Mutation and Microsatellite Burden Predict Response to PD-1 Inhibition in Children with Germline DNA Replication Repair Deficiency

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Abstract

Cancers arising from germline DNA mismatch-repair or polymerase-proofreading deficiencies (MMRD and PPD) in children harbour the highest mutational and microsatellite insertion/deletion (MS-indel) burden in humans and are lethal due to inherent resistance to chemo-irradiation. Although immune checkpoint inhibitors (ICI) have failed to benefit children in previous studies, we hypothesized that hypermutation caused by MMRD and PPD will improve outcomes following ICI in these patients. ICI treatment of 45 progressive/recurrent tumours from 38 patients revealed durable objective responses in the majority, culminating in 3-year survival of 41.4%. High mutation burden predicted response for ultra-hypermutant cancers (>100 mutations/Mb) enriched for combined MMRD+PPD, while MS-indels predicted response in MMRD tumours with lower mutation burden (10-100 mutations/Mb). Further, both mechanisms were associated with increased immune infiltration even in “immunologically-cold” tumours such as gliomas, contributing to the favorable response. Pseudo-progression (flare) was common and associated with immune activation in both the tumour microenvironment and systemically. Further, patients with flare continuing ICI treatment achieved durable responses. Our study demonstrates improved survival for patients with tumours not previously known to respond to ICI, including CNS and synchronous cancers, and identifies the dual roles of mutation burden and MS-indels in predicting sustained responses to immunotherapy.

Introduction

Accurate DNA replication in eukaryotic cells is ensured by the DNA polymerases Pol δ and Pol ϵ , which control base incorporation and proofreading, and the mismatch repair (MMR) system that undertakes post-replication surveillance.¹ Germline and somatic mutations in *POLD1* and *POLE* (termed polymerase-proofreading deficiency: PPD), or the MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*) result in DNA replication repair deficiency (RRD). RRD is a major driver of hypermutation and microsatellite instability (MSI) in several adult and pediatric cancers.^{2,3} Both germline PPD,⁴ and monoallelic germline pathogenic variants in the MMR genes (Lynch syndrome)⁵ lead to adult-onset gastrointestinal and genitourinary cancers.⁶ In contrast, biallelic loss of MMR function in the germline causes constitutional mismatch repair deficiency (CMMRD) syndrome, a highly penetrant and aggressive cancer-predisposing condition. Affected individuals typically develop cancers at a young age, most commonly malignant gliomas, gastrointestinal and haematological malignancies.^{7,8} These cancers are frequently chemo-resistant, resulting in poor survival for affected patients. Indeed, individuals with CMMRD rarely survive beyond early adulthood.⁷ The burden of CMMRD cancers is significant in areas with high consanguinity,⁹ including many developing countries and among indigenous populations.

RRD cancers are universally hypermutant due to the continuous acquisition of multiple somatic mutations. Tumour mutation burden (TMB) of cancers driven by germline RRD is 100 to 1000 times higher than MMR-intact pediatric cancers.¹⁰ Further, many MMRD tumours acquire a secondary somatic mutation in *POLD1/POLE* leading to combined MMR+PPD, characterised by ultra-hypermutation (>100 mutations/Mb).² As a result, RRD cancers harbour the highest TMB observed among all human cancers.¹⁰ Hypermutant cancers such as melanoma and lung cancer, which are driven by ultraviolet light and smoking, respectively, respond to immune checkpoint inhibitors (ICI)¹⁰⁻¹² targeting programmed death 1 (PD-1) signalling.¹³ However, despite the dramatic anti-tumour effects reported in several hypermutant adult cancers,¹⁴⁻¹⁶ these responses are sustained in only a subset of patients.^{17,18} The role of TMB in determining the nature and duration of response is not well-established¹⁹⁻²¹. Other studies have also raised questions regarding the role of TMB and PD-ligand 1 (PD-L1) expression as robust biomarkers of response to ICI.^{20,22-24} In contrast, MMR-deficient colorectal carcinomas are responsive to ICI due to excess MSI²⁵⁻²⁸, suggesting that biomarkers like TMB²⁹, MSI or PD-L1 expression may not be individually sufficient to drive immune responses following ICI across diverse cancer types^{19,21}.

Most cancers, including hypermutant brain tumours are considered ‘immunologically cold’ and are unresponsive to ICI.³⁰ Importantly, ICI did not result in significant responses in multiple large pediatric clinical trials and is considered ineffective in the management of solid tumours in childhood and adolescence.^{24,31-34} Finally, for all solid tumours receiving immunotherapy, the distinction between true tumour progression and an inflammatory pseudo-progression is a major challenge and a barrier to effective therapy³⁵⁻³⁸.

Despite the lack of response to ICI observed in children in previous studies, we hypothesized that cancers originating from germline RRD may benefit from ICI due to their excess mutational load¹². We further postulated that cancers driven by MMRD-only, PPD, or combined RRD (MMRD+PPD) will respectively exert their own unique mutational spectrum, driving local and systemic immune reactions, which would help shed light on the mechanisms of both response and pseudo-progression following ICI.

