM kinase activity and its chromatin bound DDR factors (e.g., 53BP1 and H2AX), while dispensable for V(D)J recombination in otherwise wild type cells, become essential for chromosomal NHEJ during V(D)J recombination in XLF-deficient cells (205–207). Indirectly, ATM-related repression of GSK3β and cyclin D3 also plays an important role in thymocytes and pre-B cells (208, 209). DSBs generated by both V(D)J recombination and CSR induce ATM-dependent phosphorylation of GSK3β, which is a constitutively active kinase known to promote cell death (209, 210). The inactivation of GSK3β by DSB-initiated Ser^{389} phosphorylation protects B cells during V(D)J recombination and CSR that are required for antigen-specific IgG antibody responses following immunization. During T cell development, GSK3β phosphorylation created by V(D)J recombination also promotes survival of DN3 thymocytes undergoing TCRβ rearrangements, mimicking the results described in mice harboring deficiency in several key DDR factors, including ATM, NBS1 and H2AX (209, 211, 212).

DDR in CSR and SHM

The DDR is also essential for additional adaptive immune responses that occur after antigen exposure in germinal center B cells. V(D)J recombination-rearranged immunoglobulin (Ig) variable regions are further modified by the process of SHM, after which antibodies with highest affinity are selected. While in CSR, the constant regions of immunoglobulin genes are excised and rearranged to produce other isotypes (e.g. IgA and IgG) from the initially expressed IgM or IgD isotypes (213, 214) (**Figures 2B, C**). Both CSR and SHM are initiated by B cell-specific, activation-induced cytidine deaminase (AID), a member of the apolipoprotein B mRNA editing enzyme catalytic polypeptide like (APOBEC) family of deaminases, which converts cytosine to uracil on single-stranded DNA or RNA (215, 216). Various DDR pathways are then involved in both the generation of strand breaks and their repair.

During SHM, AID deaminates a particular trinucleotide sequence in ssDNA of transcriptionally active genes, leaving behind numerous uracil residues and producing predominantly nucleotide substitutions in rearranged V genes on the heavy- and light-chain loci, and switch (S) regions, which precede most C genes on the heavy chain locus (217, 218). The mutagenic outcome of uracil lesions can then be determined by one of the following DDR responses: (i) Uracil can act as a template for replication, resulting in a fixed C-T transition mutation; (ii) U-G mismatches can be recognized by the error-prone MMR machinery, in which the MutSα complex (MSH2-MSH6) detects the mismatch and recruits MutLα complex (MLH1-PMS2) to nick the DNA, followed by the recruitment of Polη (DNA polymerase η) to generate mutations (219, 220); (iii) Non-canonical BER initiated by uracil DNA glycosylase (UNG) can be used to recruit proliferating cell nuclear antigen (PCNA) at the lesions, and low-fidelity polymerases such as Polη, which can increase mutations during replication of common DNA fragile sites (221), then can be recruited by PCNA ubiquitination and utilized by both MMR and BER resulting in mutagenic repair (149, 222–224). The nick generated by the UNG-dependent BER pathway is particularly important for CSR, as UNG1 knock out largely abolishes CSR (225).

During CSR, DSBs are generated in the switch regions that are subsequently ligated by either the canonical NHEJ pathway or A-EJ pathway which involves XRCC1, MRE11, plus FEN1 (which threads and removes DNA flaps) and Pol theta for which there are inhibitors (114, 149, 226–230). Although many details of this important pathway remain to be determined, it has been suggested that UNG removes AID-incorporated uracil to create an abasic site which is then cleaved by apurinic/apyrimidic endonuclease (APE) to create an SSB. Two closely spaced SSBs on opposite DNA strands can create a DSB (213, 231, 232). Indeed, UNG inhibition sensitizes cells to high APOBEC3B deaminase and to floxuridine (5-FdU), which are toxic to tumor cells through incorporation of 5-FU into DNA (233, 234).

Much of what we have learned about CSR has come from disruption of DNA repair genes in mice leading to immunodeficiency characterized by the production of IgM (the first spliced constant region) but not IgA or IgG (products of CSR) (136). As reviewed recently by Zha and colleagues (191), the most dramatic defects (>90% reduction) in CSR have been

observed in mice lacking the tumor suppressor p53-binding protein (53BP1). In contrast, more modest defects (50-70% reduction) occur in animals lacking MDC1, H2AX, Ku, XRCC4, LIG4 and mice lacking DNA-PKcs, Artemis or ATM have only a minor defect (<10%) in CSR (191, 235–239). Yet, a recent report revealed that defects of 3'-flap endonuclease XPF-ERCC1 in B cells impairs A-EJ-mediated CSR by impeding joining of resected 3′ flap DSB ends (240). Since 53BP1 and Shieldin both block resection and promote NHEJ, loss of either would be expected to promote resection over NHEJ (241–245). However, even loss of LIG4, which abolishes NHEJ, decreases CSR by only 70% (236, 238). A major feature of CSR is removal of large regions of chromatin between the switch regions to be joined. 53BP1 plays a role in looping DNA at telomeres and is required for rejoining of distal joins in V(D)J recombination (246–248), suggesting that long-range conformational changes in DNA may be disrupted in 53BP1-deficient cells, possibly explaining the importance of 53BP1 in CSR (249, 250). Indeed, loss of components of the shieldin complex, which protects DSB ends to mediate 53BP1-dependent repair, also yield defects in CSR (242, 245, 250–252). Alternatively, 53BP1 also recruits PTIP, an evolutionarily conserved chromatin regulator that binds γH2AX, acts as a major effector of ATM and ATR signaling mechanisms, and is also implicated in CSR (253, 254).

Besides the direct usage of DDR pathways, there are several indirect links between DDR elements in CSR and SHM. For instance, targeting HR by RAD51 inhibitor reduces AID expression, hampering the repair of AID-initiated lesions (255). Interestingly, indirect links reach into RNA-binding proteins such as the autism-associated protein vigilin, which interacts with RAD51 and BRCA1, so its depletion impairs their recruitment to DSB sites (256).

DDR INHIBITION IN ANTITUMOR IMMUNITY

Emerging evidence supports the idea that DDR inhibition in tumor cells remodels the inflammatory microenvironment (10, 257). Impaired DDR typically enhances the tumor foreignness by increasing the number of tumor cell mutations/neoantigens (10, 258). When examined by CIBERSORT analysis through the TIMER2.0 web server (259, 260), the mRNA levels of many DDR factors, such as *RPA1*, *Ku70*, *Ku80*, *MRE11A*, *RAD50*, *NBS1*, *PRKDC*, *RAD51*, *PARG* and *XRCC4*, were negatively associated with cytotoxic CD8+ T cells infiltration levels across various cancer types (**Figure 3A**). Indeed, as exemplified in prostate adenocarcinoma, significant negative correlations between gene expression and cytotoxic T cell infiltration levels were found in 19 genes of the 22 DDR related genes we tested (**Figure 3A, B**). These findings suggest enhanced anticancer immunity in tumors with lower DDR factor expression and imply substantial potential benefits from DNA repair inhibitors. Thus, inhibitors of these DDR factors, such as poly(ADP-ribose) glycohydrolase (PARG) inhibitors that impact DNA break repair and replication fork restart, may be employed to activate the innate immune response (261).

Antitumor immune responses can be promoted and utilized to treat cancer *via* immune checkpoint blockade with use of agents such as PD-1/PD-L1 and CTLA-4 inhibitors (29, 262, 263). The DDR also offers attractive targets for inhibition (264, 265). Preclinical and clinical efficacy of DDR inhibition in cells with a defective DDR genetic background, are exemplified by the success of PARP inhibitors in *BRCA1/2*-mutated advanced cancers and of inhibitors to the PARG in cancer cells (261, 266, 267). Emerging evidence has progressively unveiled the involvement of the DDR in antitumor immunity by enhancing STING-dependent immune responses, further supporting the immune-modulatory role of DDR inhibition in anticancer immunity (**Figure 4**) (134, 135, 268, 269).

The most studied DDR inhibitors in anticancer immunotherapies are those directed against PARP (PARPi). In line with the usage of PARPi in DDR-deficient tumors (266), PARPi combined with immune checkpoint blockade, including PD-1/PD-L1 and CTLA-4, exerts remarkable efficacy in tumors with BRCA1/2 or ERCC1 mutations *via* STING-dependent immune responses and infiltration of cytotoxic T cells into tumor (50, 51, 54, 270). There are also findings suggesting that PARPi, with anti-PD-1 inhibitors, have strong therapeutic potential regardless of BRCA1/2 status (49, 271, 272), although the mechanisms involved remain unclear. Besides the STING-dependent pathways, PARPi also increased PD-L1 expression in breast cancer cell lines through inhibition of GSK3β (273), which provided the rationale for combining PARPi with PD-L1 or PD-1 immune checkpoint blockade, a strategy that has been tested in clinical trials (49, 271, 274).

Recently, many other inhibitors targeting DDR components have been developed and are in preclinical study. Recently, several of them, including inhibitors of DNA-PKcs, ATM, ATR, CHK1 and WEE1, have entered into clinical trials (275). Inhibitors of DNA-PKcs promote radiation sensitization through inhibition of NHEJ (276). Their importance in modulating the innate immune response have also been demonstrated. ATR inhibition can further increase cGAS-positive micronuclei and cytokine production in PARPi-treated cancer cells (12). Significantly, inhibition of DNA-PK with AZD7648 resulted in IFN-dependent inhibition of tumor growth following IR in immune competent mouse models, indicating that inhibition of DNA-PK in combination with radiotherapy could lead to durable immune-mediated tumor control in cancer patients (277).

Another important application of DDR in antitumor immunotherapy is the usage of the DDR status as biomarkers to select the patients who are targetable to immune checkpoint blockade. Currently, only a subset of patients respond to immune checkpoint blockade. Predictive biomarkers for reliable response could better guide therapeutic choices (104). As DNA repair deficiencies that promote genome instability are relatively common among tumors, mutational signatures and DDR biomarkers may identify features associated with response to immune-directed therapies. For instance, MMR

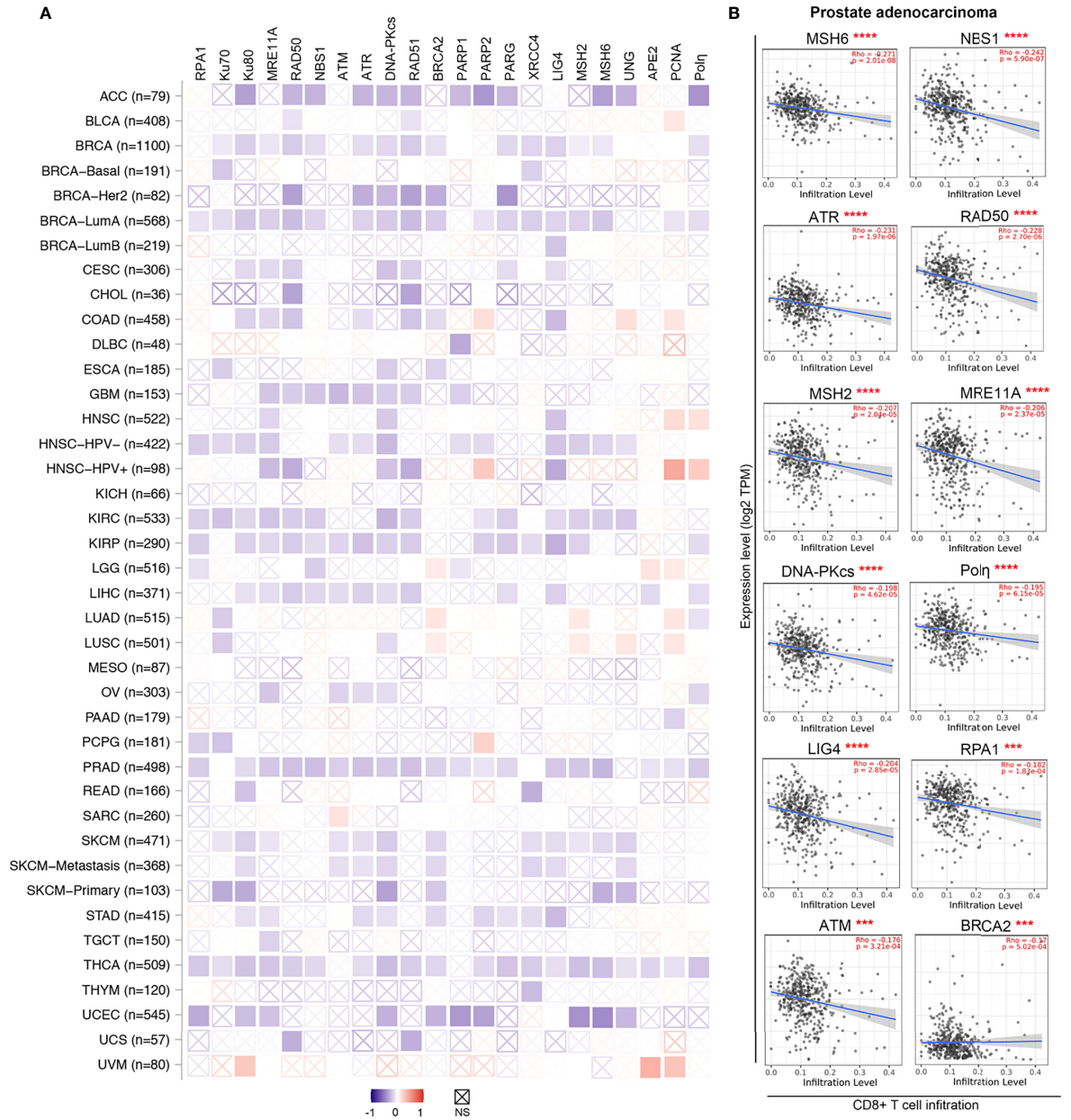

FIGURE 3 | DDR factors negatively associate with CD8+ T cells infiltration levels in diverse cancer types. **(A)** A heatmap based on the CIBRSORT method shows the purity-adjusted Spearman's rho of DDR factors with CD8+ T cells across various cancer types. The boxes with indicate non-significant p values (p>0.05). The figures was made using the TIMER2.0 web server based on CIBRSORT analysis (http://timer.cistrome.org/). **(B)** Detailed correlation between DDR factors and CD8+ T cells in prostate adenocarcinoma (PRAD) from panel **(A)** The purity-adjusted Spearman's rho and p value are labeled in red. ***p < 0.001; ****p < 0.00001.

status was reported to predict response to the PD-1 inhibitor pembrolizumab in a phase 2 study of 41 patients with progressive metastatic carcinoma (278, 279). Also, loss of BRCA1 and defects of MMR in tumors resulted in many somatic mutations, leading to continuous renewal of neoantigens, increased immune response gene expression, and enhancement of immune surveillance (20, 270, 278, 280). In non–small cell lung cancer, deleterious mutations in several DDR-related genes correlated with pembrolizumab clinical efficacy (281). A high mutation level causing a high load of tumor neoantigens suppresses immune evasion. Whereas aneuploidy of large chromosomal regions (arm and whole-chromosome), which cause somatic copy number alterations (SCNAs) and consequent protein imbalances, can weaken cytotoxic immune cell infiltration (282). Importantly, blockade of the immune system PD-1/PD-L1 inhibitory pathway can restore exhausted immune responses as an effective immunological strategy to overcome immune evasion by chronic imbalances and infections (283). For

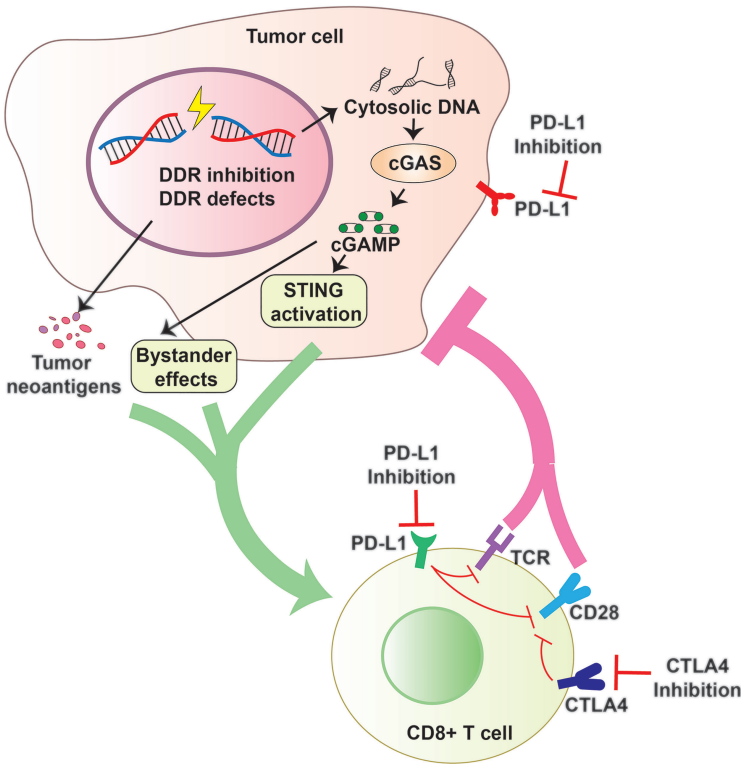

FIGURE 4 | DDR Inhibition and Antitumor Immunity. DDR Inhibition and DDR defects can increase cytosolic DNA that activates the cGAS to generate cGAMP and promote tumor neoantigen production. cGAMP can activate cell intrinsic STING pathway and spread the immunity to bystander cells. All these factors contribute to an inflammatory tumor microenvironment and promote the recruitment of cytotoxic CD8+ T cells and constrict cancer growth effectively. Combining DDR inhibition (such as PARP or PARG inhibition) with Immune checkpoint blockade (including PD-1/PD-L1 or CTLA4 blockade) may be a promising strategy with the potential to improve survival outcomes.

monoclonal antibodies used to block checkpoint molecules, such as PD-1 and PD-L1, to activate immune cells to kill tumor cells more effectively, it may be worth adding designed features such as metal ion binding sites to add to their capabilities or removing free cysteines to improve their stability (284–286).

SUMMARY AND PROSPECTS

The DDR shapes how the innate immune system responds to tumors, as well as how the adaptive immune system is recruited to sites of malignancy. Consequently, the interconnections of the DDR and the immune system, which maintain genomic fitness and pathogen protection, can be utilized to improve cancer therapeutic strategies (5, 135, 287–291). Yet, defining how the DDR impacts immune responses has remained challenging as immune activation can evidently be triggered by different types of DDR components including DNA damage sensors, transducers, and effectors (292).

Here, we assessed current molecular and mechanistic data showing how the DDR induces and impacts immune responses. At present, cancer immunotherapy is less widely used than surgery, chemotherapy, or radiation therapy. As only a subset of patients respond to immune checkpoint blockade, enhancements from defining and modulating the DDR along with reliable predictive biomarkers of response are needed to guide and improve therapeutic strategies. DNA repair deficiency is common among tumors, and emerging experimental and clinical evidence suggests that features of genomic instability are associated with response to immune-directed therapies. We propose that advancing all successful cancer therapies will benefit from elucidating key molecular and mechanistic relationships linking DDR, DNA damage outcomes, and immune responses. In fact, the efficacy of conventional chemotherapy and radiotherapy can depend in part upon induction of innate and adaptive immunity.

In innate immunity (**Figure 1**), MRN (along with its associated ATM and ATR kinases) and DNA-PK complex, which co-regulates DNA DSB repair, can serve as master cytosolic DNA sensors to initiate innate immune response. DNA-PKcs expression with validated immune biomarkers can guide patient selection for DNA-PKcs targeting strategies, DNA-damaging agents, and their combination with an immune-checkpoint blockade (293). Analogously, ATM inhibition induces tumor growth delay and overcomes tumor resistance to anti–PD-1 therapy (294). In addition, other DDR components interact with and promote cytosolic DNA sensing pathways or RIG-I–mediated RNA sensing signaling to trigger innate immune response. Whereas mice and other model systems

have proven to be of great value for testing these molecular mechanisms, it is critical to consider possible impacts from the far higher DNA-PKcs levels in human cells compared to laboratory mice (104).

Most immune-related DDR components and immune responses converge upon the STING-IFN signaling pathway, which plays a crucial role in cancer cell immune-surveillance. In adaptive immunity (**Figure 2**), DDR pathways (including MMR, BER, NHEJ, and A-EJ) are required for V(D)J recombination, CSR and SHM processes, which are critical to lymphocyte development. From a pathology standpoint, DDR modulates anticancer immunity *via* both innate and adaptive immunity, with the underlying molecular mechanisms being increasingly defined. Such knowledge is likely broadly applicable to human disease, including cancer, infectious disease and atherosclerotic disorders. For instance, SARS CoV-2 proteins, can hijack the human immune response to pathogens and the DNA damage repair system, thereby damaging both innate and adaptive immunity (295, 296). Furthermore, the results of targeting endonuclease V, a ROS response and structure-specific nuclease that cleaves DNA and RNA at inosines as a regulator of innate immune responses, suggests blocking such DDR-related epitranscriptomic modifications to ameliorate carotid atherosclerosis and ischemic stroke (297–299).

For advanced immunotherapeutic strategies, DDR defects plus the increased mutation load in tumor cells produce tumor-specific neoantigens. So chemical tools to alter the DDR in predetermined ways can leverage the full power of cancer immunotherapy. Importantly, advances in structural biology for combining atomic resolution structures with X-ray scattering and computation for solution conformations and assemblies are providing critical enabling methods to define and target dynamic complexes that can generally control mutation rates (66, 300–302). We propose here that the dynamic DNA-PK and MRN-activated ATM and ATR are potential master keys to unlock DDR and their immune system roles. As DNA-PKcs, ATM, and ATR inhibitors are already being evaluated in clinical trials as sensitizers of chemotherapy and radiotherapy, we suggest that these kinases may be both a predictive biomarkers and therapeutic targets for immunotherapy in future clinical trials.

To effectively use such master keys, it will be important to better define the molecular mechanisms orchestrating their activities in DDR and immune system outcomes and their potential as biomarkers for prognosis. We know that with molecular mechanistic knowledge, examination of DDR status can provide informed predictive biomarkers for patient selection and therapeutic approaches (135). Moreover, like immune checkpoint inhibitors, DDR inhibition strategies show great potential to improve cancer treatment efficacy by harnessing their immunomodulatory effects for radiation and chemotherapies, immune checkpoint blockade, and combined therapeutic strategies.

AUTHOR CONTRIBUTIONS

ZY and YS contributed equally to write the original draft. JT and SL-M contributed equally to the conceptualization and revising. All authors listed have made direct and substantial contribution to this work, and approved the final manuscript.

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GLOSSARY

5-FdU	floxuridine
53BP1	p53-binding protein
8-OHG	8-hydroxyguanosine
A-EJ	alternative end joining
AgR	antigen receptor
AID	activation-induced cytidine deaminase
APE2	Apurinic/apyrimidinic endodeoxyribonuclease 2
APOBEC	apolipoprotein B mRNA editing enzyme catalytic polypeptide like
ATM	ataxia telangiectasia mutated
A-T	Ataxia-telangiectasia
BCL10	B cell CLL/lymphoma 10
BER	base excision repair
BLM	bloom syndrome RecQ like helicase
BRCA1/2	breast-cancer susceptibility gene 1/2
BS	bloom syndrome
CARD9	caspase-recruitment domain
CHK1	checkpoint kinase 1
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
cGAMP	2'-3'cGAMP
cGAS	cyclic GMP-AMP synthase
CSR	class-switch recombination
DAMPs	damage-associated molecular patterns
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DSBs	double-strand breaks
EEPD1	endonuclease/exonuclease/phosphatase family domain containing 1
dsDNA	double-stranded DNA
ERCC1	excision repair cross complementary gene 1
EXO1	exonuclease 1
EXO5	exonuclease 5
FANCD2	Fanconi anemia complementation group D2
FEN1	flap structure-specific endonuclease 1
GSK3β	glycogen synthase kinase 3 beta
GRB2	growth factor receptor bound protein 2
H2AX	H2A histone family member X
HDP-RNP	HEXIM1-DNA-PK-paraspeckle components-ribonucleoprotein complex
HIV	human immunodeficiency virus
HMGB1	high mobility group box 1
HR	homologous recombination
HSPA8	heat shock protein family A (Hsp70) member 8
HSV-1	herpes simplex virus 1
Ig	immunoglobulin
IRF3	interferon regulatory factor 3
IFI16	IFN-inducible protein 16
IFN	interferon
LAT	Linker of Activation of T cells
LIG4	DNA ligase IV
LINP1	lncRNA in nonhomologous end joining (NHEJ) pathway 1
IL7	interleukin 7
IR	ionizing radiation
MAVS	mitochondrial antiviral signaling protein
MDC1	Mediator of DNA damage checkpoint 1
MMR	mismatch repair
MLH1	MutL homolog 1
MRE11	meiotic recombination 11 homolog 1
MRN	MRE11-RAD50-NBS1
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MUTY	MutY DNA glycosylase
NBS1	Nijmegen breakage syndrome protein 1
NEIL	endonuclease VIII (Nei)-like proteins
NER	nucleotide excision repair
NF-κB	nuclear factor kappa B subunit 1
NHEJ	non-homologous end joining
OGG1	oxoguanine DNA glycosylase
p53	tumor protein p53
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PARPi	PARP inhibitors
PAMPs	pathogen associated molecular patterns
PCNA	proliferating cell nuclear antigen
PD-1/PD-L1	programmed cell death protein 1/ programmed cell death ligand 1
PIKK	phosphatidylinositol 3-kinase (PI3K)-related kinase
PMS2	PMS1 homolog 2
Polη	DNA polymerase η
pre-BCR	pre-B cell receptor
PRRs	pattern recognition receptors
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
RAD50	ATP-binding cassette (ABC)-ATPase 50
RAD51	ATP-binding cassette (ABC)-ATPase 51
RAG	recombinase activating gene
RIG-I	retinoic acid-inducible gene I
RPA	replication protein A
ROS	reactive oxygen species
RSS	recombination signal sequences
SARS CoV-2	severe acute respiratory syndrome coronavirus-2
SCID	severe combined immunodeficiency
SHM	somatic hypermutation
SCNAs	somatic copy number alterations
SIDSP	STING-independent DNA sensing pathway
SSBs	single-strand breaks
STING	stimulator of interferon genes
TBK1	TANK-binding kinase 1
TCRβ	T cell receptor beta
TdT	terminal deoxynucleotidyl transferase
TREX1	three prime repair exonuclease 1
WEE1	WEE1 G2 checkpoint kinase
XLF	XRCC4-like factor
XRCC1	x-ray repair cross-complementing group 1
XRCC4	x-ray repair cross-complementing group 4
UNG	uracil DNA glycosylase
V(D)J	variable, diversity, and joining.

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REVIEW
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The Emerging Roles of Pellino Family in Pattern Recognition Receptor Signaling

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The Pellino family is a novel and well-conserved E3 ubiquitin ligase family and consists of Pellino1, Pellino2, and Pellino3. Each family member exhibits a highly conserved structure providing ubiquitin ligase activity without abrogating cell and structure-specific function. In this review, we mainly summarized the crucial roles of the Pellino family in pattern recognition receptor-related signaling pathways: IL-1R signaling, Toll-like signaling, NOD-like signaling, T-cell and B-cell signaling, and cell death-related TNFR signaling. We also summarized the current information of the Pellino family in tumorigenesis, microRNAs, and other phenotypes. Finally, we discussed the outstanding questions of the Pellino family in immunity.

Keywords: Pellino1, Pellino2, Pellino3, TLR, NLR, RIP, PRRs

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INTRODUCTION

Immune responses are mainly divided into innate immunity and acquired immunity. Innate immunity can respond rapidly to pathogens as the first line of defense mediated by macrophages, dendritic cells, neutrophils, epithelial, and endothelial cells. It utilizes germ-line encoded pattern recognition receptors (PRRs) to detect conserved microbial components known as pathogen-associated molecular patterns (PAMPs) or endogenous 'alarmins' released during infection and inflammation. Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene I-like receptors (RLRs), C-type lectin receptors (CLRs), DNA sensors, and melanoma 2-like receptors are not part of the PRRs (AIM-2-like receptors). Mammalian TLRs recognize bacteria and nucleic acids and sense inflammation caused by bacteria through binding to ligands on the cell surface and in the nuclear body. NLRs and RLRs, on the other hand, detect nucleic acids in the cytosol (1–3). PRRs mediate their biological functions by activating transcription factors such as nuclear factor−κB (NF-κB), activator protein-1 (AP-1), and interferon-regulatory factors (IRFs) to drive proinflammatory and interferon (IFN) gene expression (1–4).

Ubiquitination, a posttranslational modification involving the conjugation of the 76 amino acid proteins to the lysine residue of other proteins, is catalyzed by the sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. Ubiquitin contains seven lysine residues and one N-terminal methionine (M1) residue, each of which can be attached to another ubiquitin moiety. The presence of these lysine residues and the M1 forms a variety of ubiquitin chains (K6-, K11-, K27-, K29-, K33-, K48-, K63-, M1-linked ubiquitin chains and mixed ubiquitin chains), which are recognized by substrate proteins with linkage-specific ubiquitin-binding domains to trigger multiple biological functions such as K48- and K11-linked

chains for protein degradation, M1 or K63-linked chains for signal transduction (5). The substrate specificity of ubiquitination is mainly determined by E3s, which directly catalyzes ubiquitin transfer from E2s to the substrates (6). In particular, the Pellino family, a novel E3 ubiquitin ligase family (7–9), has been implicated in the regulation pattern recognition receptors (PRRs) signaling pathway of immunity.

Pellino (*Drosophila* Peli, Human PELI, Mouse *Peli*), first discovered in *Drosophila*, is a novel and evolutionarily conserved protein with 424 amino acid residues and an estimated molecular weight of 47 kDa (10). The Pellino family-related sequences are conserved in different species (11, 12). The identical sequence shared between *C. elegans* and *Drosophila* is 47%, and between *C. elegans* and Human is 40% (12). In mammals, the Pellino family has three sequence-conserved members, Pellino1 (13), Pellino2 (14), and Pellino3 (two splicing variants Pellino3a and Pellino3b) (15), located on chromosomes 2, 14, and 11 (13) respectively, with an amino acid length ranging from 418 to 479 (16). Mouse Pellino1 and Pellino2 possess 75% sequence similarity, whereas Pellino3 shares 84 and 85% similarity with Pellino1 and Pellino2, respectively (13). Each member of the Pellino family shows a highly similar primary structure with a C-terminal RING-like domain mediating K11, K48, and K63 linked conjugation of polyubiquitination (7) and a cryptic phosphothreonine-binding N-terminal hidden split forkhead associated (FHA) domain attached by a "wing" or appendage structure (16) (**Figure 1**). The "wing" can interact with phosphothreonine residues of proteins such as interleukin-1 receptor-associated kinase 1 (IRAK1) and interleukin-1 receptor-associated kinase 4 (IRAK4), which in turn phosphorylate Pellino1, Pellino2, and Pellino3 (9, 16, 24, 28–31).

The Pellino family was thought to be a kind of "scaffolding" protein in the signaling process of Toll-like receptors and interleukin-1 receptors (TLR/IL-1R) (15) by interacting with multiple intermediates such as IRAK4, IRAK1, TGF-beta activated kinase 1 (TAK1), TAK1 binding protein 1 (TBK1), receptor-interacting protein kinase (RIPK or RIP) and TNF receptor-associated factor 6 (TRAF6) (15, 32–38). Subsequent research showed that the Pellino family acted as a novel interesting new gene (RING) E3 ubiquitin ligases (7, 14, 39) rather than scaffold proteins (15). Similar to classical C3HC4 RING structure, the carboxyl termini of the Pellino family possesses two stable Cys-Gly-His motifs and two conserved Cys-Pro-X-Cys motifs, which determine and characterize the feature of the RING class of E3 ligase (12). The Pellino family exerts their E3 ubiquitin ligase activity through its phosphorylation form. Some proteins can phosphorylate the Pellino family, such as IRAKs (IRAK1 and IRAK4), TAK1, TBK1, and IκB kinase ϵ (IKKϵ) (15, 32–36). Upon stimulation by interleukin-1(IL-1), tumor necrosis factor α(TNFα), lipopolysaccharide (LPS) or polyinosinic–polycytidylic acid [poly (I:C)] (18, 35), Pellino1 can be fully activated by phosphorylation at some different sites (Ser-76, Thr-86, Thr-288, or Ser-293) or a combination of other sites (Ser-78, Thr-80, and Ser-82) (18, 31). As a critical family of E3 ubiquitin ligases, the Pellino family can mediate K11, K48, and K63 linked polyubiquitination (7). Pellino1 can combine with E2 conjugating complex ubiquitin-conjugating enzyme 13 (Ubc13)–ubiquitin E2 variant 1a (Uev1a) to catalyze the formation of Lys63-linked polyubiquitin (K63-Ub) chain, with UbcH3 to catalyze the formation of K48 polyubiquitination chain(K48-Ub), and with UbcH4, UbcH5a or UbcH5b to catalyze the formation of K11 and K48 polyubiquitin ubiquitination chains (30). Inducing the formation of K63-Ub chains to ubiquitylate IRAK1, IRAK4, myeloid differentiation factor88 (MyD88), receptor-interacting protein kinase1 (RIP1), and receptor-interacting protein kinase 2 (RIP2) (14, 22, 26, 30, 36, 37, 39, 40) demonstrate that Pellino family is a novel RING E3-ubiquitin ligase (14, 39). In addition to interacting with IRAK4, IRAK1, TAK1, TBK1, and TRAF6 (15, 32–36), each member has unique binding partners. Pellino-1, but not Pellino2 or Pellino3, has been reported to interact with MyD88 (20) and TBK1 (35). Similarly, only Pellino3 was associated with NF-κB-inducing kinase (NIK) (15, 39). Some other proteins can also interact with the Pellino family, such as Smad6/7 (20, 21), BCL10 (23), and caspase-8 (26, 27) (**Figure 1**). Upon diverse stimulation, the key biological and cellular function of the Pellino family has been identified in the innate immune system (17, 31, 41–43), namely, initiating NF-κB (44) and mitogen-activated protein kinase (MAPK) (22) to regulate the production of inflammatory cytokine and interferons (IFNs) (41), mediating cell death *via* receptor-interacting serine/threonine kinases (RIPs), and other phenotypic changes of cells and tissues (45–47).

THE ROLES OF THE PELLINO FAMILY IN PATTERN RECOGNITION RECEPTOR SIGNALING

IL-1R, TLRs, and NLRs were involved in innate immunity to mediate the production of inflammatory cytokines (48, 49) and interferons (50). Each member of the Pellino family is crucial to PRR signaling pathways. We divided these pathways into five categories: (i) MyD88-dependent TLR/IL-1R signaling, (ii) TRIF-dependent interferons induction signaling, (iii) RIP-dependent signaling, (iv) NLR-related signaling, and (v) B-cell and T-cell signaling due to some key proteins, i.e., Myd88, TRAF6, TAK1, Toll/IL-1 receptor domain-containing adaptor inducing interferon-beta (TRIF), TBK1, RIPs, and NLRs in the signaling conduction (51).

In the *Drosophila* genome, Pellino interacts with and regulates plasma membrane MyD88-K48-Ub turnover to balance Toll-mediated immune signaling positively or negatively (52, 53). An ancestral Pellino protein from helminth species binding and poly-ubiquitinating human IRAK1 displays its E3 ligase activity and conservative function (54). In mammals, the production of proinflammatory interleukin-1 β (IL-1β), IL-6, C-X-C motif chemokine ligand 8 (CXCL8), and IFNs regulated by Pellino1, Pellino2, and Pellino3 demonstrate the key roles of the Pellino family in TLR/IL-1R signaling (25, 55–57). All of the TRIF, RIP1, RIP3, NLRs, and the Pellino family participate in the activation of NF-κB and MAPK/ERK kinase kinases (MEKKs) signaling to regulate cell survival, apoptosis, and necroptosis (4, 25, 37, 38, 57).

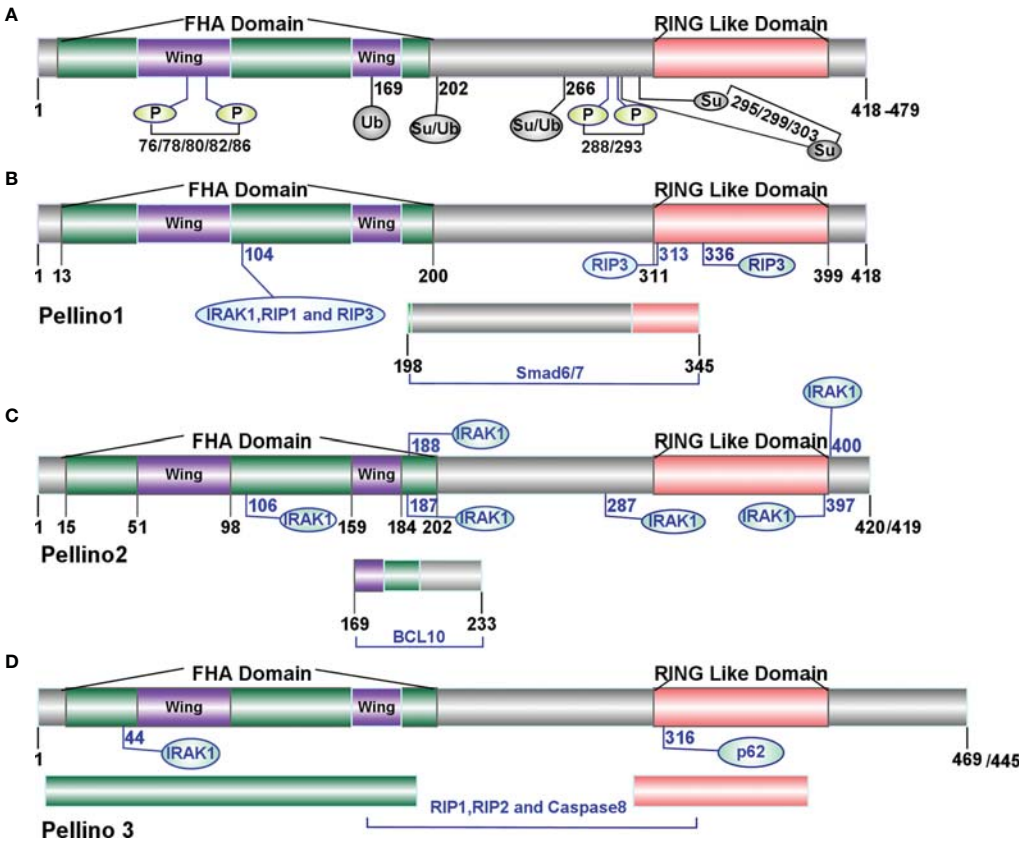

FIGURE 1 | Molecular features of the Pellino family (9). **(A)** The structure of the Pellino family. In mammals, the Pellino family comprises three family members (Pellino1, Pellino2, and Pellino3) with an amino acid length ranging from 418 to 479. The Pellino family shows a highly similar primary structure with a C-terminal RING-like domain mediating K11, K48, and K63 linked conjugation of polyubiquitination and a cryptic phosphothreonine-binding N-terminal hidden split forkhead associated (FHA) domain attached by a "wing" or appendage structure. IRAK1 and IRAK4 can phosphorylate the Pellino family on Ser-76, Ser-78, Thr-80, Ser-82, and Thr-86. Individual site Ser-76, Thr-86, Thr-288, or Ser-293 or a combination of Ser-78, Thr-80, and Ser-82 is necessary to activate the Pellino family (17) fully. IKKε/TBK1 activates Pellino1 *in vitro* by phosphorylating Ser76, Thr288, and Ser293 (18). **(B)** The sites of Pellino1 interacting with other proteins. The amino acids length of Pellino1 is 418 in both humans and mice. The 104th site of the FHA domain and 313th/336th sites of the RING-like domain are crucial to K48-linked polyubiquitylation of IRAK1, RIP1, and RIP3 (19). The region between residues 198 and 345 is essential to the interaction between Pellino1 and Smad6/7 (20, 21). **(C)** The sites of Pellino2 interacting with other proteins. The amino acids length of Pellino2 is 420 in humans and 418 in mice. The points 106, 187, 188, 287, 397, and 400 are essential to the interaction between Pellino2 and IRAK1 (16, 22). The range between residues 169 and 233 is essential to the interaction between Pellino2 and BCL10 (23). **(D)** The sites of Pellino3 interacting with other proteins. The amino acid length of Pellino3 is 469 in humans and 445 in mice. Residue 44 is essential for the binding of Pellino3 to IRAK1 (24). Residue 316 is essential to Pellino3 autophagy-dependent degradation *via* p62 (25). The FHA and RING-like domains are responsible for the interaction between RIP1 and caspase-8 (26, 27).

Pellino Family in MyD88-Dependent TLR/IL-1R Ssignaling

TLR/IL-1R family possesses an intracellular conserved Toll/IL-1R (TIR) domain which can allow the recruitment of the adapter MyD88 for the transduction of signals (58). In this section, we mainly focus on the function of the Pellino family in MyD88-dependent TLR/IL-1R signaling.

Pellino Family in MyD88-Dependent IL-1R Signaling

IL-1 is an important endogenous pyrogen and proinflammatory cytokine that can regulate hematopoiesis, recruit and activate neutrophils, macrophages, T and B-lymphocytes, and mediate inflammatory responses (59, 60). IL-1 induces signal conduction *via* IL-1R and IL-1R-accessory proteins to recruit MyD88-dependent signaling cascades, namely, IRAK4, IRAK1, IRAK2, TRAF6, and TAK1, that leads to the activation of the MAPKs and NF-κB (4, 61).

Pellino1, Pellino2, and Pellino3 can interact with IRAK1, TRAF6, and TAK1 (8, 32–34). Being upstream of TAK1 and downstream of IRAK1, Pellino1 is critical for the IL-1R-MyD88 dependent pathway through interaction with the IRAK4–IRAK1–TRAF6 complex (33). During this process, the catalytic activity of IRAK1 and IRAK4 is required for IL-1-stimulated activation of Pellino1 in Mouse embryonic fibroblasts (MEFs) (35). Aside from Pellino1, Pellino2 also interacted with IRAK4 (14, 57). Pellino3 physically interacts with IRAK1, TRAF6,

TAK1, and NIK in HepG2 and 293 cells in an IL-1-dependent manner (15). Pellino1 and Pellino2 can replace TRAF6 to generate K63-Ub chains, activate TAK1, or induce IL-8 production *via* MyD88-IL-1β signaling in IL-1R cells that express E3 ligase-inactive TRAF6 (40).

The Pellino family is associated with inflammatory mediator production (35, 62). Pellino1 knockdown can lead to a reduction in IL-1β-induced expression of proinflammatory cytokines in the bronchial epithelial cells (BEAS-2B) (62) and inhibit IL-1-mediated NF-κB activation and thus repress the production of IL-8 (33). Furthermore, *Drosophila* mothers against decapentaplegic protein 6 (Smad6) and Smad7 can bind to Pellino1 *via* mad homology (MH2) domains to mediate growth factor-β (TGF-β). It inhibited IL-1R signaling by preventing Pellino1 from forming a complex with MyD88, IRAK1, IRAK4, and TRAF6, which further suppressed IL-1β induced NF-κB activation and production of proinflammatory cytokines (20, 21). Evidence shows that Pellino1 plays a critical role in IL-1R signaling *via* MyD88, leading to NF-κB activation and proinflammatory cytokine expression. However, this conclusion is contradictory to other studies. Due to indistinct variations in NF-κB activity and expression of TNF-α, IL-6, or C-X-C motif chemokine ligand 10 (CXCL10) in mouse embryonic fibroblasts between wild type (WT) and Pellino1 knockout (KO) mice, Pellino1 is overlooked or unnecessary for the IL-1R pathway (37). A similar phenomenon can be observed in Pellino1 knockdown airway primary epithelial cells with the insignificant expression of proinflammatory cytokine CXCL8 induced by IL-1 (62). Furthermore, inactive-IRAK1induces Pellino1 significantly impaired E3 ubiquitination ligase activity with a modest effect on MAPK and NF-κB activation upon IL-1 (31). All the results indicate that Pellino1 may not be necessary for inflammation production in the MyD88 dependent IKK–NF-κB activation pathway. Whether Pellino1 is necessary for IL-1R may be controlled by cell type. Pellino2 also plays a critical role in IL-1R-mediated inflammatory production and post-transcriptional control (22), and it may be a positive regulator in the IL-1R pathway. The successive K63 and K48 ubiquitination of IRAK1 and TAK1 are required for Pellino2 to regulate IL-8 promoter activity by an NF-κB-dependent manner in the human embryonic kidney (HEK) 293-EBNA cells and the mouse embryonic fibroblast cell line C3H10T1/2 (22, 32). Upon K63 ubiquitination (22) of IRAK1 by Pellino2, the intermediate complex Pellino2–IRAK4–IRAK1–TRAF6 interacts with membrane-bound pre-associated TAK1-TGF-β activated kinase 1/MAP3K7 binding protein 1 (TAB1)-TAB2, which results in the formation of complex II (TAK1 complex, IRAK–TRAF6–TAK1–TAB1–TAB2), and IRAK1 degradation, induced by K48-linked ubiquitination of degradation. This is followed by translocation of TRAF6–TAK1–TAB1–TAB2 (complex III) from the membrane to the cytosol. TAK1 is activated and eventually leads to transcription factors activation of NF-κB, AP-1, and Elk-1in MAPKs (8, 22, 34). Pellino3 can also participate in the IL-1R signaling in HepG2 and 293 cells in an IL-1-dependent manner (15). However, Pellino3b activates JNK leading to the activation of c-Jun and Elk-1 (8, 15), and activates p38MAPK leading to cAMP-response element-binding protein (CREB) activation (24) instead of NF-κB (15). Mechanistically, upon IL-1 stimulation, upregulated Pellino3b interacts with and inhibits TAK1 complex releasing from membrane to cytosol, leading to attenuation of TAK1-dependent NF-κB activation due to Pellino3b induced K63-polyubiquitination and IL-1 induced K48 polyubiquitination competing for IRAK1-K134 ubiquitination site (8). Pellino3 activates p38MAPK *via* interacting with IRAK1, TRAF6, and TAK1. It also promotes translocation of p38 substrate MAPK-activated protein kinase (MK2) from the nucleus to the cytoplasm and activates the transcription factor CREB in a p38 MAPK-dependent manner (24). The ability of Pellino3 to activate p38 MAPK appears to be unique in the Pellino family (**Figure 2**).

For downstream signaling, Pellino1 leads to the activation of NF-κB (33) but not c-Jun N-terminal kinase (JNK) (24, 33, 34) in HEK293 cells. Mouse Pellino2 is required for NF-κB activation in mouse embryo fibroblast cells (32) and is involved in JNK signaling, which leads to AP-1 and the effect of ETS-like 1 transcription factor (Elk-1) activation in HEK293 cells (34). Pellino3 promotes c-Jun and Elk-1 activation in JNK signaling in HepG2 human hepatoma cells (15) and acts as a promoter to activate p38 MAPK in HEK293 cells (24) instead of NF-κB (15). Pellino3b, an alternative splicing variant of Pellino3, can negatively regulate IL-1-induced and TAK1-dependent NF-κB activation in synoviocytes (8) (**Figure 2**). As a conserved E3 ubiquitin ligase family, each member activates the same or different transcription factors to regulate different downstream pathways. Perhaps each member of the Pellino family has a different division of labor upon IL-1.

Pellino Family in MyD88-Dependent TLR Signaling

All TLRs mediate the signal conduction *via* Toll/interleukin-1 receptor (TIR) like IL-1R. Upon stimulation of ligands, several TLRs such as TLR2 and TLR4 recruit MyD88, IRAKs, TNF receptor-associated factor 3 (TRAF3), and TRAF6 to activate TAK1, leading to the activation of MAPK and NF-κB (3, 63). Upon LPS binding, TLR4 recruits MyD88, TRAF6, TRAF3, and cellular inhibitors of apoptosis proteins (cIAPs). There are two downstream signaling pathways for TRAF6. One is to activate TAK1 leading to MAPK and NF-κB activation (64–67). The other is to induce proinflammatory cytokines by stabilizing cIAPs *via* K63-Ub and then the TRAF3 K48-Ub degradation leading to the production of c-Rel (6).

An overall brain proteomes study in Pellino1 knockout mice showed that Pellino1 was involved in promoting antigen presentation, enhancing activities of adaptive and innate immune cells (68) with the contribution to microglial activation, neuroinflammatory responses, and neurological deficits through the activation of NF-κB and MAPK (42, 64, 66). Pellino1 positively regulates the production of inflammatory factors in MyD88-dependent TLR signaling (69, 70) as MyD88 deficiency hindered the expression of Pellino1, NF-κB, IL-1β, IL-6, Beclin-1, and cyclooxygenase-2 (COX-2) in a cerebral ischemia/reperfusion (I/R) mouse model (70). Pellino1 also positively regulates the MyD88-dependent pathway by promoting K63 linked polyubiquitination of IRAK1, TBK1, TRAF6, and TAK1 to active the MAPK and NF-κB signaling pathways *via* TLR2 and

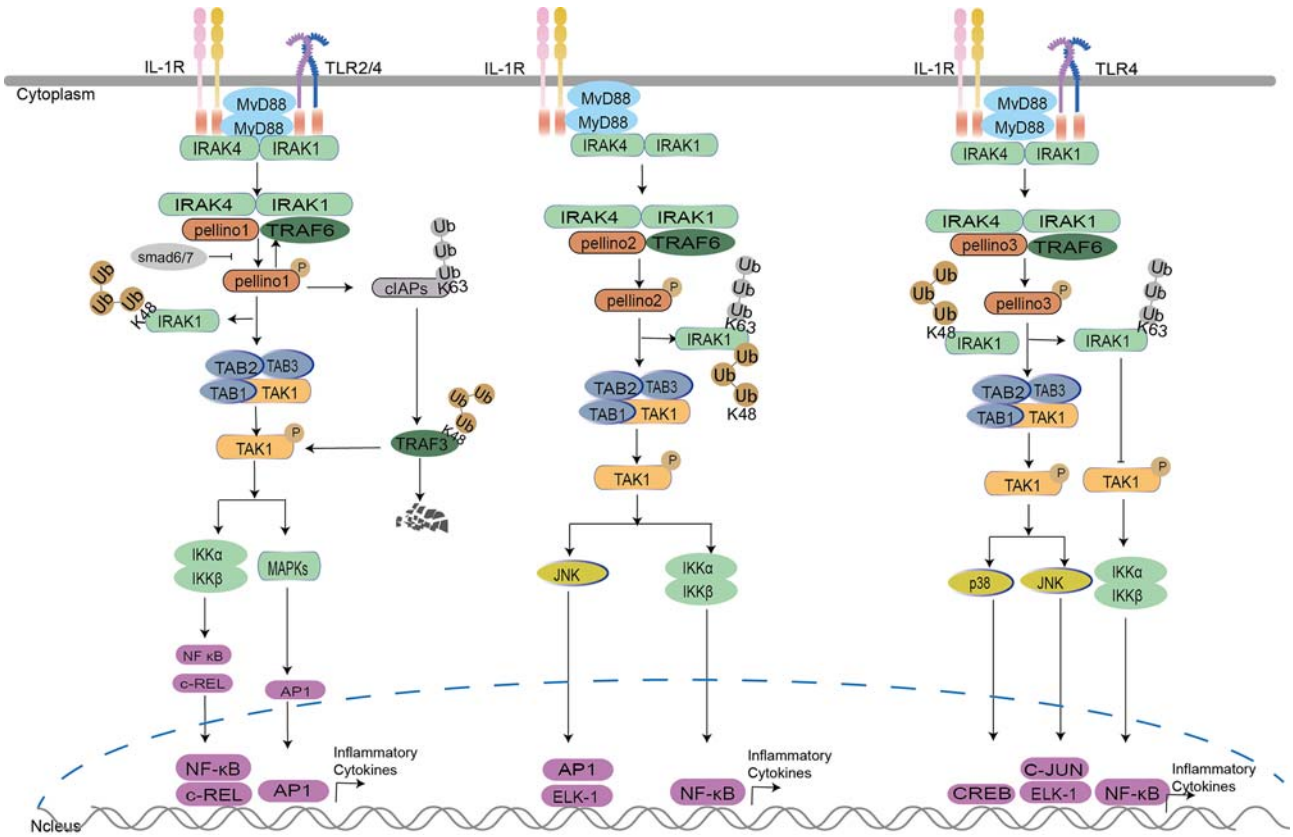

FIGURE 2 | Pellino family in the Myd88-dependent TLR/IL-1R signaling. Upon IL-1 stimulation, Pellino1, Pellino2, and Pellino3 can interact with IRAK1, TRAF6, and TAK1 (8, 32–34). Pellino1 mediates the degradation of IRAK1 by K48-Ub, leading to the activation of TABs and TAK1 with the ultimate activation of NF-κB. Pellino2 leads the activation of NF-κB and JNK by successive K63-Ub and K48-Ub of IRAK1 and activation of TAK1. Pellino3 mediates the activation of JNK and p38 by K48-Ub, which leads to IRAK1 degradation and negatively regulates NF-κB activation by IRAK1 K63-Ub. In the TLR pathway, TRAF6 induces cIAPs K63-Ub to enhance TRAF3 K48-Ub degradation, elevating proinflammatory cytokine production by interrupting TRAF3 induced K48 ubiquitin-dependent degradation of c-Rel. Pellino1 can also mediate cIAPs K63-Ub to accelerate the production of proinflammatory cytokines in microglia. In macrophages, Pellino3 represses NF-κB activation by inhibiting TRAF6 downregulation. It also inhibits IRAK1 degradation *via* K63-Ub competing with K48-Ub of IRAK1, hindering NF-κB activation.

TLR4 pathway (69, 71). Upon LPS stimulation, the expression of Pellino1 is upregulated (69, 71, 72), possibly by increasing levels of p65 and phosphorylated IKKα/β in microglia (73). Upregulated Pellino1 activates microglia and enhances NF-κB production, MAPK phosphorylation, and proinflammatory cytokines in LPS-induced TLR4 signaling by increasing TRAF6 K63-linked ubiquitination (64–66). In addition, Pellino1 promotes K63-Ub of TRAF6 in the spinal cord to enhance morphine treatment (65). However, Pellino1 is dispensable for inflammatory responses in astrocytes (66). TRAF3 degradation contributes to the production of inflammatory factors in the MyD88-TLR pathway. Pellino1 was discovered to mediate K63 ubiquitination of cIAP, resulting in cIAP K48 ubiquitin ligase activity, ubiquitin-dependent degradation of TRAF3 (41, 74, 75) activation of microglia-mediated chemokines, and proinflammatory cytokines *via* the MyD88-dependent MAPK pathway (42, 66, 75). Pellino1 is also involved in several neurogenic diseases. Upon trans-activating protein (Tat), Pellino1 induces autophagy, interrupts expression of tight junction protein zonula occludens1 (ZO-1), and increases the permeability of the blood–brain barrier (BBB) by triggering K63-Ub of Beclin1 (76). Pellino1 also impairs microglial amyloid β-protein (Aβ) phagocytosis through promoting CCAAT enhancer-binding protein β (C-EBPβ) degradation in Alzheimer's disease (AD) (77). In Parkinson's disease, upregulation of Pellino1 by α-synuclein leads to the degradation of lysosomal-associated membrane protein-2 (LAMP2) and the buildup of autophagy with decreased autophagy flux by microglial exosomes (78).

Although mediated by LPS, the Pellino family plays a different role in endotoxin tolerance in macrophages. Endotoxin tolerance abrogated Pellino1 induction by LPS in macrophages (69, 71, 72) but an enhanced expression of Pellino3 (79). Elevated transcription of TNFα and IL-6 driven by TLR2/4 and also increased expression of C–C motif chemokine ligand 5 (CCL5) driven by TLR4 were observed in Pellino3-deficient human myeloid leukemia mononuclear cells (THP-1) in response to TLR agonists (79). The Pellino3 inhibits TRAF6 downregulation by reducing IRAK1 degradation *via* K63 polyubiquitination, which competes with K48 ubiquitination, resulting in NF-κB

suppression (36) in J774.1 cell lines. This is consistent with prior Pellino3b results (8).

Several interesting results are discussed in the Pellino family-related MyD88-dependent TLR/IL-1R signaling (**Figure 2**). IRAK1, TRAF6, and MyD88 are crucial to the Pellino family, and the IRAK1 is responsible for the activation of the Pellino family. Pellino1 and Pellino2 can replace TRAF6 to generate K63-Ub chains. MyD88 mediates the Pellino1 expression level. Whether there is a similar phenomenon in Pellino3 needs further research. Upon the same IL-1 stimulation, the members of the Pellino family display different roles in regulating the IL-1R pathway. Pellino1 and Pellino2 are positive IKK activation regulators; however, Pellino3 is a negative regulator. This phenomenon is also present in TLR signaling upon LPS stimulation. Pellino1 significantly induced proinflammatory cytokines in microglial cells but showed no inflammatory responses in astrocytes.

Interestingly, the downregulation of Pellino1 and upregulation of Pellino3 were observed upon LPS induced endotoxin tolerance in macrophages. However, each member of the Pellino family can mediate the IKK activation or MAPK activation; not all the members act as positive roles in the signaling. Perhaps the cell type is critical in determining which member is accountable for the associated pathway, and this should be researched further.

Pellino Family in TRIF-Dependent Interferon Induction Signaling

TRIF plays a critical role in activating NF-κB *via* a MyD88-independent pathway in TLR3 and TLR4 signaling (80, 81). In addition to NF-κB activation, TRIF can also stimulate TANK binding kinase1 (TBK1) and IKKε kinases to activate interferon regulatory factor (IRF) transcription factors that drive the expression of antiviral type I IFNs (80, 82). Upon LPS, poly(I:C), and viral double-stranded RNA stimulation, TRIF is recruited to promote TRAF3-dependent activation of TBK1 to activate IRF3/7 leading to the induction of IFN expression (82, 83). IRF3 and IRF7 are the most important transcription factors regulating type I IFN expression (80). Pellino1 possesses a novel function in human viral pathogen infection (41, 62, 84, 85) depending on TRIF. Pellino1, as a TLR3 positive regulator (18, 37, 86), is involved in modulating the production of proinflammatory cytokines (37, 86) and induction of IFN-I in the TLR3 pathway (41, 44, 86, 87). The deficiency of Pellino1 leads to inhibition of TLR3 and proinflammatory cytokines production but without impairing IFN antiviral induction under virus stimulation and TLR3 agonists in mice and primary bronchial epithelial cells (PBECs) (62, 86). It seems that Pellino1 is dispensable for IFN induction. However, further studies showed that Pellino1 is upregulated by TRIF, TBK1, and IKKε (18, 52, 69) *via* a TRIF-dependent manner in the TLR3 pathway but not the IRAKs-coupled and MyD88-dependent pathway (37, 62). Perhaps there is a priority for Pellino1 to decide which pathway to participate in. IKK ε and TBK1 can enhance the activation of Pellino1 depending on IRF3 (18) and K63-linked polyubiquitination of TBK1 (52, 55). As a new IRF3-dependent gene, Pellino1 enhances the interaction of IRF3 with the IFNβ promoter to promote IFN production (44). In detail, Pellino1 interacts with deformed epidermal autoregulatory factor 1 (DEAF1) independent of its E3 ligase activity, followed by DEAF1 binding to IFNβ promoters IRF3 and IRF7 to promote IFNβ gene transcription and IFNβ secretion in MEFs (88). The protein Bid can upregulate Pellino1 and enhance Pellino1 interaction with TBK1, leading to IRF3 production (73, 89).