To address these hypotheses, we conducted a large, registry-based study, leveraging systematically-collected data gathered both retrospectively and prospectively through the International RRD Consortium^{2,7,10,2,39}. This enabled us to evaluate outcomes and predictors of response to anti-PD-1 therapy in children with cancers driven by germline RRD. Uniquely, this also provided us the opportunity to investigate the efficacy of ICI in individuals with synchronous malignancies who are otherwise excluded from conventional clinical trials.

Results

Thirty-eight patients who developed 45 cancers were treated with PD-1 inhibitors between May 2015 and March 2019 and followed by the International RRD Consortium registry study group (Methods and Extended Data Table S1). While guidelines for treatment were provided by the consortium, and regular communication was organized with the treating team for ongoing radiological monitoring, response assessment, and management of adverse effects (Methods), the patients were not treated prospectively as part of a clinical trial. The PD-1 inhibitor used was either nivolumab (n=34, 75%) or pembrolizumab (n=11, 25%), as per availability and choice of the treating team. All patients had germline RRD, diagnosed as CMMRD (n=28, 74%), Lynch (n=8, 21%), or PPD (n=2, 5%) syndromes (Extended Data Table S1). Median age at treatment was 12.1 years (range: 3.1-28.1) for patients with CMMRD, and 15.7 years (range: 8.5-43.4) for those with Lynch syndrome (p=0.07). Seven cancer types were included and classified into 3 major groups: central nervous system (CNS) tumours (n=31, 69%; disseminated: 2, 6%), non-CNS solid tumours (n=11, 24%; disseminated: 7, 64%), and haematological malignancies (n=3, 7%) (Fig.1a). The majority (n=43, 93%) of cancers were

progressive/recurrent after failure of first-line therapy (Extended Data Table S1). Three patients with gastrointestinal cancers received ICI directly following surgery: two of whom had synchronous CNS tumours and one who had metastatic disease. Data cutoff for outcomes was October 2019.

Responses and/or stable disease were observed in 25/45 (55.5%) tumours, with most of the responses (n=20; 80%) being sustained at a median follow-up of 1.87 years at the time of this analysis. Central radiological review (RANO and RECIST criteria; Methods)^{40,41} revealed complete response in 6 (17%), partial response in 9 (25%), stable disease in 7 (19%), and progressive disease in 14 (39%) (Fig.1b,c). Among the 7 patients with synchronous malignancies, responses in both tumours were seen in one patient, and at least in one tumour in 4 patients (Fig.2a). The three patients with haematological malignancies had progressed at a median time of 4.5 months after starting ICI therapy.

Of note, 12 (27%) tumours exhibited early radiological findings of edema and enhancement, suggestive of peri-tumoural inflammation or tumour progression (Fig.1b,d and 2a). This phenomenon occurred at a median of 34 days (range 7-74) from treatment initiation and has been termed tumour ‘flare’.⁴² These patients presented with acute clinical deterioration with headache, bone or abdominal pain, depending on the location of their tumours. Eight patients (6 with CNS and 2 with non-CNS solid tumours) stopped therapy and died. Importantly, 4 patients (3 with CNS and 1 with non-CNS solid tumour) who continued to receive ICI with adequate supportive care subsequently demonstrated objective responses. Because this suggested pseudo-progression,^{35,38,43} these tumours were studied in more detail.

Estimated 3-year overall survival (OS) was 41.4% (95% CI: 38.5, 44.2) (Fig.2b), with 18 (47%) patients being alive at the time of last follow-up (Fig.2a). This is noteworthy considering the refractory nature of their cancers. Analysis by cancer type revealed that non-CNS solid tumours had a significantly better survival compared to CNS tumours (Fig.2c and Extended Data Fig.1d; p=0.01). Nevertheless, the OS of 39.3%, (95% CI; 36.3, 42.3) and PFS of 26.9% (95% CI; 23.2, 30.6) for patients with recurrent/progressive CNS tumours is a dramatic improvement compared to the historically rapidly fatal outcomes (Fig.2c,d and Extended Data Fig.1c)^{7,9,12,44}. All patients with non-CNS solid tumours continuing ICI had durable responses and are alive at a median follow-up of 2.6 years (range: 0.38-3.5). Remarkably, 13 patients with CNS tumours continued to survive for an additional 9.6 months (median; range: 1.5-27) after radiological progression (Fig.2a,d and Extended Data Table S1). Collectively, these data suggest that late and continued responses to immunotherapy possibly due to the dynamic clonal evolution and obligatory mutation accumulation in RRD cancers¹⁰.

Molecular determinants of response to immunotherapy

To better understand the molecular determinants of response of RRD cancers to ICI, biopsy specimens and blood samples were collected before and during therapy from the patients for centralized analysis (Methods). Whole exome analysis of tumours (n=39, Fig.3) revealed high variability in the number of single nucleotide variants (SNV), including ultra-hypermutation (median: 233.8 mutations/Mb, range: 3.4-912), which was associated with tumour genotype. MMRD-only cancers (n=16) had significantly fewer SNVs (median: 15.8 mutations/Mb) than MMRD+PPD cancers (n=23, median 391.4 mutations/Mb; $p < 0.0001$; Fig.4b). This correlated with germline status, as 21 (67.7%) cancers in CMMRD patients harboured MMRD+PPD (median: 398.98 mutations/Mb), while all cancers in individuals with Lynch syndrome lacked somatic PPD (median: 21.76 mutations/mb; $p=0.03$). Both cancers originating from germline PPD, one colorectal carcinoma (P13; ICI.29) and one glioblastoma (P17; ICI.33) had acquired somatic MMRD resulting in ultra-hypermutation (Fig.2a and 3).

Analysis of COSMIC signatures, which reflect the imprints of the underlying RRD mutational processes,^{45,46} revealed that signatures 6 and 26 were enriched in MMRD-only cancers, and that all tumours in patients with Lynch syndrome exhibited signature 6. In contrast, signatures 14 and 15 were frequent in cancers in patients with CMMRD, and signature 14 was enriched in tumours with MMRD+PPD, highlighting the unique and potential diagnostic role of these signatures in determining germline predisposition in RRD cancers.⁴⁵

As questions exist regarding the contribution of tumour-intrinsic characteristics such as mutation load and MS-indels to ICI response, we sought to determine whether response is modulated by a dynamic interaction between these factors.⁴⁷ TMB correlated significantly with both response and survival. This was true for tumours with higher total SNVs/Mb ($p=0.003$, Fig.4a), as well as non-synonymous variants, synonymous variants and total indels (Extended Data Fig.2a-d). Both response and survival were also associated with a higher tumour neoantigen load (Extended Data Fig.2e). Remarkably, response and survival were predicted by RRD status. Cancers with MMRD+PPD (median: 392.7 mutations/Mb) had higher likelihood of response compared with cancers with MMRD alone (median: 15.7 mutations/Mb) ($p<0.0001$) (Fig.4b).

Recent work has shown that response in MMRD tumours is related to the degree of genome-wide MS-indels.^{3,48,49} Across our entire cohort, total MS-indels, calculated by MS-mutect (Methods),⁵⁰ were not predictive of tumour response, but still predicted improved patient survival (Fig.4c). We did not find a significant association between high MS-indel and

TMB ($p=0.47$), thereby suggesting independent roles for both carcinogenic mechanisms for likely different tumour subsets. We therefore hypothesized that MS-indels are immunogenic in MMRD-only cancers, where the contribution of TMB is less dominant. To test this, we analyzed MMRD-only and MMRD+PPD tumours separately (Fig.4d). In MMRD-only cancers, total MS-indels were higher in responders than in non-responders, and were significantly associated with survival ($p=0.025$, Fig.4d). This was not observed in MMRD+PPD cancers with high TMB (Fig.4d). Further, in MMRD-only cancers, high TMB failed to predict response ($p=0.52$). Combining the prediction models for both these types of RRD cancers (MMRD-only and MMRD+PPD), high SNVs and total MS-indels together strongly predicted improved survival (Fig.4e, $p=0.0024$).

To determine whether SNVs and MS-indels drive response within a more homogeneous cancer type, we interrogated both these genomic signatures in CNS tumours. Both components contributed independently to the response and survival (Extended Data Fig.3c,g). In multivariate analysis (Methods), high mutation burden remained the most significant predictor for response ($p=0.01$) and OS ($p=0.05$) (Extended Data Table S2 and S3), highlighting the important contribution of a combined MMRD+PPD status in determining response to ICI. Collectively, these data suggest dual roles for TMB and MS-indels in determining immunotherapy responses in RRD cancers.

Immune microenvironment and response to therapy in childhood RRD cancers

Next, we examined whether the RRD subgroups affect the tumour micro-environment and response to therapy. We tested multiple immune markers using immunohistochemistry for immune cell infiltration (CD3, CD4, CD8 and CD68) and checkpoint ligand (PD-L1) expression (Methods, Fig.5 and Extended Data Fig.4). All immune markers were scored blindly by our central pathologist (CH, Methods).

PD-L1 expression correlated with both response and improved survival ($p=0.04$, Fig.5a). Overall, increased lymphocytic infiltration within the tumour microenvironment correlated with response (Extended Data Fig.4a,b). Specifically, high CD8-T-cell infiltration predicted both response and improved OS ($p=0.0002$, Fig.5b). All non-CNS solid tumours including those with MMRD-only, harboured high MSI, exhibited high CD8+T-cell infiltration (Extended Data Fig.5a,b), and responded to ICI. (Extended Data Fig.5a-c). This corroborates previous reports in which MMR-deficient gastrointestinal tumours had high CD8+ T-cell infiltration.⁵¹ High T-cell infiltration was also observed in CNS tumours which are traditionally considered an ‘immune-privileged’ site.⁵² CNS tumours with a high mutation burden and

MMRD+PPD not only had increased CD8+ T-cell infiltration, but also significantly higher expression of PD-L1 ($p < 0.04$; Fig.5c). This suggests that in the setting of ultra-hypermutation driven by combined MMRD+PPD and high genomic MS-indels, the increased activation of the immune microenvironment can explain the remarkable responses seen in the CNS tumours. Indeed, in these CNS tumours, high CD8+T-cell infiltration was associated with improved OS (Extended Data Fig.6a,b). Collectively, tumours exhibiting high expression of immune markers and a favorable genomic profile (high SNVs or MS-indels) had a 3-year OS of 87.8% (95% CI; 84, 91.5) as compared to 33.2% (95% CI; 27.2, 39.2) for tumours lacking these biomarkers ($p = 0.005$, Fig.5d).