Contrary to the above conclusion of upregulating IFNs level, Pellino1 negatively mediates the induction of IFNs in microglia *via* TRIF dependent TLRs upon the stimulation of poly(I:C), LPS, and the RNA virus in the CNS (41). Perhaps due to this, Pellino1 allows the entry and replication of West Nile Virus (WNV) in mouse macrophages, human neurons, and microglia (84), and the enhancement of ZIKA virus (ZIKV) vertical transmission and neuronal loss *in vitro* and *in vivo* (85). However, Pellino3 does not act as a mediator of proinflammatory cytokine expression in response to TLRs but as a key regulator to control TRIF dependent type I interferon expression in the TLR3 pathway by negatively regulating activation of IRF7 but not IRF3 (87). This was demonstrated in Pellino3 deficient animals, which had increased resistance to encephalomyocarditis virus and enhanced type I interferon expression but not proinflammatory cytokines in response to TLR3 activation (87). The possible mechanism is that the TLR3 induces the Pellino3 level, which interacts with and ubiquitinates TRAF6. This modification suppresses the ability of TRAF6 to interact with and activate IRF7 leading to downregulation of type I interferon expression (87). More interestingly, Pellino3 inhibits LPS-induced IFNβ expression in oxidation-low-lipoprotein (Ox-LDL) induced macrophage-derived foam cells *via* IRAK1/IRAK4/Pellino3/scavenger receptor-A1(SR-A1) dependent mono-ubiquitination of TRAF family member associated NF-κB activator (TANK) (90). In detail, Ox-LDL activates IRAK1 and Pellino3, which provokes mono-ubiquitination of the adaptor TANK in TRAF3-containing signaling complex, leading to the failure of LPS-induced TBK1 recruitment, IRF3 activation, and IFNβ expression in macrophage-derived foam cells (90).

In the TRIF-dependent interferon induction signaling (**Figure 3**), Pellino1 and Pellion3 display polar functions in the production of IFNs (41, 44, 86, 87). The role of Pellino1 in the induction of IFNs seemed to be unclear. First, Pellino1 is dispensable or required to produce IFN in PBECs and MEFs. IFN-β induction is attenuated in myeloid cells and MEFs expressing a Pellino1 mutant lacking E3 ligase activity. Second, the fact that Pellino1 deficiency profoundly promotes IFN-β expression in microglia and Pellino1-deficient mice display heightened IFN-I levels demonstrate a potentially negative role of Pellino1 in the induction of IFN-β. The above results were confusing. The Pellino1 might play a different role in different cell types, and this needs to be further investigated. Unlike puzzled Pellino1, Pellino3 serves as a negative regulator of IRF7 but not IRF3 in TLR3 upon viruses (87). Perhaps there is a hypothesis that the Pellino family may follow a not yet clear priority rule to determine which member is responsible for the regulation of IFNs. So far, there is no report on Pellino2 and IFN production. More attention should be paid to the discrepancy of the Pellino family.

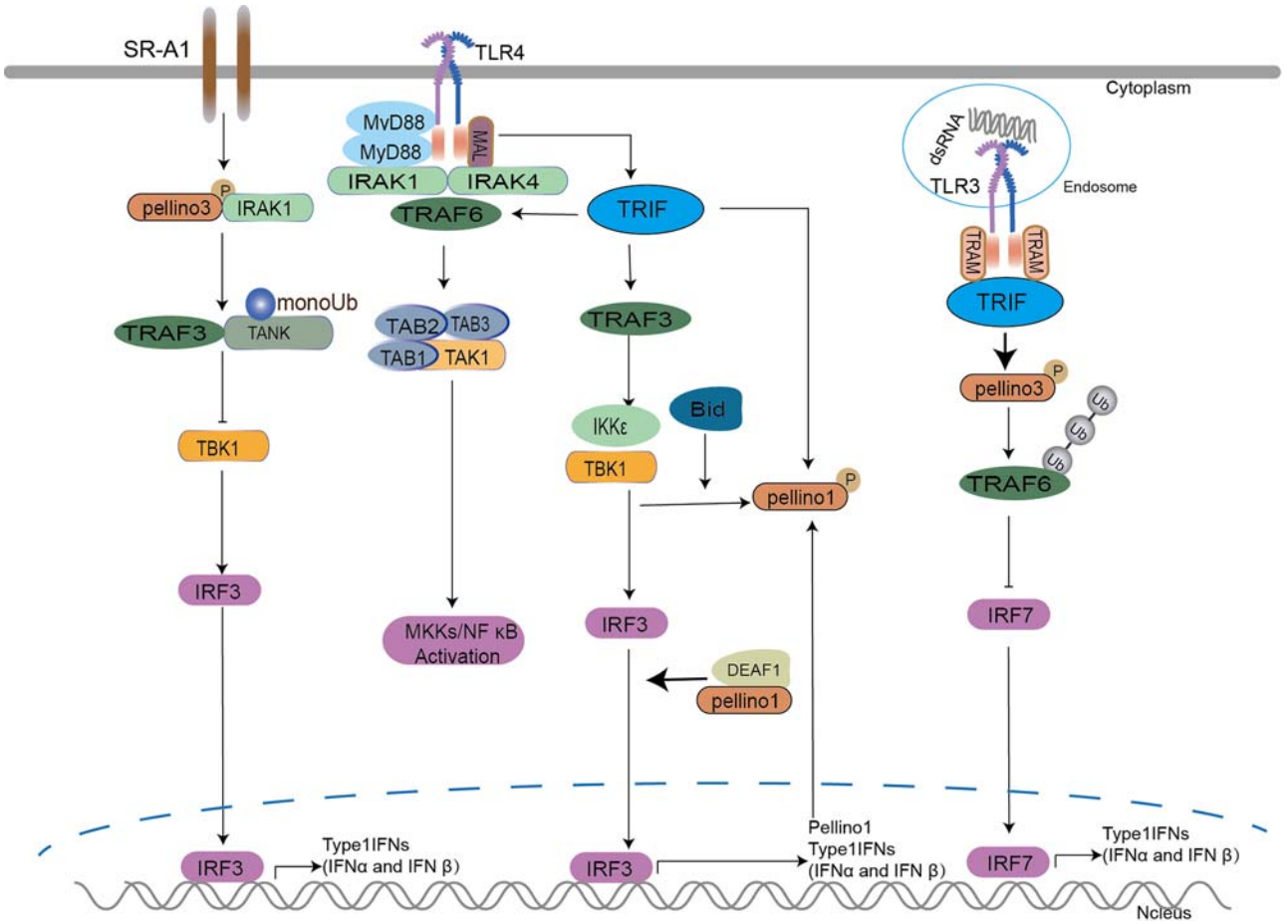

FIGURE 3 | The Pellino family in TRIF-dependent interferon induction signaling. Pellino1 is required for interferon production upon viral double-stranded stimulation and is upregulated by TRIF, TBK1, and IKKε. IKKε and TBK1 enhance the activation of Pellino1 depending on IRF3 and K63 linked polyubiquitination of TBK1. Pellino1 interacts with DEAF1 independent of its E3 ligase activity and leads to the binding of DEAF1 and IFNβ promoters (IRF3 and IRF7) for IFNβ gene transcription and secretion. Bid upregulates Pellino1 and enhances the interaction of Pellino1and TBK1, leading to IRF3 production.

Pellino Family in RIP-Dependent Signaling

RIP1 was initially discovered as an adapter kinase involved in the transduction of TNFR signals. It is required for the suppression of nuclear factor kappa-B kinase (IKK) activation and apoptosis *via* a RIP homology interaction motif (RHIM) in TRIF-dependent signaling (73, 91, 92) and TNF signaling in the absence of TRIF. In the TRIF-dependent pathway, RIP1 ubiquitination induced by poly(I:C) is required for IKK activation (92). The discovery of the kinase RIP3 (93, 94) and its substrate mixed lineage kinase domain-like protein (MLKL) (95, 96) leads to an awareness of this pathway. However, TRIF does not employ RIP1 to initiate IRFs (91, 92), and RIP3 is not required for NF-κB activation in TLR signaling (97). Both TNF/RIP1/RIP3/MLKL signaling and TRIF/RIP1/RIP3 pathway participate in the activation of NF-κB/MEKKs in cell survival, apoptosis, and necroptosis (4).

Pellino1 mediates RIP1 K63-Ub to active NF-κB signaling in the TRIF-dependent TLR pathway to maintain cell survival (37, 38, 98). Under LPS and dual hypoxia stimulation, destabilized Pellino1 (Ser39 phosphorylation and turnover) induced by death-associated protein kinase 1 (DAPK1) leads to the release of TRIF-RIP1 signalosome to recruit caspase-8 and induces tubular damage and cell apoptosis in acute kidney injury (AKI) model (98). The binding of Pellino1, RIP1, and TIF inhibits tubular damage by hindering cell apoptosis. In the TRIF-independent RIP pathway, IKK-related kinases activate Pellino1 in TNFα-stimulated mouse embryonic fibroblasts (MEFs) (35). According to an intriguing study, Pellino1 acts as a dual regulator of necroptosis and apoptosis. Pellino1 acts as a pro-necroptosis K63-ubiquitin ligase role in necroptosis by forming RIP1 and RIP3 complex in a RIP1 kinase activity-dependent way but as an apoptosis inhibitor by reducing expression levels of cellular FADD-like interleukin-1β converting enzyme inhibitory protein (c-FLIP) in MEFs cells stimulated by TNFα (56). In contrast to the previous result, Pellino1 might prevent HeLa cells aberrant necroptosis by causing RIP3 hyperactivation and further degradation *via* K48-linked polyubiquitylation (19). The results reflect the different roles of Pellino1 in normal and cancerous cells. However, Smad6 can block the interaction between Pellino1 and RIP1 to inhibit NF-κB (84, 85). Pellino3 is also proved to be a novel

regulator of cell survival upon TNFα. Pellino3 can impair TNFα-induced complex II formation and caspase-8-mediated RIP1 cleavage *via* interacting with RIP1 and caspase 8, leading to the inhibition of apoptosis *in vitro* and *in vivo* (27).

In the RIP-dependent signaling (**Figure 4**), both Pellino1 and Pellino3 were involved in two types of programmed cell death: apoptosis and necroptosis. It seemed that the K63-Ub of RIP1 is crucial to cell fate. For instance, stimulations of TLR3 and TLR4 induce the interaction of RIP1 and TRIF followed by recruitment of Pellino1, which mediates K63-linked polyubiquitylation of RIP1, leading to recruitment of TAK-1 for NF-κB induced cell survival (19, 37). Upon TNFα, the K63-linked polyubiquitylation of RIP1 is also necessary for the NF-κB pathway and cell survival (99). Pellino1 can also mediate RIP1 K63-linked polyubiquitylation on TNFα. Pellino1-induced RIP1 K63-linked polyubiquitylation appears to be a critical factor in cell survival, apoptosis, and necroptosis. Current studies show that Pellino1 only induces RIP1 K63-linked polyubiquitylation to trigger necroptosis but is not necessary for necrosome formation. Perhaps, the different interaction sites between RIP1 and Pellino1 decide the signal conduction. Interestingly, Pellion1 plays almost the exact opposite role in necroptosis in different cell types, enhancing necroptosis in normal cells (56) and preventing necroptosis in Hela cells (19). As an important role in controlling complex II formation in response to TNF, Pellino3 can interact with the complex II components, caspase-8, and RIP1, to inhibit cell death. Pellino3 plays a critical role in inhibiting the pro-apoptotic effects of TNF independent of NF-κB (27). This is consistent with previous reports indicating that Pellino3 may negatively regulate IL-1-induced and LPS-induced activation of NF-κB (8, 36). More efforts are needed to unravel the roles of the Pellino family in cell survival and death.

Pellino Family in NLRs Related Signalings

NOD1, NOD2, and NLR Family Pyrin Domain Containing 3 (NLRP3) are involved in the anti-infection process by activating

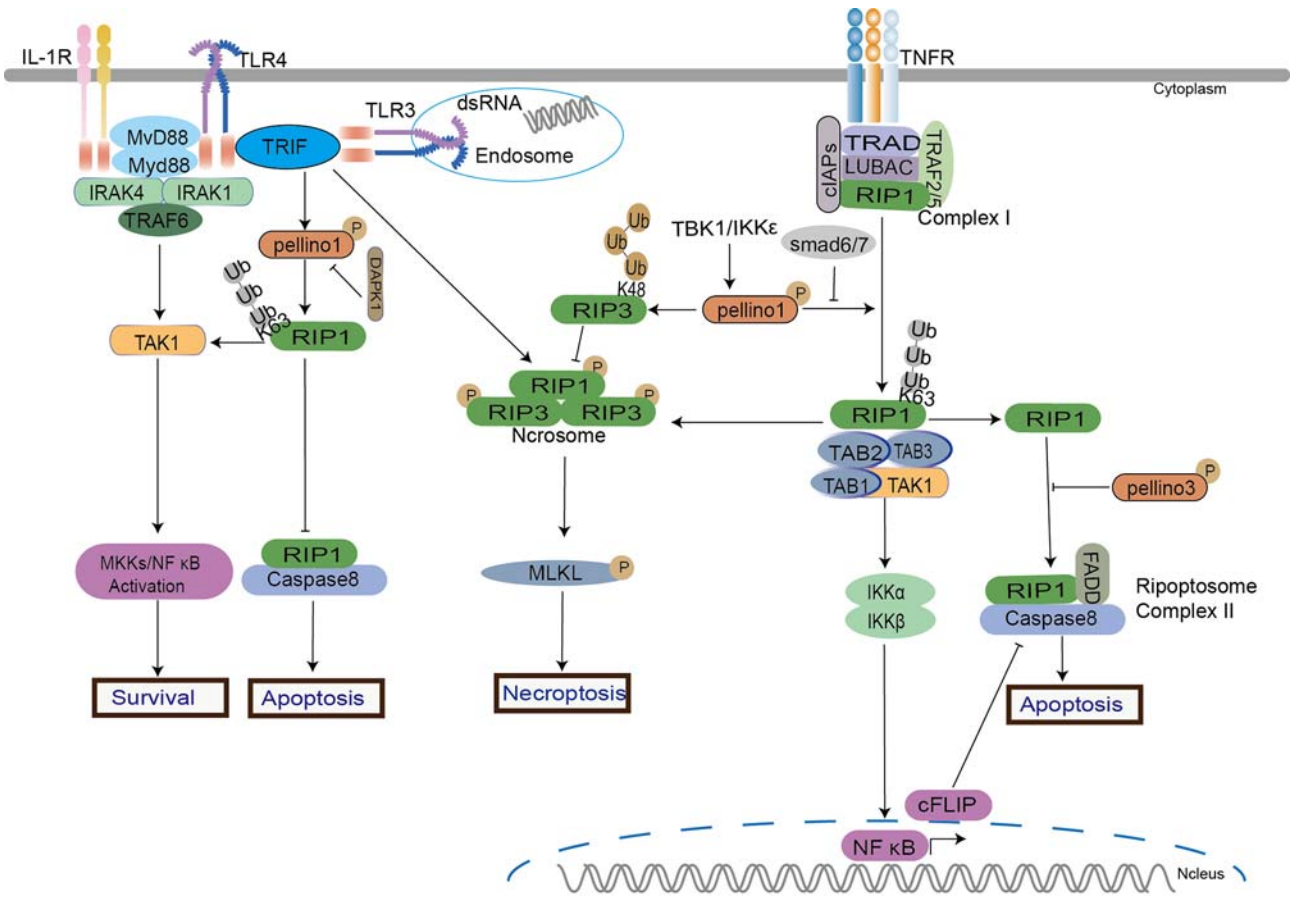

FIGURE 4 | Pellino family in RIP-dependent signaling. Pellino1 induces the ubiquitination of RIP1 and RIP3 to regulate NF-κB activation in cell survival, apoptosis, and necroptosis in TNFα, TLR3, and TLR4 signaling. Pellino1 targets RIP1 by K63-Ub to active NF-κB to maintain cell survival. Under the dual stimulation of LPS and hypoxia, Pellino1 releases the TRIF-RIP1 signalosome to recruit caspase-8 and induces tubular apoptosis *via* DAPK1-mediated Pellino1 destabilization. Upon TNFα, Pellino1 is a dual modulator in necroptosis and apoptosis. Pellino1 plays a positive role in necroptosis by K63-Ub to form RIP1 and RIP3 pro-necroptosis complex in a RIP1 kinase activity-dependent way but as an apoptosis inhibitor by reducing expression levels of c-FLIP. Smad6 blocks the interaction between Pellino1 and RIP1 to inhibit NF-κB activation. Pellino1 can induce RIP3 hyperactivation and degradation *via* K48-Ub to inhibit necroptosis.

the NF-κB signaling pathway, type I IFN signaling pathway, autophagy-related pathway, and pyroptosis pathway (100, 101).

In NOD2 related signaling, Pellino3 exerts a protective function *via* NOD2 in chemical drugs induced models of colitis (26). Pellino3 promotes magnesium-dependent phosphatase (MDP) induced K63-Ub of RIP2 and recruits TAK1 and IKK complexes to active NF-κB and MAPK in an inhibitor of apoptosis family of protein (IAP)-independent manner to maintain cell survival (26) (**Figure 5**).

Two pathways are involved in IL-1β secretion, TLR/IL-1R-mediated upregulation of precursor pro-IL-1β and NLR-induced activation of caspase-1 that cleaves pro-IL-1β to yield mature IL-1β secretion (102). Pyroptosis is a novel programmed cell death featured by IL-1β secretion (103). In NLRP3-related pyroptosis, the oligomerization of NLRP3, pro-caspase-1, and the inflammasome adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) causes pro-caspase-1 to be converted into active caspase-1, which then cleaves pro-IL-1β and pro-IL-18, resulting in the maturation and secretion of proinflammatory cytokines (104). Pellino1, Pellino2, and Pellino3 are demonstrated to mediate the release of IL-1β and IL-18 in cell pyroptosis (57, 105, 106). A new study reveals that Pellino1 is required for NLRP3-induced caspase-1 activation and IL-1β maturation (106). Pellino1 increases NLRP3 inflammasome activation, which results in IL-1β production, by facilitating ubiquitination of the inflammasome adaptor ASC K63, enhancing the ASC/NLRP3 interaction and ASC oligomerization (106). Pellino2 is also essential for the priming and activation of inflammasome to induce pyroptosis (57, 105). In Pellino2 deficient macrophages, the activation of the NLRP3 inflammasome is suppressed (57). Pellino2 isolates IRAK1 from NLRP3 *via* ubiquitination and mediates K63 ubiquitination of NLRP3 to increase NLRP3 activation for mature IL-1β generation in mice and bone marrow-derived macrophages

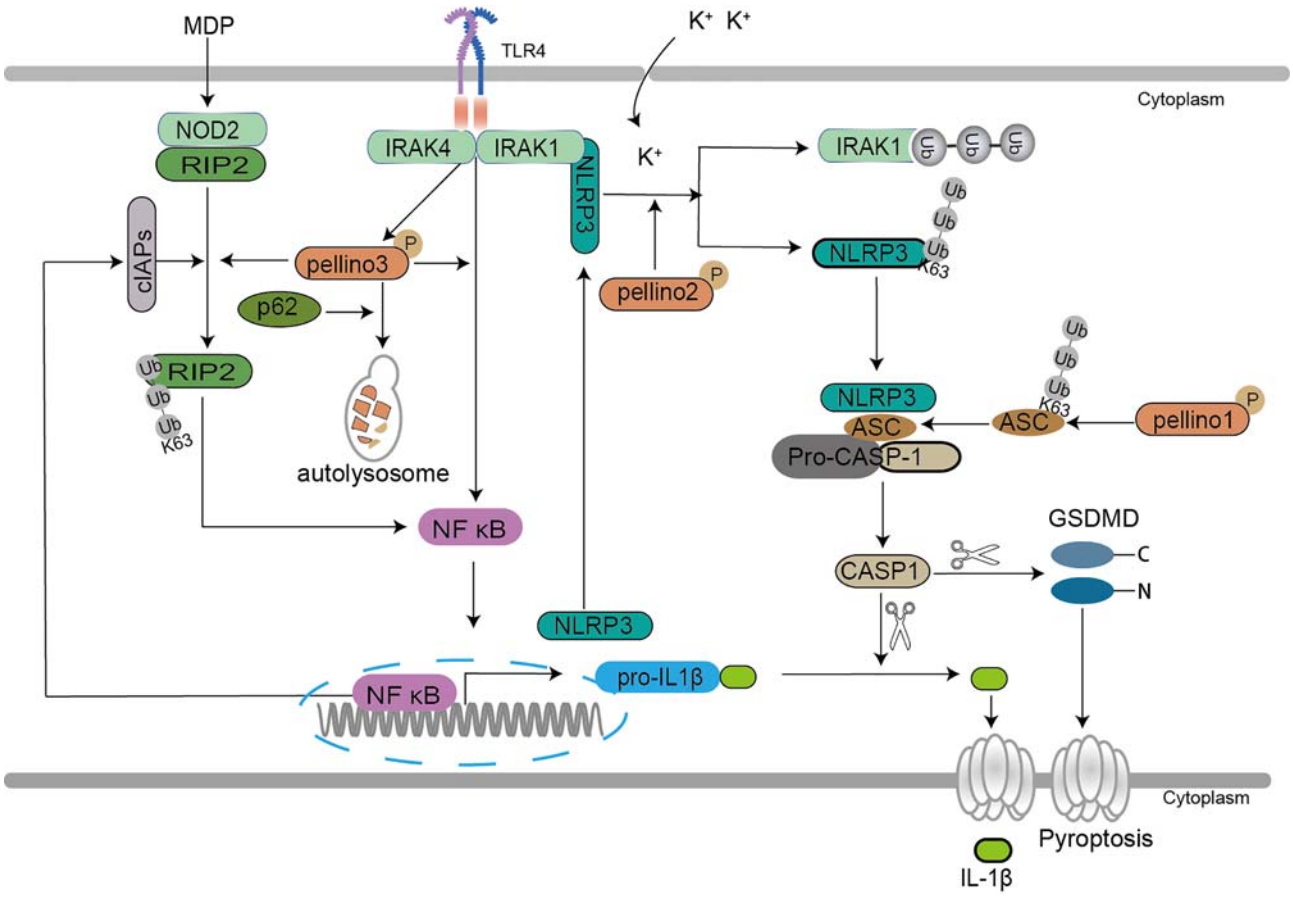

FIGURE 5 | Pellino family in the NLR-dependent signaling. Pellino1 mediates the K63 ubiquitination of inflammasome adaptor ASC, which enhances the ASC/NLRP3 interaction and ASC oligomerization to facilitate NLRP3 inflammasome activation leading to induction of IL-1β secretion. Pellino2 induces IRAK1 isolation from NLRP3 *via* ubiquitination and mediates K63 ubiquitination of NLRP3 to promote the activation of NLRP3 for mature IL-1β production in response to LPS. Pellino2 can co-localize with NLRP3 and ASC during inflammasome activation in macrophages upon the effect of potassium efflux. Pellino3 acts as a potential partner of SQSTM1/p62, which leads to Pellino3 autophagy-dependent degradation in TLR4-signaling, thereby impairing Pellino3-dependent pro-IL-1B proinflammatory expression. Pellino3 promotes MDP-induced K63 ubiquitination of RIP2 and recruits TAK1 and IKK complexes to active NF-κB and MAPK in an IAP-independent manner to maintain cell survival.

(MDMs) in response to LPS (57). Further studies show that Pellino2 can co-localize with NLRP3 and ASC during inflammasome activation in macrophages upon the effect of potassium efflux (105). Both Pellino1 and Pellino2 are implicated in NLRP3-mediated pyroptosis, demonstrating the importance of the Pellino family in pyroptosis. Furthermore, the autophagy-dependent degradation of Pellino3 induced by sequestosome-1 (SQSTM1/p62) hindering IL-1β secretion upon LPS offers a strong backup for the roles of the Pellino family in pyroptosis (25).

The above results show that the Pellino family is crucial to ubiquitin-dependent inflammasome activation and inflammatory release (**Figure 5**). Previous studies reported that the Pellino family is a key mediator for activation of NF-κB (37), and NF-κB is involved in NLRP3 induction. The most surprising is that Pellino1 deficiency did not reduce the induction of NLRP3 expression (106). Pellino1, Pellino2, and Pellino3 may play a role in the division and cooperation to mediate NF-κB activation, inflammasome activation, and inflammatory release in pyroptosis. In NOD2 related signaling, Pellino3 is still a protective regulator to maintain cell survival, consistent with Pellino3 in TNF signaling. Perhaps Pellino3 may differ from Pellino1 and Pellino1 in special cells and contexts. More efforts are needed to reveal the roles of the Pellino family in programmed cell death.

Pellino Family in B-Cell and T-Cell Signaling

In addition to the above functions in immunity, Pellino1 shows a potent negative function in T cell and B cell activation (43, 107). Under normal circumstances, Pellino1 is highly expressed in mouse splenic B cells and T cells (107). Pellino1 inhibits T cell activation and prevents autoimmunity by ubiquitinating c-Rel, a downstream important protein in NF-κB activation, with specific K48-Ub (107). Pellino1 is seemed to be unique for T cell activation and maintenance of peripheral immune tolerance for its high expression in lymphocytes (107). Pellino1 deficiency promoting B cell activation hints that Pellino1 negatively regulates B cells specifically in response to poly(I:C) and noncanonical NF-κB stimulation (43, 108). Pellino1 inhibits noncanonical NF-κB activation and alleviates lupus-like disease in systemic lupus erythematosus by K48 ubiquitination of NIK to downregulate nuclear p52 and Rel B (43) (**Figure 6**).

Ubiquitination has emerged as a critical mechanism regulating T cell and B cell activation (109). Pellino1 is critical in regulating IKK activation by TRIF dependent TLR signaling, although it is largely dispensable for IKK activation in MyD88-dependent TLR/IL-1R (37). However, in B-cell and T-cell **(Figure 6),** the reason it is dispensable to active IKK by TCR signals may be the degradation of c-Rel induced by Pellino1specific K48 ubiquitination (107). The noncanonical NF-κB pathway critically regulates B cell activation and antibody production. It is reported that TRAF2-cIAPs mediated the K48 ubiquitination of NIK as E3 ligases (110–112). Pellino1 is also required for TLR-induced cIAPs ubiquitination and activation in microglia (75). So it is reasonable to assume that Pellino1-mediated NIK ubiquitination may be due to the activation of cIAPs by Pellino1.

PELLINO FAMILY IN TUMOR AND MicroRNAs RELATED SIGNALINGS

Pellino Family in Tumorigenesis

Pellino1 plays a novel role in angiogenesis, a typical phenotype in tumorigenesis (113).

As a downstream of vascular endothelial growth factor receptor 2 (VEGFR2), Pellino1 induces the AKT and MAPK activated protein kinase 2 (MK2) phosphorylation to restore cell migration potential, proangiogenic responses and the wound healing ability with VEGFR2 deficiency *in vitro* and *in vivo* (114). Further studies demonstrated that Pellino1 is a novel proangiogenic molecule directly regulated by VEGFR (115). In mouse ischemia models, Pellino1 deletion increases oxidative stress, reduces cIAP2-NF-κB cell survival, decreases angiogenic response, and lowers tissue function (116). Transgenic mice constitutively expressing human Pellino1 had a shorter lifespan, a wide range of lymphoid tumors, and prominent B-cell infiltration (117). Pellino-1 may be an oncogene in cancer based on its proangiogenic and tumor development function. The association of Pellino1 with protooncogene-MYC, B cell lymphoma 6 protein (BCL6), murine double minute X (MDMX), and p53 demonstrates the role of Pellino1 in cancer (117–120). In diffuse large B-cell lymphoma, Pellino1 directly interacts with and induces oncoprotein BCL6 K63-Ub (117). Pellino1 is required for DNA damage in the promotion of HR repair by feedback activation of ataxia telangiectasia-mutated gene (ATM) *via* NBS1 ubiquitination (121) and p53 activation upon exposure to DNA damaging agents (120). Pellino1 negatively regulates and sequesters MDMX *via* ubiquitylation in the cytoplasm and free p53 to activate responsive genes such as p21 (119). Furthermore, Pellino1 downregulation causes MDMX nuclear localization, lowers p53 activity, and speeds up c-MYC-induced carcinogenesis linked with a reduction in p53 function (119). IAP may be a positive partner of Pellino1 in regulating tumor cell survival (116, 122). High expression of Pellino1 in human lung cancer cell lines upregulates the expression of IAP proteins (cIAP1 and cIAP2) through K63-Ub, which leads to cell survival but not apoptosis (122). Pellino1 can also promote epithelial-mesenchymal transition (EMT) by inducing K63-Ub of Snail and Slug, contributing to tumorigenesis (47, 123). Fundamentally, Pellino1 causes homeostatic regulation of the mitotic cell cycle and checkpoints to be inhibited, contributing to the initiation and progression of the neoplastic chromosome aneuploidy through ubiquitination-mediated downregulation budding uninhibited by benzimidazoles related1 (BubR1) and induced mitotic dysfunction (124). This may be crucial evidence to demonstrate Pellino1 to be an oncogene.

As a positive regulator of inflammatory factors, Pellino1 induces the production of inflammatory factors followed by the change of inflammatory microenvironment leading to the transformation of normal cells to tumor cells. So it is necessary to study the inflammatory microenvironment induced by the Pellino family in normal cells, tumor cells, and even cell co-culture systems.

Pellino Family in microRNAs Related Signalings

MicroRNAs (microRNAs) are small non-coding RNAs with the capability of modulating gene expression at the post-transcriptional

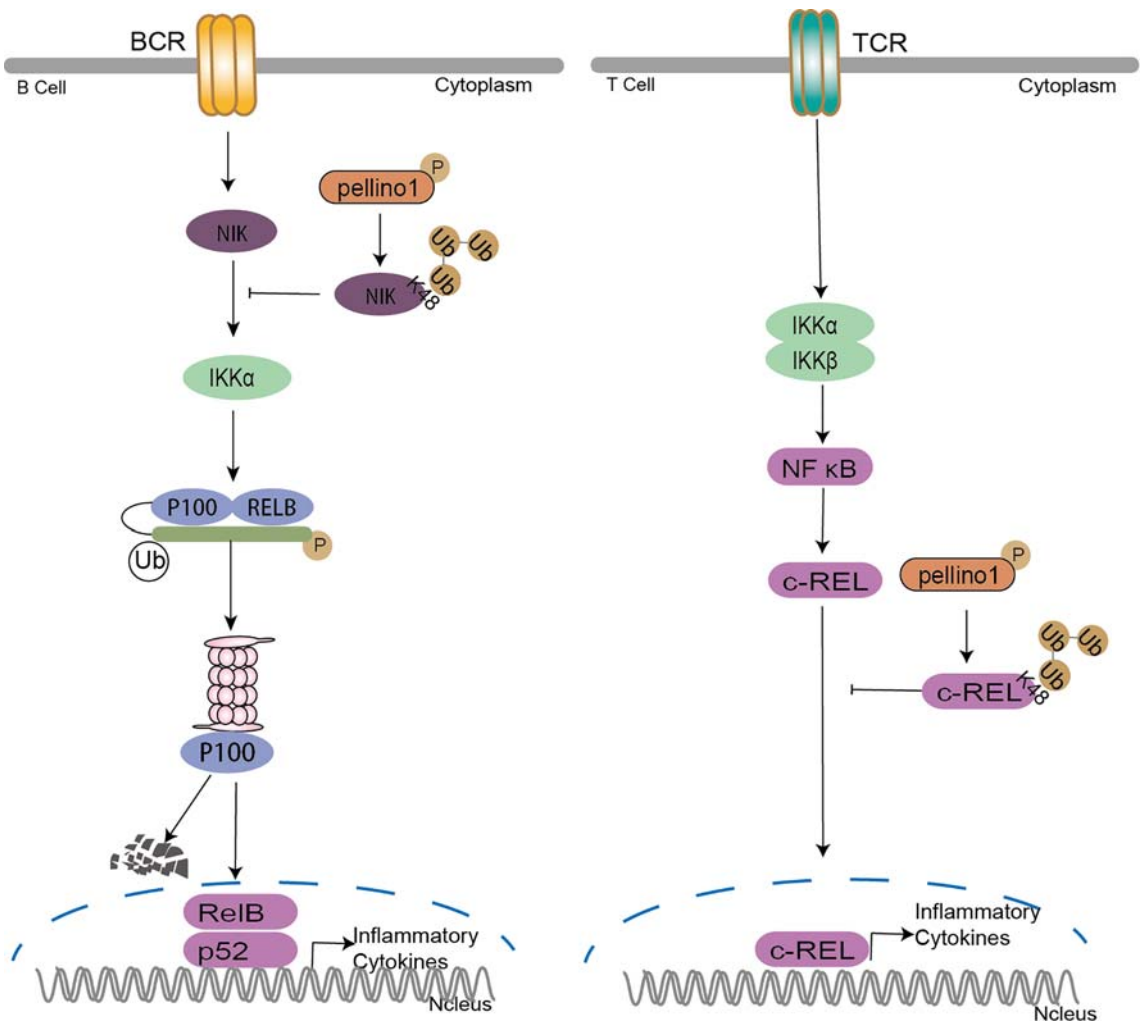

FIGURE 6 | Pellino family in B cell and T cell. Pellino1 inhibits noncanonical NF-κB activation by K48 ubiquitination of NIK to downregulate nuclear p52 and Rel B in the noncanonical NF-κB pathway. Pellino1 also negatively regulates T cell activation and prevents autoimmunity by specific K48 ubiquitination of c-Rel to inhibit NF-κB activation.

level either by inhibiting messenger RNA (mRNA) translation or by promoting mRNA degradation (125). Several microRNAs are involved in interactions with the Pellino family. MicroRNA-21 (126–128), microRNA-153-3p (129), and microRNA-155 (130, 131) are involved in T cell regulation; MicroRNA-590-5p (132), microRNA-142a-3p (133), microRNA-155-5p (133), and microRNA-744 (134) in inflammatory disease; MicroRNA-128-3p (134) in tumor disease.

A positive correlation between microRNA-21 and Pellino1 suggests that microRNA-21 and Pellino1 might be associated with autoimmune primary ovarian insufficiency (POI) patients (126). MicroRNA-21 targets the Pellino1–c-Rel pathway to promote glucose metabolism of pathogenic T helper cell 17 (TH17) cells by activating the NF-κ B with a decrease in Pellino1 and an increase in c-Rel (128). In systemic lupus erythematosus, upregulated microRNA-153-3p represses Pellino1 *in vitro*. It induces immune dysregulation by lowering umbilical cord mesenchymal stem cells (UC-MSCs) proliferation, migration, and mitigates the decrease in T follicular helper (Tfh) cells and increases T regulatory (Treg) cells (SLE) (129). MicroRNA-155 represses the expression of Pellino1, leading to the abrogation of the c-Rel, which controls cellular proliferation and CD40L expression in Tfh cells (130). MicroRNA-155 (131), microRNA-590-5p (132), microRNA-142a-3p (133), and microRNA-155-5p (133) can all target and reduce Pellino1 expression, leading to the suppression of pro-inflammatory production in neuroinflammation. MiR-744 interacting with the 3' untranslated region (UTR) represses Pellino3 expression and leads to upregulation of the IFN-dependent chemokines C–C Motif Chemokine Ligand 5 (CCL5) and CXCL10 (135). In non-small cell lung cancer, levels of Pellino3 are positive to the long non-coding RNA (lncRNA) MIAT but negatively related to miR-128-3p (134). It is clear that microRNAs primarily suppress the expression of Pellino1 to modulate immune responses. More

research should be conducted to determine the association between microRNAs and the Pellino family.

CONCLUSION

As a highly conserved protein and positive regulator in immunity discovered in *Drosophila* (52), the structure of Pellino in other species is also conserved, e.g., viral Pellino (136), Freshwater Prawn (137), Zebrafish (138), *Crassostrea hongkongensis* (139), and *Japonicas* (140). Viral Pellino should be studied further for a poxviral homolog of the Pellino protein capable of inhibiting Toll-like receptor signaling independent of IRAK1 and inhibiting Pellino3-mediated activation of the p38 MAPK pathway (136). The function of viral Pellino suggests that the mammalian Pellino family may act as a barrier or enhancer during viral infection.

There are two important conserved domains for the Pellino family: the FHA domain promoting phosphorylation with IRAKs (16, 24, 28–31)and the RING domain, which determines E3 ligase features (7, 14, 39). The FHA domain in the Pellino family differs from the classical FHA domain by containing an additional appendage or "wing" that is formed by two inserts in the FHA region (16). Interestingly, multiple IRAK phosphorylation sites in the "wing" region and the importance of this appendage region for IRAK binding remain to be experimentally addressed. More interesting is that different domains can interact with the same protein. Pellino1 can interact with RIP3 depending on the FHA and RING-like domains (19, 37). The FHA and RING-like domains are responsible for Pellino3 interacting with RIP1, RIP2, and caspase-8 (26, 27). These suggest that the activation of different sites may be a key factor in determining the cell to survive or be dead dependent or independent on the RIP family.

There are some conflict points about the Pellino family in regulating PRR signalings. In contrast to the positive role in regulating proinflammatory cytokine induction (37), Pellino1 negatively regulates T-cell activation in autoimmunity (107); and promotes microglia-mediated CNS inflammation (75) by negatively regulating type I interferon induction and antiviral immunity in the microglial cells (41). Pellino1 and Pellion3 display polar functions in the induction of IFNs (41, 44, 86, 87). Unlike peripheral macrophages expressing Pellino1, Pellino2, and Pellino3, microglia predominantly express Pellino1 (75). The induction of IFNs in MEFs and peripheral cells induced by Pellino1 deficiency showed no significance (37). However, IFN-β induction is attenuated in myeloid cells and MEFs expressing a Pellino1 mutant lacking E3 ligase activity (44). The more intriguing aspect is that Pellino1 performs different roles in necroptosis and apoptosis in the same cell, as a critical modulator of TNF-α-mediated cell death pathways, enhancing necroptosis and inhibiting apoptosis by modifying K63-Ub of RIPK1 with the inconstant expression of c-MYC and c-FLIP (56). These indicate that specific tissue expression of Pellino1 may promote their specialized roles in specific cells. According to the current studies, the Pelino1 tissue expression level is from high to low in peripheral blood, leukocytes, placenta, lung, liver, kidney, spleen, thymus, skeletal muscle, brain, small intestine, colon, and heart (33). Pelino3 tissue expression level is from high to low is brain, testis, heart, liver, lung, placenta, stomach, kidney, spleen, small intestine, colon, and muscle (15). Perhaps the tissue expression levels of Pellino1 and Pellino3 may be a clue to explain the polarized function of the Pellino family. More attention should be paid to Pellino2 and Pellino3 for a better understanding of the roles of the Pellino family.

Several studies have shown that Pellino1 acts as an oncogene role in tumorigenesis to maintain cell survival (116, 122) and even upregulates other oncogene levels, e.g., Bcl6 and c-Myc (122). As a positive regulator of inflammatory factors, Pellino1 induces the production of inflammatory factors followed by the change of inflammatory microenvironment leading to the transformation of normal cells to tumor cells. It is necessary to study the inflammatory microenvironment induced by the Pellino family in normal cells, tumor cells, and even cell co-culture systems.

Only a few proteins have been reported to mediate the Pellino1, e.g., Smad6/7 (20, 21), DEAF1 (88), Bid (89), and DAPK1 (98), which positively or negatively regulate the Pellino family. A new study reports six novel interaction partners of Pellino-2 in the liver cells, insulin receptor substrate 1 (IRS-1), NIMA-related kinase 9 (NEK9), tumor necrosis factor receptor-associated factor 7 (TRAF7), roundabout homolog 1 (ROBO-1), and disheveled homolog 2 (DVL-2) (141). More efforts are needed to study the expression and binding partners of the Pellino family in both the immune cells and non-immune cells. Understanding the regulatory mechanism between the Pellino family and other proteins can assist us in acquiring a comprehensive knowledge of the cross-talk among PRRs signaling.

This paper mainly reviews the roles of the Pellino family in the PRR signaling pathways. According to a flow of studies, we can preliminarily infer that the Pellino family has indeed been involved in the PRRs related pathways with the major function of regulating IFNs and inflammatory factors leading to cell survival or death. Maybe the different cell types and ligands stimulation play crucial roles in the Pellino family-related PRRs signalings. However, there are still many contradictory phenomena that cannot be explained very well. The Pellino family might play different roles in different cell types and contexts. Currently, Pellino1 has attracted a lot of attention and more efforts will be needed to study Pellino2 and Pellino3 in order to have a better understanding of the whole family in immunity. The future focus is to probe a more detailed and clear mechanism of the Pellino family in the immune system to improve related immune diseases.

AUTHOR CONTRIBUTIONS

EZ contributed ideas, writing the initial manuscript and creating the figures. XL revised the manuscript, approved the final version and received research support. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Immune Checkpoint Inhibitors in Tumors Harboring Homologous Recombination Deficiency: Challenges in Attaining Efficacy

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Cancer cells harbor genomic instability due to accumulated DNA damage, one of the cancer hallmarks. At least five major DNA Damage Repair (DDR) pathways are recognized to repair DNA damages during different stages of the cell cycle, comprehending base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ). The unprecedented benefits achieved with immunological checkpoint inhibitors (ICIs) in tumors with mismatch repair deficiency (dMMR) have prompted efforts to extend this efficacy to tumors with HR deficiency (HRD), which are greatly sensitive to chemotherapy or PARP inhibitors, and also considered highly immunogenic. However, an in-depth understanding of HRD's molecular underpinnings has pointed to essential singularities that might impact ICIs sensitivity. Here we address the main molecular aspects of HRD that underlie a differential profile of efficacy and resistance to the treatment with ICIs compared to other DDR deficiencies.

Keywords: immune checkpoint inhibitors, homologous recombination, DNA damage repair, mismatch repair, oncology

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INTRODUCTION

The central DNA Damage Repair (DDR) pathways comprise base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ), which are collectively responsible for repairing DNA damages during different stages of the cell cycle (1). In tumor cells, defects on DRR pathways, by one hand, works as a source of genetic diversity and mutations that are beneficial for tumor evolution. On the other hand, it exposes the tumor cell to fragilities not observed in normal cells. In this context, the functional status of the DDR system has long been recognized as a biomarker for a broad range of treatments (2).

Different therapies could take advantage of DDR pathways' defects to induce additional tumor genetic structural damage, as with radiotherapy, cytotoxic chemotherapies, or targeted DNA repair mechanisms such as PARP inhibitors, to enhance tumors cells' lethality (3). In addition, recently,

mismatch repair-deficient (dMMR) tumors have consistently been shown to harbor greater immunogenicity and be highly effective to immune checkpoint inhibitors (ICIs) (4, 5). Consequently, dMMR granted accelerated approval by the FDA to ICIs agnostic use to treat advanced solid tumors (6).

From this point onwards, understanding whether this effect also extends to other DDR pathways started to be deeply investigated. Although any type of DDR dysfunction can lead to the accumulation of tumoral mutations, there is a wide variety in burden and type of mutations, depending on the DNA level each repair mechanism actuates (7). However, the impact of those different pattern of mutations on immunogenicity and, consequently, on the response to immunotherapy is still a matter of debate.

The benefits achieved with ICIs in tumors with dMMR have prompted efforts to extend this efficacy to tumors with HRD, which are highly sensitive to chemotherapy or PARP inhibitors and expected to be highly immunogenic. Nonetheless, molecular underpinnings of HR defects have pointed to singularities that might impact antitumor immune response and ICIs effectiveness. This review will summarize the main molecular aspects of HRD that underlie a differential profile of efficacy and resistance to the treatment with ICIs compared to other DDR deficiencies.

DDR in Current Clinical Practice

Mismatch Repair

The most significant evidence linking DNA repair deficiency with ICIs activity stems from tumors with a deficiency in mismatch repair (MMR) (dMMR). Roughly 18% of endometrial cancers, 11% of ovarian cancers, and 4% of metastatic colorectal cancer present with mutations or epigenetic silencing in genes comprising the MMR system (8). In a phase II clinical trial evaluating pembrolizumab in a set of treatment-refractory dMMR tumors, the response rates were as high as 40% to 70% (9). The studies Checkmate 142 (10) and Keynote 164 (11), which evaluated nivolumab and pembrolizumab, respectively, led to ICIs' first approval, in dMMR tumors, for colorectal cancer previously submitted to chemotherapy. In addition, the Keynote 177 study (12) currently supports pembrolizumab use in the first-line setting of colorectal cancers. Finally, the Keynote 158 study (6) led to pembrolizumab approval for previously treated dMMR tumors irrespective of histology. Such an efficacy led to MMR status evaluation in current clinical practice for a broad set of other tumor histologies wherein this DDR deficiency can also be noticed, such as stomach, biliary tract, pancreas, prostate, and small intestine cancer (13).

Homologous Recombination

Homologous recombination (HR) is the most likely DDR mechanism found when considering a non-selected histology-based population (14). It is a crucial pathway to repair double-strand DNA breaks due to its error-free repairing system that relies on an intact sister chromatid instead of the non-homologous end joining (NHEJ) process (7). The incidence of pathogenic HRD varies according to histology, staging, and previous treatment burden (15). Notwithstanding, HRD is currently most recognized in tumors for which PARP inhibitors are currently approved based on a biomarker-guided *BRCA* or HR loss of function: ovarian cancer (40-50% with HRD), prostate cancer (20-25%), breast cancer (18%), and pancreatic cancer (12%) (16–20). Recently, many other malignancies were also shown to have a high incidence of HRD, such as endometrial (34%), biliary tract (28%), bladder (23%), hepatocellular (20%), and gastroesophageal cancer (20%) (14).

In contrast to the high clinical efficacy of ICIs in MMR deficient tumors, the clinical benefits are not consistent with an HRD. In phase II KEYNOTE 100 study, response rates with pembrolizumab in patients with advanced ovarian cancer were less than 10% among those harboring an HRD, with no statistical difference found when comparing BRCA-mutated versus wild-type counterparts (21). Despite other HRD genes being currently tested in ovarian cancer through NGS platforms, no prospective clinical data have evaluated their differential effectiveness, such that all available clinical data stem from BRCA-mutated tumors. Moreover, in phase III Keynote 119, patients with previously treated triple-negative breast cancers - approximately 50% of whom have HRD – derived no benefit from pembrolizumab compared to chemotherapy concerning response rate or survival (22). Although this study was not designed to evaluate patients with breast cancer having HRD specifically, both those ovarian and breast early clinical data shed light on a significant difference in clinical efficacy compared to what is seen early on with ICIs for dMMR tumors. In addition, those evidence has ultimately contributed to shifting strives for various ICIs combinations that are now undergoing prospectively to overcome such immune restoration mitigation – through anti-PD-1/PD-L1 –, which is taking place in the presence of HRD and remain underrecognized.

DDR and Immunogenicity

Deficient DDR processes that predispose to genetic alterations at the DNA sequence level, such as in dMMR, have the highest potential to elicit antigenicity due to the vast number of mutation-associated neoantigens (23). Since it has been shown that only a tiny fraction of predicted neo-epitopes are presented through MHC-I to enable T-cell responses (24, 25), it seems likely that tumors with a higher number of tumor mutation burden (TMB) are more likely to present with neoantigens that effectively stimulate the immune system (26).

Extensive mutational assessments have demonstrated enrichment in single- and multi-nucleotide variants (SNVs and MNVs) in tumors with dMMR, resulting in a high TMB, generally higher than 17 mutations/Mb (27). In the rare inherent genetic condition of bi-allelic germline dMMR, tumors can display >250 mutations/Mb (28). Due to dMMR tumors' high immunogenicity, ICIs are substantially effective in various settings, thus warranting approval on an agnostic indication basis. Regarding other hypermutated tumors, yet non-dMMR, a TCGA analysis has shown that somatic mutations in polymerase epsilon (POLE) or delta POLD1 also comprise a DDR deficient group with high TMB (29). Like in dMMR, impairment in the proofreading capability of *POLE* and *POLD1* leads to genetic alterations at the DNA sequence level. Pathogenic somatic mutations in the proofreading exonuclease

domain of *POLE* confer similar phenotypes regardless of the tumor tissue type, resulting in a large mutation rate, especially TCT>TAT and TCG>TTG transversions and, more rarely, concomitant microsatellite instability (30). Although somatic mutations in POLE have been identified in 2–8% of colorectal cancer and 7–15% of endometrial carcinoma (31), there are little data available reporting ICIs efficacy in these DDR populations due to their low incidence and the absence of systematic screening in daily practice (32, 33). Interestingly, extensive mutational profiling of 21.074 patients from 23 cancer types and subtypes suggested that *POLE/POLD1* mutation was not independently associated with survival to ICIs treatment after adjusting for TMB. The study concludes that mutations in the proofreading domain of *POLE/POLD1* are more likely to result in DNA repair defects featuring extremely high TMB, which contribute to more significant benefits from ICI treatment (34).

Tumors with HRD also have a higher mutational load and predicted neo-epitopes than those without DDR deficiencies (35). Intriguingly, when considering patients with high TMB tumors that are not MMR, POLE, or POLD1 deficient, there is no difference in survival compared to patients with low (<10 mut/Mb) TMB tumors also submitted to ICIs therapy (36). Although such an analysis did not specifically evaluate HRD, it emphasizes that TMB alone should not be considered a biomarker of sensibility to ICIs. Furthermore, the accumulation of genetic errors at the DNA strands' breaks level leads to a different set of a mutational landscape than DNA sequence alterations that characterize dMMR tumors (37), thus supporting that a high TMB in the presence of HRD may not correlate with the same efficacy seen in MMR or *POLE/POLD1* deficiencies.

Pan-tumor studies have shown that patients with genetic alterations classified as HR deficient frequently present with a high number of small deletions (indels) with flanking microhomology at the breakpoint, in addition to copy number variations (CNVs) (38). Notably, a pan-cancer TCGA analysis demonstrated that the levels of CNVs inversely correlated with a cytotoxic immune signature and clinical benefit from ICIs therapy (39). Moreover, when comparing tumors having a similar oncogenetic driver background but differing with respect to a *BRCA1* or *BRCA2* mutation, there is a significant difference in the levels of CNVs between each of these different HR deficient subtypes, in addition to a distinct set of immunoregulatory genes and ICIs efficacy (40). Conversely to *BRCA2* tumors, those with *BRCA1* deficiency presented with an immunoregulatory infiltrate and a limited response to ICIs. Moreover, another in-depth TCGA analysis also pointed to the coexistence of anti-tumoral immune transcripts downregulation, such as IFN-γ related genes, with the upregulation of immunosuppressive markers related to myeloid tolerogenic cells activity in *BRCA1* mutated breast cancers (41). Altogether, these data suggest that the tumoral HR-related genetic modifications could differentially regulate immune responses.

The molecular mechanisms supporting why CNVs or other specific genetic features associated with HRD mitigate immune responses remain unclear. Speculative hypothesis resides on large-scale mutational alterations leading to protein imbalance that impair tumor signals needed for cytotoxic immune cell infiltration or to deregulation of tumor signaling pathways that ultimately regulate immune cell recruitment (39). For a proper tumor antigen presentation, extensive integrity within the large HLA complex and the whole antigen processing machinery should be met (42). That complexity highlights the various vulnerable points that might lead to a dysfunctional tumor antigen presentation. The presence of CNVs can be associated with impaired antigen presentation owing to proteotoxic stress. Accordingly, the increased flux of unstable wild-type proteins may saturate critical chaperones and the proteasome complex while generating more self-peptides that ultimately place neoantigens at a further competitive disadvantage for loading onto limiting MHC protein (43).

Somatic copy number variation may also hinder tumor antigenic recognition through the downregulation of MHC I molecules. Extensive TCGA analyses demonstrate that loss of heterozygosis (LOH) in any MHC I genetic complex loci frequently accompanies tumors harboring chromosomal instability owing to alterations in cell cycle checkpoint genes such as *TP53*, in addition to HR deficient genes. Furthermore, tumor models with genomic instability frequently evolve with DNA hypermethylation silencing of genes belonging to the antigen presentation through MHC class I pathways (44). It is also noteworthy that a non-linear correlation between HLA-I LOH, TMB, and neoantigen burden has been suggested, such that HLA-I LOH is a frequent immune evasion mechanism in tumors overall, except for those with an either low or high (>30 mut/Mb) TMB, the latter of which are commonly represented by MMRd tumors (45).

In order to leverage neoantigen load and, thus, tumor recognition by immune cells, ongoing prospective studies are now evaluating PARPi added to ICIs in various HR deficient scenarios. Although it is attempting to speculate that further inducing inflammation in a somewhat immune-excluded tumor might restore anti-tumoral immune responses, some concerns may still be set. As aforementioned, neoantigen presentation's multifaceted and complex processes may hamper tumor recognition despite efforts to enhance immunogenicity by fostering tumor mutations, particularly in settings where at least a non-low tumor mutation burden and neoantigen load predominates. In such conditions, immune-tolerance likely occurs due to multiple coexisting mechanisms such as dysfunctional neoantigen presentation and CD8+ cells exhaustion mediated by cell-cell interactions and other non-ligand-receptor interactions that lead to immune resistance. As such, none of these mechanisms would be reversed by the primary intention of using iPARP to enhance tumor neoantigen load. Furthermore, PARP inhibitors in the presence of HR defects could foster the emergence of subclonal mutations that contribute to establishing intratumor heterogeneity under the pressure of the immunoediting process (46, 47). Indeed, intratumor heterogeneity has also been associated with ICIs resistance (48).

DDR and PD-L1 Expression

Cancer cells with dysfunction at the DNA strand break repairing apparatus increase the rate of DNA repair basal activity to establish

genome stability, particularly in the presence of constant cell proliferation. When molecular cascades featuring the homologous repair system are operating, checkpoint kinase 1 (Chk1) activation can also trigger the STAT1 – STAT3 – IRF1 signaling pathway, inducing PD-L1 expression in tumor cells (49). This model of tumor intrinsic PD-L1 expression, which is dependent on oncogenetic tumor features, has been defined as constitutive to distinguish the so-called acquired expression, in which tumor cells express PD-L1 in response to IFN-γ expression mediated by antitumor lymphocyte activity (50). The DNA repair signaling pathway ATR/Chk1/STAT3 can also upregulate CD47 and, through the engagement of SIRPα, suppress the capacity of antigen-presenting cells (APCs) to phagocytose and cross-present (51).

Concerning the constitutive tumor PD-L1 expression, ICIs may poorly correlate with response and survival, paradoxically predicting less benefit with the anti-PD-1/PD-L1 blockade in some tumors. Although PD-L1 expression is strongly correlated with clinical benefit in non-small cell lung cancer, tumors with EGFR activated-mutations, which can upregulate PD-L1 expression (52), do not derive benefit from ICIs' treatment (53). Likewise, in the context of PD-L1 expression mediated by HRD, *BRCA1* mutated breast cancer has been demonstrated to have a higher PD-L1 tumor score than *BRCA2* mutated, even though clinical efficacy is inferior (41, 54). Not only do these data support that the PD-L1 expression does not represent a perfect biomarker for ICIs response across all tumor settings, but also suggest that a non-canonical tumor PD-L1 expression (i.e., constitutive) might even associate with mechanisms of immune resistance.

The HR-driven constitutive PD-L1 expression, which occurs in a non-canonical fashion, irrespective of effector T cells activity, might mitigate ICIs efficacy by hypothetical mechanisms. Firstly, and simplistically, a sufficient lymphocyte infiltration to be restored by ICIs is essential for an effective immune response to take place. Indeed, tumor immune infiltrates (TIL) are a known biomarker predicting clinical efficacy to ICIs in various tumors (55, 56). In this regard, the simple fact of witnessing PD-L1 expression does not guarantee that this results from the positive pressure (i.e., INF-γ driven) of the presence of an immune infiltrate. Secondly, even in the presence of an adequately primed effector immune infiltrate, the constitutive tumor PD-L1 expression fomented by HRD could provide an overwhelming pool of ligands to the PD-1-expressing immune cells that might occupy the tumor microenvironment. Therefore, this could help to polarize immune responses towards a suppressive spectrum, as exemplified by the PD-L1 persistent inducement of FOXP3 expression (FOXP3 ^{high}) in PD-1+ T-cells (57), which are characteristically associated with a decreased capacity to reinvigorate into anti-tumoral responses despite ICIs' activity (58, 59). Lastly, the constitutive expression of PD-L1 may also provide evolutionary metabolic advantages to cancer cells by fostering tumor glycolysis and, in turn, impacting immune cells' metabolic fate (60). As such, the PD-L1 expression in cancer cells can directly regulate tumor metabolism through Akt/mTOR signaling, independently of the PD-1 engagement, therefore upregulating tumor glycolysis that leads to microenvironment glucose deprivation and lactic acid concentration (**Figure 1**).

DDR and Metabolic Reprogramming

Tumor cells with DDR defects have a high requirement to restore DNA damage through compensatory pathways. Ataxia-telangiectasia mutated (*ATM*) and DNA-dependent kinases (*DNA-PK*) are crucial proteins to recognize DNA damage and initiate repair signaling cascades. Besides their function in DNA strand-break repair, these proteins can remodel cancer metabolism through upregulation of glucose transporter (*GLUT*) channels and pyruvate kinase M2 (*PKM2*) enzyme, thus fostering tumor glycolysis (61). Hyperactivation of glycolysis is one of the hallmarks of cancers and has been implicated in immune evasion owing to nutrient competition and toxic metabolites accumulation, such as lactic acid (60). Furthermore, ATM activity can also induce glucose-6-phosphate dehydrogenase (*G6PD*) expression, which is fundamental to enable the pentose phosphate pathway (*PPP*) (62). The oxidative PPP generates ribose-5-phosphate, a precursor for nucleotide synthesis, and reduces the potential in the form of NADPH, which is needed for nucleotide biosynthesis and lipogenesis (**Figure 1**). Previous studies also demonstrated that *BRCA1* mutation and PARP1 activity also influence tumor metabolism. The *BRCA1* lack in breast cancer was associated with increased glycolytic metabolism than *BRCA1*-WT (63, 64). Furthermore, it was demonstrated that PARP1 works as a transcriptional coactivator for *PKM2* driving the expression of glycolytic genes (GLUT and LDH) in tumor cells (65). However, the role of metabolic changes induced by *BRCA1* and PARP1 on primary resistance to ICIs remains unknown.