Tumour flare is an immune reaction to therapy

To determine etiology of the flare phenomenon, we first analyzed the genomic and immune markers of these tumours. Cancers developing flare had pre-treatment genomic and immune characteristics similar to the responders without flare (Methods; Extended Data Fig.7a-d). We then compared the pre- and on-therapy tumours in two patients who had further surgical debulking at flare. Transcriptomic analysis and immune inference using single state deconvolution (Methods) revealed an increase in the overall immune cell expression at flare (Fig.6a,b). Notably, transcriptome signaling revealed that activated CD8+ T-cells were significantly increased in both samples following ICI. Using T-cell receptor clonotype analysis (TCR, Fig.6c,d), we observed a dramatic increase of T-cell repertoire in the post-treatment samples as compared to their baseline. One sample demonstrated an increase in both clonality and diversity of the T-cell population, with some original clones expanding during flare (Fig.6c), whereas for the other sample, there was reduction in diversity but significantly heightened clonality in the T-cell population (Fig.6d). Additionally, both increased CD8+ T-cell infiltration and PD-L1 expression were observed in both tumours during flare when compared to their pretreatment samples (Fig.6e,f and Extended Data Fig.8a,b). These observations suggest a pre-existing (specific) immune response and further ICI-driven (non-specific and specific) intra-tumoral immune expansion at flare.

We next investigated whether this immune activation could be observed systemically. We performed serial flow cytometry analysis of blood samples from multiple patients, prior to treatment initiation and within the first 90 days of initiation of ICI (Methods). Flare was associated with an expansion of peripheral CD8+T-cells expressing Ki67 (a marker of proliferation) and 4-1BB (a member of the TNF receptor family) (Extended Data Fig.9a-d and Fig.6g). Detection of Ki67+ CD8+ T-cells has been previously reported to be associated with

superior response to PD-1 blockade.⁵³ 4-1BB is well-known as a marker of T-cell activation and has co-stimulatory activity for activated T-cells.^{54,55} Flare was associated with higher proportion of 4-1BB+ CD8+ T-cells in the peripheral blood as compared to non-responders, as well as responders without flare (Fig.6h). Studies have previously shown that 4-1BB+ CD8+ T-cells correlate with response to PD-1 blockade.⁵⁶ Importantly, we did not find an increase in the 4-1BB+ CD4+ T-cell population (Extended Data Fig.9e,f), supporting our hypothesis that CD8+ T-cells expressing 4-1BB reflect the expansion of a tumour-specific response. Taken together with the immune profile of the tumour microenvironment, we conclude that flare following PD-1 blockade is associated with proliferation of tumour-antigen reactive T-cells.

Discussion

Our study reveals dramatic responses to PD-1 blockade and an associated improved survival for relapsed/refractory hypermutant cancers in children and young adults with germline DNA replication repair deficiency. Several insights can be derived from the sustained responses in different tumour types, and the contributions of TMB, MS-indels and the microenvironment to both response and flare.

The unique responses to ICI in children with germline RRD are different from previous observations by several groups. First, our data starkly contrast with the lack of ICI efficacy in childhood cancers in general³²⁻³⁴, as well as specifically for progressive paediatric brain tumours⁵⁷. The lack of response in paediatric cancers, which is independent of PD-L1 expression²⁴ or systemic immune activation^{24,33}, has been attributed to the low mutation burden^{2,10,12}, a different immunomodulatory role of the gut microbiome⁵⁸ at a very young age^{24,32,34}, low expression of major histocompatibility complex⁵⁹ and the predominance of macrophages in the tumour micro-environment^{60,61}. Some of these causes may need to be re-examined in view of our data.

Second, more than half of RRD paediatric CNS tumours had partial to complete objective responses, resulting in a median survival of 2 years when ICI was continued. This is remarkable, as historically, rapid progression with a median post-relapse survival of merely 2.6 months has been reported in children with RRD high-grade glioma.⁶² In contrast, hypermutant gliomas in adults fail to respond to ICI. These are different from RRD gliomas as they harbour relatively lower TMB with late acquisition of sub-clonal mutations during tumorigenesis possibly due to treatment-related secondary MMR deficiency, which is associated with less MS-indels, and lack of robust neoantigens.⁶³⁻⁶⁵ This is accompanied by multi-layered immunosuppression with a non-inflamed tumour microenvironment dominated

by a myeloid infiltrate, exhausted T-cell phenotype, and secretion of suppressive cytokines/molecules and hostile physical factors (hypoxia, acidosis and nutrient deprivation)^{63,64}.

Third, all patients who continued ICI therapy for non-CNS solid tumours including disseminated cancers responded and are alive at a median follow-up of 2.6 years. This compares favourably with MMR-deficient cancers in older patients (median age: 60 years, versus 12.3 years in our study), in whom late failures were noted, resulting in 50-55% survival at 2 years.^{26,27,66,67}

Collectively, the dramatic responses and favourable outcome observed in childhood RRD cancers can be explained by several key biological features. First, the earlier onset¹⁰, combined with significantly higher mutations², MS-indels³, and neoantigen burden drive CD8+T-cell activation which is especially robust in children and adolescents when compared to older patients⁶⁸⁻⁷⁰. Second, the additional loss of the polymerase-proofreading mechanism confers genomic mutational signatures (both MS-signatures³ and COSMIC signatures 10, 20, and 14) which may play unique immunogenic role in determining response and survival. Third, the exceptionally high rate of obligatory and continuous accumulation of mutations in combined MRRD+PPD cancers¹⁰ likely confers ongoing immunogenicity, contributing to immune-surveillance leading to both the durable and the delayed responses observed in our cohort.

Additional insights from this study include the ongoing and delayed responses observed in tumours that progressed on ICI therapy. This resulted in the difference between progression-free (post-ICI) and overall survival for patients who continued treatment after a second progression while on ICI therapy (Fig.2d and Extended Data Fig.1b,d). Additionally, responses in a tissue-agnostic manner were seen in a patient with synchronous tumours exhibiting favourable genomic and immune biomarkers for response (P01). As cancer immune surveillance is different than the irreversible resistance which occurs upon progression after chemo-radiation approaches, these data support the exploration of neoadjuvant^{71,72}, maintenance, and combinatorial uses of ICI in these patients, to limit toxicities and improve effectiveness of first-line strategies.

There is increasing evidence that responses to ICI cannot be predicted by single biomarker.^{73,74,19,75} Our data confirm that this is true in RRD cancers which are driven by dysfunction in both SNV and MS-indel repair. Although initially TMB was thought to be the sole contributor to immune response in hypermutant cancers²², indels and MS-indels have been suggested to be the main drivers of response to ICI in MMR-deficient cancers^{3,49,76}. We add a

new dimension to this concept by revealing that in cancers driven by MMRD-only, TMB is relatively lower, and MS-indels drive response, while in MMRD+PPD cancers, the role of MS-indels is attenuated, and TMB is the major driver of response. The dual roles of both mutational mechanisms also affect the microenvironment, with upregulation of PD-L1 and infiltration of CD8⁺ T-cells. Importantly, the combination of both genomic mechanisms and immune markers are powerful predictors of survival in RRD cancers and should be incorporated as combined biomarkers in future clinical trials.

Paradoxically, this hyperactivation of the immune microenvironment can be detrimental, because tumour flare, which indicates both specific (pre-existing) and non-specific (new) expansion of TCR clones, can be misinterpreted as tumour progression and lead to premature treatment abandonment. Flare is quite common in RRD cancers, especially when tumour burden is high.¹² These flare responses are very different from hypermutant adult glioblastomas originating as a result previous chemo-radiotherapy⁶⁵ where hyper-progression is reported.^{37,77} Our data support the development of novel functional imaging techniques⁷⁸ and minimally invasive biomarkers^{79,80} to better predict tumour response, and innovative strategies to modulate the immune response. This is important, as continuation of immunotherapy can result in late responses and long-term survival for patients developing tumour flare.

Despite the limitations of a registry study, this is the first description of a large cohort of children and young adults with previously recurrent/progressive fatal germline DNA replication repair deficient cancers demonstrating impressive responses to PD-1 blockade.⁸¹ Although longer follow-up is required to determine whether immunotherapy can be a curative strategy for RRD cancers, the sustained responses and lack of late relapses in a significant number of patients in this cohort are encouraging. This study also sheds light on the complex interplay between the tumour genomic status, microenvironment, and the systemic immune response, especially in the context of extreme mutation and MS-indel burdens. Future trials should prospectively analyse the roles of germline versus somatic RRD, and the components of the RRD machinery, to identify patients who are likely to derive maximal benefit from anti-PD-1 immunotherapy. Lastly, our study highlights the impact of studying a genetic cancer syndrome to understand general cancer processes⁸² and deriving direct therapeutic implications for patients.

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Author contributions:

DM, UT and EB planned the study. AD and UT wrote the manuscript. AC and MS reported the radiology. NA and CH reported the pathology. SS, AS and TP were responsible for the bioinformatics analysis. JC, YM and GG were responsible for the microsatellite indel analysis. SS and PO were responsible for the flow cytometry studies. VB and ME were involved in patient enrolment and sample coordination. Those involved in patient management included EB, DM, VC, LFN, LS, SC, MO, RD, GAC, SC, VL, AR, MO, GM, SL, AB, VM, EO, RLDM, AD, MS, CF, DS, DS, KC, SC, MM, PT, DZ, BG, AVD, NH, DG, RM, KEN, MZ, OM, DM, TL, AL, DM, TS, LYY, JK AB, LH, SLF, SZ, IS, DS, PD, MT, AK, DT, DM, AV, MA, CD, MA and UT. All authors have reviewed and agreed to the contents of the manuscript.