DDR and STING

A dysfunctional HR status predisposes cancer cells to DNA strands fragmentation in the presence of additional DNA damaging factors, such as radiotherapy, chemotherapy, or PARP inhibitors. Furthermore, DNA instability can occur spontaneously owing to the high tumor cell turn-over coupled with cell cycle checkpoints suppression and enhanced metabolic stress due to tumor metabolism deregulation and microenvironmental hypoxia. This background predisposes to frequent cytosolic DNA exposure in cancer cells. The cytosolic DNA activates the stimulator of IFN genes pathway (*STING* pathway) and *IRF3* activity, thus inducing the transcription of IFN type I and chemoattractive cytokines (CXCL10 and CCL5), which mediates monocytes and neutrophil recruitment in an antigen-independent manner (66). Although type I IFN is a known contributor to T cell priming by inducing MHC I antigen cross-presentation in APCs, there have been growing insights linking STING-IFN molecular pathways to mechanisms mitigating effective immune responses (67, 68). Accordingly, an enhanced baseline STING-IRF3 activity can promote the sustained recruitment of monocytes in response to CXCL10 and CCL5 chemokines, inducing a chronic myelocytic inflammatory infiltrate that could further contribute to establishing an immune tolerogenic state (69). Those constant levels of DNA damage featuring HR deficient tumors can also activate an alternative STING pathway through ATM-TRAF6 driven transcription of TGF-β that promotes protumor M2-like

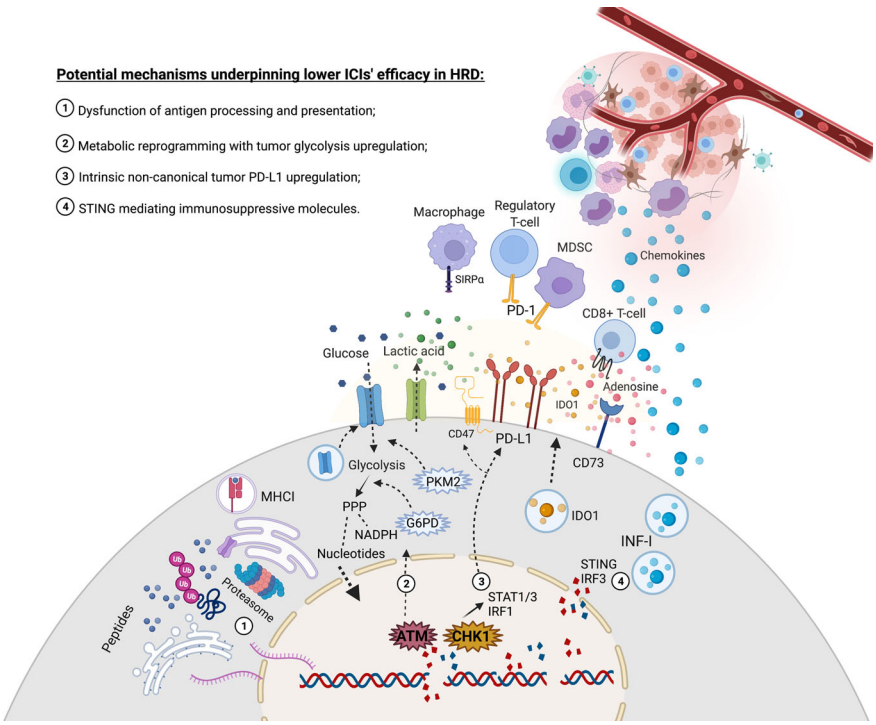

FIGURE 1 | Increased rate of DNA double strands breaks due to deficiency in HR might evolve with molecular events that lead to challenges in restoring immune responses through immune checkpoint inhibitors. (1) DNA double-strand breaks association with CNV and large structural genetic alterations contribute to an increased flux of unstable mRNA and, ultimately, proteins that may saturate critical chaperones and the proteasome complex, thus leading to a dysfunctional tumor antigen presentation. (2) ATM plays a central role in recognizing DNA strand breaks but can also upregulate glycolysis and PPP to replenish nucleotides and NADPH supply for the upcoming anabolic reactions to restore DNA damages. This metabolic regulation might deprive glucose in the tumor microenvironment and export acid lactic, impacting immune responses. (3) *CHK1* is crucial to repair strands breaks but may also activate the STAT1-STAT3-IRF1 signaling pathway that contributes to upregulating PD-L1 expression. (4) Cytosolic DNA censoring can lead to STING-IRF3 production of IFN-I, which might recruit monocytes that will be further exposed to a range of tolerogenic stimuli in the tumor microenvironment. Moreover, the STING signaling pathway might induce IDO1, and the expression of IFN-I might upregulate CD73, thus contributing to producing inhibitory molecules in the tumor microenvironment. Created with BioRender.com.

macrophage and Treg cell differentiation, respectively (70). Lastly, the *STING* signaling pathway can also contribute to establishing a tolerogenic tumor microenvironment by inducing immune-suppressive soluble factors. An increase in IDO expression was shown to occur in STING mediated fashion when in the presence of mild tumor antigenicity (71). Moreover, the augmented IFN-α expression has been shown to upregulate the ectonucleotidase CD73 and leverage adenosine production in a tumor microenvironment wherein DDR might be fostering ATP production (72) (**Figure 1**).

SUMMARY AND FUTURE PERSPECTIVES

The data summarized in this review suggest that HRD tumors have a differential profile of efficacy and resistance to ICIs' treatment compared to other dMMR. Each DDR deficient pathway could lead to the emergence of a singular tumor mutational background, but the correlation between such a range of mutational patterns and the response to ICIs remains unclear. Furthermore, various mechanisms potentially impacting immune responses could emerge from the increased DDR pathways activity, which leads to tumor metabolic rearrangements and microenvironmental recruitment of immune-suppressive factors. The TMB status may not be a pan-cancer predictive biomarker for immunotherapy response, and the incorporation of tumor DDR pathways might be necessary for future genomic biomarker refinements. As such, it would be interesting to carry out studies on tumors harboring different defects in DNA repair pathways.

AUTHOR CONTRIBUTIONS

SBS and LMC conceived the work. SBS, CW, SW and LMC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Efficient DNA Repair Mitigates Replication Stress Resulting in Less Immunogenic Cytosolic DNA in Radioresistant Breast Cancer Stem Cells

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Cancer stem cells (CSCs) are a major cause of tumor therapy failure. This is mainly attributed to increased DNA repair capacity and immune escape. Recent studies have shown that functional DNA repair *via* homologous recombination (HR) prevents radiation-induced accumulation of DNA in the cytoplasm, thereby inhibiting the intracellular immune response. However, it is unclear whether CSCs can suppress radiation-induced cytoplasmic dsDNA formation. Here, we show that the increased radioresistance of ALDH1-positive breast cancer stem cells (BCSCs) in S phase is mediated by both enhanced DNA double-strand break repair and improved replication fork protection due to HR. Both HR-mediated processes lead to suppression of radiation-induced replication stress and consequently reduction of cytoplasmic dsDNA. The amount of cytoplasmic dsDNA correlated significantly with BCSC content (p=0.0002). This clearly indicates that HR-dependent avoidance of radiation-induced replication stress mediates radioresistance and contributes to its immune evasion. Consistent with this, enhancement of replication stress by inhibition of ataxia telangiectasia and RAD3 related (ATR) resulted in significant radiosensitization (SER37 increase 1.7-2.8 Gy, p<0.0001). Therefore, disruption of HR-mediated processes, particularly in replication, opens a CSC-specific radiosensitization option by enhancing their intracellular immune response.

Keywords: immunogenic cytosolic dsDNA, radioresistance, replication stress, ATR inhibition, cellular immuneresponse, DNA repair, homologous recombination, breast cancer stem cells (BCSCs)

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INTRODUCTION

Accumulation of DNA in the cytoplasm in the cell activates the innate immune response through cyclic GMP-AMP synthase (cGAS) and binding to the activator protein stimulator of interferon genes (STING). STING induces phosphorylation and translocation of the transcription factor interferon regulatory factor 3 (IRF3) and initiates the expression of type-1 interferon (type-1 IFN). This intracellular immune response primarily serves to defend against foreign DNA but cannot distinguish it from its own cytosolic DNA. The accumulation of self-DNA in the cytosol is triggered by DNA damage and leads to the production of type-1 IFN (1). The trigger for the increased occurrence of cytosolic DNA may be a defect in DNA repair mechanisms (2, 3). This has been observed when a defect in the DNA repair pathway homologous recombination (HR) is present (3–5). Increased accumulation of cytosolic DNA and activation of the cGAS/STING pathway have also been observed in RAD51-, BRCA1-, or BRCA2-deficient carcinoma cell lines. HR is the major DNA double-strand break repair pathway of the S phase. It serves to repair direct and replication-associated DNA double-strand breaks (DSBs) in an error-free manner. In addition, factors of HR, such as RAD51, BRCA1 and BRCA2, stabilize DNA at active replication forks and protect it from degradation by nucleases such as MRE11 (6, 7). This mediates repair and restart of replication forks, prevents formation of single-ended replication-associated DSBs, and thus avoids DNA replication stress. HR is activated by the kinases Ataxia telangiectasia and Rad3-related (ATR) and checkpoint kinase 1 (CHK1). ATR is recruited to replication protein A (RPA)-bound ssDNA, which occurs at DNA replication forks in the presence of DNA damage or dNTP deficiency and at resected DNA DSBs. ATR phosphorylates CHK1 and initiates the intra-S phase checkpoint. This leads to cell cycle arrest, prevents further firing of replication origins, and CHK1 is itself also involved in protecting stalled replication forks (8, 9). Through phosphorylation of BRCA2 and RAD51, CHK1 directly initiates HR-mediated DNA repair (10). Recent studies showed that disrupting the S-phase damage response by inhibiting ATR significantly increased the amount of cytosolic DNA after irradiation in breast cancer cells (11). Thus, the S-phase DNA damage response and DNA repair by HR to avoid DSB and replication stress are critical factors for the activation of the intracellular immune response.

Tumors are composed of a heterogeneous population of cancer cells with diverse replicative, tumorigenic, metastatic, and therapy-resistant capabilities. In particular, highly plastic subpopulations of stem-like cells within the tumor bulk, termed cancer stem cells (CSCs), tumor initiating cells (TICs) or tumor stem cells (TSC) have been described for breast cancer and are now considered to drive tumorigenesis, chemoresistance, and metastasis. This is mainly attributed to their upregulated DNA damage response and DNA repair capacity. Their radiosensitivity directly correlated with the number of CSCs in xenograft tumor models (12). In fact, repeated irradiation even led to an accumulation of CSC *in vitro* and *in vivo* in HNSCC, breast cancer, glioblastoma, and pancreatic cancers (13–18). It has long been assumed that CSC, just like tissue stem cells, are mostly in a quiescent state and DNA damage is mainly repaired by classical non-homologous end-joining (cNHEJ) (19). However, for CSC in glioblastoma and breast cancer, it has been shown that only about one third of CSC are quiescent and re-enter into the cell cycle after irradiation (16, 20). In fact, a higher proportion of S/G2 phase in CSC of triple-negative breast cancer (TNBC) compared to bulk cells was observed (21). Controversial experimental data are available about the contribution of cNHEJ to radiation resistance of CSC. So far, only an increased activation of DNA-dependent protein kinase (DNA-PKcs) after irradiation has been observed in glioblastoma CSC (22, 23). Other studies, however, showed a decreased activation of DNA-PKcs and ataxia telangiectasia mutated protein (ATM) after irradiation in CSC of NSCLC or a generally decreased cNHEJ activity in glioblastoma CSC (24, 25). Most studies observed a key role of the intra-S-phase kinase CHK1 in radiation resistance in glioblastoma CSC and breast cancer (14, 15, 26, 27). Increased expression of CHK1 was shown (14, 26, 27), as well as significantly stronger phosphorylation after irradiation (15, 26, 27). Phosphorylation of CHK1 resulted in cell cycle arrest and activated DNA repair by HR (28). Several studies demonstrated a dependence of CSC on HR and its key protein RAD51 (29). Glioblastoma CSCs showed high protein expression of RAD51 and dependence of CSC on HR repair after irradiation. Accordingly, the protein expression of RAD51 significantly decreased during differentiation (30). Correspondingly, inhibition of RAD51 resulted in significant radiation sensitization of glioma CSC (31). ALDH1-positive CSC of TNBC also showed increased RAD51 protein expression compared to ALDH1-negative cells, resulting in resistance to olaparib (32). After irradiation, isolated CSC from TNBC culture showed significantly more RAD51 foci than bulk culture (21). It is unclear what role ATR plays in this context, as CHK1 is one of the major downstream targets of ATR. ATR initiates cell cycle checkpoint, both during normal progression and in response to DNA damage. Therefore, most previous observations of BCSC resistance mechanisms suggest more effective DNA repair during replication mediated by CHK1 and its upstream kinase ATR.

In addition to a more effective DNA repair capacity, mechanisms of immune evasion were observed in CSC. A decreased expression of the antigen processing gene-associated transporter (TAP) and the co-stimulatory molecule CD80 was observed in ALDH1-positive BCSC, resulting in decreased susceptibility to T cells (33). Furthermore, an increased expression of PD-L1 was observed, which also suppresses T cell stimulation (34). Recently, it was also shown that DNA-damage in S-Phase leads to the activation of cGAS/STING pathway and further increases the expression of PD-L1, counteracting T-cell stimulation by the innate immune response. This was attributed to the activation of the ATR-CHK1 signaling pathway, leading to expression of the IRF1 gene *via* STAT3 and STAT1 phosphorylation, which resulted in increased PD-L1 gene expression (35). Thus, there appears to be a direct link between DNA damage response and immune evasion triggered by HR-mediated processes and activation of DNA damage response in S phase. The observations further imply that the innate immune response, particularly in BCSC,

should be exploited by inhibiting its effective DNA repair mechanisms to successfully employ novel immunotherapies. This question is the subject of the presented study and was investigated using three TNBC breast cancer cell lines, a luminal reference cell line, their isogenic radioresistant clones, and isolated ALDH1-positive CSC.

MATERIALS AND METHODS

Cell Culture and Treatments

All cell lines used in the study were either purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) or kindly provided by Prof. Dr. H. Wikman. The MCF7 is of the luminal subtype, the MDA-MB-231 is of the TNBC subtype. The MDA-231 BR (Brain) and -SA (Sarcoma) are derivatives of the MDA-MB-231 which were originally selected with respect to their metastatic behavior in xenograft (36, 37). In Xenografts they induce a primary tumor and only brain- (MDA-MB-231 BR) or only bone metastases (MDA-MB-231 SA). All cell lines were cultivated in DMEM medium with 10% FCS, 2% glutamine and 1% penicillin streptomycin in incubators at 37°C, 5% CO_2 atmosphere and 100% humidity in cell culture flasks. ATR-inhibition was achieved by using the small molecule inhibitor VE-821 at 2 μM for 2h, for the inhibition of CHK1 the small molecule inhibitor MK-8776 was used at 2μM for 2h.

Generation of Radioresistant Clones

Cells were irradiated with 4 Gy X-rays (200 kV, 1.2 Gy/min), surviving cells were pooled, cultivated for 10-14 days and irradiated again. This procedure was repeated 10 times to a total dose of 40 Gy. Radiosensitivity was checked 14 and 42 days after the last irradiation.

Homologous Recombination Assay

HR capacity was measured by stable or transient transfection of the pDR-GFP (Addgene #26475, kindly provided by M. Jasin) plasmid, linearized by digestion with I-SceI enzyme prior to transient transfection. Briefly, 1 μg of linearized plasmid (pDR-GFP) was transfected into cells with FuGENE (Roche) at a ratio of 1:3 μg/μl according to the manufacturer's instructions. In cells with stably integrated pDR-GFP 1μg I-SceI plasmid was transfected with FuGENE (Roche) at a ratio of 1:3 μg/μl. To measure transfection efficiency, cells were transfected with pEGFP-N1 (1 μg) in a parallel approach. After 24 hours, cells were harvested, and the percentage of GFP-positive cells determined by flow cytometry. HR capacity was calculated according to GFP-positive cells (pDR-GFP) and transfection efficiency (pEGFPN1) [**Supplementary Figure S2D** (38, 39)].

DNA Fiber Assay

Exponentially growing cells were pulse labeled with 25 μM CldU (Sigma) followed by 250 μM IdU (Sigma) for 30 min each. Hydroxyurea (HU) was added for 4h between both labels. Labeled cells were harvested, DNA fiber spreads prepared and stained as described (40). Fibers were examined using an Axioplan 2 fluorescence microscope (Zeiss). CldU and IdU tracks were measured using ImageJ (40). At least 300 forks per sample were analyzed.

Clonogenic Survival

250 cells per well were seeded in a 6-well plate 6h before irradiation and were cultured for 14 days. Cells were fixed and stained with 1% crystal violet in ethanol (Sigma-Aldrich, St. Louis, MO). Colonies with more than 50 cells were counted and normalized to untreated samples. Each survival curve represents the mean of at least three independent experiments.

Immunofluorescence

Cells were seeded on culture slides. Cells were pulse labeled with 10 μM EdU for 20 minutes prior to treatment. After treatment the cells were fixed, permeabilized and blocked. Foci were detected using anti-53BP1 (Rabbit-anti 53BP1, 1:2000, Novus Biologicals), RPA (Mouse-anti RPA, 1:400, Santa Cruz), γH2AX (Rabbit-anti γH2AX, 1:250, Novus Biologicals), RAD51 (Rabbit, 1:500, Calbiochem), IFN-ß1 (Rabbit-anti IFN-ß1, 1:1000, Cell signaling) or IRF3 (Rabbit-anti IRF3, 1:400, Cell Signaling) followed by Alexa Fluor 488 goat anti rabbit IgG (Cell Signaling, 1:600), AlexaFluor 488 goat anti mouse IgG (Cell signaling, 1:500), AlexaFluor 594 goat anti rabbit IgG (Abcam, 1:600) or AlexaFluor 647 goat anti rabbit IgG (Cell Signaling, 1:600) and mounted (Vector Laboratories). EdU was stained with Alexa Fluor Azide 594 (Life Technologies, 1:500) and nuclei were stained with DAPI. Foci and fluorescence Intensity were quantified manually by capturing fluorescence images using a Zeiss Axioplan 2 fluorescence microscope equipped with a charge-coupled device camera and Axiovision software followed by quantification by Image J software. RPA/γH2AX-Foci were quantified automatically by the Aklides®-system (Medipan). Foci and fluorescence intensities of 100 cells per dose per slide and experiment were quantified.

Flow Cytometric Analysis of $CD44^{high}$/$CD24^{low}$ Cells

Cells were harvested and washed in phosphate-buffered saline (PBS) with 0.5% fetal bovine serum. Combinations of fluorochrome-conjugated monoclonal antibodies against CD44 [APC, DB105, Miltenyi Biotec, 130-095-177 (1:100)] and unconjugated CD24 [CD24-biotin, eBioSN3 (SN3 A5-2H10), eBioscience, 13-0247-80 (1:50), followed by Alexa Fluor 405 (Cell signaling, 1:500)] were used. Primary antibodies or the respective isotype controls (BD Biosciences) were added to the cell suspension, as recommended by the manufacturer, and incubated at 4°C in the dark for 20 min. The labeled cells were analyzed *via* flow cytometry.

Flow Cytometric Analysis of ALDH1-Activity

Cells were harvested, washed in PBS, incubated with ALDEFLUOR™ reagent (StemCell Technologies, Grenoble, France) and incubated at 37°C for 45 minutes. Meanwhile, 5 μl of diethylaminobenzaldehyde (DEAB), a specific ALDH

inhibitor, was added to 0.5 ml of ALDEFLUOR™-stained cells as a negative control. ALDH1-positive cells were then quantified by flow cytometry.

PicoGreen® Assay

Cells were irradiated with 8 Gy, harvested after 16 hours, washed in cold PBS and incubated with protease-inhibitors (Thermo Scientific Halt™ Protease Inhibitor Cocktail). Nuclear and cytoplasmatic fractions were separated with a nuclear and cytoplasmatic extraction reagent (Thermo Scientific NE-PER™). Cytoplasmatic dsDNA was stained using the Quant-iT™ PicoGreen® dsDNA Reagent and Kits (Thermo Scientific). A standard curve was prepared and measured together with the samples in a Spark® Microplate reader.

Statistical Analysis

Statistical analysis, curve fitting and graphs were performed using Prism 6.02 (GraphPad Software). Data are given as mean (+SEM) of 3-5 replicate experiments. Unless stated otherwise, significance was tested by Student's t-test.

RESULTS

Cytosolic DNA Correlates With Breast Cancer Cell Proportion (BCSC)

The appearance of cytosolic dsDNA is crucial for the initiation of the intracellular immune cascade (41). Cells with a DNA repair defect in HR experience increased activation of the cGAS/STING pathway and subsequent activation of the intracellular immune response due to elevated cytosolic dsDNA (4). It has not been investigated whether CSCs can suppress the induction of cytosolic dsDNA and thus an intracellular immune response, through efficient DNA repair mechanisms. To this end, the importance of the DNA damage response in relation to CSC content on radioresistance and the appearance of cytosolic dsDNA was investigated in three isogenic, triple-negative (TNBC) and one luminal cell lines and their corresponding radiation-resistant subclones. **Figure 1A** shows that TNBC cell lines have lower amounts of cytosolic dsDNA after than the luminal cell line, with only 0.55 ± 0.06, 0.74 ± 0.02 and 0.8 ± 0.01 in the MDA-MB-231 WT/BR/SA compared to the amount observed in MCF7 cells (p=0.04).

To investigate the relevance of the proportion of BCSC for this observation, ALDH1 activity was determined using the ALDEFLUOR™ assay (**Figure 1B**). TNBCs had almost twice as many ALDH1-positive cells compared to MCF7 cells (73 ± 6 versus 39 ± 1%, p<0.001), while the three TNBC cell lines examined had comparable proportions. The observed differences in CSC proportion were confirmed by further CSC markers such as plating efficency, migration ability and the proportion of CD44high/CD24low cells (**Supplementary Figures S1A–C**).

To further increase the proportion of BCSC, cell lines were repeatedly treated with ionizing radiation (**Figure 1C**), and the effects for cellular survival were analyzed (**Figure 1D**) (13, 14). Consistent with the assumption that the proportion of ALDH1-positive cells determines radiosensitivity, the initial cell lines already showed significant differences in radiosensitivity corresponding to their ALDH1-positive proportion. Accordingly, the radioresistant subclones (RR clones) of each cell line showed a marked increase in radioresistance compared to the baseline cell lines, with an increase in D37 between 1.2-1.8 (**Figure 1D** and **Figure S1D**). To confirm that radioresistance was due to the proportion of ALDH1-positive BCSCs, the ALDEFLUOR™ assay was performed (**Figure 1E**). As expected, all RR clones showed an increase in the proportion of ALDH1-positive cells. MCF7 cells showed the highest increase, approximately 25%, whereas TNBC cell lines showed only a slight increase in the already high proportion in the parental cell lines, ranging from 5% to 20%. Thus, a high proportion of ALDH1-positive BCSCs resulted in a lower incidence of cytosolic dsDNA and was consistent with the generally accepted concept that the more BCSCs present, the higher the radiation resistance (**Figure 1F**).

Radiation Resistance Of BCSC Is Mainly Mediated In S Phase

Radiation sensitivity is significantly influenced by DNA repair in addition to other factors such as proliferation, cell cycle distribution. Therefore, it was tested whether the observed radioresistance of ALDH1-positive cells was due to enhanced DNA repair. **Figure 2A** shows examples (top) and quantification (bottom) of the number of 53BP1 foci remaining after 24 h in cells that were either outside of S phase (EdU-, **Figure 2A** bottom left) or actively replicating (EdU+, **Figure 2A** bottom right) at the time of irradiation with 6 Gy. All radioresistant clones showed significantly fewer 53BP1 foci after irradiation than the parental cell lines. This difference was even more pronounced when the cells were in S phase during irradiation. Here, MCF7/RR and MDA-MB-231/RR showed the strongest reduction in 53BP1 foci compared to their parental cell lines with 4.1 ± 0.57 vs. 8.9 ± 0.81, (p<0.0001) for MCF7/RR and 3.8 ± 0.56 vs. 7.3 ± 0.79 for MDA-MB-231/RR (**Figure 2A**, bottom right). MDA-MB-231BR/RR and -SA/RR also showed a significantly lower number of 53BP1 foci than the respective parent cell line with 11.9 ± 0.8 vs. 16.8 ± 0.9, (p=0.0001) and 11.6 ± 0.8 vs. 17.0 ± 0.9, (p<0.0001). Even in cells that were not in S-phase at the time of irradiation (**Figure 2A**, bottom left), enhanced DNA repair was also detected in the RR clones, but to a significantly lower level, with 5.3 ± 0.3 vs. 6.5 ± 0.4 for MCF7/RR (p=0.02), 3.2 ± 0.4 vs. 4.8 ± 0.5 for MDA-MB-231/RR (p<0.05), 6.2 ± 0.6 vs. 8.5 ± 0.9, for MDA-MB-231BR/RR (p=0.02) and 10.4 ± 0.7 to 13.5 ± 0.6, MDA-MB-231SA/RR (p=0.0007).

These results suggest that all, but especially the DNA repair pathways in S phase are upregulated in the RR clones. Since DSBs in S phase are mainly repaired by HR, investigations were focused on the analysis of HR-dependent processes. All cell lines examined showed HR competence, evident from the successful formation of RAD51 foci formation after treatment with mitomycin C (MMC) (**Supplementary Figure S2A**) as well as successful DNA repair of the HR specific reporter construct, after both transient and stable transfection (**Supplementary Figures S2B, C**) (39). Interestingly, in the RR clones significantly higher HR capacity compared to the respective

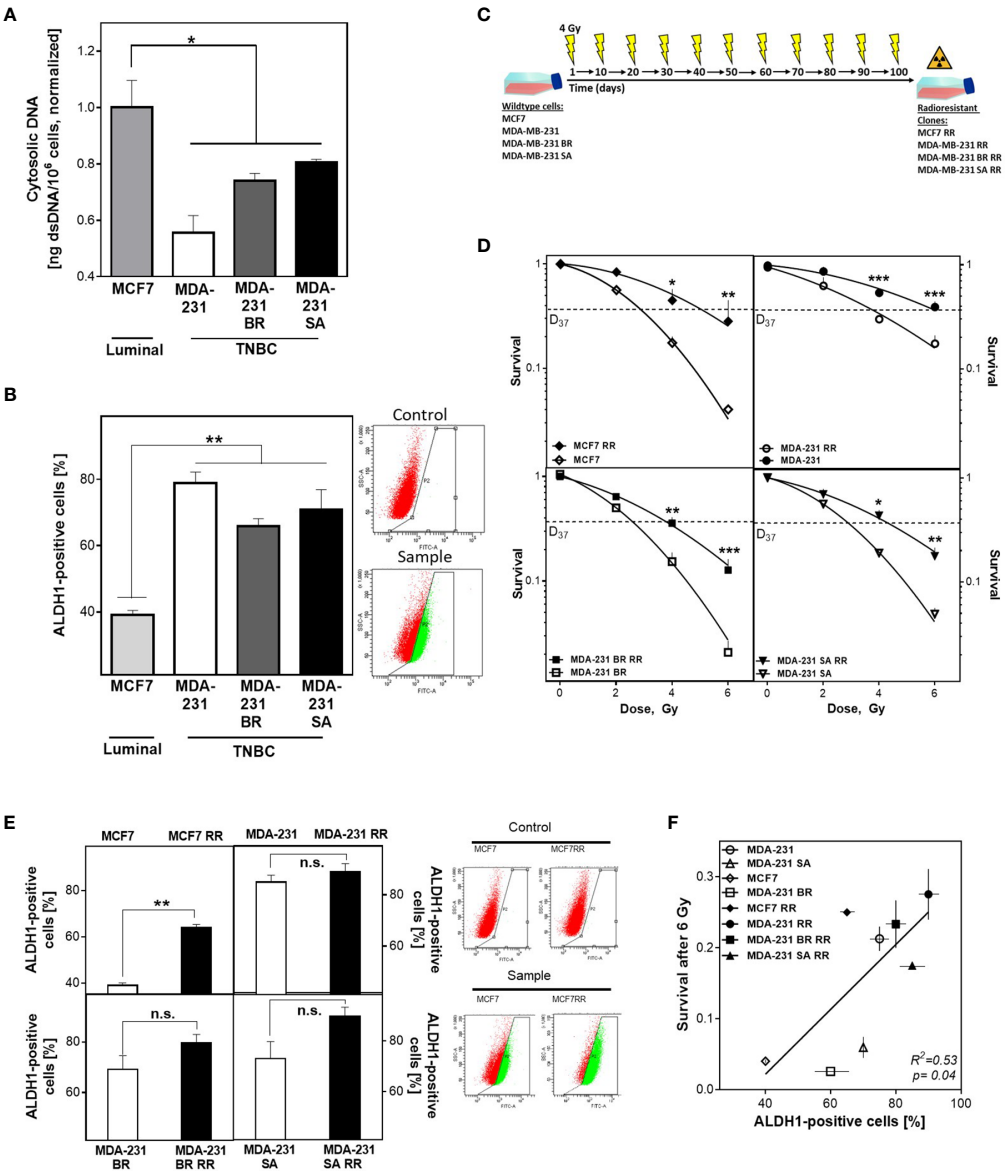

FIGURE 1 | BCSC proportion correlates with cytosolic DNA and radioresistance. **(A)** Cytosolic dsDNA in TNBC and luminal cells. Cytoplasmatic fractions were isolated, dsDNA stained with PicoGreen®reagent and quantified in a Spark® reader. **(B, E)** Detection of ALDH1 positive cells. Cells were treated with ALDEFLUOR™ reagent, harvested and the ALDH1 positive cells quantified by FACS. **(C)** Scheme to generate radioresistant sub cell lines. Cells were irradiated, pooled, and irradiated again after two weeks. The procedure was repeated ten times. **(D)** Cellular survival after irradiation. Cells were seeded 6 hours prior to treatment, irradiated with indicated doses, fixed after 14 days and the numbers of colonies was counted. **(F)** Correlation of the percentage of ALDH1-positive cells and cellular survival. Shown are means from three independent experiments ± SEM. Asterisks (*) represent significant differences (n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001; Student's t-test).

parental cell line was observed, with 1.28 ± 0.08 vs. 1.05 ± 0.05 for MCF7/RR (n.s) and 1.34 ± 0.08 vs. 0.98 ± 0.05 for MDA-MB-231/RR (n.s). MDA-MB-231 BR-RR even showed a 2-fold and MDA-MB-231 SA-RR a 3-fold increase in HR capacity with 2.0 ± 0.1 vs. 1.01 ± 0.45, (p=0.012) and 2.95 ± 0.1 vs. 1.0 ± 0.07, (p=0.003), respectively (**Supplementary Figures S2B, C**) (39).

Since HR has its highest activity in S phase (28) it was important to ensure that the observed differences in HR capacity were not due solely to differences in cell cycle distribution in favor of increased S phase content in the RR clones. **Figure 2C** shows the percentage of S phase cells for the RR clones compared to the parental cell lines. It was apparent that the RR clones all had a lower S phase content than the parental cell lines, with 24.9 ± 1.1% vs. 40 ± 5.0% in MCF7, 31.6 ± 3.0% vs. 38.5 ± 6.2% in MDA-231, 36.8 ± 8.3% vs. 44.8 ± 3.0% in BR, and 38.9 ± 7.5% vs. 42.9 ± 0.5% in SA, which was not significant in any of the cell lines.

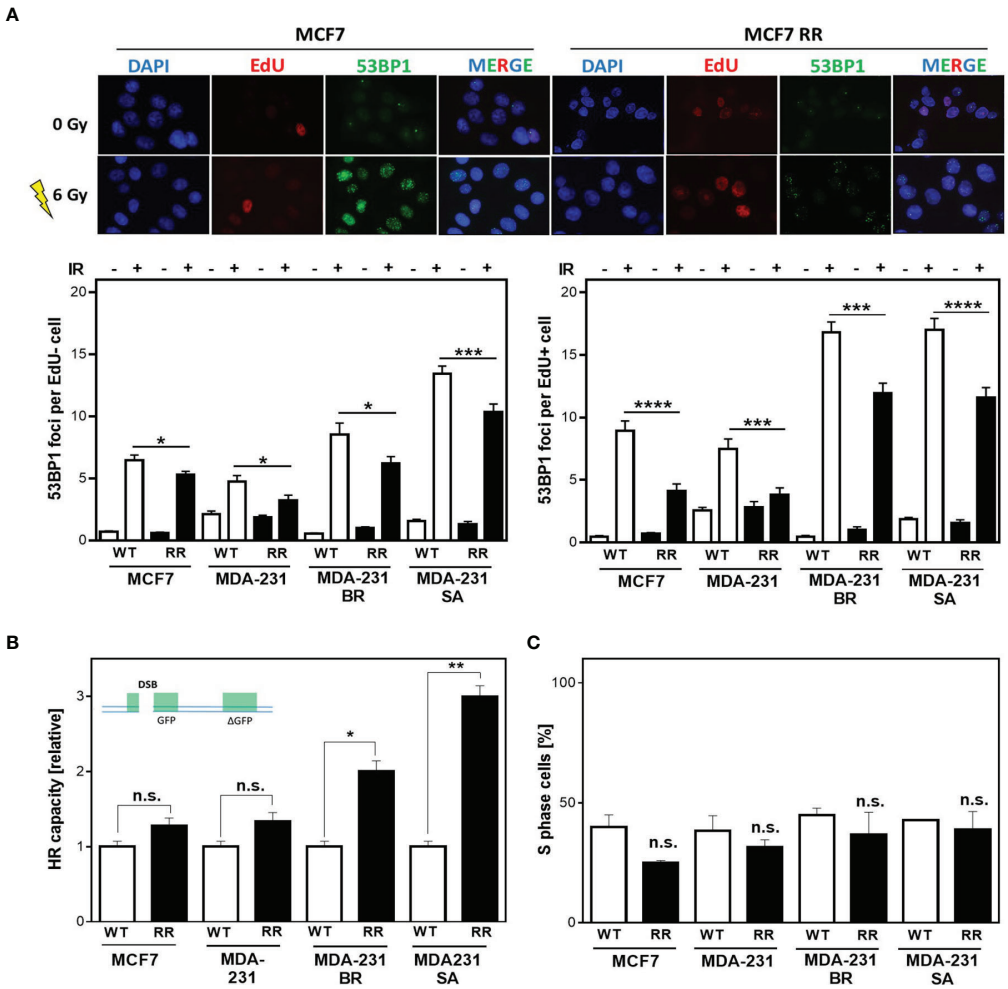

FIGURE 2 | Radiation resistance of BCSC is mediated in S phase. **(A)** 53BP1 foci (green) in non-S phase (EdU-, bottom left) and S phase (EdU+, bottom right) cells after irradiation. Cells were irradiated with 6 Gy after pulse labeling with 10 μM EdU for 20 min. Immunostaining was per-formed 24h after treatment with a specific antibody against 53BP1 and a fluorescent second anti-body. Nuclei were stained with DAPI, replicating cells were discriminated by incorporated EdU stained with the "click-it"-reaction. Foci were quantified with Image J Software for EdU+ and EdU- cells (n = 100). **(B)** HR repair of DSB. Cells were transiently transfected with the linearized DR-GFP plasmid for 24h. The number of GFP-expressing cells was analyzed by FACS and HR capacity of the radioresistant clones was normalized to the absolute HR capacity of the parental cell lines. **(C)** Percentage of S Phase cells. Exponentially growing cells were pulse-labeled with 10 μM EdU for 20 minutes, fixed and EdU stained with the "click-it" reaction. Nuclei were counterstained with DAPI. The number of EdU+ and EdU- cells was counted (n=100). Shown are means of three independent experiments ± SEM. Asterisks (*) represents significant differences (n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, Student's t-test).

Taken together, these data indicate that the observed radioresistance is largely mediated by DNA repair processes involving HR in S phase through increased HR-dependent DNA repair in RR clones. It is unclear whether this is solely attributable to more efficient double-strand break repair (DSB repair) or whether the replication-associated functions of HR are of much greater importance.

Avoidance Of Replication Stress By Functional HR Mediates Radioresistance Of BCSC

To ensure that the increased radioresistance and HR capacity was attributable to the proportion of ALDH1-positive BCSC, they were isolated by FACS sorting from the parental MCF7 as well as the radioresistant MCF7 clone (**Figure 3A**) and their radiosensitivity was determined (**Figure 3B**). There was a comparable increase in radioresistance for both ALDH1-positive subpopulations to a D37 of 4.1 ± 0.1 compared to 3.0 ± 0.2Gy for the parental MCF7 cell line and for the already radioresistant subclone with a D37 to 5.8 ± 0.1 Gy compared to 4.8 ± 0.2 Gy. The same scenario was observed for HR capacity with an increase in HR capacity in both ALDH1-positive subpopulations (**Figure 3C**). A 2-fold increase in HR capacity was seen for the ALDH1-positive cells of the parental MCF7 with 2.3 ± 0.09 vs. 1.0 ± 0.06, p=0.0002 and a 4-fold increase in HR capacity compared to the RR clone with 4.1 ± 0.1 vs. 1.0 ± 0.05, p=0.0002.

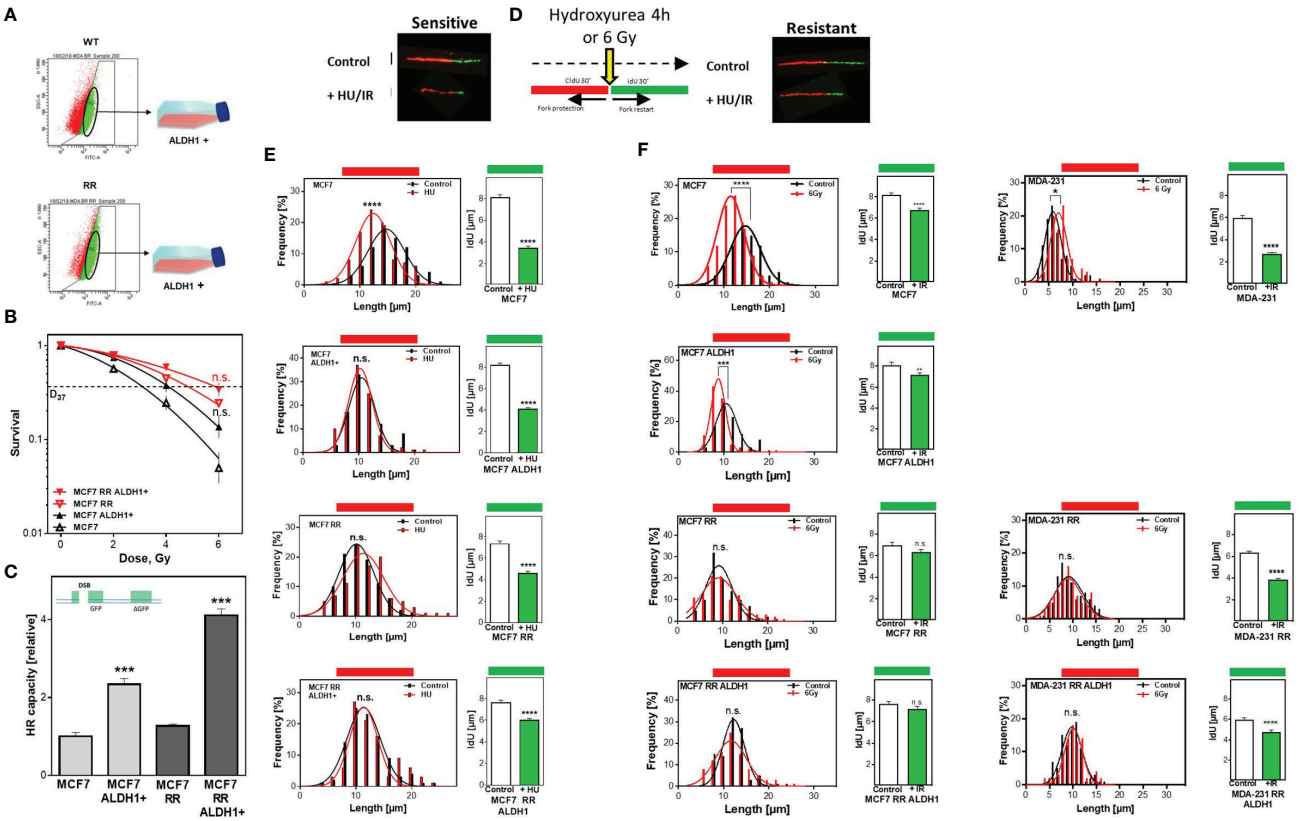

FIGURE 3 | Avoidance of replication stress by functional HR mediates radiation resistance of BCSC. **(A)** Isolation of ALDH1 positive cells. Cells were treated with ALDEFLUOR™ reagent, harvested and ALDH1-positive cells isolated by FACS sorting. **(B)** Cellular survival after irradiation. Cells were seeded 6 hours prior to treatment, irradiated with the indicated doses, fixed after 14 days and the number of colonies was counted. **(C)** HR repair of DSB. Cells were transiently transfected with the linearized pDR-GFP construct for 24h. The number of GFP-expressing cells was analyzed by FACS and HR capacity of the ALDH1 positive cells was normalized to their respective parental cell lines. **(D–F)** Replication tract lengths after HU or irradiation. MCF7 cells were sequentially labelled with CldU and IdU for 30min and either treated with HU or MCF7 and MDA-MB-231 cells irradiated with 6 Gy between both labels. DNA was spread on slides, fixed, and incorporated nucleotides were detected by immunofluorescence. The lengths of the DNA fibers were measured with the Image J software. Shown are means of three independent experiments ± SEM. Asterisks (*) represent significant differences (n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, Student's t-test).

In addition to the extensively described importance of HR for DSB repair, several studies showed that HR proteins play an essential role in stabilizing active replication forks and that their loss led to nucleolytic degradation (6, 7). To verify whether the increased HR capacity also translates into a stronger defense against nucleolytic degradation of active replication (6), both replication fork stability and restart after treatment with HU, which depletes the nucleotide pool without damage induction, were examined by the DNA fiber assay (**Figure 3D**). Parental MCF7 cells show a significant degradation of already synthesized DNA, which was manifested by significantly shorter chromatin fibers compared to the untreated control, with 11.9 ± .0.2µm vs. 14.7 ± 0.17 µm (p<0.0001) (**Figure 3E** left). In contrast, neither the radioresistant clone nor the two ALDH1-positive subpopulations showed pronounced degradation of the already synthesized DNA with 10.3 ± 0.12 vs. 10.5 ± 0.19 µm, 11.3 ± 0.32 vs. 10.8 ± 0.13 µm and 11.3 ± 0.36 vs. 11.1 ± 0.18µm, respectively. Moreover, these results surprisingly showed that the three subpopulations replicated significantly slower than the parent MCF7 cell line, with 0.84 ± 0.02 kb/min in MCF/ALDH1-positive cells, 0.81 ± 0.03 kb/min in the RR clone of MCF7 cells, 0.9 ± 0.03 kb/min in the ALDH1-positives of the RR clones compared to 1.06 ± 0.03 kb/min in wild type MCF7 cells, indicating that CSC-enriched populations exhibited significantly more endogenous replication stress than the baseline cell line.

Analysis of replication fork restart after HU removal (IdU labeling), another characteristic of functional HR (42), also showed significant differences (**Figure 3E** right). The longest time for replication restart was required by the parental cell line, evident by the shortest replication tracts with 3.4 ± 0.2 vs. 8.1 ± 0.3, (p<0.0001). Slightly faster, the ALDH1-positive cells of the parental MCF cells reached replication restart with a length of 4.1 ± 0.2 to 8.2 ± 0.2 (p<0.0001). Again, surprisingly, both the RR clone and the ALDH1-positive subpopulation derived from it were significantly faster capable to resume replication, with 4.5 ± 0.2 vs. 6.9 ± 0.3 (p<0.0001) and 6.2 ± 0.2 vs. 7.6 ± 0.2, (p<0.0001). After irradiation, a similar pattern is seen in both CldU shortening and replication restart (**Figure 3F**, left). MCF7 cells show significant shortening of CldU labeling, which is not observed in the RR clone

of MCF7 cells or in ALDH1-positive cells of either line. Similarly, replication restart is significantly faster in the populations with increased CSC content, such as the ALDH1-positive MCF7 cells, the MCF7-RR clone, and the ALDH1-positive cells of the RR clone, as revealed by significantly longer IdU strands. Interestingly, the respective cell lines showed differences in replication restart depending on their radiosensitivity (**Figure 3F**, left), the more radioresistant, the longer the IdU strand. In MDA-MB-231 cells, which already have a CSC content of about 80% in the initial population (**Figure 1B**), the RR clone and ALDH1-positive cells of the RR clone show no pronounced irradiation effect on the length of the already synthesized DNA (**Figure 3F** right CldU labeling). However, there was a clear dependence between restart of DNA replication and radiation resistance: the longer the IdU tract, the higher the radiation resistance (**Figure 3F**, far right).

Next, the question was whether differences in the ability to protect active replication forks directly impacts the number of DSB. To this end, RPA and yH2AX were analyzed in parallel after treatment with HU and irradiation (**Figures 4A, B**). It was observed that stalled replication forks resulted in single-stranded DNA in all cell lines examined (RPA foci), but significantly less frequently in the ALDH1-positive with 2.2 ± 0.2 and the radioresistant clone with 2.14 ± 0.1 than in the parental cell line with 2.8 ± 0.2, with the ALDH1-positive population of the radioresistant clone having the lowest number of RPA foci with 1.7 ± 0.2.

In parallel, the number of DSB (yH2AX) also showed significantly lower values with increase in CSC content in the cell lines studied, with the difference from MCF to ALDH1-positive MCF7 cells being most pronounced with 4.7 ± 0.3 for WT MCF7 to 1.89 ± 0.3 and only slightly reduced in the RR clone and its ALDH1-positive cells at 1.61 ± 0.07 and 1.6 ± 0.19, respectively (**Figure 4A**, right), supporting the lower replication stress observed after HU in these cell lines (**Figure 3E**).

After irradiation, however, a different pattern emerges. While all cell lines examined showed a comparable number of RPA foci, those with an increase in CSC content showed a decrease in DSB 3 h after irradiation (**Figure 4B**).

The Amount Of Cytosolic DNA Depends On The ALDH1-Positive BCSC Fraction

Next, it was of interest to determine whether the enhanced DNA repair capacity *via* HR of RR clones and their respective ALDH1-positive BCSC fractions affect the amount of cytosolic dsDNA after irradiation (**Figure 5A**). There was a significantly decreased accumulation in cytosolic dsDNA, both in the RR clone and their ALDH1-positive cells after irradiation compared with the parental cell line in all cell lines examined. Among them, MCF7 cell line showed the most obvious and MDA-MB-231 the smallest decrease of cytosolic DNA in ALDH1-positive cells compared to the baseline cell lines and their RR clones, from 1.62 ± 0.1 to 1.28 ± 0.1 and 1.08

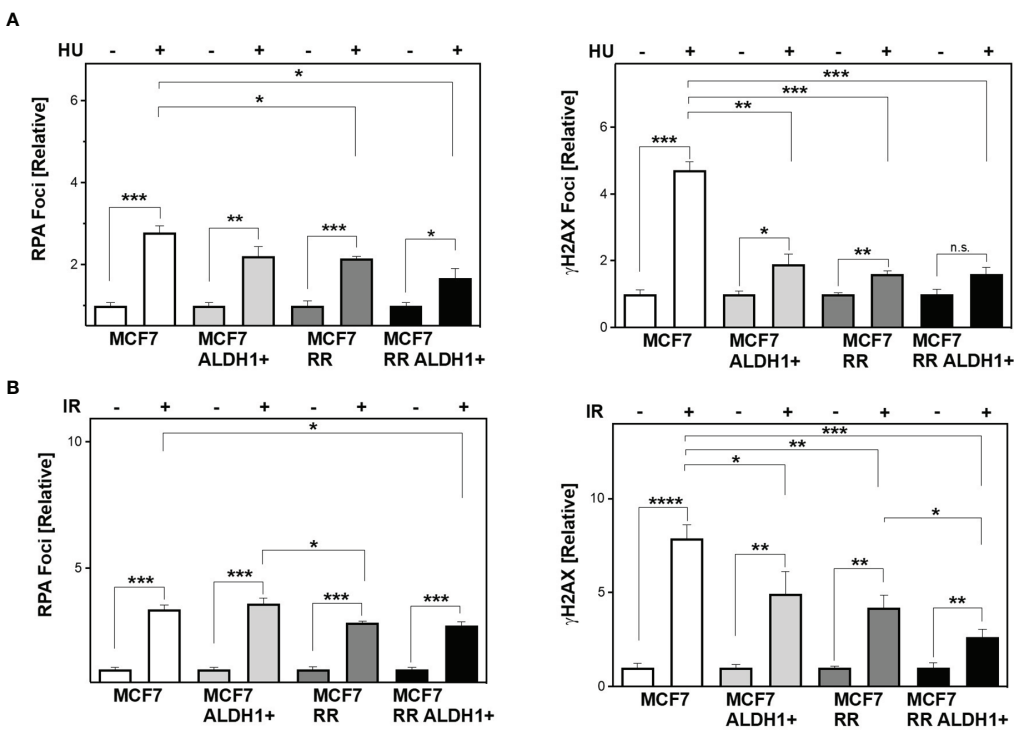

FIGURE 4 | Lower DNA replication stress leads to less DSB in BCSC after treatment. Cells were incubated with HU **(A)** for 2h or irradiated with 6 Gy **(B)**. Immunostaining was performed 3h after treatment with a specific antibody against RPA and yH2AX and fluorescent secondary antibodies. Nuclei were stained with DAPI, quantification of the foci was performed by automatic foci detection in the Aklides®-system (Medipan). For each analysis the foci of at least 100 cells were quantified. Shown are means of three independent experiments ± SEM. Asterisks (*) represent significant differences (*p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, Student's t-test).

± 0.2, respectively, p<0.05 in MCF7 and 1.57 ± 0.1 in MDA-MB-231 to 1.28 ± 0.2 and 1.2 ± 0.1 in MDA-MB-231, respectively (n.s.). The other TNBC cell lines behaved in the same manner. Thus, irradiation led to an increase in cytosolic dsDNA in all cell lines, which became lower with increasing ALDH1-positive BCSC content. Indeed, the proportion of ALDH1-positive cells correlated significantly with the amount of cytosolic DNA after irradiation with R^2 of 0.8, p<0.05 (**Supplementary Figures S3A, B**). Supporting this, IFN-ß1 showed a correspondingly lower expression in the ALDH1-positive cells compared to the RR clones and WT MCF7 and MDA-MB-231 cells after irradiation (**Figure 5B**).

DSB repair by HR and control of DNA replication stress are both dependent on the activation by the ATR-CHK1 pathway (10). To test whether radioresistance of the RR clones depended on the functionality of the S phase checkpoint, ATR was inhibited (**Figure 5C**). Notably, the RR clones were severely sensitized by ATR inhibition, whereas the parental cell lines showed only a moderate radiosensitization, with a reduction of the D37 about 1.7 Gy in the RR clone compared to only 1.0 Gy in the parental cell line, p=0.02 and p=0.004, respectively. p=0.002 (**Supplementary Figure S3C**). Additionally, the inhibition of the ATR downstream kinase CHK1 with the CHK1-inhibitor MK-8776 also led to a specific radiosensitization of the RR clones of the MCF7 and the MDA-MB-231, confirming the importance of the ATR-CHK1 signaling pathway to their radioresistance (**Supplementary Figure S3C**). Thus, radioresistant, ALDH1-positive BCSC are particularly dependent on the S phase damage checkpoint, HR-mediated DSB repair and replication fork protection.

Of particular interest was whether inhibition of ATR affects activation of the intracellular immune response after irradiation. Activation of the intracellular immune response by the appearance of cytosolic dsDNA occurs through pSTING phosphorylated and thereby activated IRF3, which is translocated by this process to the nucleus where it induces typeI IFN expression (43) (**Figures 5D, E**). As expected, irradiation alone in RR clones of both cell lines leads to a low translocation of IRF3 into the nucleus (p=0.006 and n.s.), whereas a significant increase was observed in the parental cell lines (p<0.0001 and p=0.02). Also here, the extent of nuclear IRF3 after irradiation correlated with the percentage of cytosolic DNA after irradiation (**Supplementary Figure S4A**). In contrast, inhibition of ATR alone led to a significantly higher translocation of IRF3 to the nucleus in RR clones compared to parental cells (both with p<0.0001), whereas only weak translocation of IRF3 was observed in the parental cell lines. The combined treatment of ATR inhibition with irradiation resulted in an additive increase in IRF3 translocation, with a significantly stronger expression in the RR clones (both p<0.0001). Thus, inhibition of ATR enhances the activation of intracellular immune response after irradiation in BCSC by suppressing their functional S-phase DNA damage response.

DISCUSSION

Here, we show that the increased radioresistance of ALDH1-positive BCSC in S phase is mediated by both enhanced DSB repair and improved replication fork protection due to HR. Both HR-mediated processes lead to suppression of radiation-induced replication stress and consequently reduction of cytoplasmic dsDNA. The amount of cytoplasmic dsDNA correlated significantly with BCSC content. This clearly indicates that HR-dependent avoidance of radiation-induced replication stress mediates radioresistance and contributes to its immune evasion. Consistent with this, enhancement of replication stress by inhibition of ATR resulted in significant radiosensitization. Therefore, disruption of HR-mediated processes, particularly in replication, opens a CSC-specific radiosensitization option by enhancing their intracellular immune response.

An abundance of $CD44^{high}/CD24^{low}$ and ALDH1 positive cells in the TNBC cell lines compared to the luminal MCF7 cell line was observed (**Figures 1B, E** and **Supplementary Figure S1C**). Ma and colleagues already showed an enrichment of $CD44^{high}/CD24^{low}$ cells in TNBC (44). This putative high proportion of BCSC is confirmed by work of others, who reported ~45% of $CD44^{high}/CD24^{low}$ cells in untreated TNBC and only ~5% in luminal A tumor biopsies (45, 46). Compared to Glioma with only ~2-4% of CD133+ CSC found in human specimens, the proportion in TNBC is enormous and clearly shows the relevance of BCSC in TNBC (15). For the identification of BCSC both the $CD44^{high}/CD24^{low}$ phenotype and ALDH1 activity are important, but $CD44^{high}/CD24^{low}$ is limited to a mesenchymal phenotype, whereas ALDH1 is a more general BCSC marker due to its independence from the current cell state (47, 48). This explains the higher proportion of ALDH1-positive cells in comparison to $CD44^{high}/CD24^{low}$ cells. A weakness of both markers is that they are also expressed by progenitor cells. To overcome this problem, others considered only the 1% of cells with the highest ALDH1-activity as CSC, the cells with the lowest 1% of ALDH1-activity as progenitor cells (49). Since both populations with ALDH1-activity remained tumorigenic, it was assumed - based on surface markers or ALDH1 activity – that there is no clear distinction between stem and progenitor cells.

Baumann and colleagues postulated that radiation resistance is due to the proportion of CSC (12). This is also confirmed by our data showing a clear correlation between radiation resistance and ALDH1-positive BCSC (**Figure 1F**).

It has been previously reported that repeated irradiation with fractions ranging from 4-6 Gy to a total dose of 12-56 Gy worked as a strategy to increase the endogenous CSC proportion in breast and HNSCC cell lines (13, 14). In line with this we achieved an increase in the ALDH1-positive CSC proportion and a significantly increased survival after irradiation in all investigated cell lines, independent of the molecular subtype (**Figures 1E, F**). This acquired radioresistance can be attributed to i) selection of pre-existing, radioresistant clones, ii) radiation-induced de-differentiation to a stem cell phenotype (17) and iii) alterations of DNA repair processes (14). We found that radioresistance was indeed determined by the ALDH1-positive cell fraction (**Figures 3A–C**). We also found that the ALDH1-positive cells from the MCF7 cell line were more radiosensitive than the radioresistant clone, suggesting that not only clonal selection, but also alterations of DNA repair processes due to repeated irradiation played a role. This is in line with observations in radioresistant BCSC showing a ZEB1 dependent stabilization of CHK1, mediating radioresistance (14).