Competing Interests: None

ONLINE METHODS

Study design and patients

Patients included in this retrospective registry study were identified through the International Replication Repair Deficiency Consortium (IRRDC), based at the Hospital for Sick Children (SickKids), Toronto, that has enrolled 200 patients from 45 countries since 2007. Patients with confirmed or suspected RRD were eligible to participate. Institutional ethics board approval was obtained. Consent was obtained from patients and families for participation in the study. This included submission of clinical and imaging data, as well as tissue and blood samples for centralized analysis. Germline diagnosis of CMMRD, Lynch or PPD were confirmed by the genetic counsellor from the consortium. This was done based on the results of the family history, next-generation panel sequencing of germline samples for *MMR* and *POL* genes (performed locally or centrally at CLIA-approved laboratories), and immunohistochemical (IHC) staining pattern of the tumour and normal tissues (performed at the Hospital for Sick children, Toronto). Thirty-eight patients with 45 cancers who had received treatment with anti-PD1-directed immune checkpoint inhibitor (ICI) therapy were identified and are reported here. Guidelines for ICI treatment were provided by the consortium. Regular communication was organized with the treating team to discuss clinical responses, along with ongoing radiological monitoring, as well as management of adverse effects of ICI. Monitoring for toxicity and stopping rules were as per the recommendations provided by an ongoing clinical trial (NCT02992964). However, patients were not treated prospectively as part of a clinical trial. Ultimately, the choice of the agent and the nuances of therapy remained at the discretion of the treating team. Blood samples were collected prospectively throughout the course of ICI therapy at specified time-points.

Clinical records were reviewed to obtain details regarding patient demographics, cancer diagnosis, date of initiation and completion of ICI, choice of ICI agent, and survival outcomes (including date of disease progression and/or patient death). Imaging at baseline and following initiation of ICI were obtained for central review of objective tumour response. For the remaining cases, response (or otherwise) was determined by the assessment of the local treating team. Available scans were centrally reviewed by a radiologist blind to the clinically determined response, and tumour measurements were documented according to the RANO criteria.⁴⁰ Briefly, the best tumour response was determined as the percentage change in the product of bi-perpendicular dimensions from baseline on the contrast-enhanced T1 images. Complete response (CR) was defined as the disappearance of all enhancing disease (measurable and non-measurable) sustained for at least 4 weeks, with stable or improved non-

enhancing FLAIR/T2W lesions, no new lesions and clinical stability. Partial response (PR) was defined as 50% or more decrease of all measurable enhancing lesions sustained for at least 4 weeks, with no progression of non-measurable disease, stable or improved non-enhancing FLAIR/T2W lesions, no new lesions and clinical stability. Stable disease (SD) was defined as images that did not qualify for complete response, partial response, or progression, with stable non-enhancing FLAIR/T2W lesions and clinical stability. Progressive disease (PD) was defined as 25% or more increase in enhancing lesions, with increase (significant) in non-enhancing FLAIR/T2W lesions, not attributable to other non-tumour causes any new lesions, and clinical deterioration not attributable to other causes. For patients with non-CNS solid tumours, the revised RECIST (version 1.1) was used.⁴¹ CR was defined as complete disappearance, PR as at least 30% decrease in sum of the diameters, PD as at least 20% increase in the sum of the diameters of the target lesion, and SD as lack of sufficient change to be classified as CR/ PR/ PD. Patients with objective radiological response (CR/PR) and/ or stable disease (SD) were labelled as ‘responders.’ Among those with progressive disease, patients experiencing rapid early clinical and/or radiological deterioration (with >100% increase in tumour size within 90 days of starting ICI therapy) were defined as demonstrating a ‘flare’ response and were studied in more detail. For patients able to continue ICI, subsequent imaging was reviewed to confirm response or progression.⁴³ Those with sustained clinical and/or radiological progression despite continuation of ICI treatment were classified as ‘non-responders.’ For biomarker prediction analyses, ‘responders’ also included those with an initial ‘flare,’ who continued on ICI and demonstrated delayed responses.

Whole exome sequencing (WES) and analysis

Genomic DNA from 39 tumours, along with matched germline blood samples, was extracted using the PaxGene Blood DNA Extraction Kit (Cat No./ID: 761133) for blood samples, Qiagen DNeasy Blood & Tissue Kits (Cat No./ID: 69504) for frozen tissue, MasterPure Complete DNA and RNA Purification Kit (Epicentre #MC85200) for paraffin embedded tissue). WES was performed at The Centre for Applied Genomics (TCAG), SickKids, using SureSelect Agilent All Exon v5 kit, followed by sequencing (150X) on Illumina HiSeq 2500. The software bcl2fastq2 v2.17 was used to generate raw fastq files. Alignment to the hg38 reference genome, followed by pre-processing and QC was adapted from the GATK standard pipeline, using BWA-MEM 0.7.12 (alignment), BAMQC, Picard 2.6.0 (QC). Somatic variant calling was done post-alignment, using processed bam files from tumour and matched normal samples, to call both single nucleotide variants (SNVs) and

insertion deletion (indel) variants. A consensus vcf file of shared variants across 2 or more of 4 variant callers (Mutect v1.1.5, GATK v3.6/Mutect2, Strelka v1.0.14, and VarScan2 Somatic v2.4.2) was generated for SNVs and indels separately, using VCFtools 0.1.15, and these vcfs were annotated using VEP v83. The tumour mutation burden (SNVs per megabase) from WES data was calculated by counting total number of somatic SNVs divided by total number of callable bases in megabases (~50Mb). DeconstructSigs⁸³ was used to determine COSMIC signatures⁴⁶ in the mutation spectrum within a tri-nucleotide context for each sample. All bioinformatics analyses were performed on the SickKids High Performance Cluster (HPF) and the UHN High Performance Cluster for Health (H4H).

Neoantigen calling

The Mutect2 vcfs generated for each tumour (described above) were used as input along with bioinformatically generated HLA-typing (using a consensus of HLAminer, and HLAVBSeq) of germline WES fastqs using MuPeXI,⁸⁴ to get a list of strong binding candidate neoantigens per HLA-type.

Microsatellite (MS)-indel calling

Microsatellite indels were called on the bam files of tumour and matched normal samples, using an in-house pipeline using MSMuTect. The detailed methods for this algorithm have been previously reported.⁵⁰ Briefly, repeats of five or more nucleotides were considered to be MS loci, and using the PHOBOS algorithm and the lobSTR approach, tumour and normal BAM files were aligned with their 5' and 3' flanking sequences. Each MS-locus allele was estimated using the empirical noise model, which is the probability of observing a read with a microsatellite (MS) length k and motif m , where the true length of the allele is j with the motif m . This was used to call the MS alleles with the highest likelihood of being the true allele at each MS-locus. The MS alleles of each tumour and matched normal pair were called individually, which were compared to identify the mutations on the tumour MS-loci. The Akaike Information Criterion (AIC) score was assigned to both the tumour and normal models, and a threshold score that was determined by using simulated data was applied to make the final MS-indel call.

Immunohistochemistry

Four-micron thick sections of formalin-fixed paraffin-embedded (FFPE) surgical specimens were stained using an automated stainer (Dako OMNIS) with the following primary

antibodies: PD-L1 (clone28-8, Abcam, 1/500), CD68 (Clone c8/144B, Dako OMNIS), CD8 (Clone c8/144B, Dako OMNIS), CD3 (polyclonal rabbit, Dako OMNIS), and CD4 (Clone SP35, Sigma- Aldrich, 1/50). Quantitative evaluation of the immunohistochemical stains was performed by examining each section using at least five to seven different high-power fields with the most abundant tumour-infiltrating lymphocyte areas. The tumour was considered PD-L1 positive if $\geq 1\%$ of tumour cells exhibited a circumferential and/or partial linear plasma membrane PD-L1 staining of tumour cells at any intensity.⁸⁵ The percentage of infiltrating immune system cells was estimated by manual eyeballing as none, mild, moderate, and severe (0=none, <10%=mild, 10-50%=moderate, >50%=severe). For downstream analyses, infiltration higher than the median values of the continuous data of immune infiltrates was used to classify tumours as ‘high’ or ‘low’ infiltration for each marker.

Immune inference analysis

RNA was extracted as per standard kit protocol from tumours biopsied at both, baseline and time of flare, in 2 patients (P31 and P33) and submitted for total r-RNA depletion RNAsequencing on HiSeq 2500, at TCAG. Following sequencing, the raw data were analyzed using STAR aligner followed by RSEM expression analysis to generate a gene expression matrix for each sample. This was then run through CIBERSORT,⁸⁶ a single state deconvolution algorithm, to generate immune inference data for 22 immune cell subtypes. The immune inference results from CIBERSORT were plotted using “ComplexHeatmap”⁸⁷ package on R 3.5.

T-cell receptor rearrangement repertoire profiling

Genomic DNA was extracted (methods as above) from tumours biopsied at both baseline and at time of flare in 2 patients (P31 and P33). These were transferred to the Pugh laboratory at the University Health Network (UHN) in Toronto, where library preparation and capTCRseq⁸⁸ hybrid capture were performed. Following library preparation, the samples were sequenced first on a MiSeq for QC purposes and then 300ng of each sample, pooled in a ratio of 1:1:1, was processed for a 3-step capture using target hybrid capture panel.⁸⁸ Post-capture QC was performed on a MiSeq, followed by sequencing of up to a depth of ~2 millions reads on the NextSeq. Post-sequencing the raw data were analyzed using MiXCR version 2.1.12, ‘iNext’, ‘immunarch’ R packages and Pugh Lab customized functions on R version 3.5 to look at T-cell receptor rearrangements in the form of unique clonotypes (VDJ rearranged sequences) for T-cell receptors alpha, beta, gamma and delta. As the total read depth varied across the

cohort, affecting the total successfully aligned reads, all raw fastq reads were down-sampled to ~1 million reads. QC parameters of percent aligned reads, reads used in clonotypes, final clonotype count and the total number of clonotypes per 1000 reads were considered.

Flow cytometry

Viable frozen peripheral blood mononuclear cells were incubated with Fc block (BD Biosciences) prior to staining for surface markers (anti-CD3 - clone UCHT1, anti-CD4 – clone RPA-T4, anti-CD8 – clone RPA-T8, anti-4-1BB– clone 4B4-1, anti-TIGIT – clone MBSA43, anti-Ki67 – clone 20Raj1) and viability dye (eBioscience). Cells were fixed and permeabilized for intercellular staining with the Foxp3 transcription factor staining buffer set (BD). Flow cytometry voltages were set using Rainbow beads (Spherotech) with the same setting between experiments. Samples were acquired on a BD LSR Fortessa flow cytometer and data were analyzed using the FlowJo software.