It is generally believed that CSC, similar to tissue stem cells, are mostly quiescent (19), but studies in glioma- and breast cancer cell lines showed, that only one-third of the CSC were dormant, but

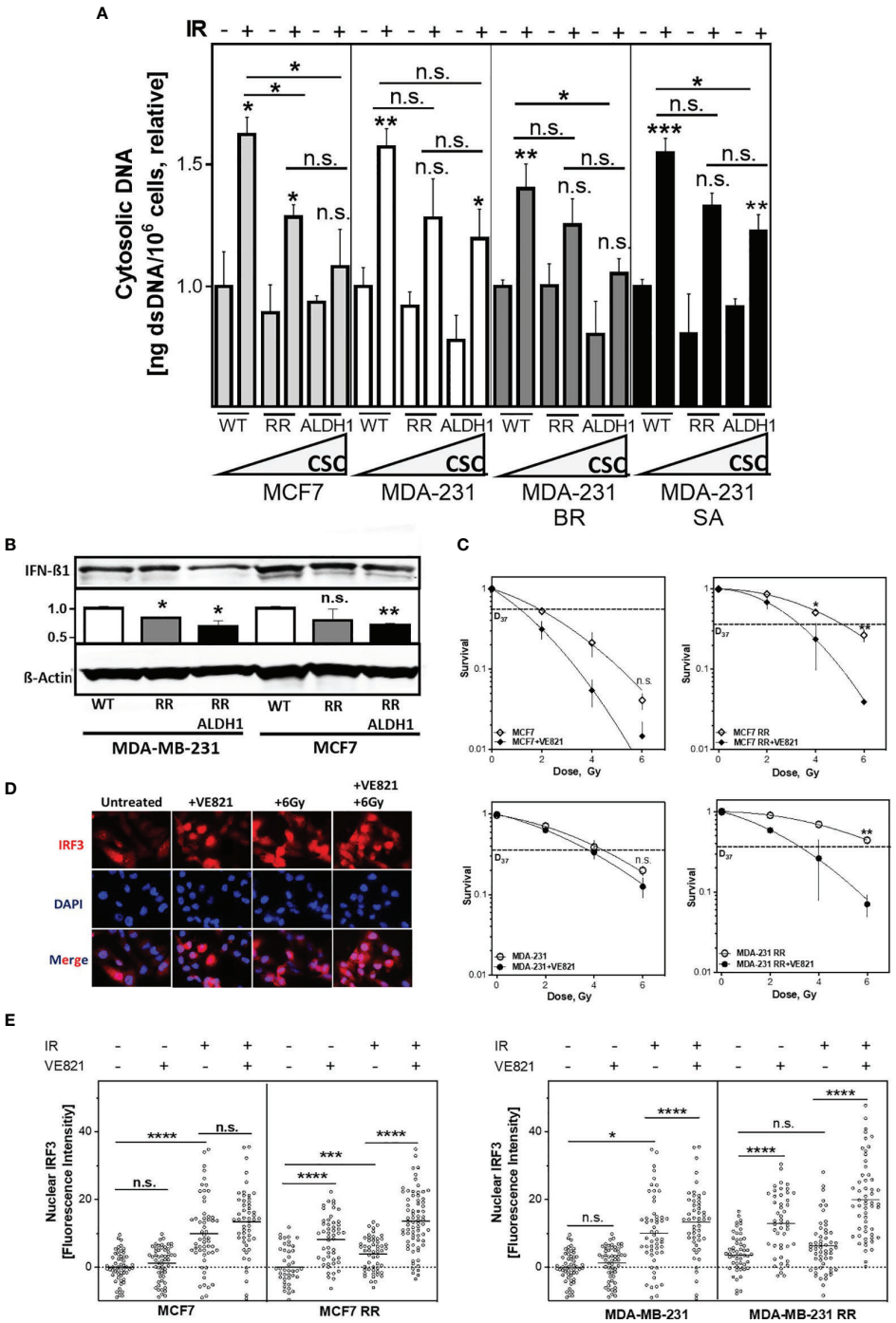

FIGURE 5 | The amount of cytosolic DNA and intracellular immune response depends on the ALDH1-positive BCSC proportion. **(A)** Relative increase of cytosolic dsDNA after irradiation. Cells were irradiated with 8 Gy, cytoplasmatic fraction isolated and dsDNA stained with PicoGreen®reagent, quantified in a Spark® reader and normalized to the untreated control or **(B)** Expression of IFN-ß1 after irradiation. Cells were irradiated with 8 Gy and proteins were extracted 24h later. Proteins were separated and transferred by Western blotting. IFN-ß was detected specific primary antibodies, followed by fluorescence-coupled secondary antibodies. ß-Actin served as a loading control. The Expression of IFN-ß was normalized to the wild type cell lines. **(C)** Cellular survival after irradiation. Cells were seeded 6 hours prior to treatment, treated with +/- VE821 2h prior to irradiation, irradiated with the indicated doses, fixed after 14 days and the number of colonies was counted. **(D, E)** Nuclear IRF3 accumulation. Cells were incubated with 1µM VE821 for 2h, irradiated with 6Gy and fixed 16h later. IRF3 was stained with a specific primary antibody, followed by a fluorescent secondary antibody. Nuclei were stained with DAPI. Fluorescence intensity (FI) of IRF3 was quantified with Image J Nuclear IRF3 was calculated by subtraction of the cytoplasmatic FI from the nuclear FI. Shown are means of three independent experiments ± SEM. Asterisks (*) represent significant differences (n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$, Student's t-test).

entered the cell cycle after irradiation (16, 20). We found that in non-S phase cells the radioresistant clones had significantly lower amounts of residual DSBs than the parental cells after irradiation, suggesting increased DNA repair by NHEJ in BCSC (**Figure 2A**, EdU-negative). Even more striking was that these differences were even more significant when the cells were irradiated during S phase (**Figure 2A**, EdU-positive). In S phase HR is the most important DNA repair pathway for the repair of frank DNA-DSB and the avoidance of DNA replication stress by replication fork protection (39). Here, we demonstrate that HR capacity is greatly increased in in the RR clones due to the ALDH1-positive cell fractions (**Figures 2B**, **3C**). These effects were not due to cell cycle changes in favor of the S phase, the RR clones provided a slightly lower S phase proportion than the parental cell lines, so the actual HR capacity of the RR clones could be higher than depicted in the figure (**Figures 2B, C**) (28). However, we demonstrate here for the first time a functional stabilization of DNA replication forks in ALDH1-positive BCSC, which further supports the increased DSB repair by HR (**Figures 3C, D**). Other than the parental MCF7 cell line the ALDH1-positive BCSC showed no replication fork degradation after HU treatment and improved replication fork restart (7). Since a functional DNA damage response (DDR) is necessary for replication fork protection (50), these results compensate the potential lack of DDR activation for measuring the HR capacity with the plasmid reconstruction assay (51). This functional HR led to a lower occurrence of DNA-replication stress markers after HU and irradiation (**Figures 4A, B**) (52). Thus, our study demonstrates the importance of ATR and CHK1, avoiding degradation of nascent DNA strands. These findings further extend observations in glioma and breast CSC (14, 25, 29, 31). The S-Phase kinase ATR and its downstream kinase CHK1 activate HR (10, 53). These kinases also regulate DNA replication processes and support replication fork protection (50, 53–55). Others observed an increased expression and activation of CHK1 after irradiation in breast- and glioma CSC (14, 15, 27). Here we show that the inhibition of ATR and CHK1 lead to a significant radiosensitization of the RR clones (**Figure 5B** and **Supplementary Figures S3C, D**), suggesting a critical role of the ATR-CHK1 signaling cascade in preventing radiation-induced replication stress and protection of replication forks in BCSC. To our knowledge this is the first study that shows a targeted radiosensitization by ATR inhibition in BCSC. Yet, similar effects were only observed in CD133+ colon carcinoma stem cells, where ATR inhibition abrogated the tumorigenicity of CD133+ CSC (56). Thus, the activation of the ATR signaling cascade mediates radioresistance in BCSC by activating HR.

It has been previously shown that efficient DSB repair and avoidance of DNA replication stress by functional HR prevents the formation of radiation-induced cytosolic dsDNA (4). Consistent with this, we show that the proportion of ALDH1-positive BCSCs significantly affects the amount of cytosolic dsDNA after irradiation (**Figure 5A** and **Supplementary Figure S3B**). The resulting lower amount of cytosolic DNA led to decreased activation of the intracellular immune response, as evidenced by decreased nuclear IRF3 levels in the radioresistant BCSC (**Figure 5C** and **Supplementary Figure S4**). This suggests that upregulated HR processes protect BCSC not only from DNA damage, but also from the activation of the intracellular immune response. This would indirectly contribute to the CSC-specific mechanisms of immune escape and complement their enhanced expression of PD-L1 (33, 34). Consequently, disruption of HR by inhibition of ATR not only resulted in a specific radiosensitization of BCSC, but also in a significantly increased translocation of IRF3 to the nucleus, thus abrogating their low activation of the intracellular immune response after irradiation alone. This is in line with other *in vitro* and *in vivo* studies showing a significantly increased the activation of the immune response, expression of inflammatory genes and the infiltration of CD8+ T-cells after combination of irradiation with ATR inhibition in comparison to irradiation alone (11, 57, 58). Thus, the inhibition of the ATR signaling cascade specifically sensitizes BCSC to irradiation and increases the activation of the intracellular immune response, potentially overcoming CSC-mediated tumor protection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization, KB and FM. Methodology, FM and AE. Software, FM. Validation, KB and FM. Formal analysis, FM and AE. Investigation, FM, AE, AK, TW. and ClP. Writing—original draft preparation, FM and KB. Writing—review and editing, KB, KR, CoP, and AD. Visualization, FM. Supervision, KB. Project administration, KB. Funding acquisition, KB. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.765284/full#supplementary-material

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REVIEW
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APE1/Ref-1 Role in Inflammation and Immune Response

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Apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1) is a multifunctional enzyme that is essential for maintaining cellular homeostasis. APE1 is the major apurinic/apyrimidinic endonuclease in the base excision repair pathway and acts as a redox-dependent regulator of several transcription factors, including NF-κB, AP-1, HIF-1α, and STAT3. These functions render APE1 vital to regulating cell signaling, senescence, and inflammatory pathways. In addition to regulating cytokine and chemokine expression through activation of redox sensitive transcription factors, APE1 participates in other critical processes in the immune response, including production of reactive oxygen species and class switch recombination. Furthermore, through participation in active chromatin demethylation, the repair function of APE1 also regulates transcription of some genes, including cytokines such as TNFα. The multiple functions of APE1 make it an essential regulator of the pathogenesis of several diseases, including cancer and neurological disorders. Therefore, APE1 inhibitors have therapeutic potential. APE1 is highly expressed in the central nervous system (CNS) and participates in tissue homeostasis, and its roles in neurodegenerative and neuroinflammatory diseases have been elucidated. This review discusses known roles of APE1 in innate and adaptive immunity, especially in the CNS, recent evidence of a role in the extracellular environment, and the therapeutic potential of APE1 inhibitors in infectious/immune diseases.

Keywords: cytokines, NF-κB, biomarker, innate immunity, DNA repair, inflammation, reactive oxygen species, oxidized DNA damage

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INTRODUCTION

APE1 - From Structure to Function

Apurinic/apyrimidinic endonuclease 1/Redox Factor-1 (APE1/Ref-1) is a multifunctional 35.6 kDa protein that responds to DNA damage (primarily DNA damage caused by oxidative stress) (1, 2). The C-terminal domain of APE1 processes apurinic/apyrimidinic (AP) sites generated by DNA glycosylase in the base excision repair (BER) pathway. The AP endonuclease activity of APE1 hydrolyzes the phosphodiester bond at these sites, generating a 3′-hydroxyl end (3′-OH) and a 5′-deoxyribose phosphate (5′-dRP) terminus. DNA polymerase β (Polβ) then removes the 5′-dRP and

inserts the correct nucleotide. DNA ligase IIIα in complex with XRCC1 seals the phosphodiester bond, terminating the BER pathway. Occasionally, several nucleotides are removed by other enzymes through a sub-pathway known as long patch repair (1, 3, 4). Although other endonucleases act in the BER pathway, APE1 is the major AP endonuclease that repairs damage caused by oxidative stress, maintaining genome integrity in mammals (2, 5).

The N-terminal domain of APE1 has redox activity and contains a nuclear localization signal in its first 33 amino acids. APE1 reduces the cysteine residues of target transcription factors (TFs) through exchange of protons with cysteine residues present in its N-terminal region (6). The functional domains of APE1 are shown in **Figure 1**. The redox function of APE1 activates TFs, such as NF-κB, p53, activator protein 1 (AP-1), hypoxia-inducible factor-1α (HIF-1α), signal transducer and activator of transcription 3 (STAT3), and early growth response 1 (EGR1) (7–12). Therefore, APE1 regulates the expression of genes that directly affect several cellular processes, including inflammatory responses (13, 14). For example, APE1 reduces HIF-1α, increasing its DNA-binding activity. This induces expression of vascular endothelial growth factor (VEGF), which promotes angiogenesis (15–17). Additionally, because APE1 regulates STAT3, NF-κB, EGR1, and AP-1, it directly influences the immune system by regulating the expression of cytokines and chemokines, including tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and IL-8 (18–21). APE1 also interacts with ERK2 rescuing ERK2 from oxidative inactivation through its redox activity (22). The MEK-ERK1/2 pathway is a critical regulator of lipopolysaccharide (LPS)-induced responses (23).

The DNA repair activity of APE1 has recently been observed to play a role in transcriptional regulation. 8-oxoguanine (8-oxoG) is the most frequent DNA lesion caused by oxidative stress (24). This lesion is removed by 8-oxoguanine glycosylase (OGG1) and APE1 in the BER pathway. The presence of 8-oxoG can delay RNA polymerase progression, inducing transcriptional arrest and initiating DNA repair. Thus, 8-oxoG functions as a repressor in transcriptional regulation of genes (25). Some studies suggest that 8-oxoG can function as an epigenetic signal that favors the expression of several genes (26–29). Pan et al. observed that TNFα treatment induces an increase in 8-oxoG and OGG1 binding in promoters of proinflammatory genes, stimulating NF-κB binding to these sites, leading to gene activation and cytokine expression (27). Corroborating these data, 8-oxoG generation in G-quadruplex promoter sites favors OGG1 recruitment, generating AP sites that are substrates of APE1. The presence of APE1 in these promoters leads to TF recruitment and gene activation (30). Similarly, demethylation of histone H3, mediated by lysine-specific histone demethylase 1A, produces H_2O_2, leading to formation of local 8-oxoG lesions. Occurrence of 8-oxoG, and recruitment of OGG1 and APE1 have been observed to enhance the DNA-binding activity of MYC to its target gene promoters, thereby increasing gene expression (31).

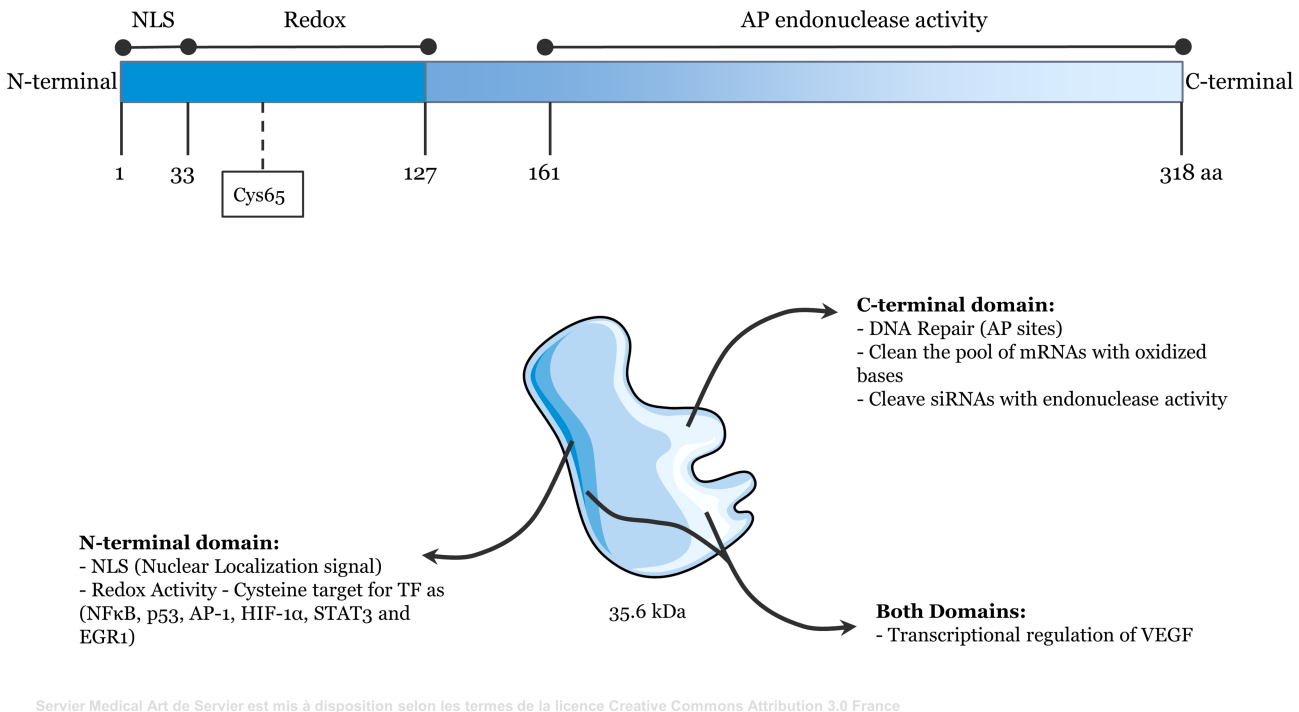

FIGURE 1 | Representative scheme of the functional domains of Apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1). The N-terminal domain (amino acids 1 to 127) contains redox activity and the nuclear localization signal. The C-terminal domain (amino acids 161 to 318) contains apurinic/apyrimidinic endonuclease activity. Both domains may be involved in the transcriptional regulation of some genes, such as VEGF.

Enzymes of the BER pathway, including APE1, are associated with active chromatin demethylation. This process is initiated by oxidation of 5-methylcytosines by ten-eleven translocation (TET). This oxidized base is removed by glycosylases such as thymine-DNA glycosylase (TDG), which generate AP sites. Thus, the endonuclease activity of APE1 is also involved in regulating chromatin and gene expression (24–27) [reviewed in (32)]. Thus, some genes, including VEGF, and cytokines, are regulated by the redox and repair functions of APE1 (33, 34).

In addition to regulating TF activity and maintaining genomic stability through DNA damage repair, APE1 plays an essential role in cell senescence by maintaining telomere stability and size through interaction with the telomere-protective proteins TRF1 and TRF2, and with POT1 (35, 36). APE1 also processes mRNAs that contain oxidized bases, thus preventing abnormal protein synthesis (37, 38). In addition to regulating mRNAs, APE1 can cleave siRNAs *in vitro* (38). APE1 has also been linked to numerous pathological processes, owing to its multiple functions in cellular homeostasis. APE1 is frequently overexpressed in cancer cells and is associated with increased resistance to chemotherapy (39). APE1 participates in signaling pathways involved in immune and inflammatory responses, which regulate gene expression of several innate and adaptive immune system mediators and is also involved in antibody production. In the following sections, the roles of APE1 and its functions in the immune system are described.

APE1 IN INNATE IMMUNITY

The mammalian immune system is divided into innate and adaptive systems. The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through germline-encoded receptors, such as pattern recognition receptors (PRRs). In the presence of PAMPs and DAMPs, cells of the innate immune system initiate an acute inflammatory response by secreting cytokines, chemokines, reactive oxidative species (ROS), and other inflammatory mediators to attract immune cells to the site of damage (40–42). ROS production plays a central role in inflammatory signaling by eliminating pathogens in phagocytic cells or acting as signaling molecules. ROS are endogenously produced in the mitochondria, peroxisomes, and endoplasmic reticulum, and by NADPH oxidases (NOX) in phagocytes and endothelial cells (42, 43). Chronic ROS exposure or imbalance between ROS and antioxidants plays a critical role in the progression of inflammatory diseases, including inflammatory bowel disease (44, 45), hepatitis (46), atherosclerosis (47), and multiple sclerosis (48).

At least two highly interconnected ROS-related processes occur in the innate immune system. First, ROS can induce an inflammatory response leading to APE1 expression. Several studies have shown that ROS induces APE1 expression and activity in different cell types, including macrophages and human gastric epithelial cell lines infected with *Helicobacter pylori* (49–51). In these cells, *H. pylori* infection and TNFα treatment induced activation of NF-κB, and AP-1 and IL-8 expression were inhibited by APE1 silencing (52, 53). APE1 inhibition also prevented H_2O_2-induced increase in IL-6 and IL-4 expression in mast cells (54). Antioxidant enzymes, such as Peroxiredoxin 1, also appear to regulate cytokine and chemokine expression in an APE1-dependent manner. Nassour et al. reported that APE1 interacts with Peroxiredoxin 1 in HeLa cells under physiological conditions or with H_2O_2 treatment. This interaction may prevent APE1 from reducing TFs, including NF-κB, and decrease IL-8 expression, attributing an APE1-dependent anti-inflammatory role to Peroxiredoxin 1 (21).

Second, the inflammatory response can induce ROS production. For example, APE1-deficient human cells infected with *H. pylori* show high Rac1 activation and NOX1 expression. Consistent with these findings, APE1 overexpression decreased ROS levels, Rac1 activation, and NOX1 expression in *H. pylori*-infected cells. APE1, through its N-terminal lysine residues, interacts with Rac1, decreasing NOX1 expression and ROS generation (55). Therefore, APE1 appears to have an inhibitory effect on ROS production. Granzyme K is a tryptase that is highly expressed in natural killer (NK) cells and is necessary for NK cell-mediated cytolysis. Granzyme K -mediated apoptosis is initiated by ROS accumulation and cytochrome C release (56). Granzyme K cleaves APE1, abrogating its antioxidant activity (57). The resultant decrease in APE1 levels correlates with NK cell-mediated apoptosis of tumor cells or virus-infected cells, indicating that APE1 is essential for maintaining cell viability.

Cytokine- and chemokine-mediated signaling is involved in both innate and adaptive immunity. These signaling molecules are often transcriptionally regulated by TFs, including NF-κB, AP-1, EGR1, and STAT3, which are activated by the redox function of APE1, which in turn increases their DNA-binding capacity (11, 12, 58). APE1 exerts a proinflammatory role in stimulating cytokine and chemokine expression. APE1 knockdown in keratinocytes treated with synthetic lipopeptide or zymosan resulted in decreased NF-κB activation and TNFα and IL-8 expression (18). Treatment of macrophages with APE1 redox inhibitor E3330 decreases NF-κB and AP-1 activation, and consequently, TNFα, IL-6, IL-12, PGE2, and COX-2 expression (19). E3330 also inhibits IL-8 expression in TNFα-induced JHH6 cells (59). E3330 (also called APX3330) is a quinone derivative and a specific inhibitor of APE1 redox functions (60–62). E3330 acts by binding to APE1 and increasing the formation of disulfide bonds between cysteine residues (Cys65 or Cys93) which are critical for redox function (61, 63), without affecting AP endonuclease activity (60–62, 64). In addition, in a gastric inflammation model of *H. pylori*, APE1 redox inhibition reduced cytokine expression, decreased immune cell infiltration, and exerted neuroprotective effects on the enteric nervous system (65). These studies demonstrate the role of APE1 redox activity as a positive regulator of cytokine and chemokine expression in innate immune system cells.

Many studies have demonstrated dual roles of APE1 in inflammation. Ectopic APE1 overexpression appears to play an anti-inflammatory role in some cells. In the macrophage-like THP-1 cell line, APE1 transfection decreased the expression of IL-6, TNFα, and IL-1 induced by oxidized LDL (66) and TNFα and COX-2 expression induced by HMGB1 (67). In addition,

using the *APE1* gene cloned in an expression vector and administered *via* retrograde renal vein injection, Maruyama et al. demonstrated that APE1 expression inhibits the development of tubulointerstitial fibrosis and modulates the immune system through different pathways, including IL-6, TNFα, and IL-1β (68). The dual role of APE1 in cytokine and chemokine expression can be attributed to specific functions of APE1 in different cell types. Yuk et al. observed a contradictory effect in the same cell line (THP-1) by analyzing the effects of APE1 overexpression and siRNA knockdown on HMGB1-induced inflammatory responses. The authors observed that siRNA-mediated inhibition decreased APE1 nuclear and cytoplasmic expression and impaired HMGB1-mediated cytokine expression and MAPK pathway activation. Furthermore, APE1 overexpression by adenoviral vectors has been reported to increase cytoplasmic APE1 expression, leading to a decrease in ROS levels, cytokine secretion, and cyclooxygenase-2 expression. A reduction in p38 and c-Jun N-terminal kinase activation and extracellular release of HMGB1 has also been observed (67). The authors suggested that APE1 compartmentalization may explain the contrasting functions described above (67). The role of cytoplasmic APE1 in the inflammatory process remains to be clarified.

APE1 deficiency in mice is associated with increased expression of inflammatory mediators in senescent cells. APE1 deficiency is also associated with decreases in the size of several organs including the brain (14). These events may be associated with senescence-associated secretory phenotype stimulation, which includes changes in the cell protein secretion profile, such as proinflammatory cytokines (IL-1α and IL-6), chemokines (IL-8), and growth factors (VEGF), many of which are regulated by the redox function of APE1 (69).

NF-κB and AP-1 are the main TFs observed in studies of APE1 expression or inhibition in inflammatory models. However, several TFs interact with the APE1 redox region. Occasionally, these factors also play a role in the inflammatory response or immunity. For example, HIF-1α, a classical target of APE1 redox activity (15, 16),is essential in glycolysis and angiogenesis. HIF-1α also participates in the immune response, and its inactivation decreases macrophage invasion, aggregation, and motility (70). APE1 redox function also regulates STAT3 transcriptional activity (7), affecting dendritic cell maturation and anti-inflammatory signaling in phagocytes and inflammatory responses related to cancer (71, 72). **Table 1** lists the principal TFs regulated by APE1, their functions, and studies reporting their involvement in the immune response.

In general, the studies cited above describe the associations of APE1 redox function or reduced APE1 expression with inflammatory regulation. However, our group recently showed that inhibition of AP site repair by methoxyamine inhibits the expression of IL-8, IL-6, TNFα, IL-10, and MCP1 in LPS-induced U937 cells (34). This treatment also decreased expression of genes involved in prostaglandin biosynthesis and MyD88-independent toll-like receptor signaling pathway genes. Reduced ELK1 expression after chemical inhibition of APE1 by E3330 or methoxyamine was also observed. ELK1 expression is regulated by ERK pathway, EGR1, and TET enzymes (82–84). In this context, our findings suggest that both redox and DNA repair activities of APE1 regulate ELK1 expression through independent but overlapping mechanisms (34). Together, these data suggest a role of DNA repair in regulating gene expression, influencing the expression of inflammatory mediators.

Figure 2 summarizes the main roles of APE1 in the inflammatory response. The potential of this response to be cell type-specific must be considered. Therefore, more studies are required to better understand the role of APE1 repair activity in the transcriptional regulation of proinflammatory genes.

APE1 IN NEUROINFLAMMATION

Increased APE1 expression in the nervous system is well-documented (85–87). APE1 expression varies in different tissues under normal physiological conditions. High APE1 levels are observed in the dentate gyrus granule cells, cerebellar Purkinje cells, and piriform cortex neurons (85). However, APE1 expression is significantly increased in the brain and spinal cord of individuals affected by diseases including amyotrophic lateral sclerosis (ALS), compared to healthy controls (88). Excessive ROS production in neurons in response to certain stimuli is associated with APE1 expression (89). ROS originate from many sources but have mainly been attributed to high mitochondrial respiration activity or malfunctioning organelles. Thus, many studies have focused on neuronal mitochondrial dysfunction during ischemia to assess the role of APE1 (90, 91).

APE1 upregulation generally protects neuronal structure and function during transient global cerebral ischemia (90, 91). This protection has been mainly attributed to its role in BER, which

TABLE 1 | Transcription factors regulated by apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1) and their functions in the immune response.

TFs	Immune system function	Function inhibited	Refs
NF-κB	Inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis	Redox and repair	(10, 27)
AP-1	Proliferation, differentiation, and apoptosis	Redox	(11)
STAT3	Dendritic cells maturation, activation, and anti-inflammatory signalization in phagocytes	Redox	(7, 72)
HIF-1	Invasion, aggregation and motility of macrophages, and energy homeostasis	Redox and repair	(16, 33, 73)
EGR1	Differentiation of myeloid cells	Redox	(74, 75)
P53	Apoptosis, antiviral defense, induction of type I IFN, enhanced pathogen recognition, and immune checkpoint regulation	Redox and redox-independent functions	(76–78)
PAX5	B lymphopoiesis	Redox	(79, 80)
PTEN	DC maturation and T cell polarization	–	(76, 81)

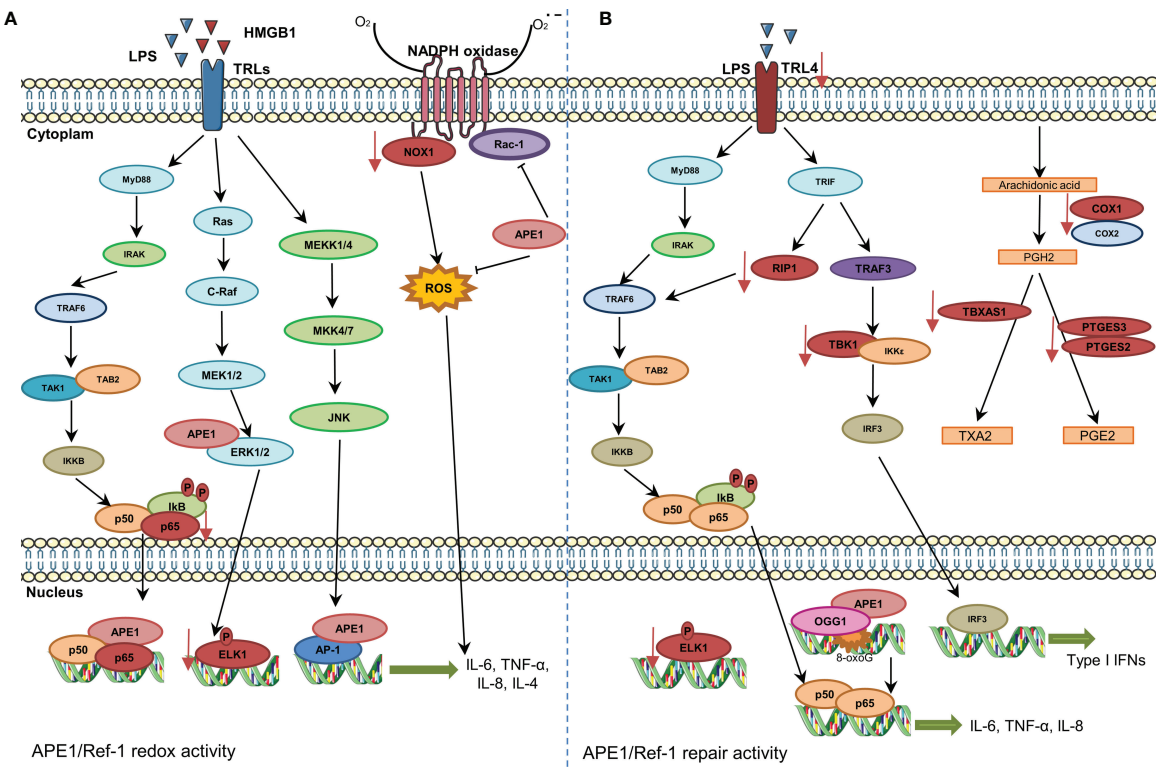

FIGURE 2 | Involvement of APE1 in expression of cytokines and chemokines and reactive oxygen species (ROS) regulation. **(A)** The stimulation of Toll-like receptors promotes NF-κB activation and its translocation to the nucleus. APE1 redox function reduces transcription factors, such as NF-κB and AP-1, and increases expression of cytokines. APE1 also inhibits Rac1 and ROS production by NADPH oxidase. Inhibition of APE1 redox function decreases the expression of NOX1, P65, and ELK1 (represented in red color). **(B)** The DNA repair activity of APE1 also regulates expression of cytokines. 8-Oxoguanine DNA glycosylase and APE1 recruitment to damaged sites is essential for downstream recruitment of transcription factors. Additionally, inhibition of APE1 DNA repair activity decreases the expression of various genes/proteins (represented in red color).

corrects damage induced by ROS. Although BER is the predominant mechanism for repairing oxidized DNA damage in neurons, APE1 also participates in non-homologous end-joining repair mechanisms in cortical neurons (92). In addition, APE1 helps regulate the nucleotide excision repair pathway to repair DNA adducts induced by cisplatin in sensory neurons (93).

Oxidative stress in neurons plays a critical role in aging and in the pathogenesis of several neurological diseases, including ALS, Parkinson's disease, Alzheimer's disease, and brain infections, such as bacterial meningitis (94–98). The inflammatory response in the nervous system is one of the primary endogenous sources of ROS in some neurological conditions, and APE1 plays an essential role in these conditions. For example, during neuroinflammation caused by pneumococcal meningitis, higher APE1 expression was observed in the cortex and hippocampus of rats than that in mock-infected animals. Rats supplemented with vitamin B6 showed reduced APE1, glutamate and ROS levels, and decreased cell death and oxidative stress during neuroinflammation (99). Furthermore, in aluminum chloride-induced neuroinflammation in rats, administration of resveratrol as an anti-inflammatory agent was associated with increased APE1 levels and reduced inflammatory responses (100).

The functions of APE1 in inflammatory responses during neuroinflammation are not entirely understood. Some studies have attributed a coactivator role to APE1 redox activity associated with NF-κB and AP-1, promoting proinflammatory cytokines, such as TNFα and IL-8 (10, 18, 52, 101). APE1 translocation from the nucleus to the cytoplasm, followed by p50 reduction, appears to be an essential mechanism for the binding of NF-κB to DNA, thereby triggering inflammation (102, 103). In rats with inflammatory pain, changes in subcellular APE1 distribution can be effected *via* intrathecal injection of E3330, leading to reduced IL-6 levels and alleviation of pain (104). Beyond reducing inflammation, changes in APE1 expression and subcellular distribution also seem to be mediated by APE1 redox function (104).

To observe the role of extranuclear APE1 in regulating neuroinflammatory processes, APE1 with a deleted N-terminal nuclear localization signal (ΔNLS-APE1) was overexpressed in hippocampal astrocytes stimulated with LPS (105). Cytoplasmic APE1 overexpression suppressed NF-κB transcriptional activity and reduced TNFα and iNOS levels, but did not reduce AP-1, showing an anti-inflammatory effect of APE1. These studies also suggested that the inhibitory effect of APE1 on LPS-induced NF-κB

activation was not mediated by IκB kinase activity. Additionally, overexpression of APE1 inhibited p300-mediated acetylation of p65 by suppressing intracellular ROS levels following LPS treatment (105). Acetylation of p65 plays a vital role in regulating the inflammatory response (106). The above study demonstrated the involvement of APE1 in this mechanism.

In summary, APE1 plays a multifunctional role in regulating neuroinflammation, acting as an activator or repressor of TFs depending on cellular redox status, APE1 expression level, subcellular compartmentalization, and post-translational modifications, exerting a proinflammatory or anti-inflammatory effect depending on cellular context.

APE1 IN ADAPTIVE IMMUNITY

The adaptive immune system is triggered by responses generated by the innate immune system upon antigen contact at the infection site. T and B-lymphocytes are involved in the adaptive response and responsible for secreting cytokines and antibodies, respectively. These cells can proliferate and differentiate into memory cells with the help of specialized cells in peripheral lymphoid organs, allowing faster and more efficient responses when encountering the same antigen a second time (107, 108).

The role of APE1 in adaptive immunity has been described in several studies (**Figure 3**). According to Akhter et al. (109), the redox activity of APE1 is essential for T helper cell 1 (Th1) response through antigen-presenting cells. The authors observed that in splenocytes from OT-II mice stimulated with ovalbumin, treatment with E3330 increased IFN-γ-producing T cells by altering functions of antigen-presenting cell, suggesting suppression of Th1 immune responses. Inhibition of APE1 redox function induced p38 MAPK activation, upregulation of *IL-12* gene expression, and IL-12 cell surface retention. APE1 redox activity also regulated Pax5a, a TF essential for B cell development. Repression of APE1 protein synthesis blocked CD40-mediated B cell activation by impairing Pax5a activity (110).

Another essential process in the adaptive immune response is class switch recombination (CSR), which is responsible for antibody diversity and is initiated by activation-induced cytidine

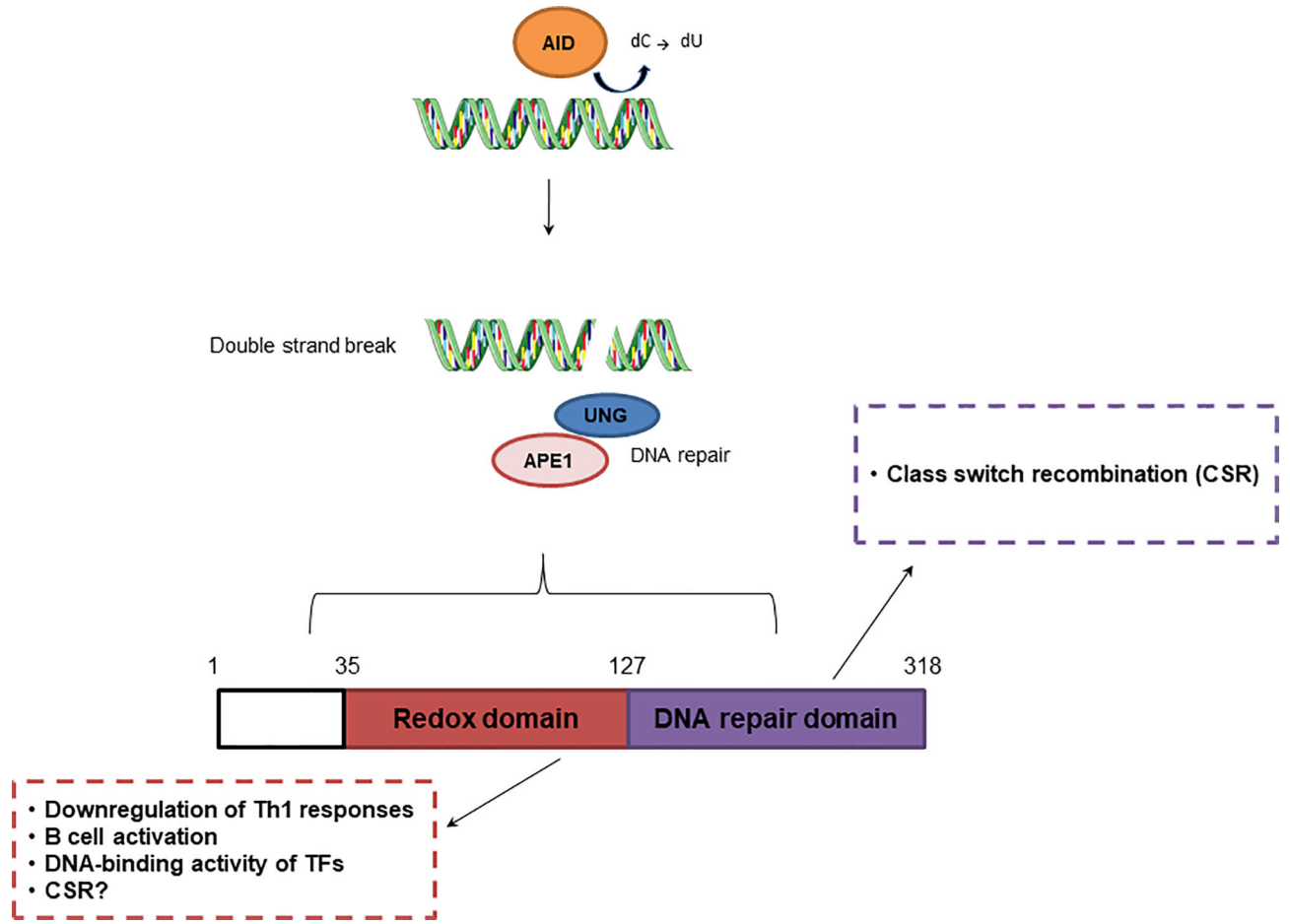

FIGURE 3 | Effect of APE1 redox and repair activities in adaptive immunity. Repair activity of APE1/Ref-1 participates in class switch recombination, while redox activity downregulates Th1 responses and regulates B cell activation.

deaminase (AID) in B cells located in the germinal center (GC) (111). APE1 endonuclease activity is involved in CSR through recognition of AP sites created by uracil-DNA glycosylase (UNG). AID recruits UNG, which converts cytosine into uracil, initiating the BER pathway. This process is crucial for IgA class switching. Although, APE1 redox function regulates CSR, this process is still observed in cells deficient in APE1 endonuclease activity (112). APE1 also mediates CSR through IL-6 signaling, and is involved in proper IgA expression (112, 113). However, once in the GC, B cells undergo somatic hypermutation (SHM), also initiated by AID, and proliferate rapidly, inhibiting the activities of UNG and APE1. APE1 expression is lower in GC B cells than in non-GC B cells and contributes to an increase in SHM (111). According to Xu et al. (114), APE1 endonuclease activity is dispensable for SHM, but may be involved in processing of DNA ends, enabling the ends to participate in CSR.

Due to the multifunctional nature of APE1, researchers have focused on roles of APE1 in tumor cells, and have demonstrated that APE1 expression is associated with poor prognosis in some cancer types including lung, liver, and gastric cancers (115–118). In patients with non-small cell lung cancer, APE1 expression is correlated with tumor-infiltrating lymphocytes, and low APE1 expression together with $CD4^+$ T cell infiltration is correlated with good prognosis, suggesting that APE1 levels regulate the function of $CD4^+$ T cells (119). APE1 was also found to be active in leukemia T cells; however, when inhibited by E3330, it promoted apoptosis and downregulation of survival genes (120). Furthermore, $CD8^+$ T cells and NK cells can release granzymes, such as granzyme A, which cleaves APE1, blocking cellular repair and leading to apoptosis (121). Thus, APE1 could serve as a molecular target for targeted therapies.

Interestingly, APE2 (a less efficient homolog of APE1) was highly expressed in splenic B cells *in vitro*. APE2 deficiency causes severe defects in lymphopoiesis, and prevents B cell progenitors from proliferating in the bone marrow, indicating that APE2 also plays a role in adaptive immunity. APE2 expression is also increased in GC B cells, and protects proliferating B cells from oxidative damage (111, 122–127). Guikema et al. demonstrated that APE1 and APE2 are essential for CSR, as a decrease in this process was observed in splenic B cells of $ape1^{+/-}$, $ape2^{Y/-}$, and double-deficient mice compared to wild type mice (122). However, the role of APE2 in CSR remains unclear, as *APE2* gene deletion in CH12F3 cells does not affect CSR even in APE1 deficient cells (125). APE2 deficient mice show decreased SHM frequency, indicating that APE2 is involved in this process. APE2 interacts with proliferating cell nuclear antigen, facilitating the recruitment of translesion polymerases to AID-induced lesions, which favors an increase in mutagenesis (111, 127). Stavnezer et al. demonstrated that downregulation of APE1 and high expression of APE2 in GC B cells are associated with error-prone repair of AID-induced lesions, and contribute to an increase in mutations in A:T base pairs (111).

APE1 AS A SEROLOGIC BIOMARKER

In recent years, several studies have shown the presence of extracellular APE1 and have suggested its potential use as a biomarker in certain clinical conditions. Although most studies have focused on cancer models which show high APE1 expression (128–131), increasing interest is focused on characterizing APE1 expression in plasma and serum in different diseases, including aging-associated disorders. Serum APE1 levels in patients with coronary artery disease and rheumatoid arthritis have been shown to be elevated compared to levels in healthy controls (132, 133). Furthermore, an experimental murine myocarditis model showed that serum APE1 levels increased until later infection, suggesting the potential use of APE1 as a valuable tool to assess myocardial injury without endomyocardial biopsy (134). Serum APE1 autoantibodies have also been detected in humans. In patients with non-small cell lung cancer, serum APE1 autoantibodies were significantly higher than those in healthy controls, and were closely related to APE1 antigen levels in tumor tissues and peripheral blood (135). Although significant evidence shows that APE1 is delivered through exosomes in response to genotoxic stresses (136), a recent study showed the endogenous hormone 17β-estradiol (E2) significantly increased APE1 secretion in plasma of ovariectomized mice (137). These data suggest that APE1 secretion may also be a natural response in cellular physiology that does not necessarily depend on stress. Therefore, the extracellular functions of APE1 require further investigation.

Some studies have suggested an essential role of APE1 in triggering cell-to-cell communication in the inflammatory response of the local tissue microenvironment. In monocytes secreting APE1 upon inflammatory challenges, extracellular APE1 treatment increased the binding of phospho-p65 to the IL-6 promoter, resulting in activation of gene expression. High IL-6 expression suggests a possible distinct signaling cascade initiated *via* cell surface binding of extracellular APE1 (20). Recently, APE1 was shown to be upregulated in aortic endothelial cells and macrophages of atherosclerotic mice, and its plasma levels were positively correlated with neutrophil/lymphocyte ratios, which indicate systemic inflammation (138). Anti-inflammatory effects have also been associated with extracellular APE1. Using a secretory APE1 adenoviral vector system, Joo et al. evaluated the role of secreted APE1 in cell culture and LPS-induced systemic inflammation in mice. Extracellular APE1 inhibited TNFα-induced VCAM-1 expression in human umbilical vein endothelial cells and LPS-induced Cox-2 expression in Raw264.7 cells. Secreted APE1 in the blood showed an anti-inflammatory effect in mice, as LPS-induced systemic inflammation was reduced together with a decrease in myeloperoxidase release and VCAM-1 expression. This anti-inflammatory effect was associated with APE1 redox function, as mutation in its cysteine residues (C65A/C93A) affected its anti-inflammatory activity. In addition, extracellular APE1 resulted in lower levels of TNFα, IL-1β, and IL-6, and chemotactic cytokines, including MCP-1, in LPS-challenged mice (139). In TNFα-stimulated endothelial cells treated with trichostatin A, an inhibitor of deacetylases, increased protein acetylation, induction of APE1 secretion, and inhibition of TNFα receptor 1, leading to a considerable reduction in VCAM-1 expression were observed. This anti-inflammatory activity may be associated with conformational changes in TNFα receptor 1

via thiol-disulfide exchange through the redox activity of extracellular APE1 (140, 141).

To date, the functions of extracellular APE1 and the secretory mechanisms involved are poorly understood. A classical secretory signal peptide is not found in APE1 (141). However, two main nonclassical mechanisms have been proposed: secretion *via* exosomes (136, 142) and ATP-binding cassette (ABC) transporter A1 (143). Furthermore, these signaling mechanisms for APE1 translocation from the nucleus to the cytoplasm and subsequent secretion into the extracellular environment seem to depend on acetylation of lysine residues (K6R/K7R) (141). Acetylation is required to direct APE1 to the plasma membrane for translocation *via* the ABCA1 transporter (143). The importance of APE1 acetylation for modulating DNA repair activity is well known (144), but the reason for acetylation of extracellular APE1 is poorly understood. Cell-to-cell communication in the extracellular environment appears to be insensitive to unmodified extracellular APE1, requiring post-translational modification to trigger responses including cell death. In triple-negative breast cancer cells, acetylated APE1 initiates apoptosis by binding to the receptor for advanced glycation end products, resulting in significant decrease in cell viability (142). Some studies have demonstrated that secreted APE1 retains redox function (140) and DNA repair activity (136). Mangiapane et al. demonstrated that APE1 is secreted through exosomes from several cancer cell lines. The authors identified APE1 p37 and APE1 p33, forms generated by proteasomal degradation, in exosomes. The two forms are enzymatically active, and under genotoxic stress, secretion of APE1 p33 is stimulated, suggesting that APE1 may be a new damage-associated molecular pathway factor, with p33 and p37 forms playing different roles. There is still much to discover about the function of extracellular APE1 and its pathways to establish APE1 as a promising biomarker with high sensitivity and specificity. However, post-translational modifications and complex interactions between APE1 and several targets limit its use as a serological biomarker for specific diseases.

APE1 SINGLE-NUCLEOTIDE POLYMORPHISMS AND IMMUNE/ INFECTIOUS DISEASES

Several APE1 variants have been identified in humans (145, 146). Most of these genetic variants are single nucleotide polymorphisms (SNPs) and some have been linked to genomic instability and carcinogenesis (147, 148). Owing to its high frequency in the human population, the most studied and cited *APE1* SNP is rs1130409 (c.444T>A). The nucleotide change (T>A) results in substitution of aspartic acid (D) for glutamic acid (E) at position 148 (D148E), located between the redox and AP endonuclease domains of APE1. Despite its high frequency in the global population (~45%, in dbSNP, NCBI) (149), the clinical significance of this SNP has not been reported in ClinVar, and its functional significance has been predicted to be benign, unknown, or nonexistent (146, 149–151). However, several associations with conditions, including sporadic colorectal, gastric and lung cancers (152–154) and infectious diseases, such as meningitis, have been reported (155). In a study on bacterial meningitis, patients carrying the D148E polymorphism had reduced levels of IL-6, IL-1Ra, IL-8/CXCL8, and MCP-1/CCL2 compared with patients not harboring the polymorphism. In addition, variant allele carriers show more DNA damage accumulation, and children with the D148E allele have a higher IgG/IgA ratio (155). These findings show that this SNP affects the role of APE1 in immunoglobulin production, DNA repair, and expression of cytokines and chemokines. Recently, it was demonstrated that the presence of the D148E polymorphism results in protein structural instability that can affect the ability of APE1 to associate with other BER enzymes (156).

Inflammatory and immune responses are also associated with DNA damage and carcinogenesis. It has been noted that DNA damage and inflammation can promote a positive feedback loop which can drive mutations and consequently, cancer development (157). Immune cells and inflammatory mediators are directly involved in tumor processes, such as angiogenesis, cell proliferation, and invasiveness (158). Meira et al. observed that alkyladenine DNA glycosylase deficiency in a mouse colitis model increased tissue damage and neoplasia development compared to control mice (159). Ulcerative colitis is a chronic inflammatory disease associated with an increased risk of cancer, and Bardia et al. observed that the genotype frequency of *APE1*-D148E was higher in patients with ulcerative colitis than in healthy controls. In addition, they also observed an increase in necrotic and late apoptotic cells and ROS levels in patients harboring this SNP (160). *APE1*-D148E is also associated with the development of colorectal cancer (161). Inhibition of APE1 redox function exerts neuroprotective effects on the enteric nervous system, as observed in a spontaneous chronic colitis mouse model (65).

R237C is another variant associated with endometrial cancer (150) and is characterized by the formation of weaker complexes with DNA and impaired association with downstream enzymes in the BER pathway, including XRCC1 and Pol β. The R237C variant showed an approximately 60% decrease in exonuclease function compared to the wild-type enzyme, and an ~3-fold reduction in 3′ to 5′ exonuclease activity (151) and AP incision capacity in nucleosomes (162), but the AP incision activity on naked DNA was not affected (162).

In a study of patients with immunoglobulin A deficiency and common variable immunodeficiency syndrome, two novel APE1 SNPs were identified: Q51H (rs1048945) and one in the 5′ UTR (rs2307490), only the latter showed an association with common variable immunodeficiency syndrome (163). Another ten polymorphisms were investigated in a study that analyzed the structural effects of amino acid changes in the APE1-DNA complex using predictive methodologies. Two of these were predicted to be deleterious variants, I64T (rs61730854) and P311S (rs1803120), and have been suggested as suitable biomarkers to evaluate the risk of certain diseases (164).

L104R and D283G are uniquely associated with ALS, also known as Lou Gehrig's disease (165), but this association needs to be confirmed. ALS is a neurodegenerative disease caused by loss of motor neurons and glial reactions. Neuroinflammation is an early event in the development of this disease. Immune system genes

C9orf72, *TBK1*, and *OPTN* are causative genes for ALS (166). Increased APE1 expression has been observed in patients with ALS (88). Furthermore, increased interaction between APE1 and NPM1, observed in patients with *C9orf72* mutations, suggests high APE1 repair activity (167). However, the roles of APE1 and SNPs in ALS development and their relationships with the immune system require further investigation.

Finally, the influence of APE1 SNPs on redox and repair activities should be further investigated. Furthermore, owing to their varied roles in the immune response, it is necessary to study the effects of APE1 variants on susceptibility to diseases associated with immune, infectious, or inflammatory components.

APE1 INHIBITORS AND POTENTIAL THERAPEUTIC DRUGS

Several compounds have been reported as APE1 inhibitors. Some of these compounds inhibit APE1 directly, while others have indirect actions. Despite the recent discovery of the involvement of AP sites in regulation of inflammatory response, inhibition of DNA repair is not the best alternative for treating inflammatory and immune disorders. Accordingly, inhibitors of endonuclease activity have been investigated for cancer treatment. The overexpression of APE1 is associated with resistance to chemotherapy. Therefore, inhibition of APE1 associated with temozolomide treatment has been used as an alternative to increase chemotherapeutic efficacy in cancer treatment (168).

Methoxyamine (MX) is an alkoxyamine derivative and indirect APE1 endonuclease activity inhibitor. MX can bind to abasic sites, thereby blocking endonuclease activity (169, 170). MX has been studied in clinical trials for the treatment of solid tumors and lymphoma (NCT01851369). Although MX decreased the expression of LPS-induced cytokines and negatively regulated genes involved in prostaglandin production in monocytes (34), the role of MX in inflammatory disorders requires further exploration. Similarly, lucanthone inhibits DNA repair activity of APE1 without affecting the redox function (39) and is in phase II clinical trials for treatment of brain metastases secondary to non-small cell lung cancer (NCT02014545).

APE1 redox function has been studied more in relation with inflammatory and immune disorders due to its role in regulating TFs. The APE1 redox inhibitor E3330 has been suggested as a potential treatment for neoplasms, as it can inhibit the growth and migration of pancreatic tumor cells (63) and also exerts inhibitory effects in other cancer types (13). A recent phase I clinical trial in patients with cancers showed that E3330 treatment was safe (171, 172). However, the therapeutic potential of E3330 in inflammatory diseases requires further exploration. The protective effects of E3330 have been observed in *in vivo* studies of liver diseases, such as alcoholic liver injury (173) and hepatitis in mice, in which E3330 treatment mitigated TNFα, AST, and ALT levels in the plasma (174). In Sprague Dawley rats, E3330 decreases IL-6 expression and inflammatory pain sensitization caused by complete Freund's adjuvant (104). Recent studies have shown that E3330 could be a promising therapeutic strategy for inflammatory bowel disease. Winnie mice with spontaneous chronic colitis treated with an APE1 inhibitor showed decreased rectal prolapse, edema, and reduced bleeding after 14 days of treatment. In addition, mice also showed decreased loss of mesenteric neurons, reduced oxidative stress, and associated DNA damage (65).

Resveratrol is a natural phenol with antioxidative, anti-inflammatory, anticancer, and anti-neurodegenerative properties (175). *In vitro* studies have shown that resveratrol can inhibit the redox activity of APE1 and decrease AP-1 DNA binding (176). However, it remains unclear whether resveratrol is a direct and specific inhibitor of APE1. In LPS-induced U937 monocytes, the addition of resveratrol did not directly affect APE1 expression, but reduced cytoplasmic localization and acetylation of APE1, contributing to downregulation of the inflammatory response (177). Another natural compound, curcumin, has also been described as an APE1 redox inhibitor. Similar to resveratrol, curcumin exhibits anti-inflammatory, antioxidative, and antineoplastic effects. An *in vitro* study showed that curcumin reduces the APE1 dependent DNA-binding of AP-1 (178). Other studies have shown that curcumin regulates APE1 expression (179, 180). Additionally, isoflavones found in soybeans, including genistein, daidzein, and glycitein, have been studied as APE1 inhibitors and potential therapeutic options for cancer. Isoflavones have been shown to suppress radiation-induced APE1 expression and decrease HIF-1α and NF-κB DNA binding in A549 cells (181). Similar results have been observed in PCa and PC3 cells (182, 183). Liu et al. observed that genistein treatment decreased APE1 expression and TGF-β1, IL-1β, TNFα, and IL-6 levels in the serum of mice with radiation-induced pneumonitis (184). Despite these findings, evidence that these natural compounds act directly to inhibit APE1 remains limited. Therefore, E3330 and its analogs are currently the APE1 inhibitors with the most potential for use in inhibiting the inflammatory response and immune system.

CONCLUSIONS

APE1 plays multiple roles in immune responses, including ROS regulation and cytokine expression in cells mediating innate immunity, including monocytes (34), macrophages (19), keratinocytes (18), dendritic cells (7), neurons (94, 95, 97, 99), and astrocytes (105), and regulation of B cell activation and CSR in adaptive immunity (112). Despite its role in cytokine expression, it is still necessary to determine whether this regulatory control extends to all cell types or is cell type-specific. Additionally, it is necessary to observe how different stimuli influence this regulation. For example, whether the effect of APE1 is the same in a bacterial-triggered response (e.g., LPS), or virus-triggered response, or by transcriptional regulation of inflammatory mediators, also needs more attention.

The recently reported secretion of APE1 in the extracellular environment also plays a role in LPS-induced inflammation (139). However, validation of the use of serum APE1 as a disease biomarker or prognostic marker requires further investigation. Identifying APE1 polymorphisms associated with immune diseases can clarify the full role of APE1 and the consequences of its malfunction in the immune system.

Finally, APE1 inhibitors have been extensively studied for cancer treatment, and some studies have also identified their potential effectiveness in inflammatory diseases (174, 178). Thus, APE1 redox inhibitors, such as E3330 may prove to be good alternatives in inflammatory diseases or in controlling inflammation in neoplastic processes.

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All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Photorepair of Either CPD or 6-4PP DNA Lesions in Basal Keratinocytes Attenuates Ultraviolet-Induced Skin Effects in Nucleotide Excision Repair Deficient Mice

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Ultraviolet (UV) radiation is one of the most genotoxic, universal agents present in the environment. UVB (280-315 nm) radiation directly damages DNA, producing cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs). These photolesions interfere with essential cellular processes by blocking transcription and replication polymerases, and may induce skin inflammation, hyperplasia and cell death eventually contributing to skin aging, effects mediated mainly by keratinocytes. Additionally, these lesions may also induce mutations and thereby cause skin cancer. Photolesions are repaired by the Nucleotide Excision Repair (NER) pathway, responsible for repairing bulky DNA lesions. Both types of photolesions can also be repaired by distinct (CPD- or 6-4PP-) photolyases, enzymes that specifically repair their respective photolesion by directly splitting each dimer through a light-dependent process termed photoreactivation. However, as photolyases are absent in placental mammals, these organisms depend solely on NER for the repair of DNA UV lesions. However, the individual contribution of each UV dimer in the skin effects, as well as the role of keratinocytes has remained elusive. In this study, we show that in NER-deficient mice, the transgenic expression and photorepair of CPD-photolyase in basal keratinocytes completely inhibited UVB-induced epidermal thickness and cell proliferation. On the other hand, photorepair by 6-4PP-photolyase in keratinocytes reduced but did not abrogate these UV-induced effects. The photolyase mediated removal of either CPDs or 6-4PPs from basal keratinocytes in the skin also reduced UVB-induced apoptosis, ICAM-1 expression, and myeloperoxidase activation. These findings indicate that, in NER-deficient rodents,

both types of photolesions have causal roles in UVB-induced epidermal cell proliferation, hyperplasia, cell death and inflammation. Furthermore, these findings also support the notion that basal keratinocytes, instead of other skin cells, are the major cellular mediators of these UVB-induced effects.

Keywords: photolesions, photolyase, nucleotide excision repair, xeroderma pigmentosum, UVB ultraviolet radiation, inflammation, cell death

INTRODUCTION

Ultraviolet (UV) radiation is the main exogenous physical factor involved in carcinogenesis, capable of directly damaging DNA by inducing the formation of covalent bonds between adjacent pyrimidines of the same DNA strand, producing pyrimidine dimers (1). The main DNA photolesions caused by UV radiation are cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6-4PPs). These lesions distort the helical DNA duplex molecule by interfering with proper base-pairing and thus interfere with essential cellular processes, such as transcription and replication (2, 3). Both DNA photolesions can be repaired by photolyases, enzymes capable of directly repairing either CPDs or 6-4PPs through a process known as photoreactivation. During this photorepair process, the photolyase binds to the pyrimidine dimer and breaks the covalent bond in a light-dependent reaction, reverting the lesion back to the original monomers (4). Moreover, photolyases act in a specific manner, with CPD-photolyases repairing only CPDs and 6-4PP-photolyases repairing only 6-4PPs. Due to their specificities, photolyases can be used as tools to study the distinct effect of each photolesion (5).

Although photolyase genes are generally found in all domains of life, they are absent in some groups, most notably placental mammals (6). Mammals remove CPD and 6-4PP lesions by the nucleotide excision repair (NER) pathway. NER is a well-conserved mechanism responsible for removing a wide variety of lesions that distort the DNA double helix structure, including those induced by the UV component of sunlight (7). Two distinct NER sub-pathways differ in DNA damage recognition: transcription-coupled repair (TC-NER) and global genome repair (GG-NER). DNA lesions at the transcribed strand of active genes stall RNA polymerase II transcription, signaling for TC-NER. DNA damage located throughout the genome are recognized by the XPC/HR23B protein complex for GG-NER (8). As a result of these distinct mechanisms, TC-NER and GG-NER differ in their capacity to recognize DNA lesions. Particularly in mice, CPDs are essentially removed mainly by TC-NER, which rescues transcription and thus promotes cellular survival, as CPDs are poorly repaired in the non-transcribed strand and non-transcribed genomic regions. In contrast, 6-4PPs are rapidly repaired in the entire genome by GG-NER (9).

Xeroderma Pigmentosum (XP), a rare recessive, autosomal genetic disorder, is mainly caused by mutations in genes (*XPA-XPG*) involved in the NER pathway (10). A milder type of XP that does not present defective NER is named XP variant (XP-V). XP-V is instead caused by mutations in the *POLH* gene, which codes for the translesion DNA polymerase eta (8). XP is characterized primarily by a marked increased risk of skin neoplasia and cutaneous hypersensitivity to UV radiation, with XP patients often displaying severe sunburn and blistering of the skin after minimal sunlight exposure (8, 11).

UV radiation can promote cutaneous inflammation, in which skin cells, especially keratinocytes, produce and activate proteins associated with pro-inflammatory processes. These include the transcription factor NF-kB, cytokines such as IL-1α, IL-1β, TNFα, as well as proteins involved in the inflammasome complex (12–14). In addition, these molecules contribute to the expression of proteins integral to the inflammatory process, such as ICAM-1 (15) and metalloproteinases (MMPs) that allow neutrophils and macrophages to enter the skin tissue and initiate inflammation.

Interestingly, the transgenic expression of CPD-photolyase and photorepair in NER-proficient mice reduces UV-induced skin inflammation, suggesting that DNA damage itself is sufficient to trigger this biological process (16). Photorepair of CPD, but not 6-4PP, in NER-proficient mice also inhibits other UV-induced effects, namely skin hyperplasia, cell death and tumorigenesis (17). Photorepair of CPD, but not 6-4PP, in cell cultures also reduces UV-induced cell death in NER-proficient cells. However, in cells derived from XP patients, photorepair by either CPD or 6-4PP-photolyase reduces the apoptotic effect of UV radiation. Concomitant photorepair by both photolyases further reduced UV-induced apoptosis, indicating that both CPD and 6-4PP lesions participate in UV-induced effects in NER-deficient models (2).

In this study, we show that *in vivo* photoreactivation through K-14 promoter driven expression of either CPD or 6-4PP-photolyase in basal keratinocytes reduces acute UVB-induced apoptosis in NER-deficient *Xpa* knockout mice. The photoremoval of either photolesion in this model also decreased UV-induced inflammation, as the expression of either photolyase diminished ICAM-1 levels and active neutrophils present in the skin of UVB-irradiated *Xpa*-deficient mice. In contrast, only CPD removal abolished chronic UV-induced skin cell proliferation and hyperplasia, with 6-4PP removal having a minor impact on these UV-induced effects. These findings indicate that both types of DNA lesions directly participate in inducing apoptosis, inflammation, and hyperplasia. Furthermore, these results also support the notion that basal keratinocytes are the key mediators of these UV-induced effects.

MATERIALS AND METHODS

Mouse Lines

Xpa knockout mice expressing CPD- or 6-4PP-photolyase were obtained by generational crossing between the $Xpa^{-/-}$ mice

described in (18) with transgenic K14-CPD-PL or K-14-64PP-PL photolyase mice (19), hereafter referred to as CPD or 6-4PP-photolyase mice, respectively. Both photolyase genes are under the control of the basal keratinocyte-specific K-14 promoter (19). All strains used in this project were initially obtained at the Erasmus Medical Center University, Rotterdam (the Netherlands) were in a C57BL/6J or hairless C57BL/6J/SKH-1 genetic background, established models for UV radiation (18, 20). *Xpa*$^{-/-}$ mice were maintained by homozygous crosses, while photolyase expressing genes and the *hairless* gene were maintained by heterozygous crosses. All animals used for experiments were 8- to 10-week-old. As there are no differences regarding UV sensitivity between males and females, mice of both genders were used for all experiments. Housing, breeding, genotyping, and experimentation were performed in accordance with the regulations established by the ethical committee for experimentation with animals of the Institute of Biomedical Sciences of the University of Sao Paulo (Protocol #121-11-03).

Genotyping was performed on mouse tail DNA followed by polymerase chain reaction (PCR) of target genes. PCR conditions of the K14-*CPD-PL* and K14-*64PP-PL* genes are described in **Supplementary Tables S1, S2**, and primer sequences in **Supplementary Table S3**.

UV Radiation and Photoreactivation of Mice

Mice were irradiated with a Philips TL12-40W UVB lamp, using a VLX-3W UV dosimeter (Vilber Loumart, Torcy, France) to measure the intensity of UV radiation. No UVC (254 nm) radiation was detected, and UVA (365 nm) radiation was below <0.05 J/m^2/s. The distance between the UVB lamp and the mice dorsal skin was 1.10 meters. Immediately after UV irradiation, mice were exposed to photoreactivating light (four white lamps Polylux XL F36W/840) for three h, positioned 40 cm above the mice. Minimal erythemal dose (MED) of *Xpa*$^{-/-}$ mice was determined as 20 J/m^2 of UVB by analyzing the macroscopic induction of erythema, wounding, skin peeling, skin thickening and pigmentation.

Chronic UV Irradiation of Hairless *Xpa*$^{-/-}$ Mice for Assessment of Tissue Hyperplasia and Cell Proliferation

Xpa$^{-/-}$ hairless mice expressing either CPD- or 6-4PP-photolyase were irradiated for 30 consecutive days, at approximately 2:00 pm, with a 1 MED UVB (20 J/m^2) dose followed by 3 h photoreactivation. Animals were observed daily for signs of distress, epidermal thickness, and pigmentation. 48 h after the last day of irradiation, mice (n=4) were euthanized and 1 cm^2 mice dorsal skin was collected. Two h prior to euthanasia, animals were intra-peritoneally injected with BrdU (5 mg) for cell proliferation analysis.

Tissue Fixation for Histology Analysis

Skin samples were fixed overnight at 4°C in 4% formaldehyde (Merck, Kenilworth, NJ, USA). Samples were then dehydrated at room temperature by sequential immersion for 1 h in each of the following solutions: PBS 1X, 50% ethanol (Merck), 70% ethanol, 80% ethanol, 90% ethanol, 2x 100% ethanol and 2x xylene (Sigma-Aldrich, Saint Louis, MO, USA). After dehydration, samples were twice incubated in paraffin 60°C for 1 h each, mounted in paraffin blocks and kept at RT until further processing. Skin tissue sections (5 µm) were obtained using a microtome, placed on Starfrost (Knittel-Glaser) slides and kept in 10% ethanol at 50°C until total fluid evaporation. For fixation on the slide, skin sections were maintained at 37°C overnight and stored at RT until staining.

Quantification of Epidermal Thickness

Tissue slides were deparaffinized and hydrated through sequential immersion in xylene (100% twice), ethanol (100% twice, 95%, 70% and 50%) and dH_2O under room temperature. Slides were then stained with hematoxylin and eosin (H&E). Stain excess was washed under indirect water flow, and tissue was subsequently dehydrated through immersion in ethanol and xylene. Slides were then mounted using Entellan and Menzel-Glass coverslips.

Epidermal thickness was quantified using Axiovert 200 (Zeiss, Oberkochen, Germany) optical microscope under a 100x objective. Epidermal thickness was defined as the distance between the end of the outer epidermal layer and the basal lamina. Invagination sites, such as sweat glands and hair follicles were not considered in this analysis. Three measurements were performed per field, using three fields in each slice, and three slices per animal, with a total of twenty-seven measurements per animal. Axiovision Rel. 4.8 (Zeiss) software was used for quantification.

Tissue Cell Proliferation

BrdU detection using immunohistochemistry was performed to quantify cell proliferation. Tissue slides were deparaffinized and hydrated as previously described, then incubated for 30 min in 50% methanol 1% H_2O_2 (30%, Merck) for endogenous peroxidase inactivation, followed by two PBS washes. Samples were then incubated in pepsin (18 U/ml) diluted in 100 mM HCl at 37°C for 30 min, followed by two PBS washes and incubation at 56°C for 20 min in 1 M HCl. pH was neutralized with 100 mM sodium borate in PBS (pH 8.5), followed by three PBS washes. Slides were incubated in blocking solution (5% FBS in 1% PBS/BSA) for 10 min, at RT, followed by incubation with anti-BrdU (M0744, DAKO), diluted 1:100 in blocking solution overnight at 4°C. Slides were washed in PBS and incubated for 1 h with HRP anti-mouse (Sigma-Aldrich, A9044), diluted 1:100 in blocking solution. Substrate reaction was done with 3,3'-Diaminobenzidine (DAB, Spring) until nuclei were stained. Counter staining was performed with hematoxylin (Merck). Slides were mounted with Entellan and coverslips. Images were obtained with Axiovert 200 Optic Microscope (Zeiss) under 100x objective using Axiovision Rel. 4.8 (Zeiss) software. We performed three blind measurements per skin tissue of BrdU$^+$ basal and suprabasal cells, analyzing 3 slices per animal. Quantification of

BrdU-positive cells was performed by calculating the ratio between stained basal layer cells and total basal layer cells, while quantification of suprabasal BrdU-positive cells was performed considering the ratio between these cells and the total number of basal layer cells.

Immunohistochemistry for the Detection of Photolesions

CPD and 6-4PP were immunodetected in skin tissue sections to confirm that the photolyases expressed in the mice models were repairing their respective photolesions. Tissue slides were deparaffinized and hydrated as previously described, then incubated in 18 U/ml pepsin diluted in 100 mM HCl at 37°C for 30 min. Slides were washed twice using PBS, then incubated at 56°C for 20 min in 1 M HCl, followed by three PBS washes. Tissues were then incubated for 20 min in blocking solution (5% FBS in 1% PBS/BSA) at RT. After blocking, tissues were incubated overnight at 4°C in anti-CPD (TDM-2, Cosmo Bio, Tokyo, Japan) or anti-6-4PP (64M-2, Cosmo Bio), diluted 1:1000 and 1:300, respectively, in the blocking solution. Slides were then washed twice with PBS and incubated in secondary antibody anti-mouse IgG conjugated with Alexa fluor 555 for 90 min. Slides were washed twice with PBS, followed by counterstaining with DAPI fluoroshield (Sigma-Aldrich) solution. Images were obtained with Axiovert 200 Optic Microscope (Zeiss) under 40x objective using Axiovision Rel. 4.8 (Zeiss) software.

Acute Irradiation of *Xpa*$^{-/-}$ Mice for *In Vivo* Assessment of Inflammation and Cell Death

Photolyase-expressing *Xpa*$^{-/-}$ mice were anesthetized and shaved 24 h before irradiation. Mice were then irradiated with a single UV-dose of 200 J/m^2 (10 MED).

To assess early inflammation induced by UVB light, mice (n=4) were injected with anti-ICAM-1/DiD fluorophore (excitation 640 nm, emission 680 nm) nanoparticles to detect ICAM-1 expression 6 h after UVB irradiation (21). Furthermore, after 6 and 24 h of irradiation, mice were inoculated with the XenoLight RediJect Chemiluminescent Inflammation Probe (PerkinElmer) to detect active, myeloperoxidase-expressing neutrophils (n=2). Probe fluorescence and chemiluminescence were detected using the *in vivo* imaging system (IVIS) Spectrum (PerkinElmer), located in the Core Facility Center for Research Support of the University of Sao Paulo (CEFAP-USP). We also used the MMPSense 645 FAST probe (PerkinElmer) to detect several MMPs (2, 3, 7, 9, 12, and 13, n=2).

Mice were assessed for *in vivo* cell death (n=3) 48 h after UV irradiation using Annexin-V/DiD fluorophore nanoparticles (22), injected intravenously 2 h prior to detection by IVIS Spectrum.

When imaging, mice were kept under anesthesia using isoflurane. Image analysis and quantification was performed with Living Image 4.0 (PerkinElmer) software. Radiances were normalized using non-irradiated control mice.

Statistical Analysis

Data were expressed as mean ± standard deviation and analyzed with one-way ANOVA followed by Bonferroni's multiple comparison test. p value < 0.05 was considered significant, with "*" indicating $p \leq 0.05$, "**" $p \leq 0.01$ and "***" $p \leq 0.001$.

RESULTS

Effects of Basal Keratinocyte-Specific Photoremoval of CPD or 6-4PP on Hyperplasia and Cell Proliferation in Chronically Irradiated *Xpa*-/- Mice

NER-deficient *Xpa*$^{-/-}$ mice show hypersensitivity to UV radiation, as low UVB doses can produce excessive skin abrasion (19). Thus, establishing a Minimal Erythemal Dose (MED) was necessary to determine a biologically relevant dose for the experiments. The MED for *Xpa*$^{-/-}$ hairless mice was determined to be 20 J/m^2, used as a reference dose henceforth. To assess the role of CPDs and 6-4PPs on the induction of hyperplasia, mice (n=4) received over 30 days a single daily dose of 20 J/m^2 UVB radiation followed by 3 h exposure to photoreactivating light to allow photorerepair by the respective photolyase, an exposure time previously shown to remove both photolesions by transgenic photolyase expression in basal keratinocytes (19), (**Supplementary Figure S1**). Epidermal thickness was quantified using H&E-stained sections (**Figure 1A**). *Xpa*$^{-/-}$ mice not expressing any photolyase under chronic UV radiation developed hyperplasia, while CPD photorepair in keratinocytes inhibited this UV-induced effect, evidencing the causative role of CPD lesions for UVB-induced hyperplasia. Curiously, removing 6-4PPs in keratinocytes also affected UVB-induced hyperplasia, decreasing but not abrogating this chronic UV response in *Xpa*$^{-/-}$ mice.

UVB-induced cell proliferation was also analyzed in these chronically irradiated mice (**Figure 1B**). CPD photoremoval in keratinocytes again prevented the UVB-induced cell proliferation effect both in basal and suprabasal epidermal layers. Similarly, the removal of 6-4PPs attenuated this effect in the basal layer and fully inhibited it in the suprabasal epidermal layer of *Xpa*$^{-/-}$ mice, corroborating the hyperplasia results.

Photorepair of Either CPDs or 6-4PPs in Basal Keratinocytes Reduces UV-Induced Apoptosis in *Xpa*$^{-/-}$ Mice

Apoptotic cell death was analyzed *in vivo* through nanoparticle probes linked to Annexin-V and DiD-fluorophore (**Supplementary Figure S2**). In addition, higher UVB dose (200 J/m^2, or 10 MED) was used to evaluate the acute, UV-induced *in vivo* effects.

Results demonstrated that 48 h after UVB irradiation immediately followed by photoreactivation, expression of CPD-photolyase in keratinocytes of *Xpa*$^{-/-}$ mice significantly reduced the apoptotic signal compared to *Xpa*$^{-/-}$ mice not expressing photolyases, suggesting the participation of persistent CPDs in UVB-induced *in vivo* cell death (n=3). Furthermore, similar results were obtained with 6-4PP-photolyase-expressing mice, suggesting that both CPDs and

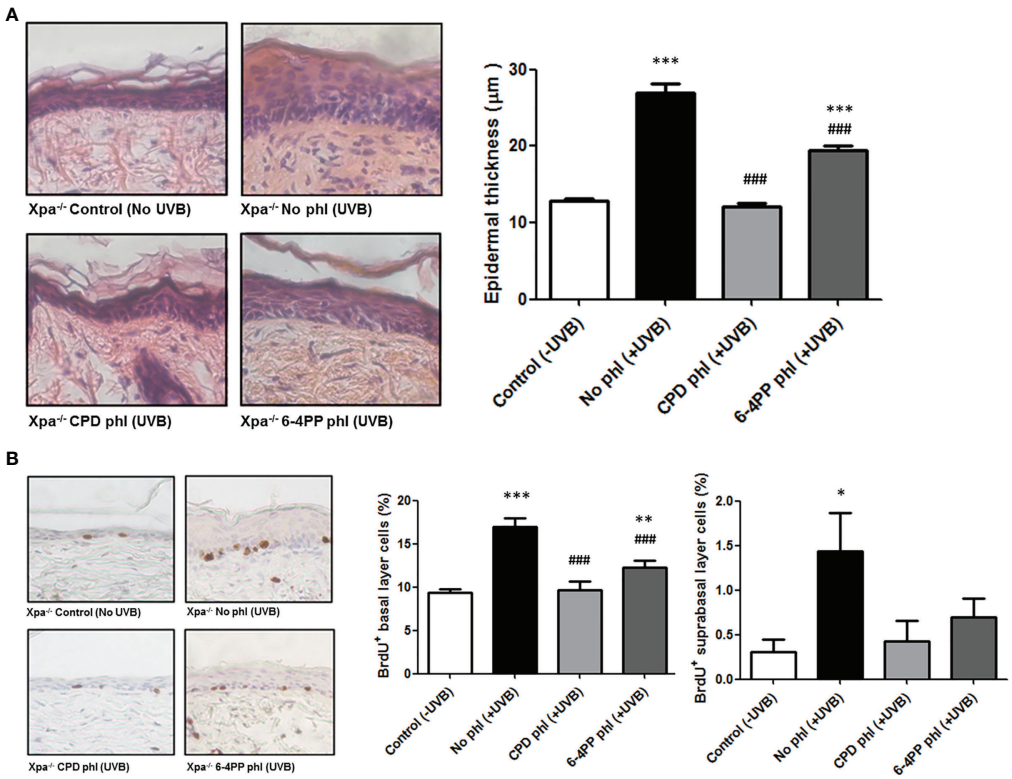

FIGURE 1 | CPD removal in keratinocytes abrogates UV-induced hyperplasia and cell proliferation, while 6-4PP removal decreases these effects in Xpa-/- mice. **(A)** Epidermal thickness of Xpa-/- mice daily irradiated or not with UVB (20 J/m2) followed by photoreactivation of CPD- or 6-4PP-Photolyase (phl) for 30 consecutive days. Quantitative analysis of epidermal thickness was performed by perpendicular measurements of the tissue extension in skin sections stained with H&E (n = 4), with representative images (40x) shown. **(B)** Quantification of cell proliferation in the basal and suprabasal layers of chronically UV-irradiated Xpa-/- mice, with representative images (40x). Tissues were stained for BrdU+ cells by immunohistochemistry counterstained with hematoxylin (n = 4). Asterisks (*) indicate a statistically significant difference between the designated group and the negative, Xpa-/- non-irradiated, controls, while the pound signs (#) indicate a significant difference between the designated group and the positive control group, Xpa-/- mice with no photolyase UVB-irradiated. "*" or "#": p<0.05, "**" or "##": p<0.01, and "***" or "###": p<0.001.

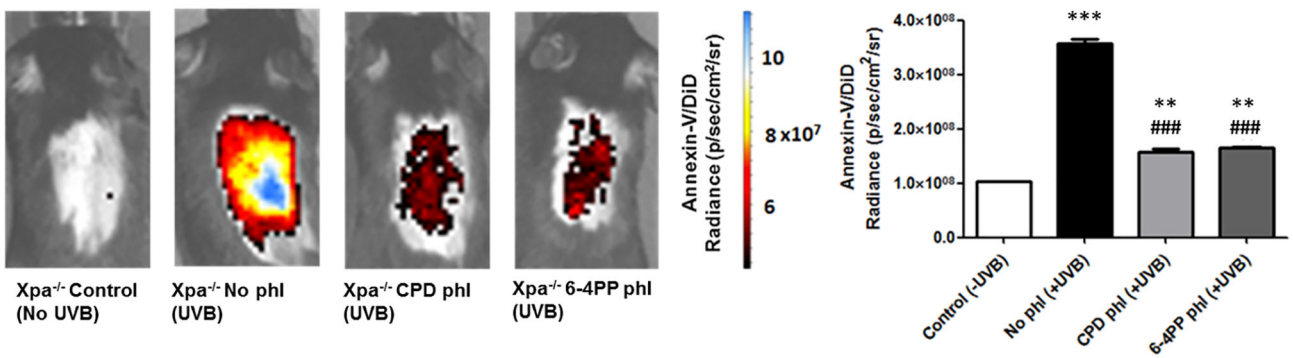

FIGURE 2 | *In vivo* effect of keratinocyte-specific photorepair of CPDs and 6-4PPs on UVB-induced apoptosis in Xpa-/- mice. Apoptosis was analyzed by *in vivo* imaging using Annexin-V/DiD fluorophore containing nanoparticles, 48 h after UVB (200 J/m2) irradiation. Radiance of DiD containing nanoparticles were quantified in the central region of Xpa-/- mice exposed dorsal skin using Living Image 4.0 software, n = 3. Asterisks (*) indicate a statistically significant difference between the designated group and the negative, Xpa-/- non-irradiated, controls, while the pound signs (#) indicate a significant difference between the designated group and the positive control group, Xpa-/- mice with no photolyase UVB-irradiated. "*" or "#": p<0.05, "**" or "##": p<0.01, and "***" or "###": p<0.001.

6-4PPs participate in apoptosis triggering events following UV irradiation in these NER-deficient mice (**Figure 2**).

UVB-Induced Inflammation in *Xpa*$^{-/-}$ Mice Is Reduced by CPD or 6-4PP-Photorepair in Basal Keratinocytes

UVB-induced inflammation was measured 6 and 24 h after UVB (200 J/m^2) irradiation and photoreactivation. Using *in vivo* probes (**Supplementary Figure S3**), we measured the expression ICAM-1, a cell surface protein responsible for neutrophil adhesion. We also used a commercial chemiluminescent probe for detecting myeloperoxidase (MPO), an enzyme highly expressed by active neutrophils and a key mediator of inflammation-dependent oxidative stress. Interestingly, both CPD or 6-4PP removal in keratinocytes decreased ICAM-1 levels (n=4) 6 h after UVB irradiation (**Figure 3A**). Similarly, CPD and 6-4PP photoremoval also lessened the infiltration of active neutrophils in the skin, as measured by *in vivo* MPO expression 6 and 24 h (n=2) after UVB irradiation (**Figures 3B, C** respectively), as well as shown in H&E-stained skin sections (**Supplementary Figure S4**), which indicates that these two photolesions participate in the inflammatory event of leukocyte tissue extravasation following UV irradiation.

Neither CPD nor 6-4PP Photoremoval Altered MMP Activation in UVB Irradiated *Xpa*$^{-/-}$ Mice

Matrix Metalloproteinases (MMPs), enzymes that modulate innate immunity, and tissue remodeling were measured *in vivo* using a commercial fluorescent probe capable of detecting active

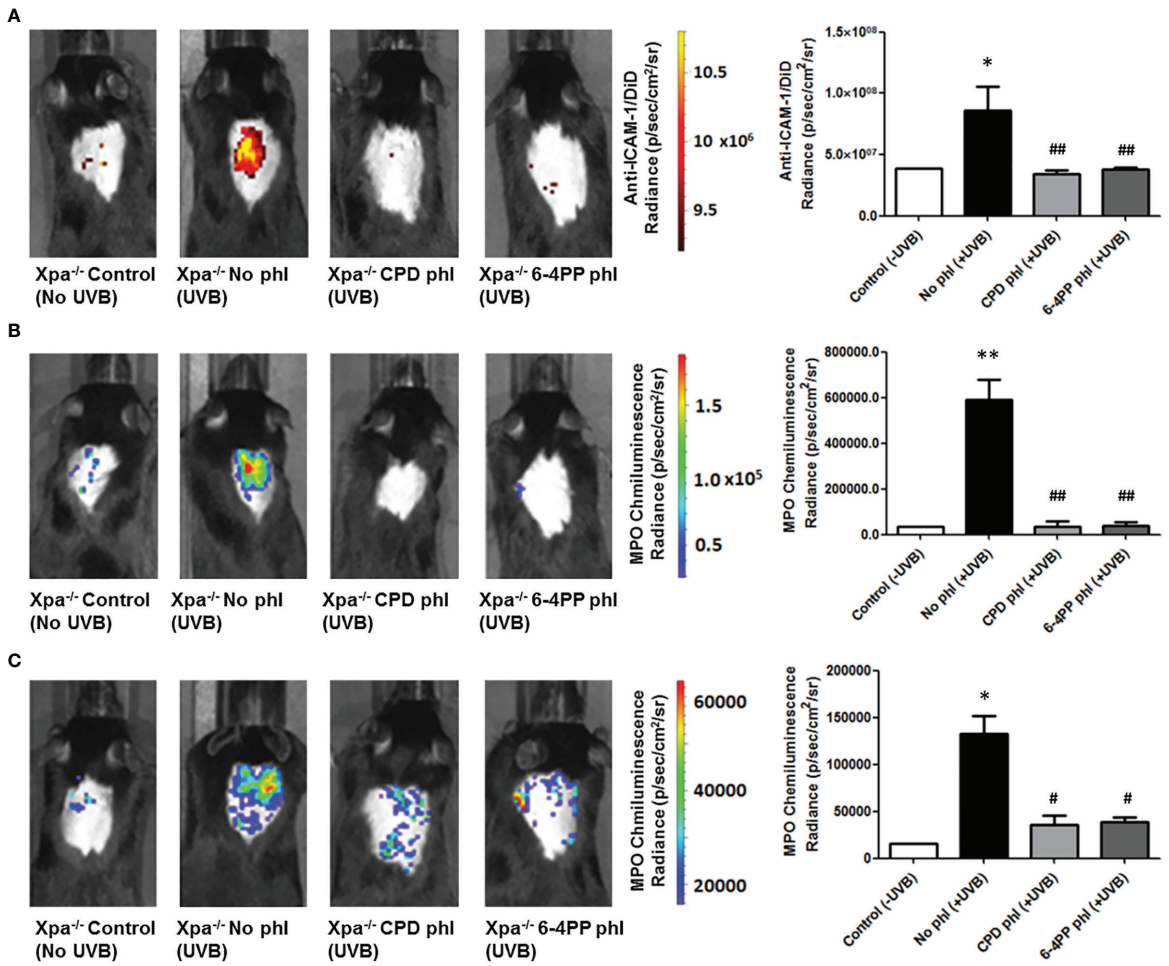

FIGURE 3 | Leukocyte infiltration and activation in vivo imaging of Xpa-/- mice expressing CPD or 6-4PP-photolyase in basal keratinocytes. **(A)** *In vivo* imaging of ICAM-1, an inflammation marker, coupled to a DiD fluorophore containing nanoparticle. Imaging was performed in photolyase (CPD- or 6-4PP-phl) expressing Xpa-/- mice 6 h after irradiation with 200 J/m2 UVB, n = 4. **(B, C)** MPO, an active neutrophil marker, was measured *in vivo* 6 **(B)** and 24 h **(C)** after UVB radiation of Xpa-/- mice by using a chemiluminescent probe, n = 2. Radiance and luminescence were measured using Living Image 4.0 software. Asterisks (*) indicate a statistically significant difference between the designated group and the negative, Xpa-/- non-irradiated, controls, while the pound signs (#) indicate a significant difference between the designated group and the positive control group, Xpa-/- mice with no photolyase UVB-irradiated. "*" or "#": p<0.05, "**" or "##": p<0.01.

members of the MMPs, including MMP2, 3, 7, 9, 12, and 13. Surprisingly, unlike our previous results, the removal of neither photolesion significantly reduced the UVB-induced MMP tissue presence 24 h after radiation (n=2). Although there was a trend towards lower MMP levels after CPD photorepair, it was not significant (p=0.1488) (**Figure 4**).

DISCUSSION

In this work, we used NER-deficient, *Xpa*$^{-/-}$ mice to investigate the *in vivo* effects of transgenic photolyase-mediated lesion-specific removal of CPDs or 6-4PPs in basal keratinocytes after UV radiation. We observed that removal of CPDs strongly prevented UVB-induced hyperplasia and cell proliferation, while the removal of 6-4PP reduced these effects to a lesser extent. In cultured cells, UVB-induced photolesions suppress cell proliferation by stalling cell cycle progression through p53-dependent mechanisms (23, 24). *In vivo*, however, basal keratinocytes display a different response to photolesions, as previously reported using NER-proficient mice expressing CPD-photolyase (17, 19). CPDs not only upregulate the expression of genes typically associated with DNA damage response and pro-apoptotic genes, but also genes associated with cell proliferation (19).

Photorepair of 6-4PPs, however, does not prevent any UVB-induced cell proliferation in NER-proficient mice, an effect interpreted to be due to the rapid repair of 6-4PP lesions by NER (19, 25). Since CPD lesions are generated at a higher rate than 6-4PPs when DNA is exposed to UV radiation (25, 26), it is expected that, in a NER-deficient background, CPDs will be present in higher amounts than 6-4PP lesions. We thus hypothesize that the remaining 6-4PP lesions in *Xpa*$^{-/-}$ mice expressing CPD-photolyase do not exceed the necessary damage threshold to initiate cell proliferation signaling in the present study settings. Interestingly, photorepair of 6-4PPs in *Xpa*$^{-/-}$ mice also attenuated this response, further indicating that the presence of UV-induced DNA photolesions are a major factor to UV-induced cell proliferation and hyperplasia.

Furthermore, both unrepaired CPD and 6-4PP lesions also contribute to UVB-induced apoptosis in *Xpa*$^{-/-}$ mice, as the expression of either photolyase leads to a reduction in this cell death process. These results and previous *in vivo* studies using NER-proficient mice expressing CPD- or 6-4PP- photolyase (17, 19) corroborate with *in vitro* studies (2). The removal of CPD, but not 6-4PP lesions in NER-proficient cells, reduces apoptosis induction, while in XP-A NER-deficient cells, the removal of either lesion resulted in the reduction of apoptosis (2). As previously mentioned, CPD lesions are generated at a higher rate than 6-4PPs as a result of UVB irradiation (25, 26). Therefore, it stands to reason that activation of apoptotic pathways by UV irradiation depends not only on the number but also on the type of photolesion (2).

While less numerous, 6-4PP lesions cause a more pronounced distortion on the DNA molecule, i.e. generating a 44° bend of the DNA helix, contrasting to a 9° helix bend caused by CPDs (3, 27). These structural differences have a significant impact on DNA replication, which is obstructed by 6-4PP, but not CPD lesions (27). In addition, both CPD and 6-4PP lesions stall transcription by RNA polymerases in a cell-free transcription elongation system (28). In cells, RNA pol II has been shown to bypass certain DNA lesions, including CPDs, by a translesion transcription mechanism, albeit with low efficiency (29). Both replication and transcriptional stress can activate proapoptotic signaling (3, 30), and the greater distortion of the DNA molecule by 6-4PP lesions may generate different responses to polymerases (27), explaining the distinct role these lesions have in the apoptotic cell death observed in UVB-irradiated *Xpa*$^{-/-}$ mice and in XP-A cells. Interestingly, photolesions have also been implicated in skin inflammation (14, 16, 19), as DNA damage-induced replication and transcriptional stress induces pro-inflammatory cytokines (21, 31–33).

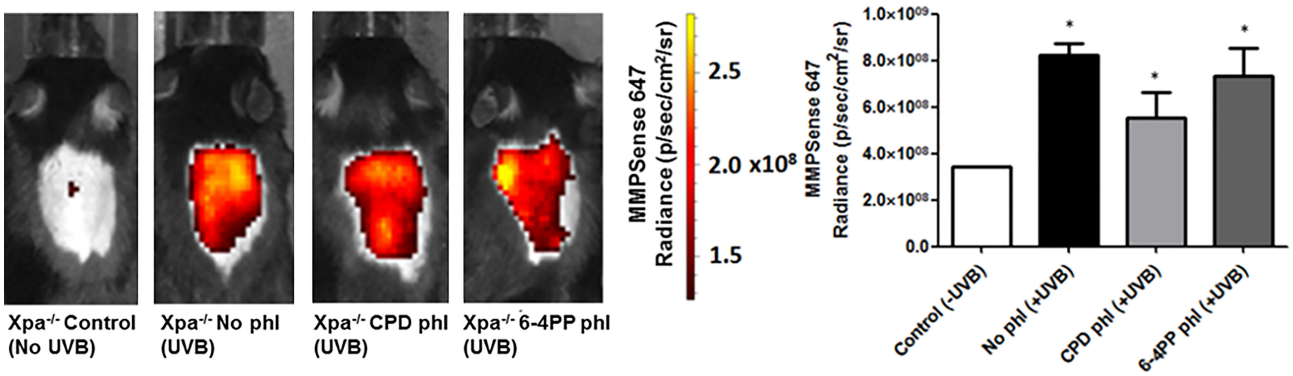

FIGURE 4 | *In vivo* effects of keratinocyte-specific photorepair of CPDs or 6-4PPs in Xpa-/- mice after a single, high UVB irradiation dose. MMPsense was used to measure in vivo the presence of MMPs in mice skin 24 h after UVB irradiation, n=2. Radiance quantifications of fluorescent and chemiluminescent probes were performed in the central region of the mice exposed dorsal skin with equivalent sized regions of interest using Living Image 4.0 software. Asterisks (*) indicate a statistically significant difference between the designated group and negative, Xpa-/- non-irradiated, controls.

UV radiation promotes inflammation through several distinct mechanisms, such as activation of the inflammasome and pro-inflammatory cytokines, as well as major inflammation-related signaling pathways such as NF-κB and p38 (34, 35). IL-1α has also been suggested to act as a DNA damage sensor, as it colocalizes with CPD lesions and is secreted after genotoxic stress. Interestingly, removal of CPD lesions in NER-proficient animals reduces the pro-inflammatory effects of UV (16, 19), again linking CPD photolesions to inflammation. Our study further characterizes this association by showing that both CPDs and 6-4PPs have a role in these effects on NER-deficient mice, as removing either lesion in basal keratinocytes caused a reduction in UVB-induced neutrophil infiltration and activation in the skin. Similar to the apoptotic response, this suggests that the inflammatory responses elicited by photolesions might also depend on the lesion type, and not only on the amount of damage. Furthermore, these results indicate that basal keratinocytes have a major role in regulating UV radiation-related inflammation, in agreement with previous studies (12, 36).

Despite the anti-inflammatory effect of photorepair, the removal of neither CPD nor 6-4PP displayed any significant effects regarding the level of Matrix Metalloproteinases (MMPs) in the skin following UVB irradiation. Notably, the methodology of the present study did not differentiate between different kinds of MMPs, as the probe used can be activated by MMP 2, 3, 7, 9, 12, and 13. Different MMPs generally have distinct effects and may participate in both inflammation initiation and resolution (37). For instance, MMP2, MMP3, and MMP9 have a role in activating the pro-inflammatory cytokines TNF-α and IL-1β, while MMP3 may also participate in the degradation of mature IL-1β depending on the context (38). Moreover, as the photolyases were expressed only in basal keratinocytes, it is possible that other cells, such as fibroblasts residing in the upper dermis layer (19), also contributed to the release of MMPs. There is also the possibility that photolyase-expressing mice presented a decrease in MMP expression, but no signal reduction could be detected due to an over-saturation of the MMP fluorescent signal caused by a high dose of UVB irradiation on *Xpa*$^{-/-}$ mice. Therefore, further studies regarding specific MMPs are required to better elucidate the role of CPDs and 6-4PPs on the induction of these molecules.

In summary, by using *Xpa*$^{-/-}$, keratinocyte-specific photolyase-expressing mice, we were able to demonstrate that both CPD and 6-4PP lesions participate in UV-related effects such as hyperplasia, cell proliferation, inflammation, and apoptosis using *in vivo* NER-deficient models, with keratinocytes having a major role regarding these effects. These results corroborate previous studies concerning photolesion effects on apoptosis and hyperplasia and have novel implications regarding DNA damage as a pro-inflammatory stimulus. These discoveries also have important implications for XP patients, incapable of repairing UV-induced photolesions. These patients have a much higher skin carcinogenesis predisposition and different mutation spectra in skin tumors (39). This could be related the pro-inflammatory effects of both photolesions, with inflammation being a critical factor in tumor progression and DNA damage due to releasing oxidizing agents (40). Furthermore, unlike NER-proficient models, in which CPD lesions are the main photolesion responsible for triggering the studied effects, NER-deficient models have both CPDs and 6-4PPs participating in these effects, with 6-4PPs possibly having a different role in XP tumorigenesis (2), with the tumors from these individuals having different causative lesions compared to the rest of the population. Additional investigations on the molecular mechanisms of the activation of the UVB effects in NER-deficient models could shed light on XP carcinogenesis and how the photolesions interact with the multitude of molecular pathways involved in these UVB-induced responses.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical committee for experimentation with animals of the Institute of Biomedical Sciences of the University of Sao Paulo (Protocol #121-11-03).

AUTHOR CONTRIBUTIONS

GK, CQ, CM conceived the study. CM and CQ. CQ supervised the work. GK,CQ and CM designed the experiments. GK, CQ, CG, WF and JdS performed the experiments. GK, WF and CM analyzed the data. GK prepared the figures and wrote the manuscript with input from CQ, CG, GH, JH and CM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.800606/full#supplementary-material

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Expression of Constitutive Fusion of Ubiquitin to PCNA Restores the Level of Immunoglobulin A/T Mutations During Somatic Hypermutation in the Ramos Cell Line

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Somatic hypermutation (SHM) of immunoglobulin (Ig) genes is a B cell specific process required for the generation of specific and high affinity antibodies during the maturation of the immune response against foreign antigens. This process depends on the activity of both activation-induced cytidine deaminase (AID) and several DNA repair factors. AID-dependent SHM creates the full spectrum of mutations in Ig variable (V) regions equally distributed at G/C and A/T bases. In most mammalian cells, deamination of deoxycytidine into uracil during S phase induces targeted G/C mutagenesis using either direct replication of uracils or TLS mediated bypass, however only the machinery of activated B lymphocytes can generate A/T mutagenesis around AID-created uracils. The molecular mechanism behind the latter remains incompletely understood to date. However, the lack of a cellular model that reproduces both G/C and A/T mutation spectra constitutes the major hurdle to elucidating it. The few available B cell lines used thus far to study Ig SHM indeed undergo mainly G/C mutations, that make them inappropriate or of limited use. In this report, we show that in the Ramos cell line that undergoes constitutive G/C-biased SHM in culture, the low rate of A/T mutations is due to an imbalance in the ubiquitination/deubiquitination reaction of PCNA, with the deubiquitination reaction being predominant. The inhibition of the deubiquitinase complex USP1-UAF1 or the expression of constitutive fusion of ubiquitin to PCNA provides the missing clue required for DNA polymerase η recruitment and thereafter the introduction of A/T base pair (bp) mutations during the process of IgV gene diversification. This study reports the establishment of the first modified human B cell line that recapitulates the mechanism of SHM of Ig genes *in vitro*.

Keywords: immunoglobulin somatic hypermutation, PCNA monoubiquitination, Ramos B cell line, USP1 inhibition, A/T mutation pathway

INTRODUCTION

The process of SHM in germinal center (GC) B cells is basically the result of two distinct molecular mechanisms taking place in separate phases of the cell cycle (1). Both mechanisms start with the introduction of uracils along the region that is subject to SHM in immunoglobulin (Ig) genes by AID-dependent deamination of deoxycytidine residues. When this occurs during S phase of the cell cycle, this leads to mutations mainly focused on dC/dG pairs creating both transitions and transversions. This mutagenic process involves either the action of uracil DNA glycosylase (UNG), which creates an abasic site by removing the uracil that is subsequently bypassed by translesion DNA polymerases (TLS pols), or through the direct copy of the deoxy-uracil (dU) by the replicative DNA polymerases (2). These dU could be either generated during S phase or as shown recently generated during G1 of the cell cycle and survive until S/G2 due to the activity of Fam72a that reduces UNG levels in G1 (3, 4). Conversely, the mutations are spread on the surrounding A/T bases using an error-prone gap filling reaction by DNA polη (5–7). This latter is initiated by MSH2/MSH6 recognition of the dU:dG mismatch, followed by the action of exonuclease 1 (EXO 1), which creates the single-stranded gap in a process called noncanonical mismatch repair (ncMMR) (8–10). Furthermore, the monoubiquitination of proliferating cell nuclear antigen (mUb-PCNA) is a major posttranslational modification (PTM) required for the generation of mutations at A/T base pairs during SHM (11). PCNA undergoes monoubiquitination (mUb) mainly in response to replication fork stalling (12). This PTM orchestrates polymerase switching that favors the recruitment of TLS polymerases for lesion-bypass DNA synthesis during replication (13). However, in the case of SHM of Ig genes, how PCNA is monoubiquitinated in G1 in the absence of DNA lesions and how it participates in the specific recruitment of polη remain unknown. PCNA that forms the eukaryotic DNA sliding clamp is an auxiliary factor of DNA polymerases. Active PCNA is composed of the association of three monomers in a ring-shaped structure (14). In mammalian cells, to ensure cell survival, PCNA is modified at a conserved site, K164, *via* a single ubiquitin polypeptide moiety by RAD6 and RAD18 in response to various DNA damaging agents (15). When the lesion is bypassed, the ubiquitin polypeptide is removed mainly by ubiquitin-specific protease 1 (USP1) to allow the recruitment of high-fidelity DNA polymerases and resume replication. Previous work from H. Jacobs's group (The Netherlands Cancer Institute, Amsterdam) has shown that a mouse expressing PCNA with a lysine-to-arginine mutation at residue 164 preventing the mUb displays a phenotype similar to polymerase η and mismatch repair-deficient B cells with a strong reduction of somatic mutations at A/T bases in Ig V region (IgV) associated with a compensatory increase at G/C mutations (11). A similar result was obtained using knockout mice for PCNA expressing exogenous PCNA with the K164R mutation (16). However, in activated B lymphocytes, the situation is particular since the PMT of PCNA takes place in the absence of undamaged DNA *via* an unknown mechanism, and the main goal is to generate mutations (17).

The search for lymphoid cell lines that could provide a tractable system for investigating *in vitro* the process of SHM in general and, more specifically, the process of A/T mutagenesis in particular started several decades ago (18–22). Many mouse and human B cell lines have been identified. Among them, the human Burkitt lymphoma cell lines CL-O1, BL2 and Ramos have been extensively studied. These cells were transformed in the germinal center (GC) during the process of antibody affinity maturation (23). Induction of somatic mutations in CL-01 cells requires cross-linking of the BCR and T cell contact through CD40/CD40 ligand and CD80/CD28 co-engagement. The BL2 cell line undergoes V_H diversification on culture in the presence of an anti-immunoglobulin and coculture with activated T cells (18) or through simultaneous aggregation of three surface receptors, IgM, CD19 and CD21 (21, 22). Ramos cells, however, diversify the IgV domain constitutively during culture (19, 20, 24). IgV gene diversification in both cell lines exhibits the major hallmarks of *in vivo* Ig SHM: the mutations are (I) largely base substitutions (II) targeted to transcribed V genes and especially concentrated at selected hotspot motifs RGYW/WRCY (R: purines, Y: pyrimidines and W: A or T) (III) dependent on AID activity and (IV) biased for transitions over transversions. However, despite the presence of intact components of the Ig A/T mutational machinery (24), the major drawback of these cells remains their inability to efficiently perform A/T mutagenesis. Therefore, they display a mutation pattern biased toward G/C mutations (80 to 90%), thus greatly limiting their use for elucidating the mechanism of A/T mutagenesis. In this report, we discovered that in Ramos cells, the paucity of A/T mutations is due to an imbalance in the ubiquitination and deubiquitination of PCNA, the latter being predominant. The inhibition of the deubiquitinase responsible or the expression of a constitutive fusion of ubiquitin to PCNA significantly increases the rate of A/T mutations, thus reviving the SHM A/T mutagenesis pathway and consequently providing the first *in vitro* system that can be used to elucidate the A/T mutagenesis process.

MATERIAL AND METHODS

Plasmids, Plasmid Construction and Cell Transfection

The His7-Ub-PCNA-K164R dsDNA fragment was synthesized by Eurofins Genomics. To avoid USP1-dependent deubiquitination, the C-terminal Gly codon of the ubiquitin gene was replaced by Arg, and PCNA Lys164 was replaced by Arg to avoid endogenous ubiquitination. The synthetized open reading frame was cloned into the plasmid vector pcDNA3.1.puro (Thermo Fisher) to make an N-terminal (mUb-PCNA-) fusion protein. pcDNA.3.1.zeo (Thermo Fisher) expressing full-length human pol eta full-length dsDNA was previously described (25). The pIRES-Hygro2 vector (Clontech, Palo Alto, CA) expressing full-length human AID was a gift from CA Reynaud (21). Ramos cells were transfected with the desired plasmid by electroporation (Amaxa) according to the manufacturer's protocol. Stably transfected clones were selected

with the appropriate antibiotic. Stable transfectants were isolated and further propagated in medium containing 600 ng/mL puromycin (*In vivo*Gen) for the cells expressing His-mUb-PCNA, 150 μg/mL Zeocin (*In vivo*Gen) for clones expressing exogenous POLH or 500 μg/mL hygromycin (Roche, Mannheim, Germany) for clones expressing exogenous AID.

Cell Lines and Culture Conditions

We cultured Ramos and both Burkitt lymphoma and mantle cell lymphoma cells in RPMI 1640 +GlutaMAX medium (Gibco) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (Invitrogen). The non-B cells were cultured as reported in the literature. Human tonsils were obtained as discarded material from routine tonsillectomies. B cell isolation was performed as described in (26).

Cell Treatments

For H2O2 treatment, cells were exposed to 1 mM H2O2 (Sigma–Aldrich) for 20 min at 37°C in MEM without FCS. After treatment, the cells were washed once with PBS and incubated in complete medium prior to harvesting. For UVC light treatment, the culture medium was removed, and cells in a dish were exposed to 254-nm UV irradiation at a dose of 10 J/m^2. The culture medium was immediately added, and the cells were returned to incubation.

For immunoblotting, cells were collected 2 h later, and total protein was extracted and analyzed using SDS–PAGE and western blot.

Inactivation of the *POLH* and *AID* Genes in Ramos Cells

For gene deletion, a pair of single guide RNAs (sgRNAs) were designed with the CRISPOR program. One plasmid expressing both gRNA, Cas9 and green fluorescent protein (LentiCRISPRV2GFP, Plasmid # 82416 Addgene) was nucleofected into Ramos cells using Amaxa (Lozano) according to the manufacturer's protocol. At 24 h after transfection, GFP cells were sorted with BD FCAS Aria II and plated into single clones in 96-well plates. Individual clones were genotyped by PCR to identify mutated clones by insertion or deletion. Candidate clones were further confirmed by Sanger sequencing and western blot. Guide RNA sequences: pol *eta* gRNAfor: 5'-GGTGAGGTTAGCTTTCCCAC-3' and pol *eta* gRNARev: 5'-GTGGGAAAGCTAACCT-CACC-3', AICDA gRNAfor: 5'-GTGGAATTGCTCTTCCTCC-3', AICDA gRNARev: 5'-GGAGGAAGAG CAATTCCAC-3'. A vector expressing full-length human polη was described previously (27), and a vector expressing full-length human AICDA was described in (21) and used for complementation of the KO cell lines. polη Zeocin- and AID puromycin-resistant clones were selected with 150 μg/mL Zeocin (Roche, Mannheim, Germany) and 600 ng/mL puromycin (Invitrogen), respectively.

Analysis of SHM in Ramos Cells

Genomic DNA was isolated after 42 days of culture and cell sorting. The rearranged $V_{H4}DJ_{H6}$ region was amplified with two rounds of PCR using Phusion DNA polymerase (Thermo Fisher Scientific), the primers Vh4.1 for 5′- CAGGTGCAGCT ACAGCAG -3′ and Jh6.1Rev 5′- GCTGA- GGAGACGGT GACC -3′ for the first round and the primers Vh4.2 for 5′- TGGGGCGCAGGACTGTTGAA -3′ and Jh6.2 Rev 5′- GACCGTGGTCCCTTGGCC -3′, for the second round. The conditions for the first PCR amplification were 98°C for 2 min, 20 cycles at 98°C for 10 s, 70°C for 20 s and 72°C for 20 s, and for the second PCR, 30 cycles at 98°C for 10 s and 72°C for 30 s. For amplification of the constant Cmu 2-4 region, we used the primers Cmu. for 5'- CGGACCAGGTGCAGGCTGAGGCC -3' and Cmu. Rev 5'- CTCCCGCAGGTTCAG CTGCTCCC -3' with the following program 98°C for 2 min, 35 cycles at 98°C for 10 s and 72°C for 20 s. The PCR products were gel-purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned with CloneJET PCR cloning kit (ThermoFisher scientific). Plasmid DNA extracted from individual bacterial colonies and sequencing using Sanger sequencing were performed by Eurofins Genomics.

Flow Cytometry and Sorting

Cells were collected and labeled with anti-human IgM-FITC antibodies (Ref# 31575; Invitogen) at 4°C for 20 min and then washed with PBS/1% BSA. To estimate the percentage of IgM-negative cells, FACS analyses were performed using a BD Accuri C6 flow cytometer (BD Biosciences). Cell sorting of IgM-negative cells was performed using a FACSAria III or Influx (BD Biosciences).

Proliferation and Cell Cycle Analyses

Approximately 2x10^6 cells were pelleted by centrifugation at 1200 rpm for 5 min. After Centrifugation, cells were washed in cold PBS and resuspended in PBS. The suspended cells were transferred dropwise into 4.5 mL of 70% ethanol and then fixed overnight at 4°C. The ethanol-suspended cells were then collected, washed and resuspended in 50 mg/mL propidium iodide (Sigma, P 4170)/0.1% (v/v) Triton X-100 staining solution with 100 μg/mL RNase A in the dark for 1 h at 37°C. A BD Accuri C6 flow cytometer (BD Biosciences) was used for analysis of cells. For cell proliferation, at day 0, 10^4 viable cells were seeded in 48 plates in 200 μL of complete medium and incubated at 37°C and 5% CO2. Cells were counted at 24, 48, 72 and 96 h in the presence of Trypan blue using a Countess II FL automated cell counter (Life Technologies). All experiments were done in triplicates.

Western Blotting and Cellular Fractionation

Samples were collected and placed on ice in a lysis solution [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol] containing 0.5% SDS and 2 mM PMSF with a protease inhibitor cocktail (Sigma P-8340, 1:100). Cellular proteins were resolved on a 12.5% SDS–PAGE gel. The membrane was incubated for 1 h at room temperature in 5% skim milk in PBS with 0.05% Tween-20 (PBST), and the membrane was probed with anti-PCNA PC10 (Ref # sc56; Santa Cruz), anti-alpha tubulin (Ref# MA1-80017; Thermo Fisher Scientific), anti-actin (Ref #MA1-744; Thermo Fisher Scientific), anti-Vinculin (clone 7F9, Ref# 14-9777-80; eBioscience), anti-AID (Ref #14-959-82; Thermo Fisher

Scientific), anti-polη (Ref# ab17725; Abcam), anti-FancD2 (Ref# sc20022; Santa Cruz), anti-USP1 (Ref # ab108104Ref; Abcam), anti-Msh2 (Ref #A300-451A; Bethyl), and anti-Msh6 (Ref # A300-022A; Bethyl) antibodies. Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody.

Nickel Beads Pull-Down

Whole-cell extracts were prepared in lysis buffer without EDTA supplemented with benzonase. The extracted proteins were adjusted to 20 mM imidazole and incubated with Ni-NTA-agarose (Qiagen) overnight at 4°C. Beads were then washed three times with the same lysis buffer without EDTA and containing 30 mM imidazole. Following the last wash, the beads were resuspended in 2X Laemmli buffer and boiled at 98°C for 5 min. The bound proteins were analyzed by immunoblotting using the indicated antibody.

RESULTS

Low PCNA Monoubiquitination in Ramos Cells After Treatment With Genotoxic Agents Correlates With a High Amount of USP1 Deubiquitinase

Ramos cells lines, like most Burkitt lymphoma cell lines that undergo SHM in culture, display a strong bias in favor of mutations at G/C over A/T (18, 19, 28, 29). To date the reasons of such bias remain unknown. During SHM, A/T mutation induction requires, on the one hand, the activity of several factors, including AID, DNA mismatch repair proteins (MSH2/MSH6), polη and, on the other hand, the monoubiquitination of PCNA. We and others have shown previously that Ramos cells express unmutated full-length cDNA of *AID, UNG, POLH, PCNA, MSH2, MSH6* and *EXO1*. In addition, the cells are both BER and MMR proficient (22, 24, 30). Thus, there were no mutations, no lack of expression and no obvious evidence of dysfunction of any of these factors in Ramos cells. On the other hand, mUb of PCNA at the conserved K164 site is necessary for the recruitment of polη (11, 16), which is the sole mutator of A/T bases in the normal physiological context during SHM (7). We therefore asked whether the ubiquitination pathway of PCNA is deregulated in these cells. In mammalian cells, PCNA is monoubiquitinated by RAD6 and the RAD18 ubiquitin ligase complex in response to UV irradiation or other genotoxic agents; therefore, we used this property to investigate the induction of mUb-PCNA in Ramos cells under these conditions. To do so, we treated the cells with either UVC light or H_2O_2 and monitored the monoubiquitination of PCNA by western blotting. As shown in **Figure 1A**, mUb-PCNA was not or hardly detectable in either Ramos or BL2 cells (even after long exposure) compared to non-Burkitt MRC5 and U2OS cells, which showed clear mUb regardless of treatment. These results could suggest that the monoubiquitination of PCNA in response to genotoxic stress is defective, weak or inefficient in Ramos cells. Nevertheless, monoubiquitination of PCNA is a reversible process, and its removal is catalyzed by the deubiquitinase USP1. Thus, the balance between the opposing actions of specific ubiquitin ligases and USP1 ultimately determines the ubiquitination status of PCNA. Therefore, we next asked whether the deubiquitination reaction is predominant due to abnormal expression of USP1 in Ramos and BL2 cells compared to normal B cells. To answer this question, we analyzed the expression of USP1 by western blotting in Ramos and several other Burkitt lymphoma (BL) and non-BL cell lines. To establish a comparison scale, we used tonsillar B cells, which represent physiological counterpart cells,

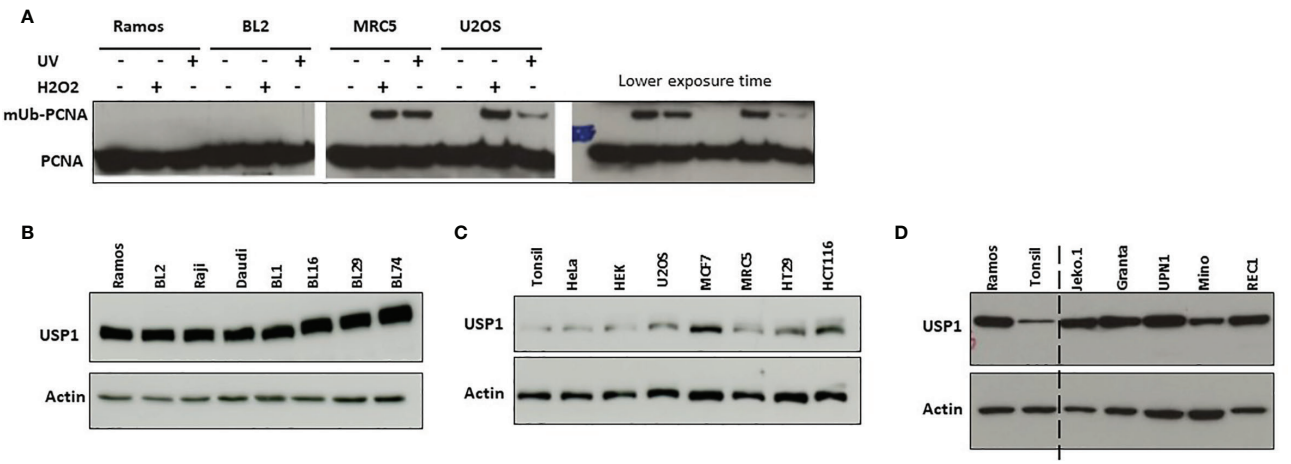

FIGURE 1 | Induction of monoubiquitination of PCNA and USP1 expression in Ramos and BL2 cells. **(A)** B cell lines Ramos and BL2 cells (left pannel) and non-B cells MRC5 and U2OS cells (right pannel) were exposed to UVC light (10 J/m^2) or incubated in the presence of H_2O_2 as indicated in the M&M. Total protein extracts were prepared 2 hours post-treatment, separated by SDS–PAGE and analyzed by immunoblotting using an anti-PCNA antibody. **(B, C)** Protein extracts were prepared from the indicated cell lines and analyzed as described above using an anti-USP1 antibody. In B–D actin was used as loading control. **(D)** Protein extracts from Ramos and Tonsillar B cells were loaded side by side to facilitate comparison.

to estimate the physiological quantity of USP1 expressed in B cells. As shown in **Figures 1B–D**, Ramos and all BL cell lines analyzed expressed high levels of USP1 compared to tonsillar B cells. Interestingly, with the exception of MCF7 cells, the expression in all other non-BL cells remained within the range of B cell physiological expression (**Figure 1C**). In addition, it should be noted that in BL cells, high USP1 levels do not depend on their EBV status since both EBV-positive (Daudi, BL16 and Raji cells) and EBV-negative (Ramos, BL2, BL1, BL29, BL74) BL cells have similar levels. Similar elevated expression of USP1 was found in human B cells derived from mantle cell lymphoma (**Figure 1D**). Together, these data suggest that the high expression/activity of USP1 in Ramos and probably most BL cell lines is responsible for the observed low mUb status of PCNA and consequently could explain the origin of the alteration in the A/T mutation pathway.

USP1 Inhibition Increases the Half-Life of PCNA Monoubiquitination in the Ramos Cell Line

We have shown above a possible imbalance in PCNA mono-Ub/de-Ub reactions due to the elevated expression of USP1. We therefore anticipated a higher ongoing deubiquitination reaction in those cells mimics PCNA monoubiquitination deficiency. To test this hypothesis, we first treated Ramos cells for 3 to 24 hours with increasing concentrations of ML323, a selective inhibitor of USP1, and monitored both the efficacy and toxicity of the drug. As shown in **Figure 2A**, after 3 hours of treatment, we detected a dose-dependent increase in the levels of mUb-PCNA. At 24 hours, treatment with 10 μM ML323 maintained a detectable fraction of mUb-PCNA in the cells. Exposure to higher concentrations induced rapid and greater mUb-PCNA at 3 hours, but this increase was followed by a sharp decrease at 24 h, probably due to the toxicity of the drug at high concentrations (**Figure 2B**). Indeed, while more than 95% of cells remain alive in the presence of 10 μM ML323 at 48 hours, exposure to 30 μM kills 60% of the cells at 24 hours and more than 90% at 48 hours of treatment. Incubation with 20 μM killed 10% of the cells at 24 hours and 30% at 48 hours. Similar results were obtained with the BL2 cell line (data not shown). We therefore decided to use a dose of 10 μM as an effective and nontoxic concentration for the next experiments. In parallel to its role in the process of TLS, USP1 participates in the Fanconi anemia pathway through monodeubiquitination of FANCD2 during DNA interstrand crosslink lesion repair (31, 32). As expected, treatment of both Ramos and BL2 cells with ML323 led to the detection of a clear band that corresponded to mUb-PCNA and mUb-FANCD2 even in the absence of any genotoxic treatment (**Figure 2C**). Furthermore, the combination of USP1 inhibition and UVC irradiation further increased mUb-PCNA levels (**Figure 2D**). Together, these results confirm that in Ramos cells, the PCNA monoubiquitination reaction is efficient and UV-inducible. We conclude that the observed absence of mUb-PCNA in Ramos cells is the consequence of high ongoing monodeubiquitination reactions that result from higher USP1 expression and activity.

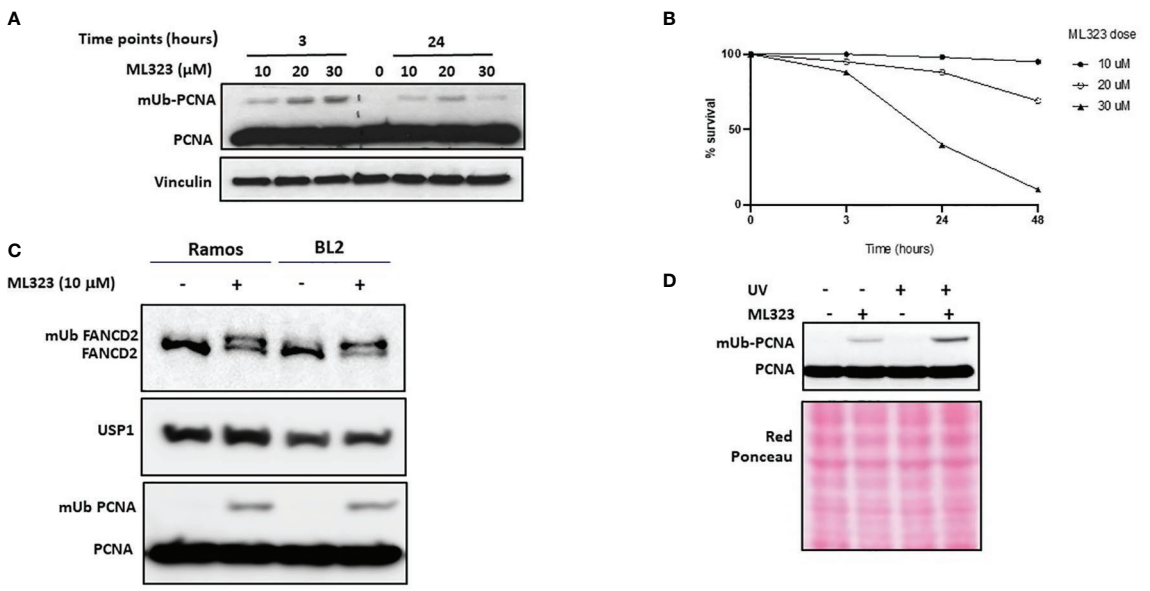

FIGURE 2 | Inhibition of USP1 increases the monoubiquitination of both PCNA and FANCD2. Ramos cells were incubated with increased doses of ML323, and the monoubiquitination of PCNA and the cell toxicity of the drug at the indicated times were determined by SDS–PAGE and immunoblotting in **(A)** and using a cell survival assay in **(B)**, respectively. The data in B represent the mean of three independent experiments. **(C)** Ramos and BL2 cells were treated for two hours with 10 μM ML323, and monoubiquitination of PCNA and FANCD2 was analyzed by SDS–PAGE and immunoblotting. **(D)** Ramos cells were treated with UVC, ML323 or both, and the monoubiquitination of PCNA was analyzed as described above. Vinculin and red Ponceau were used as loading controls.

Inhibition of PCNA Deubiquitination Increases the Rate of A/T Mutations

To assess whether increasing the mUb-PCNA half-life can restore the rate of A/T mutations during SHM, we treated Ramos cells with 10 μM ML323 continuously for 6 weeks to allow the cells to accumulate a sufficient number of mutations. To maintain elevated levels of mUb-PCNA throughout that period, we added fresh medium containing the drug every 2 days. Ongoing SHM in Ramos cells in culture generates diverse IgM-loss subclones without affecting cell viability due to the occurrence of stop codons, indels and frameshift mutations in the V_H (19). Therefore, the detection of IgM-negative cells by fluorescence-activated cell scanning (FACS) provides a quick read-out and convenient semiquantitative measure of SHM. During the 6 weeks of treatment, surface IgM was assessed by FACS (**Figure 3A**). We observed an accumulation of IgM-negative Ramos cells over time in both the presence and absence of ML323. However, USP1 inhibition further increased the percentage of IgM-negative cells. Of note, BL2 cells that do not undergo constitutive SHM do not show IgM-negative cell accumulation even in the presence of ML323. This suggests that the increase in cellular mUb-PCNA levels quantitatively participates in the processes of SHM that generate the IgM-population. At the end of treatment, the IgM- cells were FACS sorted, and VH4 segments were PCR amplified, cloned, and sequenced to appreciate whether the treatment impacts only quantitatively the process of SHM or causes a mutation pattern change or both. Interestingly, the data presented in **Table 1** show both quantitative and qualitative changes. USP1 inhibition in three independent experiments (E1-3 **Table 1**), led not only to an overall increase in unique mutation frequency in treated versus nontreated cells (0.19 versus 0.086 mut/100 bp) caused mainly by the increase of number of mutation per sequence (**Figure 3B**), but also to significant increases in the rate of A/T mutations. Indeed, while the rate of A/T mutations remained at approximately 13% (7 to 18%) for the nontreated cells, the rate increased significantly to 31% (24 to 38%) in treated cells (**Table 1**). Collectively, these results suggest that an increase in the availability of mUb-PCNA in the cell is sufficient to promote the induction of A/T mutations and further confirm that the high turnover of mUb-PCNA is responsible for the low rates of A/T mutations during SHM in Ramos cells.

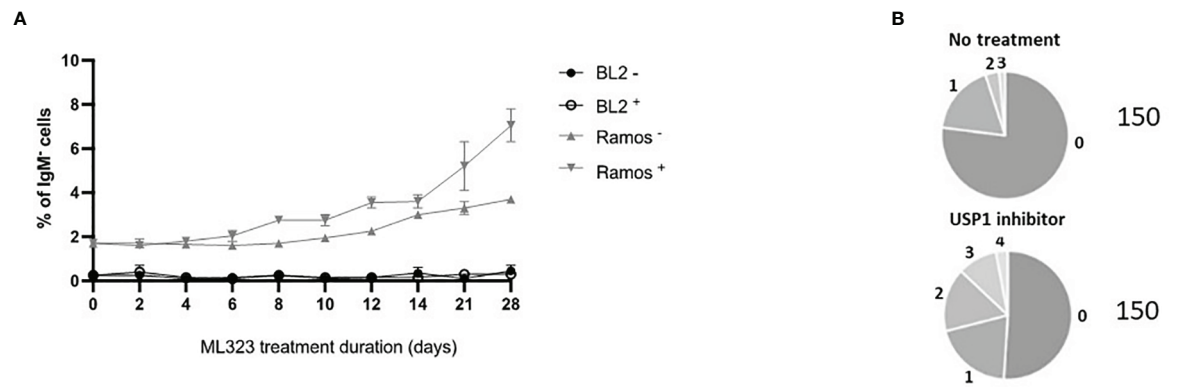

FIGURE 3 | Treatment of Ramos cells, but not BL2 cells, with a USP1 inhibitor increased IgM loss compared to that of nontreated cells. **(A)** Cells were treated with 10 μM ML323 (+) or DMSO (–), and the percentage of surface IgM was measured by FACS at the indicated time points. **(B)** Sequence analysis of SHM in the amplified Ramos VH region. Relative amounts of sequences with the indicated number of mutations (from 0 to 4) are given in the pie charts. The total number of analyzed sequences is indicated in the right of each chart and corresponds to the pool of the three experiments reported in **Table 1**.

TABLE 1 | Somatic mutations in VH4 sequence (338 bp) from Ramos cell treated or not with USP1 inhibitor.

IgM⁻ cells(6 weeks)		Total nucleotides sequenced*	Number of mutated sequences(Unique)	Number of substitutions	Mutation frequency (per 100 bp)	Number of AT/GC mutations	% AT/GC	AT % mean value(SD)
Ramos	E1	16900	10	15	0.089	1/14	6.7/93.3	13.6 (6.219)
	E2	16900	13	16	0.095	3/13	18.75/81.25	
	E3	16900	11	13	0.077	2/11	15.4/74.6	
Ramos + ML323 (USP1i)	E1	16900	18	25	0.148	6/19	24/76	33 (7.810)
	E2	16900	25	35	0.201	13/22	37/63	
	E3	16900	30	42	0.249	16/26	38/62	

*E1 means experiment number 1. E1, E2 and E3 are independent experiments. *50 clones. P= 0.0282; Two-tailed P value in unpaired t test for the AT% mean value comparison; SD. standard deviation.*

Mimicking mUb-PCNA in Ramos Cells by Ubiquitin-PCNA Fusion: Validation of the Experimental Approach

Artificial Ub and PCNA fusion proteins have been successfully used in both yeast and mammalian cells to mimic native ubiquitinated PCNA (33–36). To avoid the use of inhibitors that could interfere with the physiological functions of the cell, we provided an exogenous modification by stably expressing mUb-PCNA fusion. The human PCNA K164R mutant sequence was used for this construction to prevent additional *in vivo* PCNA ubiquitination at the K164 residue during Ig SHM. We next added N-terminal fusion with 7x His-tagged ubiquitin to mimic PCNA-K164 monoubiquitination and to facilitate purification and analysis. Finally, to prevent its cleavage by USP1 in the cell, the C-terminal glycine-glycine residue was removed from the Ub polypeptide in the fusion construct (**Figure 4A**). Stable clones were obtained after plasmid transfection and puromycin selection. First, we performed several experiments to show that mUb-PCNA fusion expression does not perturb cell growth and participates in DNA replication as the endogenous. (i) We showed that the constitutive expression of mUb-PCNA does not modify the cell cycle and does not affect cellular proliferation (**Figures 4B, C**), indicating that mUb-PCNA fusion proteins do not affect DNA replication. (ii) We verified that the exogenous mUb-PCNA fusion protein was able to interact with endogenous PCNA to form a physiological homotrimeric ring. To this end, we used nickel beads to pull down exogenous mUb-PCNA containing a poly-His tag in the N-terminal region of ubiquitin and searched for the presence of endogenous PCNA in the pulled down fraction. As expected from previous studies (35), **Figure 4D** shows that the pulled down fraction contains similar quantities of endogenous and mUb-PCNA, consistent with the fusion proteins being able to interact equally with the untagged protein molecules. Together, these data demonstrate that USP1-resistant mUb-PCNA fusions behave similarly to the endogenous form. Therefore, Ramos cells expressing such a construct can be confidently used to study *in vitro* the mechanisms of SHM.

Expression of Mono-Ub PCNA Fusion Protein Is Sufficient to Increase the Rate of A/T Mutations

Taking into account the aforementioned validations, we measured the impact of mUb-PCNA expression on the rate and pattern of SHM. After 6 weeks in culture, genomic DNA was extracted from several clones, and VH4 segments were PCR amplified, cloned, and sequenced. As shown in **Table 2A**, the clones expressing exogenous mUb-PCNA showed an increase of mutation frequency as seen above after the use of USP1 inhibitor, with an increase of both mutated sequences and number of mutation/sequence (**Figure 4E**). Interestingly we observed an increased rate of A/T mutations of approximately 30% (22 to 40%) compared to nonexpressing clones, which remained around 10% (5-15%). It should be noted that, as reported before, there is variability in the rate of A/T mutations in the different clones (discussed below); nevertheless, in general, the mutating clones expressing mUb-PCNA show significantly higher A/T mutation rates compared to nonexpressing clones. We next compared the mutation profile of both clones expressing and non-expressing

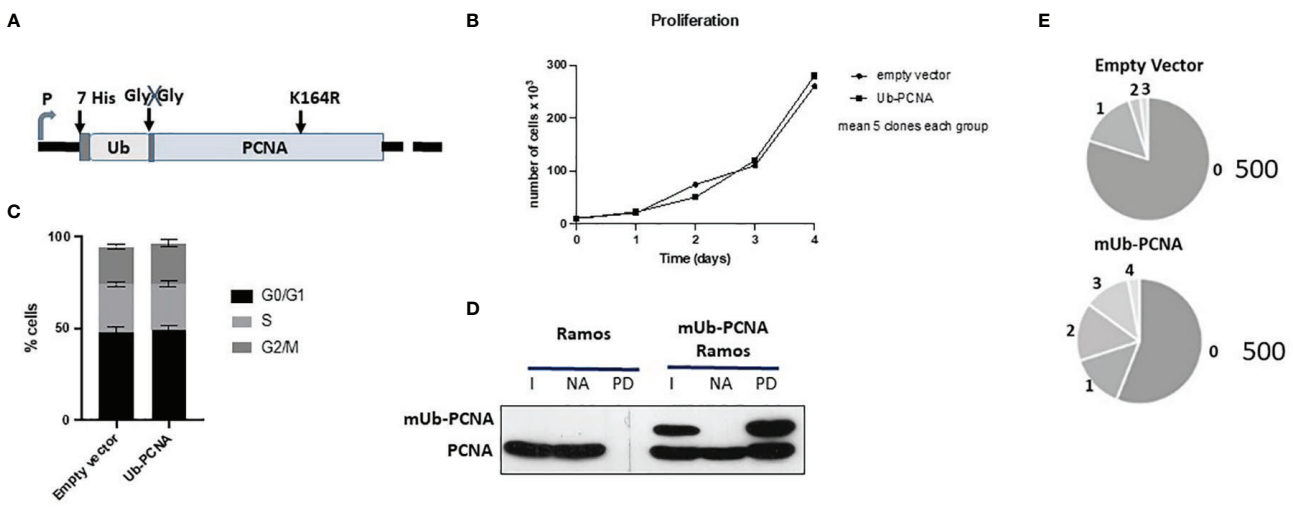

FIGURE 4 | Schematic diagram of the mUb-PCNA fusion construct and validation in Ramos cells. **(A)** Schematic diagram of the mUb-PCNA fusion construct used. **(B)** Cell proliferation analysis. Cell growth was estimated by counting the viable cells on the indicated days. **(C)** Cell cycle analysis by DNA content estimation with flow cytometry and represented as histograms showing the relative percentage of cells at the indicated phases of the cell cycle. The data in B and C represent the mean of three independent experiments. **(D)** His7-UbPCNA was pulled down with nickel beads, and the fraction pulled down was analyzed by SDS–PAGE and immunoblotting using an anti-PCNA antibody. I, input; NA nonadsorbed, PD pull down. **(E)** Sequence analysis of SHM in the amplified Ramos VH region. The pie segments represent the proportion of clones that contained the specified number of mutations (from 0 to 4) indicated. The total number of analyzed sequences is indicated in the right of each chart and corresponds to the pool of the data obtained from the five clones reported in **Table 2A**.

TABLE 2A | Somatic mutations in VH4 sequence (338 bp) from mUb-PCNA expressing and non-expressing Ramos clones.

IgM$^-$ cells (6 weeks)		Total nucleotides sequenced*	Number of mutated sequences (unique)	Number of substitutions	Mutation frequency (per 100 bp)	Number of AT/GC mutations	% AT/GC	AT % mean value(SD)
Ramos + Ub-PCNA	R4.16	33800	55	90	0.266	36/54	40/60	30.38
	R2.4.6	33800	52	79	0.234	27/52	34/66	(6.861)
	R1.16	33800	40	70	0.207	20/50	28.5/71.5	
	R3.2	33800	37	62	0.183	17/43	27.4/72.6	
	R3.7	33800	35	50	0.148	11/39	22/78	
Ramos control (empty vector)	R13.5	33800	17	20	0.060	1/19	5/95	9.18
	R2.4.8	33800	21	24	0.071	3/24	12.5/87.5	(3.571)
	R1.12	33800	20	25	0.074	3/23	13/87	
	R2.5	33800	26	30	0.089	2/28	6.7/93.3	
	R3.8	33800	19	25	0.074	2/23	8.7/91.3	

**100 clones.*

P= 0.0003 (two-tailed P value, unpaired t test) for the AT% mean value comparison; SD, standard deviation.

TABLE 2B | Somatic mutations in Constant μ-region sequence (550 bp) from mUb-PCNA expressing Ramos clones after six weeks and three months in culture.

IgM$^-$ cells		Total nucleotides sequenced* ($x10^3$)	Number of mutated sequences (unique)	Number of substitutions	Mutation frequency (per 100 bp)($x10^{-3}$)	Number of AT/GC mutations	% AT/GC	AT % mean value
Ramos + Ub-PCNA	R4.16	55	0	0				
6 weeks	R2.4.6	55	1	1	1.8	0/1		
	R1.16	55	1	1	1.8	1/0		
	R3.2	55	0	0				
	R3.7	55	0	0				
Ramos + Ub-PCNA	R4.16	55	1	1	1.8	0/1		
3 months	R2.4.6	55	1	1	1.8	1/0		
	R1.16	55	2	2	3.6	0/2		
	R3.2	55	0	0				
	R3.7	55	1	2	3.6	0/2		

**100 clones.*

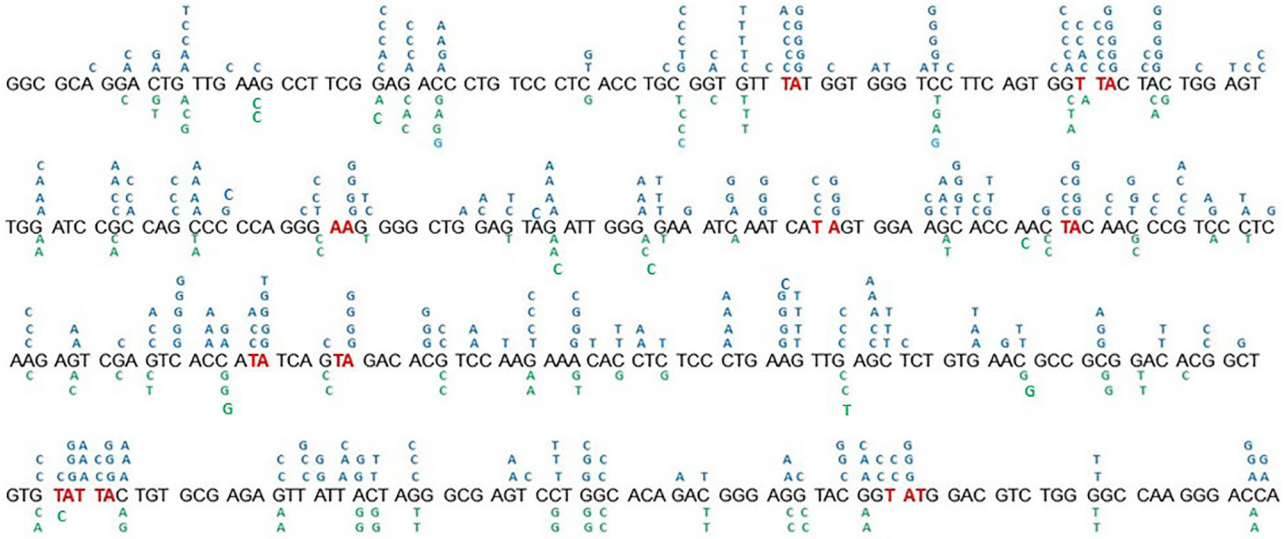

FIGURE 5 | Distribution of point-mutations along the amplified Ramos VH region. Independently occurring base substitutions are indicated at each nucleotide position. The Pol h hotspots (WA/TW) targeted following the expression of mUb-PCNA are in indicated in red. The figure represent the pool of base substitution obtained from the clones indicated in **Table 2A**. The Nucleotide Substitutions in blue indicated above the Ramos VH sequence are from the 5 clones expressing mUb-PCNA and those below in green are from the five control clones.

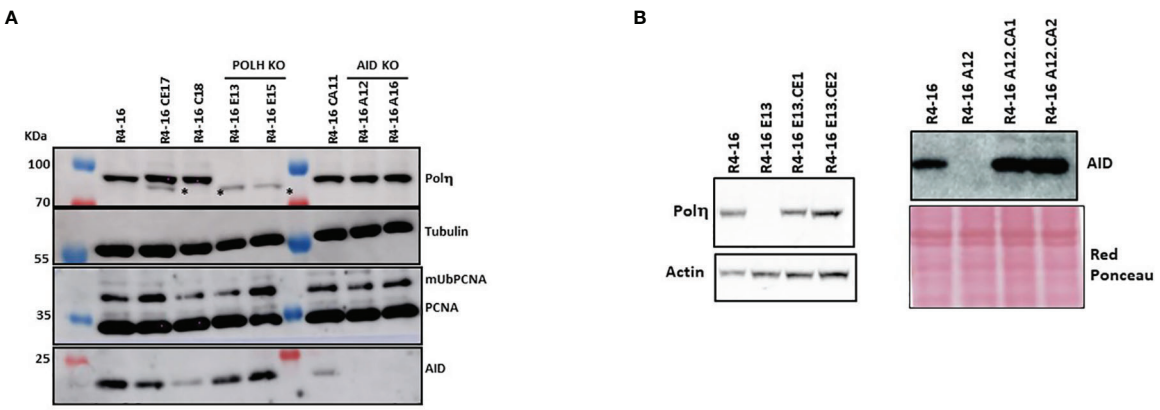

FIGURE 6 | Inactivation and restoration of polη and AID in the R4-16 clone. CRISPR/Cas9 was used to inactivate and gRNA editing were used to inactivate polη or AID. **(A)** The absence of the corresponding proteins was confirmed in two selected clones by immunoblot analysis. **(B)** KO clones were reconstituted by stable expression of POLH or AID cDNA. Left panel: R4-16E13-CE1 and R4-16E13-CE2 are two clones derived from the human polη deficient clone R4-16E13 after transfection with polη-expressing vector. Right panel: R4-16A12-CA1 R4-16A12-CA2 are two clones derived from the AID-deficient clone R4-16A12 after transfection with the human AID-expressing vector. Actin and red Ponceau staining were used as loading controls. *Nonspecific band.

mUb-PCNA expressing and non-expressing mUb-PCNA. The distribution of point-mutations along the amplified Ramos VH region presented in **Figure 5** showed in addition to an increase of number of mutations, differences in their base substitution characteristics. Indeed, we observed that most of the A/T mutations induced following mUb-PCNA expression (base substitution in blue) are targeted to the described polη hotspots (WA/TW). Furthermore we observed within these hotspots (red color) a clear A to G and T to C transition bias (**Figure 5**). Since the preferred mutation of Polη when copying normal DNA is the incorporation of Gs opposite Ts, thereby generating T to C and A to G transition mutations, thus strongly suggesting that these mutations are introduced by polη.

Together, these results indicate that the expression of stable mUb-PCNA is sufficient to retrieve the A/T mutagenesis pathway in Ramos cells.

Induction of A/T Mutations Is AID- and POLH-Dependent in Ramos-Ub Cells

Since polη is the major A/T mutator in both mice and humans during SHM, we asked whether the induced A/T mutations following the expression of mUb-PCNA are generated through a genuine SHM process that requires AID activity and are polη-dependent. To this end, we inactivated the genes encoding AID (*AICDA*) or polη (*POLH*) using CRISPR/Cas9 technology in the Ramos R4-16 subclone. It should be noted that, among the clones with increased rates of A/T mutations we chose clone R4-16 to continue our investigations, for two main reasons: (i) it displays a higher rate of A/T mutations and (ii) it is stable and retained ongoing SHM when maintained in culture for up to 3 months. The absence of AID and POLH protein expression was validated by immunoblotting (**Figure 6A**). POLH-deficient clones were grown for 6 weeks, and the expressed V(D)J was amplified and sequenced. **Table 3** shows that in the polη-deficient clones, A/T mutations decreased to the level of the wild-type Ramos cell population. Induction of A/T mutations was restored upon re-expression of human *POLH* cDNA in these clones to frequencies comparable to those observed in the Ramos mUb-PCNA parental clone (**Figure 6B**, left and **Table 3**). In addition, inactivation of *AID* completely abolished both A/T and G/C mutations. Similarly, the re-expression of *AID* restored the mutations to the level of the parental clone (**Figure 6B**, right and **Table 3**). Altogether, these results confirm that the A/T mutations induced following mUb-PCNA expression result from a genuine immunoglobulin V gene diversification mechanism initiated by AID and generated by polη activity.

Absence of Genome Wide Mutagenesis

Since mUb-PCNA preferentially recruits low-fidelity TLS polymerase, we wondered whether the constitutive expression of mUb-PCNA could also promote mutations elsewhere in the genome. To answer this question, on the one hand, we sequenced the μ-constant region known not to be targeted by physiological SHM and on the other hand estimated the mutation rate at the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) locus[17]. As shown in **Table 2B**, mutations did not accumulate in Cμ even after 3 months in culture. Furthermore, we found a similar mutation rate at the HPRT locus, approximately 1.5 to 2.1 X 10^{-7} mutations per locus and per generation, determined by fluctuation analysis of resistance to 6-thioguanine in both clones expressing or not expressing mUb-PCNA (data not shown). Collectively, these results allow us to conclude that the constitutive expression of mUb-PCNA in Ramos cells induces and targets A/T mutations to the Ig locus and does not induce a general mutator phenotype.

DISCUSSION

We report the establishment of a cell line that can be used to address *in vitro* the mechanism of A/T mutations during the process of somatic hypermutation of Ig genes. The Ramos Burkitt cell line has been widely used to study the mechanism

TABLE 3 | Somatic mutations in VH4 sequence (338 bp) from cultured Ramos clones knocked-out for *POLH* or *AID* in the mUb-PCNA background.

IgM⁻ cells(6 weeks)		Total nucleotides sequenced*	Number of mutated sequences(Unique)	Number of substitutions	Mutation frequency (per 100 bp)	Number of AT/GC mutations	% AT/GC	AT % mean value
Ramos + Ub-PCNA *POLH* KO	R4.16-E13	33800	18	24	0.071	4/20	16.7/83.3	17.25
	R4.16-E15	33800	23	28	0.083	5/23	17.8/82.2	
Ramos control (empty vector)	R4.16-E17	33800	53	80	0.237	33/47	41/59	38.8
Ramos + mUb-PCNA *POLH* KO + hPOLH	R4.16-E13 CE 1	33800	42	51	0.151	16/35	31.4/68.6	32.65
	R4.16-E13 CE 2	33800	45	62	0.183	21/41	33.9/66.1	
Ramos + mUb-PCNA *AID* KO	R4.16-A12	33800	2	2	0.006	1/1	ND	ND
	R4.16-A16	33800	1	2	0.006	0/2	ND	
Ramos control (empty vector)	R4.16-A11	33800	50	82	0.243	30/52	36.6/63.4	38.8
Ramos + mUb-PCNA *AID* KO + hAID	R4.16-A12 CA1	33800	21	29	0.085	8/21	27.6/72.4	31.65
	R4.16-A12 CA2	33800	26	28	0.083	10/28	35.7/64.3	

**100 clones; ND, none detected; CE complemented with exogenous pol η CA complemented with exogenous AID.*

of SHM *in vitro* (19, 37, 38). Ramos cells exhibit most of the features of SHM *in vivo* except that the spectrum of mutations displays a deficiency in A/T mutations. Indeed, 85 to 90% of the mutations are at G/C residues compared to *in vivo*, where mutations are distributed equally on the G/C and A/T bases. We have demonstrated in this study that the paucity of A/T mutations in Ramos cells is due to the high activity of USP1, which favors the deubiquitination of mUb-PCNA. To bypass this limitation, we either selectively inhibited the activity of USP1 or constitutively expressed mono-Ub PCNA. In both cases, we increased the rate of A/T mutations at the expressed Vh gene. Finally, we showed that these mutations are genuine SHM, depending on both AID and polη. The SHM mechanism depends on the deaminase activity of AID, which converts deoxycytidines (dCs) to deoxyuridines (Us) in both strands of DNA. The processing of the U/G mismatch, thus created by several DNA damage responses, is generated at the site of the leading to G/C mutations, both transitions and transversions, and A/T mutations around the lesion, resulting in similar rates of mutation at both A/T and G/C bp. Although the current knowledge of DNA repair mechanisms in mammalian cells can explain the spectrum of G/C mutations following high rate deamination of dC in S phase of the cell cycle regardless of its origin, introduction of mutations at the A/T bases that occurs mainly in G1 phase of activated B cells is more challenging. Furthermore, this pathway is restricted to the diversification of Ig genes during the maturation of the immune response, it results from diverted DNA repair factors and remains poorly understood to date. Investigations to decipher the underlying molecular mechanisms are limited by the absence of a cellular model able to faithfully reproduce the mechanism of both G/C and A/T base mutagenesis.

In this report, we used a Ramos cell line that constitutively diversifies its rearranged immunoglobulin V gene during *in vitro* culture. This ongoing process does not require the help of activated T cells, added cytokines, or B cell antigen receptor signaling. However, these cells mainly use the G/C mutation pathways to diversify their Igs. The absence or low rate of the A/T mutagenesis process led to the neglect of this model. In B cells, the U/G mismatch is recognized by Msh2-Msh6 and recruits exonuclease I (ExoI), which creates a long single-stranded gap. Then, polη is recruited *via* mUb-PCNA, leading to error-prone gap filling (7, 11, 39). The specific activity of polη when copying undamaged DNA, which copies Ts with very low fidelity, generates the majority of mutations at A/T bases away from the AID-modified C base. We therefore postulated that either one or several components of the A/T pathway are missing or malfunctioning in Ramos cells.

We showed that the paucity of A/T mutations is due to the high activity of ubiquitin-specific protease 1, which is responsible for the short half-life of mUb-PCNA. We have shown that the inhibition of USP1 activity using ML123, a potent and specific inhibitor, increases the fraction of cellular mUb-PCNA in Ramos cells, which in turn favors an increase in the rate of A/T mutations at the Ig locus. These data indicate that although the reaction of PCNA ubiquitination is intact in Ramos cells, its deubiquitination rate is higher, greatly shortening its half-life and thus limiting its participation in the A/T mutagenesis pathway. Of note, elevated expression of USP1 has been reported in several human cancers, including osteosarcoma, non-small-cell lung cancer, and breast and colorectal cancers (40–42). This study reports for the first time elevated expression of USP1 protein in both Burkitt lymphoma and mantle cell lymphoma.

Since PCNA is not the only USP1 target in mammalian cells and to avoid any nonspecific or off-target effects, we substituted the use of USP1i, which served as proof of principle, by stably expressing the mUb-PCNA fusion in Ramos cells. Indeed, artificial Ub and PCNA fusion proteins have been successfully validated and used in both yeast and mammalian cells to mimic native ubiquitinated PCNA and bypass the requirement for PCNA monoubiquitination in response to UV DNA damage (34–36). We showed that the stable expression of USP1-resistant mUb-PCNA in Ramos cells resulted in an increase in the rate of Ig A/T mutations without affecting cell proliferation or the cell cycle profile. mUb-PCNA pull-down experiments in Ramos show that the latter interacts with endogenous PCNA and participates in the formation of the sliding ring, suggesting that this fusion protein fulfills cellular function(s) similar to endogenous monoubiquitinated PCNA. This result is in agreement with previous reports showing (i) that yeast cells can survive mUb-PCNA fusion in the absence of endogenous PCNA and (ii) that in mammalian cells, mUb-PCNA fusion can be loaded onto DNA and is able to protect host cells from UV-induced DNA damage, with characteristic TLS activities, thus mimicking endogenous PCNA monoubiquitination (36). Of note the analysis of the mutation pattern show that beside the high increase of A/T mutations, we observed also a small increase of G/C mutations. This latter was not expected since *in vivo* the analysis of SHM in PCNAK164R expressing cells show mainly an impact on A/T mutations. Although we cannot propose any obvious explanation, we observed within the G/C mutation an increase of C to G and G to C (107 out of a total of 238 G/C mutations in the presence of mUb-PCNA (45%) versus 57/137 (39.4% in its absence); this could be partly attributed to higher recruitment of Rev1 in the presence of constitutive expression of USP1 resistant mUb-PCNA. The permanent availability of USP1-resistant mUb-PCNA into the cells may provide mUb-PCNA more time, although to a lesser extent, to recruit other TLS that are normally not recruited under physiological conditions where PCNA is continuously ubiquitinated and de-ubiquitinated.

Importantly, we demonstrate that the introduced A/T mutations in Ramos cells expressing mUb-PCNA fusion are genuine SHM for several reasons: i) are mainly targeted to WA hotspots with a strong A to G and T to C transition bias which is strictly attributable to the enzymatic specificity of pol η ii) they depend on the A/T pathway master genes *AID* and *POLH*. Importantly we found that both A/T and G/C mutations are absent in the AID-deficient clones, and polη deficiency leads to low A/T mutation even in the presence of constitutive expression of mUb-PCNA. iii) they are strictly targeted to the expressed V gene. Indeed, no mutations were detected in the constant μ-region, and the constitutive expression of mUb-PCNA does not

lead to an overall increase in genome-wide mutagenesis as assessed by the rate of mutations at the *HPRT* locus. These results are in agreement with previous studies showing that the expression of mUb-PCNA fusion does not cause an increase of spontaneous mutations (34, 35)

Although the mUb-PCNA fusion has been used to study the mechanism of DNA damage tolerance in response to replication-blocking lesions induced by a variety of genotoxic agents (in human and yeast cells), this study is the first to address its role in the specific recruitment of polη during the mechanism of Ig A/T base mutations that occur in the absence of any DNA lesion, by DNA gap filling in instead of bypass and likely independently of replication stress or fork stalling signaling.

The Ramos BL cell line used in the laboratory was first described as a population that constitutively mutates its rearranged V(D)J region at a rate of 2 to $3x10^{-5}$ mutations/bp/generation (19). In this study, we found that the expression of mUb-PCNA fusion modifies both the pattern and the rate of mutations. Similar to what has been shown previously (43), the analysis of several individual subclones expressing mUb-PCNA isolated from the original population after transfection revealed the presence of 15 to 20% nonmutated clones and that almost 35 to 50% of clones progressively lost the ability to mutate their V region over time when maintained in culture (1 to 3 months) regardless of the expression of AID and/or mUb-PCNA. The remaining 20 to 30% display variable ongoing mutation rates ranging from 2×10^{-5} to 6×10^{-4} mutations/bp/generation. Despite this finding that suggests a clonal variation and instability of Ramos clones that should be taken into account when working with this cell line, the expression of mUb-PCNA increased the mutation rates of A/T bases in all clones that continue to undergo SHM when maintained in culture. Finally, we observed that in the clones with the highest mutation rates, the A/T mutations did not reach 45 to 50%, as expected. This could be partially attributed to the decrease in the number of AID hot spots in the V(D)J sequence over time compared to the corresponding germline sequence due to the ongoing SMH. It would be interesting to use the recombinase-mediated cassette exchange system established by MD Scharff's lab (38) to exchange endogenous V(D)J in Ramos cells expressing mUb-PCNA, analyze the rate and pattern of A/T and C/G mutations and compare them to the *in vivo* pattern.

In summary, we have established and validated a cell model with few limitations that reproduces the full process of somatic hypermutation of Ig genes *in vitro* and can be used to answer questions concerning the A/T mutation pathway that cannot be addressed otherwise. For example, how, in activated B cells, ncMMR activity is targeted to Ig loci and becomes active in G1 phase of the cell cycle? While mUb-PCNA is required for pol η recruitment for TLS in response to replication-blocking UV lesions, it remains unclear how polη is selectively recruited to fill in the gap created by ncMMR activity. How are the other DNA polymerases, both error-free and error-prone, actively excluded? We expect that these questions and others will be addressed with the use of this *in vitro* cellular model.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SA designed and supervised the study. LL, DB, MG, MM, ZK, and ED performed experiments. SA, LL, DB, and AS analyzed and curated the data. SA, LL, ED, and AS participated in the writing of the paper. All authors read, reviewed, and approved the final manuscript.

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The Role of DNA Repair in Immunological Diversity: From Molecular Mechanisms to Clinical Ramifications

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An effective humoral immune response necessitates the generation of diverse and high-affinity antibodies to neutralize pathogens and their products. To generate this assorted immune repertoire, DNA damage is introduced at specific regions of the genome. Purposeful genotoxic insults are needed for the successful completion of multiple immunological diversity processes: V(D)J recombination, class-switch recombination, and somatic hypermutation. These three processes, in concert, yield a broad but highly specific immune response. This review highlights the importance of DNA repair mechanisms involved in each of these processes and the catastrophic diseases that arise from DNA repair deficiencies impacting immune system function. These DNA repair disorders underline not only the importance of maintaining genomic integrity for preventing disease but also for robust adaptive immunity.

Keywords: immunological diversity, immunodeficiency, antibodies, DNA damage, DNA repair

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INTRODUCTION

A functional immune system is defined by a diverse repertoire of cells, surface receptors, and antibodies needed to effectively respond to pathogenic challenges (1). Endogenous DNA damage is a potent driver of disease and aging (2), can trigger innate immune responses, and drive loss of cells *via* apoptosis, necrosis, and senescence (3–5). However, deliberate DNA damage is necessary for vertebrates to respond to the limitless variability of pathogen-related antigens (6, 7). Programmed DNA double-strand breaks (DSB) that occur in B and T cell receptor genes are necessary for lymphocyte development and maturation (6, 8, 9). These programmed DNA breaks occur at specific sites and serve as critical intermediates for rearrangements required for V(D)J recombination (**Figure 1**) (9). Through this process, the nearly 10^{12} B and T cells in an individual express millions of unique combinations of antibody and T-cell receptor genes (10). Immune repertoires of any two individuals may overlap by only a fraction of a percent even though these repertoires are formed by Variable, Diversity, and Joining gene segments that are shared by all humans (11, 12). The diversity between two individuals at the immunoglobulin loci is greater than their germline diversity.

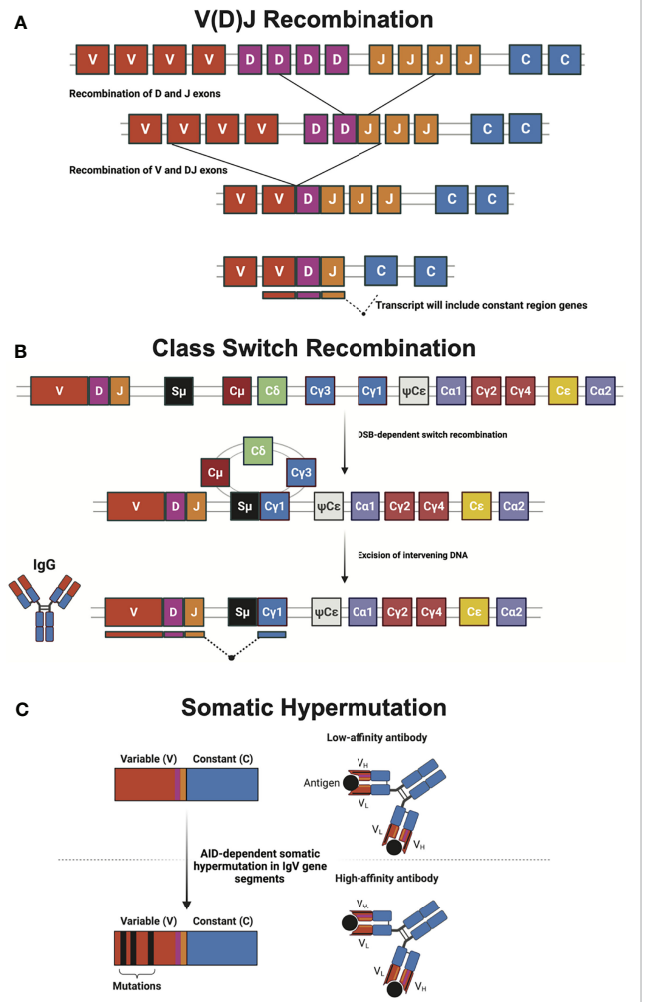

FIGURE 1 | Mechanisms of generating diversity in adaptive immunity. **(A)** V(D)J recombination relies upon RAG-mediated recombination for the rearrangement of immunoglobulin and T cell receptor variable (V), diversity (D), and joining (J) gene segments during lymphocyte development. Many enzymes involved in non-homologous end joining (NHEJ) and other DNA repair mechanisms are required to correct the programmed DNA double-strand breaks (DSB) that initiate gene segment rearrangement. **(B)** Class-switch recombination (CSR) of the immunoglobulin heavy chain locus swaps antibody isotype *via* recombination of different constant (C) regions. CSR requires activation-induced cytidine deaminase (AID) to initiate a DNA DSB break at the switch (S) region, which is subsequently repaired by classical and alternative NHEJ. The schematic shows a CSR event that leads to the production of IgG antibody isotype. **(C)** Somatic hypermutation (SHM) utilizes AID-dependent programmed mutations in the variable region of antibody gene segments to create a large number of antibodies with goal of creating greater affinity for antigen. Antibody heavy (V_H) and light (V_L) chains, as well as antigen (black circle) are illustrated. Figure created with BioRender.com.

Antibodies (immunoglobulins) directly neutralize pathogens and their gene products (13). In addition, antibodies recruit cellular effectors of immunity to eliminate pathogens and tumor cells. During development, variable regions of the immunoglobulin (*Ig*) locus undergo V(D)J recombination of both the heavy (*IGH*) and light chains (*IGL*) to generate 10^{11} to 10^{14} novel combinations of genetic material (13–15). Upon stimulation, further diversifications of *Ig* genes can be induced by Class Switch Recombination (CSR) and Somatic Hypermutation (SHM) (**Figure 1**). Antibody effector function is governed by its antibody class or isotype. In response to antigen stimulation and costimulatory signaling, programmed DNA damage in the constant region of the *IGH* locus of mature B cells initiates CSR, causing cells to undergo antibody class switching (13, 16). This allows antigen-activated B cells, which are initially IgM^+ or IgD^+, to change heavy chain constant domains and express one of the other isotypes encoded downstream in the locus, thus altering antibody function and tissue distribution (6).

Germinal center B cells undergo affinity maturation in lymphoid tissue germinal centers to generate high-affinity antibodies that enable a more effective humoral immune response (17, 18). This process relies upon SHM to generate single point mutations in the *IGH* and *IGL* loci (19, 20). While CSR acts on the constant region of the *IGH* and *IGL* loci, SHM is directed at the variable region. CSR and SHM act in concert to create high-affinity immune responses to each pathogen encountered. VDJ, CSR, and SHM are absolutely dependent on intentional but tightly regulated induction of DNA damage at discrete areas of the genome (20). Multiple components of the DNA repair machinery: sensors, binding proteins, kinases, helicases, recombinases, nucleases, polymerases, and ligases are required for the resolution of the programmed DNA damage that occurs in each of these processes (19, 21). This review highlights the pathophysiological consequences caused by mutations in genes encoding these DNA repair enzymes required for immune diversification.

V(D)J RECOMBINATION

V(D)J recombination is the process that assembles the variable domain of immunoglobulin and T-Cell Receptor (TCR) genes *via* DNA rearrangements (22). V(D)J recombination increases the sequence heterogeneity of a defined gene fragment during the early stages of lymphocyte development. It shapes the immune system repertoire by forming T-cell receptors and immunoglobulins in immature B cells. V(D)J recombination involves multiple DNA repair proteins, including DNA-PKcs, Ku70, Ku80, XRCC4, DNA Ligase IV, and the Cernunnos-XLF protein, all required for non-homologous end-joining of DSBs. Initiation of V(D)J recombination requires lymphoid-specific DNA recombinases RAG1 and RAG2, which recognize recombination signal sequences that flank all V, D, and J gene units and as a complex introduce site-specific DSBs (23–26). MRE11, RAD50, and EXO1 repair proteins are then needed to join the broken DNA ends and resolve the DSB. Mutations in DNA repair factors that participate in V(D)J recombination can severely impact immune function. Mutations in the above genes encoding the above DNA modifying proteins cause varied effects on T and B cell immune cell repertoires. Immunological diseases that arise from DNA repair defects impacting V(D)J recombination are discussed below.

Severe Combined Immunodeficiency

Severe combined immunodeficiency (SCID) is a rare genetic disorder characterized by impaired development of the immune system and absence of T and B lymphocytes. Mutations in human DNA repair genes *RAG1*, *RAG2*, *DCLRE1C, PRKDC, NHEJ1*, and *LIG4* cause SCID (27). These genes all encode proteins that incise (RAG1-RAG2 complex), excise (*DCLRE1C* Artemis protein) or participate in NHEJ DSB repair (*PRKDC*/DNA-PKcs, *NHEJ1*/XLF4, and LIG4). Loss of function mutations in *RAG1/2, PRKDC*, *KU70*, or *KU80* preclude T and B cell development, leading to SCID (26, 28) (**Table 1**).

Artemis deficiency, caused by null mutations in *DCLRE1C*, also causes SCID. Artemis is an exonuclease essential for the repair of DSBs *via* non-homologous end-joining (NHEJ) and plays a critical role in V(D)J recombination. Artemis mutations create a broad spectrum of phenotypes that range from SCID to antibody deficiency (29, 44, 45). NK cell number and function are unaffected in Artemis-deficient SCID patients. However, these patients commonly have radiation sensitivity consistent with a DSB repair defect (44). The impact of a mutation on NHEJ repair and capacity can vary between individuals with mutations in *DCLRE1C* and do not correlate well with clinical severity (46).

DNA-PKcs is a key component of DNA Protein Kinase complex (DNA-PK), which plays a critical role in NHEJ. Artemis is a substrate for DNA-PKcs kinase activity and phosphorylation is required for its nuclease activity that cleans up broken DNA ends. Artemis binds DNA-PKcs and the Artemis-DNA-PK complex cleaves 5' and 3' overhangs of hairpins generated by the RAG complex. Mutations in *PRKDC* can also impair Artemis activation or its ability to bind DNA ends during DSB repair. DNA-PK also has a role in recruiting other NHEJ proteins like XRCC4 and LIG4 to DSBs. As the NHEJ pathway is critical in V(D)J recombination, hypomorphic and null mutations in *PRKDC* lead to dysfunction in the development of T and B cells. *PRKDC* mutations were only discovered relatively recently in a SCID patient that exhibited

TABLE 1 | DNA repair deficiency-induced immunological disorders.

Pathway	Disease	Genes	Description	Refs
V(D)J Recombination	Severe Combined Immunodeficiency (SCID)	*RAG1*, *RAG2*, *DCLRE1C, PRKDC, NHEJ1, LIG4*	SCID patients have T and B lymphocyte deficiency. At least 4 diseases can be distinguished by clinical phenotypes and the gene affected.	(18)
V(D)J Recombination	SCID with ARTEMIS deficiency	*DCLRE1C*	Subclinical immunodeficiency: reduction of naïve T cells with increased terminally differentiated T cells due to a reduction in T-cell proliferation. Some patients have reduced B-cell numbers. Hypomorphic mutations in *DCLRE1C* can cause atypical SCID, Omenn syndrome, Hyper IgM syndrome, or inflammatory bowel disease.	(29)
V(D)J Recombination	SCID with Ligase IV deficiency	*LIG4*	Microcephaly and neurodevelopmental delay. T- and B-lymphocytopenia and varying degrees of hypogammaglobulinaemia often associated with high IgM due to defective CSR. Some patients present with features of Omenn's syndrome and autoimmunity.	(30)
V(D)J Recombination	SCID with Cernunnos-XLF deficiency	*XLF*	T and B-cell lymphopenia, growth retardation, microcephaly, and increased sensitivity to ionizing radiation.	(31)
V(D)J Recombination	SCID with DNA-PKcs deficiency	*PRKDC*	Radiosensitive, growth retardation, microcephaly, and immunodeficiency due to profound T and B cell lymphopenia.	(32, 33)
V(D)J Recombination	Ataxia Telangiectasia (A-T)	*ATM*	Progressive cerebellar degeneration leading to ataxia, telangiectasia*, immunoglobulin deficiency (IgA), lymphopenia (T cells), recurrent sinopulmonary infections, radiation sensitivity, premature aging, and a predisposition to cancer, especially lymphomas. Other abnormalities include poor growth, gonadal atrophy, delayed puberty, and insulin resistance, ataxia: abnormal control of eye movement and postural instability. Telangiectasia: abnormal, tortuous blood vessels (*telangiectasia not present in all A-T patients)	(34)
V(D)J Recombination and, CSR	Ataxia Telangiectasia-like disorder (ATLD)	*MRE11*	Lack of specific functional antibodies causing minimal immunodeficiency, ataxia, and dysarthria.	(35)
V(D)J Recombination	Nijmegen breakage syndrome	*NBN*	Progressive microcephaly presenting *in utero*, dysmorphic facial features, mild growth retardation, mild-to-moderate intellectual disability, and, in females, hypergonadotropic hypogonadism. Immunodeficiency (decreased T cells and reduced IgG/IgA) and a high incidence of pediatric malignancies, mostly lymphomas and leukemias.	(36, 37)
CSR and NHEJ	RIDDLE syndrome	*RNF168*	Radiosensitivity, Immunodeficiency, Dysmorphic features, and Learning difficulties, increased serum IgM and reduced IgG levels.	(38, 39)
CSR, SHM, BER	Hyper IgM Syndrome Type 5	*UNG*	Elevated serum IgM with low IgG and IgA, increased susceptibility to bacterial infections and lymphoid hyperplasia.	(40)
CSR and SHM	Hyper IgM Syndrome Type 2	*AICDA*	Elevated serum IgM levels, low IgG, low IgA. lymphoid hyperplasia, and recurrent infections.	(41)
CSR and MMR	*PMS2* or *MSH6* deficiency	*PMS2*	Elevated serum IgM and low IgG and IgA, recurrent infections, cafe-au-lait spots. Associated with Lynch Syndrome and colorectal and endometrial cancer.	(42, 43)

symptoms similar to patients with *RAG* or *DCLRE1C* mutations (32). The patient was practically devoid of B and T cells while NK cell numbers were normal. The patient did not display signs of microcephaly or intellectual disability observed in other DNA repair disorders impacting the immune system (32).

DNA ligase IV syndrome, which has features of SCID, is caused by a *LIG4* deficiency. This rare autosomal recessive disorder is characterized by microcephaly, abnormal facial features, sensitivity to ionizing radiation, and SCID (30). Only 30 patients with Ligase IV syndrome have been described, and while they all are sensitive to ionizing radiation (47), they exhibit a broad spectrum of clinical features. Patients typically exhibit low T and B cell numbers and low serum Ig levels, resulting in immunodeficiency (30).

Ataxia Telangiectasia

Ataxia Telangiectasia (A-T) is a genetic neurodegenerative disorder that is characterized by progressively cerebellar atrophy with impaired coordination of voluntary movements (ataxia), the development of reddish lesions of the skin and mucous membranes due to dilation of blood vessels (telangiectasia), and immune dysfunction (cellular and humoral immunodeficiency resulting in increased susceptibility to infections, cancer and malignancies, in particular lymphoid malignancies) (34). A-T is caused by mutations in the AT-mutated *(ATM)* gene, the gene product of which is a key component of the DNA damage response. Mutations in *ATM* cause aberrant V(D)J recombination and apoptosis during lymphocyte development, resulting in patients having immunoglobulin deficiencies and lymphopenia (48–50). A-T patients with inactivating mutations in *ATM* sporadically have T cell prolymphocytic leukemia (T-PLL), B cell chronic lymphocytic leukemia (B-CLL), and mantle cell lymphoma (MCL) (51). CSR deficiency is also characteristic of A-T, resulting in high serum IgM levels, with low IgA and IgG levels (35).

Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive syndrome of chromosomal instability mainly characterized by microcephaly at birth, SCID, and a predisposition to malignancies. It is caused by mutations in *NBN*, which encodes NBS1 (36, 52). NBS1 forms a multimeric complex with MRE11 and RAD50 nuclease (MRN complex) *via* its C-terminus. The function of NBS1 is to recruit and retain the complex at sites of DNA damage by directly binding to histone H2AX, a histone phosphorylated by PI3-kinase family members such as ATM, in response to DNA damage. The MRN complex facilitates the rejoining of DBSs predominantly by homologous recombination repair rather than NHEJ (52, 53). NBS patients have variability in immunodeficiency, as the number of $CD8^+$ T cells could be normal, elevated, or considerably reduced, with decreased $CD4^+$ T cell counts. However, universally there is an increase in unresolved recombination-mediated breaks in *IGH* and a compensatory proliferation of mature B cells as absolute B cell numbers are decreased, consistent with a V(D)J recombination defect (36, 37).

CLASS SWITCH RECOMBINATION

The ability of the immune system to fight and eliminate a wide array of pathogens is made possible by the production of a variety of antibody isotypes, each with unique effector functions. Naïve B-cells produce only membrane-bound antibodies IgM and IgD. Following infection, naïve B cells are activated and can be induced to undergo CSR (13, 18, 54). CSR occurs in the DNA encoding the constant region of *IGH* (16). Here, deletional recombination occurs between DSBs intentionally introduced at switch (S) regions between *IGH* constant region genes (18) (**Figure 1**).

The process of introducing DSBs begins with activation-induced cytidine deaminase (AID), which demethylates cytosines to uracil at immunoglobulin switch regions (55, 56). Next, uracil-DNA glycosylase (UNG), a component of the base excision repair (BER) pathway, excises the uracils, leaving abasic sites that are further processed to create DNA single strand breaks (SSB) (57, 58). If SSBs occur in both strands of the DNA in close proximity, then a DSB results. DNA mismatch repair (MMR) can also create DSBs following AID-induced demethylation (16). MMR recognizes U:G mismatches and resects single-stranded DNA created by mismatch-induced DNA unwinding. If there is a SSB on the opposite strand in the resected region, then a DSB is introduced. The DSBs at the switch regions are recognized, recombined, and then repaired using primarily NHEJ, similar to VDJ recombination (59). In CSR, alternative end-joining (A-EJ) also plays a role in repairing DSBs (60). In contrast to the classical NHEJ (c-NHEJ), A-EJ is a relatively slower and more error-prone process that relies upon annealing at microhomologies. A-EJ is also considered as a prominent source of genome instability (59). A-EJ is substantially less efficient than NHEJ but enables CSR in c-NHEJ-deficient cells (60). Many factors including, stage of the cell cycle, also influence which repair pathway is utilized (61). Some DNA repair factors have distinct contributions in A-EJ versus c-NHEJ. For example, 5-Hydroxymethylcytosine binding, ES cell-specific-protein (HMCES) is dispensable for c-NHEJ but the significant CSR defect observed in HMCES-deficient primary B cells is due to its downstream role in A-EJ (62). Elevated end-resection, non-productive interchromosomal translocations and inversions were observed during sequence analysis of CSR junctions of kinase-dead DNA-PKcs but not DNA-PKcs-deficient B cells (63). ERCC1-XPF, whose role in CSR is not fully understood, removes non-homologous 3' overhangs that result from annealing at microhomologies during A-EJ (64).

While most CSR-related diseases (discussed below) result from non-functional CSR proteins, the initiation of AID-induced damage outside of the *IGH* locus can lead to translocations and B cell lymphomas (65–68). Beyond AID's role in CSR, it also participates in a phenomenon called locus suicide recombination (LSR) which abolishes B cell function (69, 70). In LSR, AID initiates recombination between the most upstream *IGH* switch region (Sμ) and a "switch-like" region near the 3' regulatory region resulting in the deletion of the *IGH* constant region, rendering the B cell non-viable. Although its regulation is not well understood, the balance between CSR and LSR may play a critical role in B cell fate. These studies illustrate

the deleterious aspects of AID-mediated recombination that yield non-productive antibodies and B cell death (69, 71).

DNA Repair Syndromes Affecting CSR

DNA repair is critical for antibody diversification through CSR, which is evident in the numerous CSR-related diseases caused by mutations in DNA repair proteins (**Table 1**). When CSR is not functioning properly, individuals exhibit immunodeficiency due to an impaired ability of B-cells to switch to IgA, IgG, and/or IgE production. The characteristic phenotype of CSR-related diseases is elevated serum IgM levels with low IgA, IgG, and/or IgE levels (6, 13). There is substantial variation in clinical phenotypes both within a disease and between diseases with impaired CSR. For example, a study of patients with MSH6 deficiency found that one patient had elevated IgM levels and reduced IgG, four had elevated IgM and normal IgG, two had normal IgM and reduced IgG, and one had normal IgM and normal IgG (72).

Although AID is not technically a DNA repair protein, its intentional introductions of DNA damage are crucial for the initiation of CSR. Mutations in *AICDA*, the gene that encodes AID, cause hyper-IgM syndrome (HIGM) type 2 (41). Patients with HIGM type 2 typically present with elevated serum IgM levels with low IgA and IgG levels (73, 74). Following the replacement of cytosine DNA bases in switch regions with uracil by AID, BER and MMR proteins play critical roles in producing DSBs. Mutations in *UNG*, coding for the BER protein UNG, result in the HIGM Type 5 (**Table 1**). Like HIGM type 2, this syndrome is characterized by high serum IgM levels, low IgG levels, and low IgA levels (40, 42). Additionally, patients with PMS2- and MSH6-driven MMR deficiency exhibit defective CSR, which is also the case in MLH1 and MSH5-deficient mice (**Table 1**) (43, 72, 75, 76).

Defects in DSB recognition and signaling proteins can cause CSR-related immunodeficiency. The MRN complex (MRE11-RAD50-NBS1) recognizes DSBs and activates ATM, the key transducer of signaling in response to DSBs. Ataxia Telangiectasia (A-T), Ataxia Telangiectasia-Like Disorder (A-TLD), and NBS, caused by mutations in *ATM, MRE11*, and *NBS1*, respectively, all lead to CSR defects (34, 35). NBS patients have a defect in CSR as well as VDJ recombination. A-T and A-TLD share many clinical phenotypes such as ataxia, dysarthria, and abnormal eye movements. However, A-T and NBS result in more similar immunodeficiency phenotypes than A-T and A-TLD. Patients with A-T and NBS often exhibit elevated serum IgM levels, low IgA levels, and low IgG levels (**Table 1**) (77–79). In contrast, A-TLD patients exhibit very mild immunodeficiency, with reductions in some specific antibody isotypes observed (35, 80). RNF168 is another protein involved in signaling and repair protein recruitment following recognition of DSBs (38). RIDDLE syndrome is caused by *RNF168* mutations and is characterized by defective CSR resulting in low serum IgG levels (39). Mutations affecting critical NHEJ proteins often cause CSR-related immunodeficiency. Low or absent serum IgA and IgG levels are common in Cernunnos-XLF- and DNA-PKcs-deficient patients (81, 82). In addition, DNA Ligase IV deficiency often results in low serum IgG levels (30) (**Table 1**).

SOMATIC HYPERMUTATION

SHM is another example of intentional DNA damage being induced to enable antibody diversification in germinal center B cells. SHM introduces point mutations in the *Ig* locus primarily in the antibody variable (V) region that codes for the antigen-binding site of immunoglobulin heavy and light chains (**Figure 1**). This allows for the production and selection of B cells with high-affinity antibodies (17, 83, 84). The mutation frequency in SHM is a million-fold higher than the basal genome mutation rate. How B cells restrict SHM to the V region while maintaining genome-wide integrity is not well understood. AID initiates antibody affinity maturation through SHM, analogous to initiating CSR. Centroblast B cells in the germinal centers of lymphoid organs express large amounts of AID to initiate SHM (85). Numerous point mutations occur at both the site of uracil incorporation and proximal nucleotides through three predominant mechanisms: replication, BER, and MMR. Uracil incorporated by AID can persist into the S phase during which DNA replication can result in C to T (or G to A) transition mutations (86). However, replication accounts for less than half of all the mutations incorporated during SHM (83). Error-prone non-canonical BER and MMR can combine to diversify mutations introduced during SHM (87, 88). Similar to CSR, SHM-associated uracils are excised by UNG creating an abasic site during BER. Abasic sites are then bypassed by an error-prone translesion synthesis (TLS) DNA polymerase, like Rev1, which can introduce C:G transversion (88, 89). Alternatively, a non-canonical MMR pathway can recognize and repair AID-induced U:G mispairs. This pathway utilizes the error-prone TLS DNA polymerase η, which primarily creates mutations at A:T base pairs (88, 90, 91). Inactivating mutations in *AID* can result in HIGM type 2 and *UNG* mutations can result in HIGM type 5 (41, 57, 92). In both conditions, the patients have defects in CSR and SHM and are susceptible to infections (**Table 1**).

CONCLUSION

While genotoxic injury is looked upon as unfavorable, it is quite beneficial for certain processes like meiotic recombination and immunological diversity. Deliberate induction and repair of DNA damage serve as a catalyst to expand our immune repertoire. V(D)J and class-switch recombination yield unique antibody combinations and establish effector function (6). Both pathways incorporate many components of the DNA damage response, recombinases, and enzymes from NHEJ repair pathway in addition to other components of the DNA repair machinery, including helicases, nucleases, polymerases, and ligases. Lastly, intentional *de novo* mutations in the variable region of immunoglobulin genes by SHM create high-affinity antibodies. While DNA repair-deficient murine models have been used to explore disease mechanisms and driver events in tumorigenesis, samples from DNA repair disorder patients have provided great insight into the functional consequences of

impaired DNA damage on diversification and development of the adaptive immune system. Future exploration to investigate immune perturbations in other monogenic diseases of DNA repair may provide insight into other DNA repair mechanisms that contribute to immune responses.

AUTHOR CONTRIBUTIONS

PG, YWX, and MJY prepared the figure and table. PG, YWX, MJY, ELT, and LJN wrote the manuscript. The order of co-first authorship was chosen alphabetically. All authors contributed to the article and approved the submitted version.

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REVIEW
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Targeting PARP1 to Enhance Anticancer Checkpoint Immunotherapy Response: Rationale and Clinical Implications

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Reinvigorating the antitumor immune response using immune checkpoint inhibitors (ICIs) has revolutionized the treatment of several malignancies. However, extended use of ICIs has resulted in a cancer-specific response. In tumors considered to be less immunogenic, the response rates were low or null. To overcome resistance and improve the beneficial effects of ICIs, novel strategies focused on ICI-combined therapies have been tested. In particular, poly ADP-ribose polymerase inhibitors (PARPi) are a class of agents with potential for ICI combined therapy. PARPi impairs single-strand break DNA repair; this mechanism involves synthetic lethality in tumor cells with deficient homologous recombination. More recently, novel evidence indicated that PAPRi has the potential to modulate the antitumor immune response by activating antigen-presenting cells, infiltrating effector lymphocytes, and upregulating programmed death ligand-1 in tumors. This review covers the current advances in the immune effects of PARPi, explores the potential rationale for combined therapy with ICIs, and discusses ongoing clinical trials.

Keywords: cancer, immunotherapy, DNA damage, immune response, PARP (poly(ADP-ribose) polymerase

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Abbreviations: 53BP1, p53-binding protein 1; AEs, Adverse events; APCs, Antigen present cells; ARID1A, AT-rich interactive domain-containing protein 1A; ATM, Ataxia telangiectasia mutated; ATR, Ataxia Telangiectasia Rad3-related; BC, Breast cancer; BRCA, Breast cancer gene; BRCA1, Breast cancer gene 1; BRCA2, Breast cancer gene 2; cGAS, Cyclic guanosine monophosphate–adenosine monophosphate synthase; CHK1, Checkpoint kinase1; CHK2, Checkpoint kinase2; CRs, Complete responses; CT, Chemotherapy; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; DCR, Disease control Rate; DDR, DNA damage response; DLT, Dose-limiting toxicities; dMMR, Defective mismatch repair; DNA-PKcs, DNA-dependent protein kinase; DOR, Duration of response; HER2, Human epidermal growth factor 2 receptor; HR, Homologous recombination; ICIs, Immune checkpoint inhibitors; IFNAR, Interferon-α/β receptor; IFNs, type I interferons; IKK, IkappaB kinase; IRF3, Interferon regulatory transcription factor 3; Ku70/80, Ku heterodimer protein; MRE11, Meiotic recombination 11; MSI, Microsatellite instability; MSS, Microsatellite stable; NAD+, Nicotinamide; NBS1, Nijmegen breakage syndrome 1; NF-κB, Factor nuclear kappa B; NHEJ, Non-homologous end-joining; OC, Ovarian cancer; OR, Objective Response; ORR, Overall response rate; OS, Overall survival; PARP, Poly (ADP-ribose) polymerases; PARP1, Poly (ADP-ribose) polymerase 1; PARPi, Poly-ADP-ribose polymerase inhibitors; PD, Progressive disease; PD-1, Receptor of programed death 1; PD-L1, Programmed death ligand-1; PFS, Progression-free survival; PR, Partial response; RAD50, RAD50 homolog; RPA, Replication protein A; SCLC, Small Cells Lung Cancer; SD, Stable disease; SOC, Serous ovarian cancers; STAT1, Signal transducer and activator of transcription 1; STING, Stimulator of interferon genes; SWI/SNF, Switch/Sucrose-Nonfermentable; TAAs, Tumor-associated antigens; TBK1, TANK-binding kinase 1; TCR, T-cell receptor; TFAM, Mitochondrial transcription factor A; Th1, T helper 1; TILs, Tumor-infiltrating lymphocytes; TTR, Time to tumor response; XLF, XRCC4-like factor; XRCC1, X-Ray Repair Cross Complementing 1; XRCC4, X-ray cross-complementing protein 4.

INTRODUCTION

Cancer immunotherapy has revolutionized the field of oncology by demonstrating that the use of immune checkpoint inhibitors (ICIs) alone or in combination with other therapies prolongs the survival of patients with advanced disease, including melanoma, genitourinary, lung, gastric, and more recently breast cancer (1–4). However, the efficacy of ICIs varies depending on the type of cancer and within the same tumor tissue cohort (5). Ultimately, the benefits of ICI therapy in the overall population could be considered low, especially in some common tumor types, such as prostate and breast cancers (6, 7).

In this context, strategies to enhance the benefit of ICIs have focused on patient selection based on biomarkers such as programmed death ligand-1 (PD-L1) or the use of ICIs combined with other agents, including chemotherapy or targeted therapy (4, 5).

Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) are a class of drugs that inhibit single-strand DNA repair, leading to DNA damage and apoptosis (8). Notably, this process of DNA damage can modulate the antitumor immune response by activating antigen-presenting cells (APCs), infiltrating effector lymphocytes, and upregulating PD-L1 in tumors. In this review, we summarize the current knowledge on the immune-mediated effects of PARPi and the rationale for clinical trials that combine these agents with ICIs.

DNA DAMAGE REPAIR PATHWAYS AND CANCER

The genome of every cell is constantly exposed to endogenous and/or exogenous sources of DNA damage. Usually, a chemical addition or disruption to a base of DNA or a break in one or both chains of DNA strands is characterized as DNA damage (9). DNA damage mechanisms for detecting and repairing DNA, collectively termed DNA damage response (DDR), are activated to ensure cell survival (10). Thus, dysregulation and mutations in these DDR factors and their modulators have implications for human health and disease, including increased susceptibility to DNA mutations that can lead to neoplastic transformation (9, 10). High levels of replication stress often induce DNA damage in cancer cells and their survival relies on certain DNA repair pathways (11). Understanding the broader role of DDR pathways in cancers has led to the development of pharmacological interventions for cancer therapy, such as drugs targeting poly (ADP-ribose) polymerases (PARP) (12).

DNA Repair and PARP

PARP belongs to a family of 17 enzymes involved in several cellular processes, including DDR (13). Poly (ADP-ribose) polymerase 1 (PARP1), the most well-known enzyme in this family, is involved in the detection and repair of DNA single-strand breaks (13, 14). Functionally, PARP1 can rapidly detect DNA damage. The binding of PARP1 to DNA alters its catalytic domains, causing PARP1 to catalyze the post-translational polymerization of ADP-ribose units (15). PARP1 enables the auto-PARylation and PARylation of histones and other chromatin-associated proteins. Finally, PARP1 recruits additional DNA repair molecules, such as X-ray repair cross complementing 1 (XRCC1), to the site of damage, promoting the effective repair of DNA (8, 16, 17). However, when PARP fails or is pharmacologically inhibited, single-stranded breaks accumulate and become double-stranded breaks (18). Cells with an increasing number of double-strand breaks become more dependent on other repair pathways, mainly homologous recombination (HR) and non-homologous end joining (NHEJ) (19). The two main pathways involved in DNA double-strand break repair are described below:

Homologous Recombination (HR)

HR is an efficient and high-fidelity DNA repair mechanism based on a homologous template (8, 20). The HR pathway mainly occurs during the S/G2 phase of the cell cycle (21). HR is initiated by the MRN-complex, composed of meiotic recombination 11 (MRE11), RAD50 homolog (RAD50), and Nijmegen breakage syndrome 1 (NBS1), which is recruited to the sites of double-strand breaks (22). The MRN complex produces a 3 overhang of single-stranded DNA that is coated by replication protein A (RPA) to avoid DNA secondary structure formation (8, 20). Breast cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA2*) enable DNA repair protein RAD51 homolog 1 (RAD51) recombinase to displace RPA and stabilize RAD51-single-stranded DNA filaments (20, 23). These filaments invade a sister chromatid to execute the homology search, and repair-associated DNA synthesis is terminated by the generation of a double-Holliday junction, which leads to the effective repair of the DNA double-strand break (21). Therefore, tumor cells with defective HR, such as those with a *BRCA1/2* mutation, are susceptible to impairment of PARP, facilitating cell death, or can be alternatively repaired by the error-prone NHEJ pathway, resulting in genomic instability before cell death (24, 25).

Non-Homologous End Joining (NHEJ)

NHEJ repairs double-strand breaks in DNA without a template strand. Consequently, NHEJ is an error-prone double-strand break repair mechanism. It does not require a template strand and can be activated in all phases of the cell cycle (8, 26). The initial step in NHEJ is recognition and binding of the Ku heterodimer protein (Ku70/80) to double-strand breaks. The Ku-DNA complex acts as a scaffold for DNA-dependent protein kinase (DNA-PKcs) and enzymes such as X-ray cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV, which ligate DNA and mediate the ligation of the double-strand break (8, 26). In this context, PARPi in HR-deficient cells promotes NHEJ DNA repair and induces genomic instability or cell death.

PARP INHIBITORS (PARPI)

Currently, four agents (olaparib, rucaparib, niraparib, and talazoparib) are approved for the treatment of different tumors, including ovarian, breast, prostate, and pancreatic cancers. The success of PARPi in cancer treatment is believed to originate from their ability to induce synthetic lethality (27, 28). Synthetic lethality arises when the co-occurrence of two gene conditions causes cell death, whereas a deficiency in only one of the genes

does not determine cell lethality (29). Mechanistically, PARPi anticancer agents compete with nicotinamide (NAD^+) for the PARP catalytic site, inhibiting single-strand break repair (8, 30, 31). This effect promotes the accumulation of single-strand breaks that result in the collapse of the replication fork and replication associated with the double-strand break. Subsequently, tumor cells become more dependent on the HR or NHEJ repair pathways (18, 19). In tumors with HR defects, such as those with *BRCA1/2* mutations, PARPi induces synthetic lethality. *BRCA2*-deficient cells compared to *BRCA2*-proficient cells are 90 times more sensitive to PARP inhibition (18, 32). Although there is a consensus that PARPi mechanisms of action rely on inducing synthetic lethality in tumors with defective HR, more recent findings suggest that PARPi also modulates the antitumor immune response.

IMMUNE EFFECTS OF PARP INHIBITORS

Previous reports have noted an association between DDR defects or failure with the activation of anticancer immunity through the response-dependent type I interferon (IFN) pathway or *via* the accumulation of mutations and neoantigens (33–35). In this context, novel findings have demonstrated that the pharmacological inhibition of PARP can mimic this condition, dramatically affecting the balance of the immune response in the tumor microenvironment.

The DNA Damage Induced by PARPi Induces Antitumor Immune Response

DNA sensing through the cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS)/stimulator of interferon genes (STING) pathway participates in host defense by detecting aberrant entry of DNA into the cytosol (36). This pathway is classically involved in defense against viruses; however, new evidence indicates that the cGAS-STING pathway is also activated by fragments of endogenous DNA generated by cancer treatment, driving an effective antitumor immune response (36–38).

In preclinical studies, PARPi effectiveness in *BRCA1*-deficient tumors was found to be dependent on CD8 T-cell recruitment *via* intratumoral cGAS/STING pathway activation. The use of PARPi in DRR-defective tumors produces single-and double-strand breaks in DNA that bind to cGAS, leading to the production of a second messenger molecule that stimulates the adapter protein STING. STING, *via* kinases TANK-binding kinase 1 (TBK1) and IkappaB kinase (IKK), activates transcription factor interferon regulatory transcription factor 3 (IRF3) and factor nuclear kappa B (NF-κB), which translocate into the nucleus to trigger type I IFN signaling (39).

It is well known that IFNs play a central role in antitumor immunity (40). The seminal demonstration that interferon-α/β receptor (IFNAR) or signal transducer and activator of transcription 1 (STAT1) knockout mice fail to reject immunogenic tumors (41, 42). Numerous studies have shown that the expression levels of IFNs are positively correlated with CD8 + T cell lymphocyte infiltration in the tumor microenvironment (39, 43, 44). Thus, the introduction of DNA damage by PARPi can trigger the transformation of tumors from cold to hot (39). Moreover, CD8 T lymphocytes kill malignant cells upon recognition by the T-cell receptor (TCR) of specific antigenic peptides present on the surface of the target cells (45). In this context, effective antitumor immunity relies on cross-presentation of tumor antigens by APCs to CD8 T lymphocytes. APC activation requires type I IFN signaling, which can be initiated by cGAS-STING activation (40, 42, 46, 47). Therefore, cGAS-STING signaling can act as a bridge between DNA damage and the activation of anticancer immune responses.

However, in parallel, type I IFNs activate pathways that control the exacerbated inflammatory immune responses. For example, IFN-β has been shown to induce the expression of PD-L1 in tumor cells, which contributes to the immune escape by cancer cells (48). In line with our premise, PARPi induces upregulation of PD-L1 in tumor cells (49).

The Genomic Instability Induced by PARPi Triggers Antitumor Immune Response

In tumor cells, DDR failure can result in the accumulation of mutations in drive genes that produce survival advantages and accelerate tumor development (50). However, this genomic instability can encode tumor-specific neoantigens, which may make tumors more attractive to the immune response (51, 52). There is a correlation between tumor mutational burden and the likelihood of response to ICIs. Preclinical studies have shown that cancer cells with microsatellite instability (MSI) or defective mismatch repair (dMMR) grow in immune-deficient mice but are unable to grow in immune-competent mice (53). In clinics, MSI or dMMR are biomarkers for predicting responses to ICIs approved by the FDA (54). In this context, it has been discussed whether drugs that modulate DDR pathways, such as PARPis, can promote genetic instability and neoantigen formation.

It has been experimentally demonstrated that in *BRCA*-deficient cells, PARPi induces chromosomal instability typified by the accumulation of chromosomal breaks and eventual lethality *via* NHEJ (24). In another study, genomic instability and cell death induced in *BRCA1*-deficient cells by PARPi were found to be dependent on the NHEJ factor p53-binding protein 1 (53BP1) (55). Although this mechanism still needs to be further explored clinically, these primary findings suggest that the pharmacological blockade of PARP has the potential to increase genomic instability and lead to dynamic mutational profiles, resulting in the persistent renewal of neoantigens and engagements of an immune response.

THE RATIONALE FOR THE COMBINATION OF ICIS AND PARPI

Immune checkpoints represent a set of modulatory pathways essential for exacerbating inflammatory responses and maintaining self-tolerance (56). The receptors cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and PD-1,

expressed mainly in lymphocytes, and PD-L1, expressed in APCs, are part of the immunological checkpoint system (57). The interaction between CTLA-4 and CD80, CD86, or PD-L1/PD-1 reduces T cell activity, leading to suppression of the inflammatory response and preservation of tissues (57, 58). However, this mechanism favors cancer progression, enabling the escape of the anti-tumor immune response. Therefore, the use of monoclonal antibodies (mAbs) to block CTLA-4, PD-1, or its ligand PD-L1, ICIs, reactivates and drives the immune response to detect and destroy tumors by overcoming the negative feedback mechanism of the immune response (59).

Although there is no doubt that ICI therapy positively impacts cancer treatment in several neoplasms, ICIs may not be sufficient for optimal antitumor activity in some patients, particularly those with a defect in cancer antigen-specific T-cell activation or impairment of T-cell infiltration into tumors (60). Thus, efforts to enhance these responses are needed. The interaction between tumor DNA damage and the immune system plays a role in driving the response to ICI. DNA-damaging agents include chemotherapy (CT), ionizing radiation (RDT), and targeted DNA repair therapies. CT activates the immune system by inducing immunogenic cell death pathways. RT causes several types of DNA damage. DNA repair targeted agents include PARPi. In particular, combination strategies with PARPi can potentially maximize the benefit from ICIs, and its plausible synergistic effect resides in the immune properties of PARPi at different points in the cancer immune response. PARPi may facilitate a more profound antitumor immune response and synergize with ICIs by inducing DNA damage, producing a T helper 1 (Th1) immune-mediated response *via* IFN signaling, activation of APC cells, increased recruitment of effector lymphocytes, and promoting upregulation of PD-L1 in tumor cells (39, 49, 61).

In summary, DNA damage induced by a PARPi can promote antitumor immunity *via* the cGAS-STING/type I IFN/CD8 army (positive effect). In contrast, type I IFN induces PD-L1 expression and promotes tumor immune escape (a negative effect). In this context, the combination of PARPi and ICIs has particular translational appeal owing to its potent immune-stimulatory anticancer effects (**Figure 1**).

PARPI AND ICIS COMBINATION IN PRECLINICAL STUDIES

In preclinical models, PARPi has demonstrated synergy with ICIs in a variety of tumor models regardless of *BRCA1/2*-defect. It was demonstrated that PARPi-based therapy synergizes with anti-PD-1 against both MSI and microsatellite stable (MSS) colon cancer models, with a potential sensitizing effect of anti-PD-1 therapy against MSS tumors (61). In another study, PARPi led to the accumulation of cytosolic double-stranded DNA, thereby activating type I IFN-related immune response. Shen et al. (2019) (62) demonstrated the combined use of PARPi and ICIs against colon and ovarian experimental tumors, regardless of the *BRCA1/2* mutation status of the cell lines assessed both *in vitro* and *in vivo*. Furthermore, PARPi treatment upregulated PD-L1 expression *in vitro* and *in vivo* in breast cancer cell lines, xenograft tumors, and syngeneic tumors. Although PARPi attenuated anticancer immunity *via* upregulation of PD-L1, the combination of PARPi and anti-PD-L1 therapy compared with each agent alone significantly increased therapeutic efficacy (49). Investigating the effects of the PARP1/2 inhibitor niraparib in combination with ICI therapy in *BRCA*-deficient and BRCA-proficient breast cancer tumor models, it was observed that the combined regimen demonstrated synergistic antitumor activity in both *BRCA*-proficient and *BRCA*-deficient tumors. Interestingly, mice with tumors cured by single-agent niraparib completely rejected tumor growth upon rechallenge with the same tumor cell line, suggesting the potential establishment of immune memory (63).

Together, these data reinforce that PARPi in combination with ICIs may be beneficial in tumors, regardless of DNA repair status, which has important clinical implications.

PARPI AND ICIS COMBINATION IN CLINICAL STUDIES

Combination of a PARPi With Anti-PD1/PD-L1 ICIs: What Do We Already Know?

Phase I study analysis of this combination showed toxicities manageable with supportive care, and no new adverse events were noted compared with the PARPi or ICI toxicities in monotherapy (64, 65) A phase I study of solid tumors tested a combination of durvalumab, an anti-PD-L1 agent, and olaparib. Durvalumab was administered at 10 mg/kg every 2 weeks or 1,500 mg every 4 weeks, and olaparib tablets were administered twice daily. No dose-limiting toxicity was observed for durvalumab plus olaparib. Two partial responses (≥15 months and ≥ 11 months) and eight stable diseases ≥ 4 months (median, 8 months [4–14.5 months]) were seen in patients who received this combination, generating an 83% disease control rate (65).

Here, we explored clinical trials evaluating the efficacy of PARPi and anti-PD1/PD-L1 ICIs in ovarian and breast cancers. Studies with breast cancer and ovarian cancer patients, as summarized in **Table 1**, demonstrate interesting response rates with acceptable toxicity.

MEDIOLA is a phase II basket study assessing the efficacy and safety of a chemo-free combination of olaparib and durvalumab in patients with solid tumors (NCT02734004) and germline *BRCA1/2* (*gBRCA1/2*) mutations. Patients received olaparib for 4 weeks, followed by a combination of olaparib and durvalumab until disease progression. The primary endpoints were the disease control rate (DCR) at 12 weeks, safety, and tolerability. Patients with platinum-sensitive recurrent ovarian cancer (n=34) received at least one prior line of platinum therapy. The 28-week DCR was 65.6%, while the overall response rate (ORR) was 71.9%, with a total of seven complete responses (CRs). The median progression-free survival (PFS) was 11.1 months (95% CI: 8.2, 15.9), with a median duration of response (DOR) of 10.2 months. The median overall survival (OS) for all patients is not

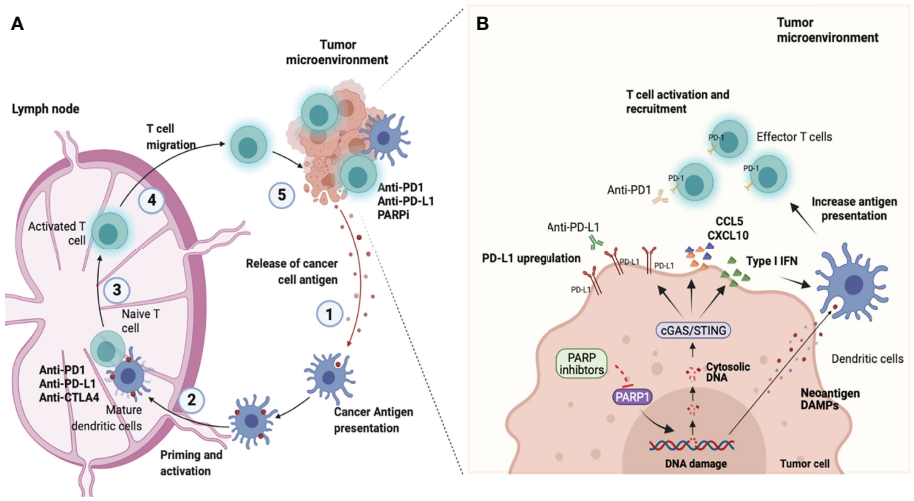

FIGURE 1 | Combining PARP inhibition and immune checkpoint blockade. **(A)** Antitumor immunity depends on a series of stepwise events. Primarily this process includes the capture and processing of Tumor-associated antigens (TAAs) by Antigen-presenting cells (APCs), such as dendritic cells or macrophages in the tumor microenvironment (step 1). Next, APCs cells presented antigen to CD8+ T cells at the lymph nodes (step 2). This process promotes the prime and activation of effector CD8 T cells (step 3). Finally, the activated effector T cells migrate from lymphocytes (step 4) and infiltrate into the tumor microenvironment to recognize and eliminate tumor cells (step 5), completing the cancer-immune cycle. However, the continued immune attack may enable cancer cells to evolve mechanisms for the escape of immune attacks. Molecules that negatively regulate T lymphocyte activation, called immune checkpoints are central players involved in tumor immune escape. In the cancer-immune cell cycle the ICIs (anti-CTLA-4, anti-PD-1, or anti-PD-L1) reactivate and drive the immune response to detect and destroy tumors by overcoming the negative feedback mechanism of the immune response acting in steps 3 and 5. **(B)** Poly-ADP-ribose polymerase inhibitors (PARPi) have effects in the early steps of the cancer-immune cell cycle. PARPi induce DNA breaks in *BRCA1/2*-deficient cells which can result in cell death or genomic instability and neoantigen formation. Furthermore, the DNA damage induces the release of DNA fragments into the cytosol which causes the cGAS/STING pathway activation in tumor cells and the production of type I IFN and chemokines (CCL5 and CXCL10). This effect culminates with paracrine activation of APCs such as dendritic cells (step 1 and 2) and with the recruitment of CD8 cells for the tumor microenvironment (step 4). Another important immune effect of PARPi is associated with the increased expression of Programmed death ligand-1 (PD-L1) in tumor cells (step 5). Therefore, the combined use of PARPi with Immune checkpoint inhibitors (ICIs) has the potential to amplify the entire cancer immune cycle (image created at Biorender).

TABLE 1 | Clinical trials evaluating the combination of PARP inhibitors and immune checkpoint inhibitors in breast cancer ovarian cancer.

Studies in Breast Cancer	Immunotherapy	PARPi	Patients	Outcome
NCT02657889 (TOPACIO/ KEYNOTE-162) Phase II	Pembrolizumab (200 mg Q3W)	Niraparib (200 mg QD)	**N=55** Advanced/Metastatic TNBC	ORR 21% with 5 CRs and 5 PRs (better *BRCA*-mutated tumors), DCR 49%
NCT02734004 (MEDIOLA) Phase II	Durvalumab (1500 mg Q4W)	Olaparib (300 mg BID)	**N=34** *gBRCAm* HER2 negative mBC	28-week DCR 47%, ORR 56%, PFS 6.7 months.
NCT03330405 (JAVELIN PARP Medley) Phase Ib/II	Avelumab (800 mg Q2W)	Talazoparib (1mg QD)	**N=34** Previously Treated advanced solid tumors	First-cycle DLT 25% ORR 8% with 1 PR, SD 50%
Studies in ovarian cancer				
NCT02571725 Phase I	Tremelimumab (10 mg/kg Q4W)	Olaparib (300 mg BID)	**N=3** *gBRCAm* recurrent ovarian cancer	No DLT or grade 3 AE ORR 100% with 3 PRs
NCT02484404 Phase II	Durvalumab (1500 mg Q4W)	Olaparib (300 mg BID)	**N=35** Platinum-resistant recurrent ovarian cancer	ORR 14% with 5 PRs, DCR 71%, mPFS 3.9 months
NCT02657889 (TOPACIO/KEYNOTE-162 Phase II	Pembrolizumab (200 mg Q3W)	Niraparib (200 mg QD)	**N=60** Platinum-resistant recurrent ovarian cancer	ORR 18% with 3 CRs and 8 PRs (irrespective of *BRCA* and HRD status), DCR 65% mPFS 3.4 months
NCT02734004 (MEDIOLA) Phase II	Durvalumab (1500 mg Q4W)	Olaparib (300 mg BID)	**N=32** *gBRCAm* platinum-sensitive ovarian cancer	12-week DCR 81%, ORR 63% with 6 CRs and 14 PRs
NCT02660034 Phase I	Tislelizumab (200 mg q3W)	Pamiparib (40mg BID)	**N=49** advanced and previously treated solid tumors	ORR 20%. RP2D

TNBC, triple-negative breast cancer; gBRCAm, germline breast cancer gene mutation; BRCA, breast cancer gene; N, number of patients; ORR, overall response rate; CR, complete response; PR, partial response; SD, stable disease; DCR, disease control rate; PFS, progression-free survival; HRD, homologous recombination deficiency; DLT, dose-limiting toxicities. RP2D: recommended phase 2 dose. QD, daily; BID, two times per day; Q2W, 2 week cycle; Q3W,, 3 week cycle; Q4W, 4 week cycle.

yet reached, with 87.0% of patients alive at 24 months (66). Thirty-four patients were enrolled in the human epidermal growth factor 2 receptor (HER2)-negative metastatic breast cancer group. The 12- and 28-week DCRs were 81% and 47%, respectively. The ORR for the overall cohort was 56%, with one patient with CR and six (19%) patients with progressive disease (PD). The median PFS was 6.7 months (95% CI: 4.6, 11.7 months). The most common grade 3 or 4 adverse events reported were anemia (11.8%), neutropenia (8.8%), and pancreatitis (5.9%) (67). Therefore, we concluded that the combination of olaparib and durvalumab was well tolerated and showed promising median PFS and DOR for ovarian cancer, breast cancer, and gBRCA1/2 mutations.

The TOPACIO/KEYNOTE-162 phase I/II study evaluated the efficacy and safety of nivolumab plus pembrolizumab in platinum-resistant recurrent ovarian cancer and metastatic triple-negative breast cancer (TNBC). This trial included patients with or without gBRCA mutations. The primary outcome was ORR. In the ovarian cancer group (n = 60), ORR 18% with 3 CRs and 8 PRs (irrespective of *BRCA* and HRD status), and DCR 65%. The median PFS was 3.4 months, with acceptable toxicity. Responses in patients without tumor *BRCA* mutations were higher than expected with either agent as monotherapy (68). Of 46 breast cancer evaluable patients, 20 (49%) achieved durable clinical benefit (any complete response/ partial response or stable disease ≥16 weeks), with stronger activity in BRCA-mutated tumors (69).

The PARPi talazoparib was also evaluated in the phase Ib/II study. Patients with advanced solid tumors who had received ≥1 prior standard of care chemotherapy regimen were treated with Avelumab in combination with Talazoparib. In phase 2 cohorts, eligible patients had metastatic TNBC (cohort 2A) or hormone receptor-positive (HR+), HER2 negative, DNA damage repair defect-positive breast cancer (cohort 2B). Patients in cohort 2A/B had received 0 to 2 prior therapies (no progression on prior platinum-based chemotherapy). The primary endpoint was the objective response. A total of 22 patients had been treated in both cohorts. In cohort 2A, 12 patients were evaluable for disease assessment: PR in 1, SD in 6, and PD in 5. All 3 patients in cohort 2B were non-evaluable for response at data cutoff. Treatment-related Adverse events (AEs) of any grade occurred in 94.7% of patients, the most common AEs were anemia, nausea, fatigue, and thrombocytopenia; 9 patients (47.4%) had grade ≥3 AEs. Therefore, Avelumab administered in combination with Talazoparib in patients with advanced solid tumors showed preliminary antitumor activity and a manageable safety profile. The study is ongoing (70).

Michael Friedlander and colleagues reported the findings of a phase 1a/b trial of the combination of a PARPi (Pamiparib) and ICI (Tislelizumab) in 49 patients with previously treated, advanced solid tumors. The results from the dose-escalation stage, phase 1a/b trial, show that the combination was generally well tolerated and associated with antitumor responses (20%) in patients with advanced solid tumors supporting further investigation of the combination (64).

Combination of PARPi With ICIs: Ongoing Studies

There are numerous ongoing trials (phases I-III) exploring the combination of PARPi and anti-PD1/anti-PD-L1 agents, and some trials with new immunotherapy agents such as TSR-022. TSR-022 is a monoclonal antibody against T-cell immunoglobulin and mucin domain molecule 3 (TIM-3) (also called HAVCR2), an immune checkpoint receptor. **Table 2** summarizes the ongoing phase III studies with a combination of immunotherapy and PARPi.

The association between PARPi and anti-CTLA-4 has been less studied. The combination of PARPi and CTLA-4 blockade is tolerable in heavily pretreated women with recurrent *BRCA*-associated ovarian cancer (62). Preliminary results of a phase I study combining olaparib and tremelimumab demonstrated evidence of therapeutic effects, supporting the ongoing evaluation of this regimen in phase II trials: NCT02571725 (71).

Targeting DNA Damage Signaling Proteins: Beyond PARP Inhibitors

The mechanisms that have inspired numerous PARPi-based combination therapies, including immunotherapy, also capitalize on the potential synergistic effects of different inhibitors of the DDR pathway, such as ataxia telangiectasia and Rad3-related (ATR), ataxia telangiectasia mutated (ATM), and Checkpoint kinase 1 (CHK1) inhibitors.

Preclinical data have demonstrated a synthetic lethal interaction between ATR and the ATM-p53 pathway in cells that respond to DNA damage. In a large proportion of cancer cells, where ATM-p53 signaling is defective, initiation of DNA replication continues and DNA damage accumulates, leading to cell death (72). It was demonstrated that combined treatment with ATR and CHK1 inhibitors leads to replication fork arrest, single-stranded DNA accumulation, replication collapse, and synergistic cell death in cancer cells *in vitro* and *in vivo* (73).

Strikingly, in addition to direct cytotoxic effects, ATM, ATR, and CHK1/2 inhibitors potentiate antitumor immunity. Inhibition of ATM/Chk2 led to replication stress and accumulation of cytosolic DNA, which subsequently activated the STING-mediated immune response (74). Vendetii et al. (2018) and Sheng et al. (2018), the ATR kinase inhibitor AZD6738 combined with radiation therapy boosted infiltration, increased cell proliferation, enhanced IFNγ production by CD8 T cells, and caused a decrease in the number of Tregs and exhausted T cells in the tumor in mouse models. Mechanistically, this study revealed that the antitumor effect of AZD6738 relied on the activation of the cGAS/STING pathway. These findings indicate that inhibitors of key DRR mechanisms, beyond PARP, promote the antitumor immune response through activation of the STING pathway (75, 76).

The proposed rational approach to enhance the efficacy of ICIs to utilize DRR inhibitors, to increase tumor DNA damage and thereby 'prime' tumors for response to immune ICIs have been explored in mouse models. The genic deletion of ATM induced IFN response and enhanced lymphocyte infiltration into

TABLE 2 | Ongoing studies with a combination of immunotherapy and PARP inhibitors.

Ongoing Phase III Studies	Immunotherapy	PARPi Agent	Patients	Outcome
NCT03740165 (KEYLYNK-001)	Pembrolizumab + CT	Olaparib (maintenance)	First-Line Treatment of Women with *BRCA* Non-mutated Advanced Ovarian Cancer	PFS
NCT04191135 (KEYLYNK-009)	Pembrolizumab	Olaparib	First-Line in Triple Negative Breast Cancer after induction CT + embrolizumabe	PFS
NCT03737643 (DUO-O)	Durvalumab +/- Bevacizumab	Olaparib (maintenance)	Newly diagnosed advanced ovarian, fallopian tube or primary peritoneal carcinoma or carcinosarcoma	PFS
NCT03598270 (ANITA)	Atezolizumab + Platinum-based Chemotherapy	Niraparib	Patients with Recurrent Ovarian Cancer	PFS
NCT03522246 (ATHENA)	Nivolumab	Rucaparib	Maintenance Treatment Following Response to Front-Line Platinum-Based Chemotherapy in Ovarian Cancer Patients	PFS
NCT03642132 (JAVELIN OVARIAN PARP 100)	Avelumab	Talazoparib	Maintenance therapy in Untreated Advanced Ovarian Cancer patients	PFS
NCT03602859 (FIRST)	Platinum-based Therapy With TSR-042	Niraparib	First-line Treatment of Stage III or IV Nonmucinous Epithelial Ovarian Cancer	PFS
Ongoing phase I/II trials				
NCT03101280	Atezolizumab	Rucaparib	Participants with Advanced Gynecologic Cancers and TNBC	AE; DLTs Recommended Dose of Rucaparib3.
NCT02849496	Atezolizumab	Olaparib	*BRCA* Mutant Non-HER2- Locally Advanced or Metastatic Breast Cancer	PFS; ORR
NCT03307785	TSR-022 & TSR-042	Niraparib	Patients with Advanced or Metastatic Cancer	DLT; AE; ORR3
NCT03565991 (Javelin BRCA/ATM)	Avelumab	Talazoparib	Patients with *BRCA* or ATM Mutant metastatic Solid Tumors	OR; TTR; DOR; PFS; OS
NCT02660034	Tislelizumab	Pamiparib	Subjects with Advanced Solid Tumors	AE; DLT; ORR; PFS; DOR; OS
NCT02484404	Durvalumab	Olaparib and/ or Cediranib	Advanced Solid Tumors and Advanced or Recurrent Ovarian, Triple Negative Breast, Lung, Prostate and Colorectal Cancers	ORR; RP2D

CT, chemotherapy; BRCA, breast cancer gene; TNBC, triple-negative breast cancer; ATM, ataxia telangiectasia mutated; HER2, human epidermal growth factor 2 receptor; AE, adverse events; PSF, progression-free survival; ORR, overall response rate; DOR, duration of response; OR, objective response; TTR, time to tumor response; DLT, dose-limiting toxicities; RP2D, recommended phase 2 dose.

the tumor microenvironment *via* cGAS/STING activation. This effect potentiated ICI therapy in mouse melanoma (B16) and breast cancer (4T1) tumors (77). In another study, tumor immunogenicity was evaluated after the pharmacological inhibition of ATM following PD-L1/PD-1 checkpoint inhibition. ATM inhibition increased the tumoral expression of type-I IFN in a TBK1- and SRC-dependent manner. Furthermore, ATM silencing increased PD-L1 expression, tumoral CD8 cells, and the sensitivity of pancreatic tumors to ICIs, suggesting that the efficacy of ICIs in pancreatic cancer can be enhanced by ATM inhibition (78). Similarly, ATM inhibition in tumors with a mutation in AT-rich interactive domain-containing protein 1A (ARID1A), a component of the chromatin-remodeling complex switch/sucrose-nonfermentable (SWI/SNF), selectively potentiates replication stress and accumulation of cytosolic DNA, which subsequently activates the DNA sensor STING-mediated innate immune response in ARID1A-deficient tumors. In patients, tumors with mutations or low expression of both ARID1A and ATM/CHK2 exhibit increased tumor-infiltrating lymphocytes and are associated with longer patient survival (74).

The combination of SRA737, an oral CHK1 inhibitor, with or without anti–PD-L1/anti-PD-1 leads to an antitumor response in multiple cancer models, including Small Cells Lung Cancer (SCLC). The combination of low-dose non-cytotoxic gemcitabine with SRA737 plus anti–PD-L1 increased the expression of type I IFN genes and chemokines (CCL5 and CXCL10*)*, and the number of CD8, dendritic cells, and M1 macrophages in the tumor microenvironment. Using the PARPi (olaparib) or the CHK1 inhibitor (prexasertib) in combination with anti-PD-L1, a significant increase in cytotoxic T-cell infiltration inducing tumor regression was observed in the SCLC mouse model (79). Mechanistically, it was demonstrated that the treatment with DDR inhibitors activated the STING/TBK1/IRF3 pathway, leading to increased levels of chemokines (CXCL10 and CCL5), which recruited and activated CD8 T lymphocytes into the tumors (80).

In the clinics the ATR inhibitor ceralasertib has been tested in phase I in combination with chemotherapy, olaparib, or an anti-PD-L1 antibody. The durvalumab plus ceralasertib combination arm enrolled 25 patients with advanced head and neck squamous cell carcinoma or non-small cell lung cancer (NSCLC). The primary objective was to recommend a phase 2 dose of ceralasertib. Of the 21 patients evaluated, one complete response and three partial responses were observed, independent of tumor PD-L1 expression. They concluded that this combination is tolerated in dose escalation, with preliminary signals of antitumor activity in patients with advanced solid

tumors (81). Berzosertib, another ATR inhibitor, has been tested in a phase IB/II study of combination chemotherapy and pembrolizumab in patients with advanced NSCLC with squamous cell histology; the estimated enrollment was 18 participants (NCT04216316).

CONCLUSION AND PERSPECTIVES

The combination of PARPi and ICIs is promising and has been explored in various clinical trials. While most studies with this combination have focused on patients with ovarian or breast cancer harboring germline pathogenic variants in *BRCA1/2* genes, other tumor histologies, including prostate cancer and pancreatic cancer, have been studied (82). Biomarkers trying to identify patients whose tumors have HR defects without germline *BRCA* mutations that could benefit from this combinatorial approach have also been explored. The results of ongoing phase III studies are awaited and can change the landscape of treatment for these patients.

AUTHORS CONTRIBUTIONS

RB-S conceived the work. CW, TC, FC, MS, and RB-S wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Human Variation in DNA Repair, Immune Function, and Cancer Risk

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DNA damage constantly threatens genome integrity, and DNA repair deficiency is associated with increased cancer risk. An intuitive and widely accepted explanation for this relationship is that unrepaired DNA damage leads to carcinogenesis due to the accumulation of mutations in somatic cells. But DNA repair also plays key roles in the function of immune cells, and immunodeficiency is an important risk factor for many cancers. Thus, it is possible that emerging links between inter-individual variation in DNA repair capacity and cancer risk are driven, at least in part, by variation in immune function, but this idea is underexplored. In this review we present an overview of the current understanding of the links between cancer risk and both inter-individual variation in DNA repair capacity and inter-individual variation in immune function. We discuss factors that play a role in both types of variability, including age, lifestyle, and environmental exposures. In conclusion, we propose a research paradigm that incorporates functional studies of both genome integrity and the immune system to predict cancer risk and lay the groundwork for personalized prevention.

Keywords: DNA repair, immunity, inter-individual variation, cancer risk, personalized medicine

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1 INTRODUCTION

Why some individuals are more susceptible to cancer than others remains a fundamental unanswered question in cancer biology. Both immunodeficiency and DNA repair deficiency are associated with elevated cancer risk. The canonical hypothesis regarding DNA repair deficiency is that unrepaired DNA damage leads to increased somatic mutations and malignant transformation of somatic cells. An alternative, underexplored hypothesis is that DNA repair deficiency increases cancer risk, at least in part, by leading to impaired immune cell function. Immunodeficiency is associated with profound defects in some DNA repair pathways, but for some, like nucleotide excision repair, how they contribute to immune function is not yet understood. Furthermore, it remains unclear how inter-individual variation in immune function and DNA repair capacity (DRC) among the general population collectively contribute to cancer risk. We propose that integrating blood-based genome integrity assays and immunophenotyping could afford improved predictions of cancer risk and ultimately open new opportunities for precision prevention and treatment of cancer.

Here we provide an overview of the current understanding of the origins of inter-individual variation in both DNA repair and immune function, and the extent to which they have been shown to contribute to cancer risk. We have structured two sections on inter-individual variation in DNA repair (**Section 2**) and immune function (**Section 3**) similarly to underscore the many parallels

between two fields that have largely developed independently. We discuss the role of each process in cancer risk, as well as genetic and non-genetic mechanisms contributing to inter-individual variation. After discussing the potential for integrating immunophenotyping and genome integrity assays into cancer risk prediction (**Section 4**), we highlight emerging technologies that are increasingly making such analyses feasible (**Section 5**), and close with a list of open questions recommendations for future studies (**Section 6**) and a brief synopsis (**Section 7**).

2 VARIATION IN DNA REPAIR AND ITS RELATIONSHIP TO CANCER RISK AND CARCINOGENESIS

2.1 DNA Repair Protects Against Cancer

Genome integrity is constantly threatened by endogenous and environmental DNA damaging agents. These agents include reactive oxygen species (ROS) generated by normal cellular metabolism, errors in DNA replication, ultraviolet (UV) light, ionizing radiation, and mutagenic chemicals (1). While unrepaired DNA damage can lead to disease by promoting cell death, transcriptional stress, senescence, and mutations (2), DNA repair limits these processes by maintaining genome integrity. Depending on the agent, DNA can be damaged in numerous ways. The types of DNA damage include base damage, single strand breaks, inter- and intra-strand crosslinks, bulky adducts, methylated DNA adducts, mismatches, and double-strand DNA breaks (DSBs). Complexes of DNA repair proteins form DNA repair machines that specialize in the removal of particular types of DNA damage, and defects in one or more of the DNA repair pathways increase the frequency of specific types of mutations in the genome (3) (**Figure 1**). As DNA damage and repair have been extensively reviewed elsewhere (1, 4, 5), we will not cover the detailed mechanisms here.

2.2 Defects in DNA Repair Are Linked to Cancer-Prone Genetic Disorders

Genome instability is a hallmark of cancer, and nearly all cancers are caused by one or more somatic mutations induced by DNA replication in the presence of DNA damage (6, 7). As our understanding of the etiology of cancer mutation signatures advances rapidly, it is becoming evident that genomic alterations in individual cancers can often be attributed to specific DNA damaging agents and DNA repair defects (3, 8–11). Historically, much has been learned from constitutional DNA repair deficiency syndromes that are associated with elevated cancer risk in humans. Below we highlight examples for several DNA repair pathways. In subsequent sections, we discuss variability in DNA repair in the general population, which is emerging as a potential predictor for cancer risk (12, 13) (14) (15).

Nucleotide excision repair defects in xeroderma pigmentosum (XP) patients are associated with an extremely high risk of skin cancers due to the inability to repair UV-induced DNA damage (16). Early studies revealed seven complementation groups that correspond to the DNA repair genes XPA, XPB, XPC, XPD, XPE, XPF, and XPG. Deficiency in the translesion DNA polymerase eta (aka POLH or XPV) also causes XP in humans (17) (18). Cells from these individuals exhibit normal NER, but are deficient for accurate replicative bypass of unrepaired UV-induced DNA damage, resulting in an increased rate of UV-induced mutagenesis. In the case of combined XP and Cockayne syndrome (XP-CS), mutations in

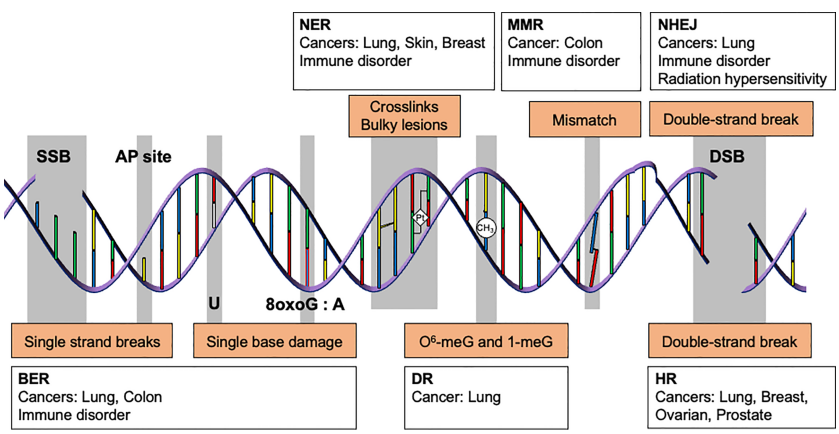

FIGURE 1 | DNA repair pathways and their association with cancer and immune disorders. Genome integrity is maintained by multiple DNA repair pathways. Depending on the type of DNA damage, specific subsets of DNA repair proteins recognize and repair the damage. For instance, single strand breaks, abasic sites, and single base lesions are primarily repaired by base excision repair (BER). Some types of alkylation damage, such as O^6-methylguanine and 1-methylguanine, are repaired by direct reversal (DR). Intra-strand crosslinks and bulky lesions are repaired by nucleotide excision repair (NER). Mismatched bases are repaired by mismatch repair (MMR), whereas double strand breaks are resolved by homologous recombination (HR) or non-homologous end joining (NHEJ). Unrepaired DNA lesions may give rise to somatic mutations and cancer. Deficiency in BER, NER, MMR, and NHEJ is also associated with immunodeficiency, which increases cancer risk(s).

XPB, *XPF*, *XPD*, or *XPG* have been detected among patients. These patients display a mild XP phenotype. Yet, despite the universality of DNA repair deficiency, skin cancers are rare except in those with mutations in *XPB* or *XPD* (19). XP-CS patients with mutations in XPG are photosensitive and have skin freckling, but skin cancers are rare. This may be in part due to very early mortality, but the severe photosensitivity phenotype that commonly accompanies XPG-CS also leads to early diagnosis and better sun protection for these patients.

Numerous diseases are associated with defects in double strand break repair. Fanconi Anemia is caused by mutations in a group of genes involved in both DSB repair and the repair of DNA inter-strand cross-links (20). Patients commonly experience immunodeficiency due to bone marrow failure and are at increased risk of acute myeloid leukemia (21). Mutation in Werner syndrome protein (WRN) predisposes to cancer. WRN is a RecQ family DNA helicase with well-established roles in both non-homologous end joining (NHEJ) and homologous recombination (HR), as well as emerging roles in base excision repair (BER) and nucleotide excision repair (NER) (22). Patients with Werner syndrome display premature aging, and have higher risks of cancer and cardiovascular disease (23–26). WRN patients develop thyroid epithelial neoplasms, melanoma, and soft tissue sarcomas, as well as leukemia and primary bone neoplasms (27). RECQL4 is involved in NHEJ (28, 29), HR (30), NER, and BER (31), and its mutation is known to induce trisomy, aneuploidy, and chromosomal rearrangements. RECQL4 deficiency is associated with several diseases, including Rothmund-Thomson syndrome (RTS), RAPADILINO syndrome, and Baller-Gerold syndrome (BGS) (32). Patients with RTS or RAPADILINO have higher risk for osteosarcoma and lymphoma (33, 34). LIG4 syndrome is caused by deficiency in Ligase IV, which is essential for NHEJ (35). Patients with LIG4 syndrome exhibit severe combined immunodeficiency due to the role of NHEJ in V(D)J recombination, a key process for antibody diversification (36). Ataxia telangiectasia (A-T) is a DNA damage response disorder caused by mutations in the Ataxia telangiectasia mutated (ATM) gene. Among other symptoms, patients with A-T experience immunodeficiency and are at increased risk for cancer, particularly lymphoid malignancies (37).

Several diseases are associated with defects in base excision repair and single strand break repair (38) (39). MutY DNA glycosylase (MUTYH) -associated polyposis (MAP) arises from germline mutation of MUTYH. Characterized mainly by the biallelic germline mutations of Y165C or G382D in MUTYH, MAP is associated with colorectal adenomas and carcinomas (40, 41). As a BER protein, MUTYH functions to remove adenine opposite 8-oxo-7,8-dihydroguanine (OG), which is left unrepaired by 8-oxoguanine DNA glycosylase (OGG1), and thereby prevent G:C to T:A transversion mutations (42). Some MUTYH variants are associated with diminished OG:A repair (43), leading to higher colorectal cancer (CRC) risk (44) (45). Defects in uracil DNA glycosylase (UNG) result in an extreme immunodeficiency known as Hyper-IgM syndrome due to the central role of this enzyme in antibody diversification (46). While UNG deficiency is too rare to allow reliable estimates of its consequences for cancer risk, in general Hyper-IgM patients suffer from higher rates of malignancy (47). Similarly, deficiency in the Nth like DNA glycosylase 1 (NTHL1) is associated with a tumor syndrome that is dominated by colorectal cancer but includes several other malignancies (48–50).

Constitutional mismatch repair deficiency is an extremely rare disease that is associated with increased risk of a wide range of malignancies (51). Lynch syndrome is another DNA repair deficiency syndrome associated with cancer. It arises from the presence of one or more mismatch repair (MMR) gene mutations (52). While the normal tissues in Lynch syndrome patients often do not exhibit detectable MMR defects, Lynch syndrome is associated with MMR-deficient cancers with high microsatellite instability (MSI) (53).

2.3 Factors That Contribute to Variation in DRC

While much has been learned from diseases associated with DNA repair deficiency, they are relatively rare and represent the extremes of inefficient DNA repair in human populations. In the general population, DNA repair gene polymorphisms, age, environmental exposures, and lifestyle are several major factors thought to give rise to inter-individual variation (12). Variation in DRC is a consequence of the collective influence of these factors.

2.3.1 Genetics

A large number of polymorphisms have been identified in DNA repair genes, and their associations with cancer imply functional consequences. While relatively few studies have investigated functional significance directly, accumulating research supports genetic variation as an important driver of inter-individual variation in DRC (**Table 1**). For example, variant alleles of *X-Ray repair cross complementing 3* (*XRCC3*) are associated with higher levels of bulky DNA adducts (59). *XRCC1* variants may be associated with either higher or lower BER repair activities (60, 62–64). *XPD* polymorphisms decrease XPD expression, with the most pronounced effect seen in older individuals (55). Some *OGG1* variants are associated with higher percentage tail DNA measured using comet assays (% tail DNA). Variant genotypes of BER and NER genes have also been associated with a wide variety of markers of genome instability. These include micronuclei and baseline %TD (58, 61, 66), chromosome breaks (62), sister chromatid exchanges (56, 60, 61), deletions and dicentric chromosomes (56), DNA adduct levels (59), overall BER repair activities (65), repair of radiation-induced damage (54) (58) (56), and repair of oxidative damage (54) (43), with cumulative effects for individuals with variant alleles in multiple DNA repair genes (57). While genetic determinants of DRC might be presumed exert similar effects on all tissues, this may not be true in light of evidence from animal models indicating tissue-dependent allele specific expression (67).

2.3.2 Aging

An age-dependent decline in DRC and DNA damage accumulation has been proposed as a key mechanism

TABLE 1 | Polymorphism in DNA repair genes and their association with genome integrity.

Genes	Genotype	DNA damage and repair activities	Ref.
Base excision repair			
OGG1	Ser326Cys; GG	Lower OGG1 activity vs. CC and CG genotypes	(54)
	Ser326Cys	Higher DNA damage vs OGG1 326 Ser/Ser genotype	(55)
		Inefficient repair of oxidative DNA damage [a]	(54)
MUTYH	G382D, Y165C, and Q324H	Less efficient in repairing 8oxoG:A mispairs vs. wild-type MUTYH	(43)
APE1	Asn148Gln	Inefficient repair of oxidative DNA damage	(54)
		Associated with repairing of X-ray induced DNA damage	(54, 56)
		Associated with mitotic delay following X-irradiation	(57)
Nucleotide excision repair			
ERCC/XPC	Lys939Gln	Associated with repairing of X-ray induced DNA damage	(58)
ERCC2/XPD	D312N in exon 10	reduced XPD expression [b]	(55)
	K751Q in exon 23	reduced XPD expression [b]	(55)
	R156R in exon 6	reduced XPD expression [b]	(55)
	312Asn	Not associated with repair of X-ray induced DNA damage	(56)
		Reduction in dicentric chromosomes and two-fold increase in translocation and chromatid exchange	(56)
	751Gln	Not associated with repair of X-ray induced DNA damage	(56)
		Reduction in dicentric chromosomes and two-fold increase in translocation and chromatid exchange	(56)
	Lys751Gln	Higher levels of bulky DNA adducts	(59)
		Not associated with higher mean SCE frequency [c]	(60)
	Gln751Gln	Higher SCE frequency vs. Lys/Lys and Lys/Gln	(61)
Single strand break repair			
XRCC1	399Gln	Lower BER activities	(60, 62–65)
		Associated with repair of X-ray induced DNA damage	(56, 58)
		Higher mean SCE frequency [c]	(60)
		Increase in deletions	(56)
	Arg399Gln	Lower irradiation-specific DNA repair rates	(54)
		Associated with mitotic delay [d]	(57)
	Arg399Gln; Gln/Gln	More chromosome breaks per cell vs. other genotypes	(62)
	Arg399Gln; AA	Higher DNA adduct levels vs. AG and GG genotypes among non-smokers	(59)
	194Trp	Higher BER activities	(60, 62–64)
	194Try	Not associated with repair of X-ray induced DNA damage	(56)
		Increase in chromatid exchange	(56)
	Arg194Try	Inefficient repair of oxidative DNA damage	(54)
	Arg194Try; Arg/Arg	More chromosome breaks per cell vs. other genotypes	(62)
	Arg280His	Inefficient repair of oxidative DNA damage	(54)
Double strand break repair			
XRCC3	Thr241Met	Higher levels of bulky DNA adducts	(59)
	241Met	Not associated with repair of X-ray induced DNA damage	(56)
		Increase in deletions	(56)

[a]*dominant effect, with repair capacity of oxidative DNA damage decreases with increasing number of variant alleles in OGG1 Ser326Cys and in combination with other gene polymorphisms (XRCC1 Arg194Try, Arg280His, and Arg399Gln, and APE1 Asn148Glu).*
[b]*either single or in combination, reduced XPD expression.*
[c]*independent of age, race, and family history of lung cancer.*
[d]*only among individuals with family history of breast cancer.*

underlying aging (68), and ongoing studies are beginning to uncover interventions that may mitigate the effects of compromised genome integrity in older individuals (69). The presence of age-dependent changes and the potential for interventions that may reverse them underline the likely role for age in inter-individual variation in DRC. Here we highlight studies testing this idea directly in human populations.

Assays that measure the accumulation of DNA damage provide indirect evidence for age-dependent changes in DRC. Peripheral blood mononuclear cells (PBMCs) isolated from older individuals have a higher frequency of dicentric and ring chromosomes (70) and a higher degree of negative supercoiling (71). Levels of single-strand breaks (SSBs) and oxidized bases (detected as formamidopyrimidine DNA glycosylase (FPG)-sensitive sites) in PBMCs are lower in younger individuals (age <65 years) when compared with older individuals (age >65 years) (72), although basal levels of SSBs and alkali sensitive sites in lymphocytes were age-independent in a separate study (73).

Direct measurements of DNA repair provide further insights into age-dependent changes in genome integrity. A study that used neutral comet assays to measure double strand break (DSB) repair and fluorometric analysis of DNA unwinding (FADU) to measure SSB repair in X-irradiated lymphocytes found diminished DSB repair in older individuals (74). Another study found that while overall rates of repair were similar, a subpopulation of repair deficient lymphocytes was significantly more abundant in older individuals (73, 74). Higher levels of DNA damage might intuitively be interpreted to reflect inefficient DNA repair, but the situation may be more complex. For example, one study found that the level of SSBs correlated positively with OGG1 activity (72), which was higher

in older individuals. The higher levels of SSBs may thus reflect the accumulation of unresolved repair intermediates downstream of BER initiation, and phenomenon that has been termed BER imbalance (75–79). Elevated OGG1 activity in lymphocytes from older individuals has been observed in additional studies (72, 80). Nevertheless, in another study OGG1 repair activity in lymphocytes was reported to decrease with age (81). The decrease in OGG1 activity was more pronounced among individuals with Cys/Cys, Ser/Cys, than with Ser/Ser genotypes at position 326, suggesting that study design and the genetic makeup of cohorts may at least partially explain the differences among studies. By contrast with OGG1, AP site incision capacity is not associated with age (82).

Evidence for age-dependent changes in DRC have also come from studies wherein cells have been challenged with DNA damaging agents. Repair replication declines in lymphocytes irradiated with ultra-violet light (UV), with the rate of UV-induced decrease in DRC estimated to be about 30% from 20 to 90 years (83). By contrast, repair replication in UV-irradiated keratinocytes is comparable between infant and older adults, suggesting that age effects may be heterogeneous across human tissues (84). Similar to the decline in repair of UV-induced damage, rejoining of chromosomes following X-irradiation decreases with age in human leukocytes (85). Consistent with higher rates of BER initiation following oxidative DNA damage, a study that compared individuals in three groups based on age of 35-39 years (Group 1), 50-54 years (Group 2), and 65-69 years (Group 3) using an ELISA assay in PBMCs following challenge with hydrogen peroxide revealed significantly higher levels of single stranded DNA in Group 3, but not Group 2, when compared to Group 1 (86). This finding is consistent with a second study that made use of comet assays (80),, as well as those in the previous section finding elevated OGG1 activity in older individuals. A rare *in vivo* study in which the epidermis of subjects was subjected to UV-irradiation followed by skin biopsies found that the efficiency in removing irradiation-induced cyclobutane pyrimidine dimers (CPD) is lower in older subjects, consistent with *ex vivo* studies in cultured primary cells (87).

Host cell reactivation assays have provided important insights into age-dependent changes in DRC. For instance, in one study skin fibroblasts from younger donors had higher efficiency in repairing UV-irradiated plasmids than those from the older donors (88). However, the same study found no relationship between age and the removal of genomic UV-induced adducts, and a second study found the repair UV-induced induced plasmid lesions was similar in skin fibroblasts from donors of age 21-96 years (89). The differences between the HCR studies in fibroblasts might reflect the relatively small samples sizes (N=8-10), which limit statistical power; a somewhat larger study (N=20) using host cell reactivation assays in fibroblasts did find an age-dependent decrease in DRC (90). In lymphocytes, repair of UV irradiated plasmids decreases with age, with an estimated average 0.61% decrease per year between 20 and 60 years of age (91). These results were consistent with a second study using HCR in lymphocytes that found an age-dependent decline in repair of UV-induced damage, which was notably absent among basal cell carcinoma cases, for whom DRC was lower than in controls at younger age (92). Another study that stands out for its analysis of pathways other than NER using host cell reactivation assays in primary skin fibroblasts indicated that both NHEJ and HR decline strikingly with age (93). Taken together, the findings suggest that age-dependent changes in DRC may depend in a complex manner on the cell type, DNA repair pathway, and the health status of the study participants.

Age-associated changes in DRC may be explained in part by the differential expression of DNA repair genes. The expression levels of excision repair cross-complementing group 1 (ERCC1) (94, 95), XPA (94), XPF (95), XRCC4, ligase 4 (LIG4), LIG3 (93), DNA polymerase delta 1 (POLD1) (88, 96), POLE, replication factor C (RFC) (88), and replication protein A (RPA) (94) decrease with age. On the contrary, the expression levels of CSA and XPG seemed to increase with age, but the change could not be confirmed by qPCR (88). There is no difference in the expression levels of proliferating cell nuclear antigen (PCNA) (88), NHEJ factors DNA PKcs, artemis, XRCC4-like factor (XLF) (93),, Ku70 and Ku80 (93, 97) and HR factors breast cancer associated gene 2 (BRCA2), meiotic recombination 11 (MRE11), RAD51, Nijmegen breakage syndrome 1 (NBS1), and RAD51 (93) among different age groups. While these studies were performed in either human PBMCs and primary fibroblasts, whether these changes in the expression of DNA repair factors resemble those in other tissues from the same individual have not been studied.

Though a detailed review of animal models is beyond the scope of this article, we note that they recapitulate several aspects of human aging biology with respect to genome integrity, including age-dependent increases in DNA damage levels (98) (99), accumulation of mutations (100, 101), and dysregulation of DNA repair (102) (103) (104) (105) (106).

2.3.3 Environmental Factors

Mounting evidence indicates that environmental exposures can alter DRC. Here we focus on two well-established examples, namely arsenic and smoking. Like tobacco smoke, arsenic is an environmental agent classified as carcinogenic to humans by the International Agency for Research on Cancer (107), and causes cancer at least in part by directly inducing DNA damage (108, 109). Exposure to arsenic is associated with chromosome aberrations in human PBMCs (110) and DNA damage (111, 112). Children with *in utero* exposure to arsenic have higher salivary 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of DNA damage caused by oxidative stress, than their unexposed counterparts (113) (114). Consistent with a key role for DNA damage in the etiology of arsenic associated malignancies, arsenic exposure is associated with a distinct mutational signature (115). Furthermore, individuals with lower DRC and those with select polymorphisms in DNA repair genes are more susceptible to arsenic induced skin lesions (109) (108) (116, 117) (110) (118).

Population studies provide extensive evidence in support of the concept that arsenic exposure leads to alterations in DNA

repair. Arsenic exposure is associated with decreased expression of *MutS homolog 2* (*MSH2*) and mutL homolog 1 (*MLH1*), though not PMS1 homolog 2 (*PMS2*) (119). Urinary arsenic concentrations are positively associated with *MLH1* promoter methylation, which is consistent with an epigenetic mechanism of arsenic-induced dysregulation of MMR (120). Arsenic exposure also leads overexpression of excision repair cross complementation group 2 (ERCC2/XPD) and less efficient NER (121). *ERCC1* expression may be influenced by arsenic exposure (122) (111), but there appear to be complex dependencies on dose, duration, and speciation of arsenic exposure (122), as well as the age of the exposed population (94, 95). Diminished expression of *XPF* and *XPB*, but increased in *XPG* expression have been associated with arsenic exposure (123). At the functional level, repair of DNA damage induced by hydrogen peroxide, ionizing radiation and 2-acetylaminofluorene (2-AAF) is impaired in arsenic-exposed individuals relative to unexposed controls (112) (117) (111). These population studies are broadly consistent with *in vitro* studies indicating that arsenic exposure synergizes with the DNA damaging effect of UV (124, 125) and inhibits repair of DNA damage induced by a variety of agents (124) (126) (127). Collectively, these findings indicate that, in addition to the direct induction of DNA damage, arsenic exposure likely sensitizes cells to the DNA damaging effects of other mutagenic agents.

Exposure to environmental tobacco smoke (ETS), also known as passive smoking, compromises genomic stability. Passive smokers have higher levels of several types of DNA damage than unexposed individuals (128). They also excrete higher levels of 5-hydroxymethyluracil (129), which is not directly induced by tobacco smoke but may result from ETS-induced oxidative stress. Though passive smoking has not been correlated with levels of 8-OHdG in serum or leukocyte DNA (128, 130), lymphocytes from passive smokers have a longer comet tail length, more Fpg-sensitive sites, and are slower in repairing H_2O_2-induced DNA damage (131). Furthermore, buccal epithelial cells of passive smokers have higher micronuclei frequency when compared to non-smokers (132). Interestingly, allele variants and expression levels of several DNA repair genes have been associated with lung cancer risk and genome instability among never-smokers, including *XRCC1* (132), *OGG1*, *XPD* (133), and *AGT* (134). A study using nasal epithelial cells further revealed that NER was among the top 6 pathways with altered gene expression in association with third hand smoking (135). While these data underscore the potential role of environmental exposures in modulating DRC, the mechanism by which passive smoking affects the activity of specific DNA repair pathways is incompletely understood.

2.3.4 Circadian Rhythm, Lifestyle, and Dietary Factors

Lifestyle factors have been shown to influence DRC. One of the most studied factors is circadian rhythm, which has been reviewed extensively (136–138) (139). It has recently been shown directly that individuals subjected to a night shift schedule exhibit diminished DRC (140). Diet can also affect the efficiency of DNA repair (141). Mounting evidence indicates that calorie restriction is associated with changes in DNA damage and repair (142). While these phenomena await more detailed study in human populations, animal models provide substantial support for the influence of diet on DNA repair. In mice, calorie restriction enhances NHEJ (143), and increases the fidelity of DNA polymerase and DNA excision repair in the liver (144). It also reverses the age-related decline in BER in brain, liver, spleen, and testis, and lowers their mutation frequency (145). In rat hepatocytes, caloric restriction altered the induction and repair of DNA damage in a manner that depended on age (146). Findings from an $Ercc1^{\Delta/-}$ mouse model of premature aging further show that dietary restriction from 10% to 30% could preserve genome integrity, mitigate premature-aging associated decline in gene transcription, and prolong their lifespan (147). This supports the hypothesis that dietary restriction may attenuate the aging process. Similarly, chronic supplementation of melatonin reduces DNA damage by upregulating APE and OGG1 (148). The underlying mechanism and whether additional DNA repair pathways are affected require further investigation. Overall, the findings in humans and animal models support a role for lifestyle and circadian rhythm in DNA repair, adding a layer of complexity to the origins of inter-individual differences.

3 VARIATION IN IMMUNE FUNCTION AND ITS RELATIONSHIP TO CANCER RISK AND CARCINOGENESIS

The immune system defends against both infection and malignancy. Based on the response time, mode of initiation, and the cell types involved, there are two immune subsystems. The innate immune system is activated rapidly upon recognition of pathogenic antigens and stress signals. It is, in part, comprised of dendritic cells (DC), monocytes, macrophages, granulocytes, and natural killer cells (NK). These cells phagocytose pathogens and activate inflammation signaling pathways and the complement cascade. The adaptive immune system, on the contrary, is more flexible in recognizing antigens. Its cellular components, including T lymphocytes (T cells) and B lymphocytes (B cells) can undergo mutagenesis to create novel and specific antigen receptors. T cells can be further subdivided into naïve T cells that recirculate between blood and lymph nodes to scout for specific antigens and memory T cells that are long lived and can mount a response to previously encountered immunogenic stimuli. Cytotoxic T cells (or $CD8^+$ T cells) secrete granzymes to induce apoptosis in target cells and pore-forming perforin to punch holes in the target cell membrane for granzymatic actions. T helper cells (or $CD4^+$ T helper cells) secrete cytokines to activate macrophages and further activate cytotoxic T cells. B cells express membrane-bound and secretory antigen-specific immunoglobulins (or antibodies) to defend against pathogens. Like NK cells, they are also involved in the

activation of $CD4^+$ T cells (149). Thus, immune response to foreign antigens depends on the specific functions of and interplay between the two immune subsystems that are comprised of a wide variety of immune cells.

Due to the presence of neoantigens that arise from genome instability and can be presented on the cell surface, cancer cells can be immunogenic. They can accordingly be recognized and eliminated by immune cells in the process of immune-surveillance (2). However, cancer cells are capable of escaping surveillance by altering antigen expression and hijacking the immune system to favor tumor growth. Through cytokine secretion, they can induce the differentiation of myeloid suppressor cells, which are inflammatory monocytes capable of inhibiting the activities of cytotoxic T and NK cells, as well as DC maturation (150). Moreover, as innate immune cells, including macrophages and neutrophils, infiltrate into tumors through chemotaxis, they can be polarized towards a pro-tumor phenotype and increase the secretion of proinflammatory cytokines to support, rather than suppress, tumor growth (151).

Current cancer immunotherapies that leverage the cytotoxicity of immune cells have proven efficacy in suppressing tumor growth. For example, NK and NKT cell populations expanded and activated *in vitro* have demonstrated potent cytotoxicity against liver cancer (152). T cells engineered with chimeric antigen receptors (CAR-T) are highly effective in targeting CD19-expressing tumors (153). DC vaccines that capitalize on the cytotoxicity of monocyte-derived DCs induce a tumor-specific immune response, although the effects differ by vaccination route and do not correlate with overall survival in phase I/II clinical trials (154). To date, immune checkpoint blockade therapies that target the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death 1 (PD-1) and its ligand PD-L1 have demonstrated improved responses and better overall survival for multiple cancers (155). Pembroluzuimab, which is an anti-PD-1 antibody, has been approved by the Food and Drug Administration to treat patients with metastatic melanoma. Another anti-PD-1 antibody, Nivolumab, has also been approved to treat patients with metastatic melanoma and patients who are previously treated for advanced or metastatic non-small cell lung cancer. These emerging therapeutic strategies form the basis for numerous ongoing clinical trials (156). For the purpose of this review, we highlight them as evidence in support of immune control of cancers.

3.1 Defects in Immune Function Are Linked to Cancer

Impaired immune function has been linked to increased cancer risk. By analogy to genetic diseases of DNA repair deficiency, patients with impaired immune function have provided insights on the role of the immune system in cancer. Numerous primary immunodeficiency disorders are associated with increased risk of malignancy (157) (158) (159) (160). Notably, since the DNA repair machinery plays integral roles in multiple aspects of immune function, some immunodeficiency disorders are caused by genetic defects in DNA repair as outlined in **Section 2.2**. In the general population, individuals with low cytotoxic activity of peripheral blood lymphocytes have higher risk of cancer (161). Immunosuppression due to organ transplantation and some viral infections are likewise factors that impair the immune response. Patients receiving immunosuppressants to prevent organ rejection have higher risk for non-melanoma skin cancer (162). This may explain why transplant recipients are generally more likely to develop cancer than those without organ transplantation (163–166). Cancers in transplant patients are also more aggressive and are associated with poor overall survival (167–169). Viral infection can suppress the immune system and increase cancer risk. Human immunodeficiency virus (HIV) -infected patients develop more aggressive cancer (164) and have higher risk for Kaposi's sarcoma, B-cell non-Hodgkin's lymphoma, and multiple myeloma (170).

Despite the strong evidence in support of a role for the immune system in controlling cancers, there are notable exceptions. Individuals with severe combined immunodeficiency due to loss of LIG4 function and those with dendritic cell deficiency tend to be susceptible to hematologic malignancies, but are not notably predisposed to solid malignancies (160, 171). Similarly, immunodeficient mice do not necessarily develop cancer. NOD scid gamma (NSG) mice have a relatively low risk of developing tumors over a life-span of about 89 weeks (172), and nude mice do not frequently develop spontaneous tumors (173), despite being highly susceptible to infection (174, 175).

3.2 Factors That Contribute to Variation in Immune Function

Inter-individual variation in immune function has been postulated as a driver of variations in cancer susceptibility. While age appears to be the most prominent intrinsic driving factor for variation in immune function, environmental exposures can also have a significant impact. Genetic variation associated with autoimmunity, inflammatory disease, and susceptibility to infection, is estimated to explain to 20-40% of the immune variation (176), leaving the remainder to be explained by other mechanisms. In this section, we review how immune function can be affected by heritable factors, and describe how environmental exposures may further explain inter-individual variation in the immune response across populations.

3.2.1 Genetics

Reminiscent of the situation for DRC, significant inter-individual differences in immune function have been reported. In a recent detailed repeated measures study, inter-individual variation in immune cell composition and plasma cytokine levels revealed that differences between individuals are generally larger than longitudinal variability within person (177). Plasma levels of the chemokine CC chemokine ligand 20 (CCL20) are negatively associated with the proportion of central memory and effector memory cells in $CD4^+$ and $CD8^+$ T cell lineages, and individuals with extremely high counts of these immune subsets are found to have low levels of plasma CCL20 and CCL22. Plasma levels of IL-16 are also negatively associated with the proportion of central memory T cells in $CD4^+$ and $CD8^+$ lineages, and $CD56^{dim}$ NK cells. Overall, plasma levels of 21 proteins accounted for nearly

80% of the variation in the abundance of central memory T cells. In a separate study, the abundance of $CD8^+CD45RO^+$ memory T cells and $CD3^+CD56^+$ NKT cells was found to vary significantly between individuals in repeated measures taken from 25 individuals over at least a three-week interval, but levels were largely stable within-person (178) Of note, differences in immunophenotype have been associated with age, sex, body mass index, and race (177, 179–183). Environmental exposure, vaccination (184, 185), and infection (186–189) can furthermore lead to within-person variation. Nevertheless, the observation that the immune cell composition and cytokine levels of an individual are relatively stable throughout a year (177) suggests that some variability may be determined by genetics or processes that occur during development.

Several lines of evidence support a role for genetics in human variation with respect to immune function. Although studies in monozygotic and dizygotic twins indicate that immune responses are dominated by non-heritable factors, numerous parameters including serum proteins and immune cell population composition are heritable (190). Single nucleotide polymorphisms (SNPs) in the IL-12B gene, which codes for IL-12p40, are associated with immune-related diseases such as psoriasis (191) and asthma (192). Eight SNPs have also been identified to be associated with IL-10 levels (181). Furthermore, studies in twins indicate that *ex vivo* lipopolysaccharide (LPS)-induced IL-1β production, as a measure of innate immunity, is heritable (180). This suggests that varying levels of LPS-induced secretion of tumor necrosis factor alpha (TNFα), which ranges widely from 0.187 to 2.714 ng/ml in healthy blood donors may be explained at least in part by genetics (193). In further support of genetic variation as a driver of differences in immune responses, a functional study using toll-like receptor (TLR) ligand-stimulated cord blood mononuclear cells has detailed the association between cytokine production and SNPs in innate immune genes (182). Taken together, the available data support a role for genetics in inter-individual variation in immune function in the general population.

3.2.2 Aging

It has long been appreciated that the immune system undergoes age-related changes, which are collectively referred to as immunosenescence and notably include the accumulation of DNA damage in immune cells (194). Although age-dependent changes in immune cell function have been reported in bone marrow (187), bronchoalveolar lavage (179), and thymus (195), this review will focus on PBMCs because they are most immediately amenable to population studies. Several studies have found age-dependent changes in total leukocyte counts (196) or the composition of leukocyte subtypes (179, 197, 198) (199) (183, 198) (**Table 2**).

TABLE 2 | Age-dependent changes in the population of immune cell subtypes.

Immune system	Cell types	Cell subtypes	Age-dependent change	Rate of change	Ref
Adaptive immune system	Total lymphocytes		Decrease	Not studied	(196)
	T lymphocytes	$CD4^+$ T cells	Slight decrease	An average of 9.8 cells/μl/year ranging from -120 to +170 cells/μl/year	(199)
		Naïve $CD4^+$ T cells ($CD45RA^+CD28^+$)	Decrease	An average of 4.3 cells/μl/year ranging from -80 to +108 cells/μl/year	(199)
			Decrease	–0.3%/year	(200)
		Treg ($CD4^+CD25^+FOXP3^+$)	Increase	An average of 1.4 cells/μl/year ranging from -4 to +10 cells/μl/year	(199)
		$CD4^+CD28^-$ T cells	Increase	An average of 1.6 cells/μl/year ranging from -23 to +60 cells/μl/year	(199)
			Increase	0.24%/year	(200)
		$CD8^+$ T cells	Decrease	An average of -1.3 cells/μl/year ranging from -163 to +69 cells/μl/year	(199)
		Naïve $CD8^+$ T cells	Insignificant change	An average of -1.8 cells/μl/year ranging from -121 to +53 cells/μl/year	(199)
		$CD8^+CD28^-$ T cells	Insignificant change	An average of 0.9 cells/μl/year ranging from -121 to +53 cells/μl/year	(199)
	B lymphocytes	Mature B cells	Insignificant change	-6.6 cells/μl/year	(199)
		Naïve B cells	No difference	-5.5 cells/μl/year	(199)
			Decrease	–0.36%/year	(200)
		Memory B cells	No difference	-0.1 cells/μl/year	(199)
Innate immune system	NK cells		No difference	An average of 25.3 cells/μl/year ranging from -180 to 100 cells/μl/year	(199)
			Increase	Not studied	(196, 201, 202)
		$CD56^{bright}$ NK cells	Decrease	Decrease from 15.6 cells/μl to 8.1 cells/μl in 60 years	(196)
		$CD56^{dim}$ NK cells	Increase	Not studied	(201)
	Monocytes		Trend of increase	Not studied	(203)
	Dendritic cells	Plasmacytoid DCs	Decrease	Not studied	(203)
		Myeloid or classical DCs	Increase	Not studied	(203)

Age-related changes in adaptive immune cells have been noted. A major study involving 177 individuals, who were sampled every six months for three years, has identified an age-dependent decrease in $CD4^+$ and $CD8^+$ recent thymic emigrant T cells and transitional B cells (183). This decrease coincides with the reduction in thymus and bone marrow activity and an increase in the inflammatory population and $CD8^+$ T cells. In particular, the proportion of $CD4^+$ T cells decreases with age (198, 199) whereas that of $CD4^+$ NKT cells increases with age [(198); **Table 2**]. Based on the expression of CD27 and CD28, T cells can be further subdivided into naïve and early-differentiated cells ($CD27^+CD28^+$) and fully differentiated ($CD27^-CD28^-$) $CD4^+$ and $CD8^+$ T cells (204, 205). A younger cohort had a significantly larger $CD27^+CD28^+$ subpopulation when compared to an older cohort (206). Similarly, based on the expression of a leukocyte common antigen isoform, CD45RA, and chemokine receptor CCR7, T cells can be subdivided into naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$), and terminally differentiated effector memory ($CD45RA^+CCR7^-$) cells (207). Within the $CD8^+$ subset, older individuals had fewer $CD45RA^+$ naïve T cells and more central memory and terminally differentiated effector cells when compared to younger individuals (208). In contrast, within the $CD4^+$ subset, a decrease in the $CD27^+CD28^+$ cells was the only difference observed in older individuals. These findings imply that the naïve T cells shift towards a more terminally differentiated subpopulation upon aging. This may limit the plasticity of the naïve T cells to differentiate and respond to novel antigens. The concomitant loss of the central memory and terminally differentiated $CD8^+$ T subsets suggests that regardless of the activation by $CD4^+$ T cells, the cytotoxic T cell response is compromised in the elderly.

Age-dependent, subset-specific changes in innate immune cell counts have been documented. The proportion of NK cells increases with age (196, 199, 201, 202) (**Table 2**) Based on the expression level of the pathogen recognition receptor CD56 (209), NK cells can be further divided into $CD56^{bright}$, which resides in the lymph node (210) and are immunoregulators due to their cytokine production capacity (211), and $CD56^{dim}$ NK cells, which have cytotoxic potential (212). The $CD56^{bright}$ subset is less abundant in cord blood when compared to adult blood (202), and decreases further with age (196). By contrast, the $CD56^{dim}$ subset increases with age (201, 203). Similarly, the proportion of monocytes increases with age (203). This monocyte population includes the classical, transitional, and $CD14^+CD16^+$ non-classical subsets. Among the non-monocytes, the proportion of myeloid-derived DCs increases with age, whereas that of plasmacytoid DCs decreases with age. In view of the age-dependent changes in immune cell composition, associating the cell count and their functions will help map the landscape of immunophenotype throughout life. Integrating this information may help identify phenotypic and functional biomarkers for immunosenescence, treatment response, or higher susceptibility to diseases including infections and cancers.

Molecular markers of immune function, including cytokine production and response to antigenic stimuli also change with age. In particular, the production of cytokines interferon gamma (IFN-γ), interleukin (IL) -4 (IL-4) and IL-6 has been shown to increase with age whereas that of IL-2, IL-10, and TNF-α decreases with age (97, 180, 183, 213). Since the cytokine production capacity of $CD4^+$ T cells is invariant with age, changes in T cell subtype composition are proposed to explain age-related changes in function (206). Interestingly, expression levels of *IL-7* are lower in nonagenarians than middle aged individuals (214). Genes in the IL7R gene network are also differentially expressed between the age groups. The fact that higher *IL-7R* expression level is associated with better prospective survival suggests a role for cytokines and immune response in longevity.

The ability of T cells to respond to mitogenic stimuli is also affected by age. Aging attenuates the proliferation of PBMCs induced by stimuli including phytohemagglutinin (PHA), concanavalin A, pokeweed mitogen, and anti-CD3 (aCD3) or anti-CD28 (aCD28) monoclonal antibodies either alone or in combination (97, 197, 213). In particular, $CD4^+$ T cells from elderly individuals have a lower proliferative response to staphylococcal enterotoxin B (206). Activated T cells also have lower induction of nuclear factor kappa B (NFkB) in response to anti-CD3, phorbol myristate acetate (PMA), and TNFα (215). Notably, treatment with phorbol dibutyrate and calcium ionophore A23187 induces higher nuclear translocation of NFkB in neonatal than adult T cells, though the composition of NFkB is similar between the two groups (216). Collectively, these results imply that T cells from older individuals are less sensitive to stimuli.

Similar to T cells, NK cells isolated from elderly individuals have diminished proliferation activity and CD69 induction following treatment with IL-2 when compared to the younger group (201). The response of $CD8^+$ $CD45RO^+$ memory T cells and $CD3^+CD56^+$ NKT cells to IL-23 also decreases with increasing age. T cell receptor repertoire diversity decreases and clonality increases with age (217). Taken together, the findings support the notion that age-dependent decrease in immune cell function, based on proliferation and cytokine production induced by antigenic stimuli, and cytotoxicity, has an impact on cancer risk. How age-dependent changes in immune function modify cancer risk warrants further investigation.

3.2.3 The Environment

Exposure to environmental agents can have major effects on the immune system (218). Given that the exposure effects have been well documented, to underscore parallels with environmental agents that affect DNA repair, we will focus on how the immune system is impacted by the same cancer-causing agents (arsenic and smoking) that were discussed in **Section 2.3.3**. We will review how environmental exposure may contribute to inter-individual variation in immune function. As with DNA damaging agents, extensive experimentation has been carried out *in vitro* and with animal models to understand the biological mechanisms underlying the immune effects of environmental exposures, but they are beyond the scope of this review.

Arsenic-induced changes in the immune system are implicated by epidemiological studies. Subjects exposed to higher levels of arsenic have higher serum levels of

immunoglobulin (Ig) A (219). Urinary arsenic levels are also positively associated with the number T helper (Th) 17 cells (220), whereas nail arsenic levels are associated with lower counts of $CD56^+$ NK cells (221), after adjusting for confounding factors. Consistent with impairment of the immune system, lymphocytes isolated from arsenic-exposed individuals have a longer average doubling time *in vitro* (222). They also secrete lower levels of cytokines, including IL-2, IL-4, IL-6, IL-10, tumor necrosis factor alpha (TNFα), and IFNγ under basal conditions (223) and following stimulation with concanavalin A (Con A) (224). Monocyte-derived macrophages isolated from the exposed individuals display abnormal morphology, diminished adhesion, and have reduced phagocytic capacity (225). These findings indicate arsenic exposure disrupts both innate and adaptive immune responses. Notably, arsenic exposure often leads to skin lesions (219) but not necessarily cancer. Whether the immunomodulation induced by arsenic contributes to excess cancer risk in exposed populations awaits further investigation.

Early life exposure to arsenic may also impact the immune system. Children with prenatal exposure to arsenic have higher risk of respiratory illness (226), and diminished cell-mediated immune function (227). Prenatal arsenic exposure alters cord blood immune cell composition, increases the proliferation of effector T and T cells, and reduces the suppression by T regulatory (Treg) cells in a dose-dependent manner (228) (229). Prenatal arsenic exposure is also inversely associated with the percentage of naive and activated T helper memory cells in cord blood, with notable sex-dependent differences in the strength of the association (230). Moreover, lymphocytes isolated from children with prenatal arsenic exposure secrete lower levels of CX3CL and tumor necrosis factor alpha following PHA stimulation (231). Proteomic analyses of cord blood further revealed aberrant levels of chemokine (C-X-C motif) ligands, macrophage migration inhibitor factor (232), and interleukins (233). This implies that the prenatal period may be a critical window of susceptibility for disruption of immune responses by environmental arsenic exposure. Nevertheless, further studies are needed to determine whether these arsenic effects can be causally linked to higher cancer risk later in life.

Smoking suppresses the immune system (234), but the impact of passive smoking is less studied. One study involving non-smoking adult volunteers has shown that serum levels of the nicotine metabolite cotinine correlate with an increase in the naïve $CD3^+$ and $CD4^+$ T cell subsets and a decrease in the memory $CD3^+$ and $CD4^+$ T cell subsets in peripheral blood (235). Other studies have focused on immune cells in the saliva and nasal lavage, which are primary target tissues due to their proximity to the exposure route of ETS. For instance, ETS is associated with a higher percentage of phagocytic cells in the saliva (236). ETS is also correlated with the level of immunoglobulin E and immunoglobulin A in nasal lavage following exposure to ragweed (237). By contrast, ETS has no effect on the levels of cytokines IL-2, IL-5, IL-13, and IFNγ in the nasal lavage. These findings indicate that ETS has differential effects on the subsets of peripheral T cells, and may induce inflammatory responses. Interestingly, parental smoking dose-dependently decreases IFNγ production in mitogen stimulated PBMC and is associated with active wheezing in children (238). In view of the above findings, exposure to ETS in children is suggested to be associated with asthma and cancer (239). In summary, the findings reported in this section underscore the significant impacts of two exemplar environmental exposures that can modify immune function, and which are associated with increased cancer risk.

2.3.4 Circadian Rhythm, Lifestyle, and Dietary Factors

As is the case with DNA repair, accumulating evidence indicates that immune function can vary substantially within an individual over the course of the day. Circulating immune cell populations undergo cyclic diurnal changes (240) (241). Among the immune cell subpopulations investigated, rhythmic changes are strongest among naïve $CD4^+$ and $CD8^+$ T cells (242, 243), and weakest, albeit still significant, among B cells (240). These observations have been made in both humans and mouse models (244), which have provided insights into how circadian rhythm regulates the trafficking of immune cells (245) (246). Notably, mice immunized with T cell dependent antigen trinitrophenyl-ovalbumin (OVA) in the evening have higher serum levels of antibodies when compared to those immunized in the morning (247). Consistent with these findings in animals, individuals receiving bacillus Calmette-Guerin vaccination in the morning exhibit stronger trained immunity and adaptive response when compared to those vaccinated in the evening (248). It is thus postulated that the timing of immunotherapy or cancer vaccine administration may affect the tumor suppressing effect. With these considerations in mind, chronotherapy is emerging as a novel research field that may improve the efficacy of cancer treatment (138).

Rhythmic changes in the immune cells are associated with levels of hormones and regulated by changes in cytokine levels and the expression of molecular clock genes (249–252). Levels of the stress hormone cortisol level peak near the time of awakening and then decline throughout the day (240). Its serum level negatively correlates with the abundance of circulating T cell subsets, including total $CD4^+$ and $CD8^+$ T cells (243). *In vitro* treatment with cortisol further shows that the suppression is most pronounced in native T cells, when compared to central memory and effector memory T cells. By contrast, the effector $CD4^+$ and $CD8^+$ T cells remain unaffected. Melatonin is the pineal hormone responsible for circadian synchronization (253) and its level peaks at night (240). Treatment *in vitro* with melatonin does not affect T cell proliferation upon simulation with Con A (254). However, higher salivary melatonin levels measured in the morning are associated with a higher percentage of HLA-DR^+ monocytes and $CD16^+$ lymphocytes, a higher CD4/CD8 ratio, lower lactate dehydrogenase activity in lymphocytes, and fewer $CD3^+$ and $CD8^+$ cells when compared to low salivary melatonin levels (255). High salivary melatonin levels in the evening are associated with a different constellation of immune system characteristics including lower phagocytic activity of granulocytes, lower CD4/CD8 ratios, and lower circulating levels of HLA-DR monocytes and $CD16^+$ lymphocytes. Moreover, melatonin inhibits the secretion of T-cell

independent antibodies (IgM, IgG1, IgG2b, and IgG3) in mice (247). These findings indicate that hormonal disruption of circadian rhythm can impact the immune response in complex ways.

Similar to the situation with DNA repair (139), animal models reveal that immune cells are subject to regulation by a circadian clock at a molecular level. For instance, rhythmic changes in the expression of clock genes including brain and muscle ARNT-like 1 (*Bmal1*), nuclear receptor superfamily 1, group D, member 1 (Rev-erbα) Period circadian regulator 1 (*Per1*), *Per2*, and *Clock* have been identified in mouse bone marrow derived macrophages (256), peritoneal macrophages (257), splenic macrophages, DCs, and B cells (256). In human $CD4^+$ T cells, rhythmic changes in the expression of clock genes are synchronized with the production of IFNγ, IL-2, IL-4, and CD40L (258). In wild type mice, serum levels of LPS-induced cytokines display rhythmic changes (259), which are lost in *Bmal1* deficient mice. Similarly, rhythmic change in serum levels of IL-6 is lost in *Rev-erbα*–/– mice (259). These findings reveal that the rhythmic control of immune function is tightly regulated by an intrinsic circadian clock, and the available data currently support a stronger role of the circadian clock in the innate immune response.

How nutrition modifies the immune system is a continually evolving field of research. Early studies focused primarily on the effects of vitamins and trace elements on the immune function have been reviewed (260). For instance, deficiency in vitamin B6 impairs lymphocyte maturation, proliferation, antibody production, and activity of T cells. It also attenuates NK cell activities. Deficiency in folate attenuates proliferation of $CD8^+$ T cells and NK cell activities. Deficiency in vitamin B12 reduces total lymphocyte counts and the number of CD8+ cells. Vitamin C has also been shown to stimulate neutrophil chemotaxis, but its anti-inflammatory effects remain incompletely understood. Deficiency in vitamin A impairs phagocytosis and increases production of IL-12 and TNFα, which promotes inflammation. Deficiency in vitamin D impairs the innate immune response. Deficiency in trace elements including selenium, zinc, copper, and iron, can also disrupt the immune system. Comparisons between high and low fat diets have revealed impacts on cytokine levels that may impact the homeostatic balance between Treg and Th17 cells (261). Children following a Mediterranean diet for a year have higher salivary levels of an anti-inflammatory cytokine IL-10 and lower levels of IL-17 (262). A variety of dietary components, including red grape polyphenols, prebiotics, probiotics and symbiotics have been suggested to boost immune function in older individuals (263). Taken together, these findings establish an important role for diet-dependent immune-modulation, which may affect cancer susceptibility, as has been recently reviewed (263–265).

Several lines of evidence support a role for exercise in modulating immune function. Regular exercise and physical fitness can delay the onset of immunosenescence and tumorigenesis (265). Exercise improves the circulation and function of innate immune cells (266–268). Although the increase in immune cells is transient, it leads to a 40-50% decrease in the number of days with upper respiratory tract infection among adults during winter season (269). By contrast, exercise routines that induce muscle and tissue injury are pro-inflammatory and suppresses immune response transiently (265). Thus, the effects of exercise on the immune system appear complex and require further investigation.

Collectively, the data presented in this section outline numerous potential non-heritable sources of inter-individual variation in immune function. Taken together with the effects of aging, genetics, and the environment, these findings are consistent with a highly dynamic model of immune function. As with DNA repair, assessments of immune function at the individual level may provide important insights into disease susceptibility, but must be carried out in a manner that takes the many sources of variability into account. In the next section, we discuss a possible strategy for surveying both immunophenotype and genome integrity in human populations.

4 POTENTIAL FOR SIMULTANEOUS PROFILING OF IMMUNOPHENOTYPE AND GENOME INTEGRITY FOR MORE ACCURATE ASSESSMENTS OF CANCER RISK

Although DNA repair and immune function are distinct biological processes, they are subject to many of the same influences, and they both play important roles in cancer susceptibility. It has long been appreciated that several DNA repair pathways play integral roles in the immune system (270) (271). Furthermore, one of the most acute consequences of exposure to DNA damaging agents is suppression of the immune system (272) (273) (273). As outlined in this review, environmental exposures such as arsenic and passive smoking, circadian disruption, and lifestyle factors can modulate both DNA repair and immune function. It is noteworthy that defects in DNA repair and immune function are two of the most prominent hallmarks of cancer (2). Accordingly, efforts are underway to perform functional profiling in human populations, with the goal of identifying biomarkers that could be used for personalized prevention and treatment of cancer. While the idea of functional profiling has been framed independently in the context of genome integrity (13), and immune function (274), we propose that considering both simultaneously would increase the accuracy and robustness of cancer susceptibility predictions (**Figure 2**).

Patients with defects in nucleotide excision repair provide an excellent example of elevated cancer risk in individuals who are deficient in DNA repair and, perhaps, immune function (275–277). XP patients have a massively higher risk of developing UV-induced skin cancers (278), but also have an increased risk of developing internal tumors including glioblastoma, leukemia, lymphoma, and lung cancer (279, 280). The prevailing hypothesis regarding cancer susceptibility, both in XP patients and among those with lower NER capacity in the general

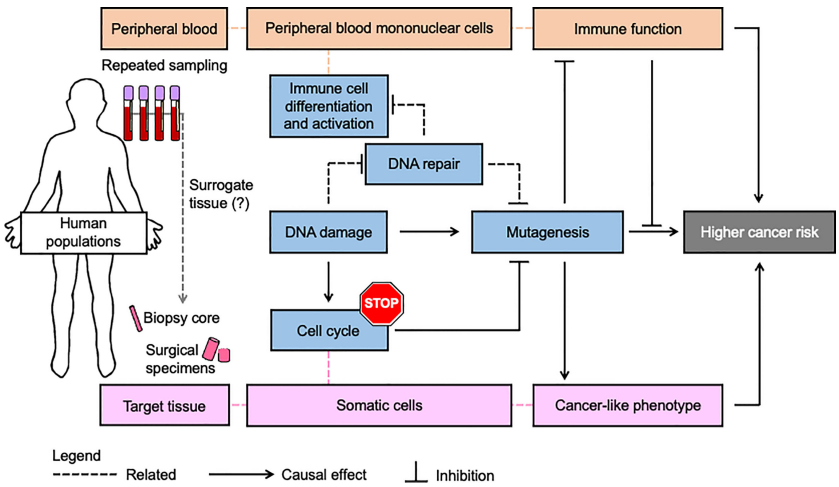

FIGURE 2 | Simultaneous assessment of genome integrity and immune function may be a more robust strategy for personalized prevention and treatment of cancer. Most population studies use blood samples to assess genome integrity and immune function because blood draws are less invasive than the procedures for collecting other tissues from human subjects. A key assumption is that fundamental processes in cancer etiology (blue boxes) as measured in blood (red boxes) are sufficiently related to be considered a surrogate for the corresponding target tissue (pink boxes). Since blood and its components are heavily involved in immune processes, this tissue can provide extensive insights into immunophenotype. Likewise, lymphocytes provide extensive insights into inter-individual variation in genome integrity mechanisms, including those underlying risk of numerous solid malignancies as reviewed herein. In addition to its role in preventing mutagenesis and immunosuppression that can be induced by DNA damage, DNA repair is extensively involved in the differentiation and activation of immune cells. Nevertheless, variation in immune function and genome integrity pathways is independent and challenging to predict from genetics and indirect genomic markers. Therefore simultaneous functional assessment of DNA repair activities and immune function in studies using blood may improve the accuracy and precision of cancer risk estimates beyond what is possible when considering either process alone.

population (14), has been that increased genome instability leads to higher rates of mutation and thus greater cancer susceptibility. However, it was noted in early case reports and small studies that XP patients also suffer from some forms of immune dysfunction (281) (275). Lymphocytes from XP patients have a larger clone size in response to allogeneic leukocytes (282), suggesting that lymphocytes of XP patients are more diverse, possibly due to a higher somatic mutational burden. Earlier studies have shown that lymphocytes from XP patients are less responsive to stimulation with mitogens (275, 277). Notably, serum from XP patients can attenuate the response of normal lymphocytes to PHA (275). A case study has also shown that DCs isolated from a trichothiodystrophy (TTD) patient with an XPD mutation have lower expression of CD86 co-stimulatory molecules and HLA glycoproteins, and are defective in stimulating native T lymphocytes (277). Notably, TTD patients commonly suffer from infections and there are several documented cases of immunodeficiency (283). Since some TTD patients do not appear to exhibit defects in DNA repair, these findings raise the possibility that NER proteins could have a role in immune function that is distinct from their role in DNA repair. NK cells from XP patients of multiple complementation groups display impaired lytic activity and lower IFNγ production in response to poly I:C stimulation, though the total NK cell count is normal (276). Moreover, XP patients have higher tolerance to the grafting of skin from a normal HLA-incompatible donor (275). Taken together, these findings suggest that innate and adaptive immune cell function may be defective in patients with nucleotide excision repair defects, but the underlying mechanism and the extent to which these findings may extend generally to patients with XP and other NER deficiency disorders remain unknown. Additional, comprehensive studies in larger cohorts of patients with NER deficiency are needed to assess whether their cancer-prone phenotypes can be explained in part by an accompanying immune defect. Such studies would also illuminate whether inter-individual variation in NER can be expected contribute to variation in immune function in the general population.

In the case of xeroderma pigmentosum variant (XP-V), patients express a truncated *POLH*, which reduces the expression and activity of DNA polymerase η (Pol η) (284). POLH is involved in translesion synthesis (TLS), which promotes tolerance of CPDs, thymidine dimers, and 8-oxoguanine lesions (285, 286). Loss of *POLH* leads to error prone-repair of CPDs by mutagenic polymerases zeta, kappa, and iota (287). Interestingly, UVA irradiation induces a mutational signature that suggests a role for basal mutagenesis induced by oxidative damage in the elevated risk for internal cancers in XPV patients (288). XP-V patients also have lower frequency of A/T mutation and higher frequency of deletion in Ig genes in activated B cells, which likely reflects the role of POLH in somatic hypermutation in B cells (289). POLH deficiency may thus drive the higher cancer incidence among XP-V patients *via* multiple mechanisms. In contrast with the severe combined immunodeficiency often associated with LIG4 syndrome due to disrupted V(D)J recombination (35), XPV patients do not

present with pronounced immunodeficiency, possibly due to compensatory activities of other polymerases in somatic hypermutation. A small group of patients deficient for a subunit of another polymerase (POLE) does exhibit immunodeficiency and points to the possibility for additional rare polymerase deficiency disorders yet to be discovered (290).

Population studies offer numerous opportunities for simultaneous investigation of immune function and genome integrity. In identifying cancer risks and associating genome instability with cancer outcomes, these studies almost exclusively rely on blood samples due to its safe and relatively less invasive sampling method when compared with other types of biopsies. Furthermore, the multitude of cellular and molecular markers of immune function in blood represent a rich source of information that can be paired with analyses of genome integrity in lymphocytes. Some studies have already taken advantage of the opportunity to measure both genome integrity and immune function in a single population. For example, it has been observed that immunosuppressive drugs suppress DNA repair in human PBMCs (291, 292). As discussed in the following section, emerging technologies have greatly increased the feasibility of simultaneous profiling of DRC and immunophenotype in human populations.

5 TECHNOLOGICAL ADVANCEMENTS THAT WILL HELP SHAPE THE FUTURE OF PRECISION MEDICINE

Significant technological advances have recently yielded functional tools for the interrogation of genome integrity and immune function. Here we review a sampling of emerging technologies that hold promise for enabling combined phenotyping with respect to DNA repair and the immune system in human populations.

As has been reviewed recently, several technologies are now available for analyses of genome integrity in human populations (13). Fluorescence-based multiplex flow-cytometric host cell reactivation (FM-HCR) assay measures the ability of live cells to repair site-specific DNA lesions (293). The assay is designed to have each fluorescent plasmid engineered to incorporate a specific type of DNA damage, including mismatches, abasic sites, oxidized bases, or DSB. The use of multiple fluorescent proteins enables multiplexing analyses for DNA repair activities. FM-HCR has thus been applied in a variety of settings, including in primary human lymphocytes (294–299).

The high throughput CometChip has been developed based on the established single gel electrophoresis assay (300, 301). Due to its 96-well format and automated image analysis, the CometChip is amenable to analyse large numbers of samples. It has recently been applied in a population study (302) and has been widely adopted for genotoxicity testing (303–305). CometChip technology has also been modified to interrogate DNA methylation status (306), levels of specific DNA adducts (307), and DNA damage in spheroids, which is also known as SpheroidChip (308).

A fluorescence-based unscheduled DNA synthesis (UDS) assay provides a substantially more convenient and user-friendly approach for measuring NER in populations. The original UDS assays used radio-labeled thymidine and autoradiography, making them laborious and inconvenient for routine clinical use (309). A new fluorescence-based method incorporates a thymidine analogue 5-ethynyl-2-deoxyuridine, which is conjugated to a fluorescent azide after UV irradiation and can be quantified by flow cytometry (310) (311). This technology is now being used to support the diagnosis of rare DNA repair deficiency disorders (312).

Single-cell whole-genome sequencing has opened up a new venue for studying somatic mutation and identifying mutational hotspots within the genome (313–316). This technology leverages single-cell multiple displacement amplification (SCMDA) procedure for detecting a full spectrum of base substitutions in a somatic cell. The technology has been used to reveal age-dependent changes in somatic mutations of B lymphocytes. The mutations in normal B lymphocytes not only resemble the COSMIC signatures in cancer (317), the data imply the age-dependent accumulation of somatic mutation is pivotal to the development B cell cancers (316). Thus, SCMDA, in combination with single-cell whole genome sequencing, is the tool for dissecting interindividual variation in mutation burdens influenced by genetics, age, environmental exposure, and lifestyle factors.

Single-cell RNA and DNA sequencing technology has advanced rapidly in recent years and found application in nearly every dimension of human biology (318). This technology analyzes the transcriptome of single cells within a heterogeneous population (319). It provides a powerful unbiased alternative to immunophenotyping approaches such as flow cytometry mass cytometry (CyTOF), which are less expensive but require labeling of surface markers and reveal little additional information at the single cell level (320) (321) (322). Single cell RNA sequencing enables the interrogation of cell-cell interactions, identification of changes during cell fate specification, and dissection of regulatory networks associated with cellular functions at single-cell level and based on cellular subtypes, which are not feasible in whole tissue analyses (323–326). Although single cell technologies remain expensive, continuous innovation raises the prospect of their eventual application in population studies. The emerging theory of clonal hematopoiesis of indeterminate potential (CHIP) describes the presence of somatic mutation in the cancer driver gene at a variant allele frequency of at least 2% in blood and bone marrow cells of a healthy individual (327–329). This process of clonal selection effectively amplifies mutations in a manner that makes them detectable by bulk sequencing. CHIP is induced by DNA damaging agents, and associated with increased risk of both leukemia and solid malignancies. It can thus be presumed to represent a molecular ruler that reflects both exposure to DNA damaging agents and the ability to repair DNA damage at the individual level.

Cellular indexing of transcriptomes and epitopes by sequencing, also known as CITE-seq, is a high throughput single-cell RNA sequencing analysis that is coupled with

epitopes to interrogate expression of cell surface proteins (330). Since immune cell subtypes express specific surface markers, which can be captured by specific epitopes, CITE-seq has been widely used for determining the transcriptome profile of specific immune cells within a heterogeneous population (331, 332). Though CITE-seq and single-cell RNA sequencing serve similar purposes, CITE-seq has a shallower sequencing depth and relies heavily on the protein expression of specific cell surface marker. Its design better fits for studying immune cells.

Historically, it has not been feasible to perform functional screens of such nuanced phenotypes as those associated with modest defects in genome integrity or immune function. But these emerging technologies, particularly when used in combination, will enable such studies. Since blood samples are routinely collected for molecular epidemiological studies that focus on either genome integrity or immunophenotyping, the tissue could be maximally leveraged to understand how both processes may interact and contribute to cancer risk. Furthermore, studies combining immunophenotyping with genome integrity assays may shed light on whether mild DNA repair deficiencies in the general population lead to increased cancer risk, at least in part, by limiting the efficiency of immune responses.

6 OPEN QUESTIONS AND FUTURE STRATEGIES FOR POPULATION STUDIES

Here we briefly propose a framework for future studies aimed at understanding the joint influence of inter-individual variation in DRC and immune function on cancer risk. We pose several questions in the field that we view as important areas to investigate, followed by broad recommendations for pursuing population studies at the intersection of immune function and genome integrity.

6.1 Open Questions

1. **Is blood a reliable surrogate for other tissues?** Blood is an extremely rich source of data, including a variety of immune cells, cytokines, circulating DNA, and small molecules that can be analyzed to assess immune function, DNA damage and repair, and environmental exposures (**Figure 3**). Because it can be collected relatively easily and in a repeated manner, sampling blood is also among the most feasible approaches for population studies. Nevertheless, circulating immune cells may not reflect the biology of tissue resident immune cells and tissue-specific microenvironments. For these reasons, whenever possible, ideal studies would include sampling the tissue of interest and, in the case of cancer studies, the tumor as well.

2. **Which immune markers are the best predictors of cancer risk and outcomes?** The emerging technologies described in the previous section provide an unprecedented opportunity for deep analysis of immunophenotypes, but because they have been developed so recently, they have only begun to be applied towards understanding the relationship between immune function and carcinogenesis. Studies surveying a broad array of immune markers are needed; these would include a census of circulating immune cells, measurements of cytokines, and tests for immune cell function.

3. **Which combinations of functional assays are the best predictors of cancer risk and outcomes?** Emerging functional assays described above and numerous established assays for immune cell activation and proliferation (333) integrate complex regulatory mechanisms and can complement 'omics approaches (genotyping, transcriptional profiling, proteomics and DNA sequencing). Functional assays for DNA repair often outperform polygenetic cancer risk scores (334), and even stronger associations are seen in limited cases where multiple functional assays for different pathways have been applied to the same set of samples (335). But it is not possible to predict which functional biomarkers provide the most useful information to support mathematical models that would predict cancer risk or cancer outcomes. Thus, cancer case-control studies should be designed to integrate as many functional assays as is feasible for the same set of subjects. Given the practical constraints of funding and expertise, biological materials should be banked appropriately to enable future analyses.

4. **How do markers of genome integrity and immune function change over the life course?** As detailed in **section 2** and **section 3**, the phenotypic markers we propose to survey with the goal of advancing personalized medicine are subject to time-dependent variation due to a variety of factors including lifestyle, environmental exposures, health status, and aging. To use these functional biomarkers as predictive tools, it will be necessary to carry out longitudinal studies wherein they are measured prospectively.

5. **Does NER contribute to immune function?** Numerous DNA repair pathways are already implicated in the mutagenic processes that occur during immune cell development and activation. In addition to those processes, emerging roles for DNA damage and DNA repair in gene regulation (336, 337), together with the growing recognition that many proteins "moonlight" in multiple roles within the cell (338), raise the possibility of as yet unrecognized mechanisms by which DNA repair pathway might contribute to immune function. By carrying out detailed immunophenotyping in individuals with profound defects in DNA repair, such as patients with XP, CS, and TTD, it can be determined whether NER deficiency, perhaps specifically which global genome (GG-NER) or transcription-coupled (TC-NER) NER subpathways, is associated with an immune disorder.

6. **Can stem cell-derived cells recapitulate DRC of primary human tissues?** A growing number of studies have found associations between DRC in blood cells and cancer risk, and the simplest interpretation is that the blood cells accurately represent genome maintenance in the tissue where the cancer develops. However, DNA repair varies with cell type and as a function of cell cycle and the tissue microenvironment. It is therefore possible that at least some of the associations between cancer risk and genome integrity as measured in immune cells is a reflection of immune cell function, rather than genome integrity in the target tissue. This question can in principle be unraveled by studies that measure DNA repair in multiple cell types from the same individual, but it likely will not be feasible to collect most

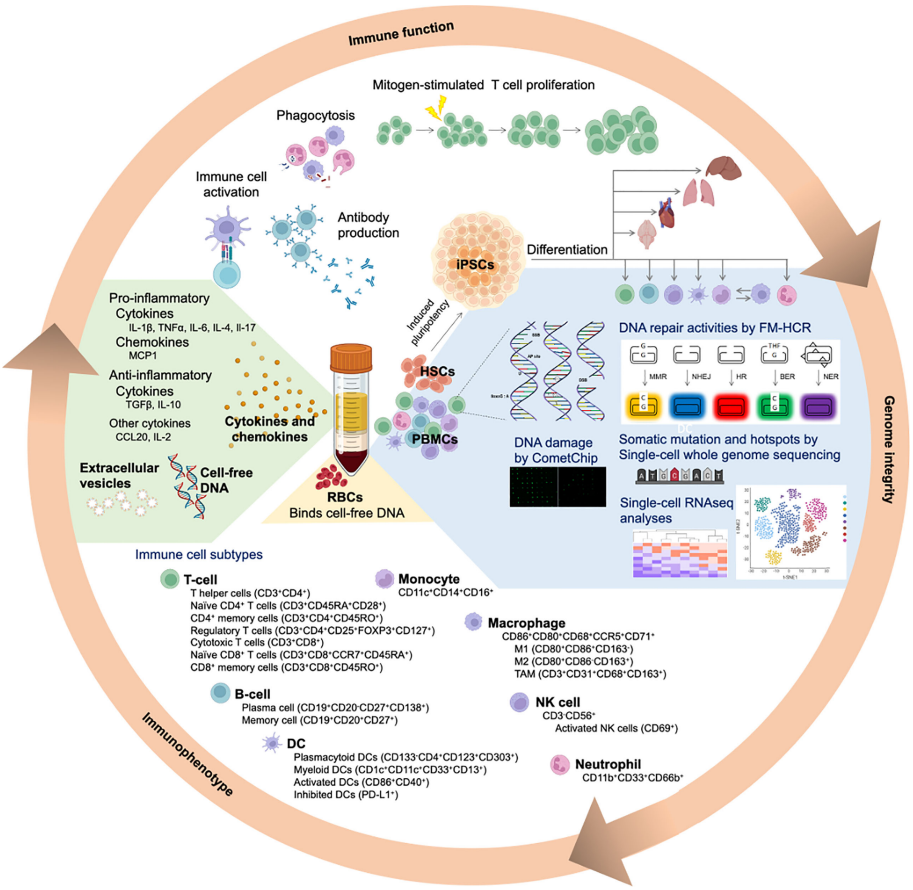

FIGURE 3 | Simultaneous assessment of genome integrity and immune function using human blood samples. Following density gradient centrifugation of peripheral blood, peripheral blood mononuclear cells (PBMCs) are enriched in the buffy coat layer. Different immune cell subtypes within the PBMC population can be further identified based on their specific cell markers. Genomic integrity of the immune cell subtypes can be comprehensively evaluated by integrating various complementary approaches. Fluorescence-based multiplex host cell reactivation (FM-HCR) evaluates the ability of cells to repair specific DNA lesions. The CometChip assay reveals the magnitude of genomic DNA damage and repair kinetics in a high throughput manner. Single-cell whole genome sequencing identifies somatic mutations, whereas RNAseq (CITE-seq and single-cell RNAseq) measure the transcriptome. Moreover, hematopoietic stem cells isolated from the blood sample can potentially be used to generate induced pluripotent stem cells (iPSCs). Upon differentiating these iPSCs into a somatic cell type of interest, it becomes feasible to obtain large number of patient-derived, tissue-specific somatic cells, which may otherwise be scarce or not feasible to obtain. Red blood cells (RBCs), which are enriched in the bottom layer, bind cell-free DNA to minimize inflammatory responses. The plasma layer contains cytokines and chemokines secreted from the immune cells. These signaling molecules can be pro-inflammatory or anti-inflammatory, depending on the cellular status and presence of antigens. Notably, cell-free DNA and extracellular vesicles (EVs) are present in the plasma.

tissues as part of a population study. By differentiating stem cells into cell types of interest, it may be possible to recapitulate physiological cell programming and make tissue-specific assessments of DRC on an individualized basis.

6.2 Recommendations

1. **Focus on human studies:** The framework we are proposing is at least in part discovery-based and centers human subjects, not biological model systems. This is a notable departure from the traditional approach more familiar to mechanistic biologists, wherein simple genetic models are used to test hypotheses before broaching the complexity of human systems. Instead, in this framework, one would first identify promising biomarkers in humans, and then follow up with confirmatory studies in model systems that best approximate the human biology. Taking the differences in telomere biology in mice and humans as an example (339), one can appreciate the value of prioritizing mechanistic characterization of biomarkers that have shown promise in human studies, and doing so in a model system that recapitulates the human biology. Though highly controlled genetic model systems such as CRISPR knockouts are not a feature of population studies, there are invaluable natural experiments and edge cases that can be leveraged for analogous purposes. For example, the phenotypes associated with rare genetic disorders that disrupt key aspects of genome maintenance and/or immune function such as those discussed in previous sections can be taken as upper or lower bounds for phenotypic variation in the general population. Likewise,

biological samples from patients undergoing therapy with immunogens, immunosuppressants, or DNA damaging agents provide opportunities to understand physiological human responses to potentially carcinogenic real-world exposures. This is particularly so when the studies are conducted longitudinally, such that functional assays can be applied to samples collected before and after the exposure. Samples from individuals participating in studies that collect detailed personal environmental monitoring data present similar opportunities, and hold the advantage of avoiding the potential bias introduced by focusing on individuals with pre-existing health conditions, as is common in clinical studies.

2. **Maximize the use and preservation of biological sample (s):** The comprehensive functional characterization of human populations we are proposing is ambitious and may require some realignment of funding agency priorities and philosophies to reach its full potential. The prioritization of hypothesis-driven research commonly constrains the scope of projects and forces researchers to make decisions to severely limit the collection and analysis of biological samples. However, as illustrated in **Figure 3**, biological samples have extraordinary potential to provide insights into the many mechanisms driving human variation. To address this mismatch in the meantime, researchers should preserve biospecimens as comprehensively as possible. In the case of blood samples, this would entail banking each of the components and preserving them in a manner that is compatible with future downstream analyses, which may require live cells, for example.

3. **Engage in team science:** Population studies that make use of emerging technologies to characterize biological samples are inherently interdisciplinary. It is generally not within the capacity of a single investigator to have the expertise needed for establishing a human study cohort, developing and applying new technologies, interpreting biological data that span multiple fields, and, when applicable, treating and evaluating patients. In addition to a diverse group of scientific and medical experts who cover the technical expertise, the team should ideally include stakeholders who stand to benefit from the research. These stakeholders can also guide the focus of the study from its inception and ensure that vulnerable and underserved populations are included.

7 CLOSING REMARKS

Many of the syndromes associated with defects in immune function or genome integrity have been discovered in recent years as genotyping technology has advanced. But these studies importantly relied upon functional characterization of variants of unknown significance, or the discovery of patients with a familiar disease of unknown etiology. The data suggest there are many more deficiency syndromes still to be discovered. Functional assays such as those outlined herein present powerful tools for identifying individuals with deficiencies in immunity or genome maintenance. By integrating these assays with modern genomics tools, it should be possible to accelerate the discovery and annotation of rare variants as well as functional associations with disease. Population studies are most easily carried out with blood, which contains the circulating cells and cytokines that can be used to define the immunophenotype. Therefore blood samples represent a largely untapped resource for analyzing both genome integrity and immune function simultaneously. Studies that compare these biological features between cancer patients and healthy counterparts will provide important clinical insights. Yet, simply surveying the complexity of the functional landscape across populations to define the range of variability is also a useful precursor to developing predictive models that incorporates the variability to explain disease susceptibility. Leveraging the advanced technologies and our current understanding of DRC, immune function, mutation, and cancer, it is timely to address these questions and improve the precision of strategies that assess and manage cancer risk for the welfare of population health.

AUTHOR CONTRIBUTIONS

Both AC and ZN developed the concept and defined the scope of the review. AC performed a literature review, organized the results, drafted the review, and composed the figures and tables. ZN supervised the development of the review and contributed to the writing and editing of the manuscript and its components.

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