Statistical analyses and reproducibility

Overall survival (OS) and event-free survival (EFS) was estimated using Kaplan-Meier statistics and determined from the date of initiation of ICI therapy. Patients without an event were censored at the date of last known contact. Uniquely in this population, several patients had multiple separate synchronous malignancies and therefore in these analyses, survival is presented for each individual cancer/tumour in addition to analyses per patient. For example, for the tumour-wise analysis, a patient experiencing an event related to one cancer diagnosis, would be shown as censored (rather than an event) for a second synchronous cancer diagnosis. Multivariate analysis was performed using binary logistic regression analysis for independent variables for predicting response. Cox regression analysis was performed for predictors of overall survival. Variables used in multivariate analysis included age, gender, site, ICI agent, higher mutations (SNVs/Mb) and higher MS-indels (than the median). Statistical analyses were performed with SPSS version 20, R version 3.5 and Python version 2.7. Statistical significance was calculated using Welch's unequal variances t-test and the Wilcoxon–Mann-Whitney test, for parametric and nonparametric data, respectively. All p-values were 2-sided, with a cut-off of 0.05 for significance. Plots generated were edited for aesthetics using Adobe Illustrator version 23.0.1.

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MAIN FIGURES AND LEGENDS

Fig. 1. Clinical response to ICI across cancer types in patients with germline DNA replication repair deficiency. (a) Distribution of tumour types across 38 patients who developed 45 tumours. **(b)** Waterfall plot of all radiological responses in non-haematological malignancies. Values show the best fractional change in the 2 dimensions from baseline measurements as per RANO and RECIST criteria. Arrows point to representative T2-weighted FLAIR and T1-weighted contrast-enhanced MRI sequences in two patients showing flare and partial responses.

Fig.2. Patient outcome and survival by tumour type. (a) Swimmer plot by patient and tumour type. **(b)** Kaplan-Meier (KM) estimates of overall survival for all patients **(c)** KM estimates of overall survival as per tumour type. Median survival for CNS tumours was 21.6 months. Median survival was not reached for non-CNS solid tumours. **(d)** KM estimates of progression free and overall survival for CNS tumours continuing ICI therapy. Note: prolonged median survival at 24 months (estimated 3-year OS=49.1%) despite initial radiological progression at a median of 9.9 months (estimated 3-year PFS=32%).

Fig. 3. Genomic biomarkers, survival and response to PD1 blockade. (a) Onco-plot summarising the genomic features from 39 available paired tumour and germline exomes, and their clinical correlates. **(b)** Response and overall survival (OS) by single nucleotide variants (SNVs) per Mb. For survival analysis, median SNV burden was used. **(c)** SNVs as a function of MMRD (blue) and MMRD+PPD (orange) status (left), and response association with both SNV and RRD status. **(d)** Response and overall survival (OS) by microsatellite indels (MS-indels). For survival analysis, median MS-indel values were used. **(e)** Response and overall survival by total MS-indel count for MMRD and MMRD+PPD cancers separately. **(f)** Kaplan-Meier (KM) estimates using combined SNVs/Mb and MS-indel in all RRD cancers. (Abbreviations: MMRD: mismatch-repair deficiency; PPD: Polymerase-proofreading deficiency; MS-indel: microsatellite insertion/ deletion.)

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907

908 **Fig. 5. Tumour immune microenvironment, survival and response to PD-1 blockade. (a)**

909 PD-L1 expression, response and survival in all RRD cancers. Cut-off is $\geq 1\%$ of cells

910 (Methods). **(b)** CD8 expression, response and survival for RRD cancers. Cut-off is $\geq 3\%$ of

911 cells (higher than the median; Methods). For both (a) and (b), the histology depicts

912 glioblastoma at 20X magnification. **(c)** Association of immune markers with SNV and RRD

913 status. **(d)** Combined immune (PD-L1 and CD8 expression) and genomic (TMB and MS-

914 indels) and overall survival in RRD cancers.

Fig.6. Characterization of the tumour flare response. (a-d) Analysis of 2 patients who had tumour-debulking prior to therapy and at the time of flare. (a, b) Total immune cell content in pre-therapy and at flare. (c, d) The corresponding CapTCR-sequencing and T-cell receptor clonotype analysis in these samples. (e, f) Immunohistochemistry for PD-L1 expression, and CD8-T-cell infiltration in the pre-therapy sample and at flare, as shown in the representative 20X images from the tumour sample in patient-1 (P33). (g) Representative flow cytometry plot showing activation of CD⁺ T-cell (TIGIT and 4-1BB) from the blood sample of a patient before treatment initiation and at flare. (h) 41BB⁺ CD8⁺ T-cells in blood from responders without flare, non-responders and flare.

926
927 **Extended Data Fig.1.** KM estimates of progression-free and overall survival for **(a)** all
928 tumours, **(b)** CNS tumours, **(c)** all solid tumours (CNS and non-CNS). **(d)** Progression free
929 survival for non-CNS solid tumours versus CNS tumours (p=0.01).

Extended Data Fig.2: Genomic biomarkers, survival and response to PD-1 blockade.
 Response and overall survival by **(a)** total synonymous variants, **(b)** non-synonymous variants,
(c) total indels, **(d)** total mutations/Mb, and **(e)** neoantigens.

Extended Data Fig.3: CNS tumours: Genomic biomarkers, survival and response to PD-1 blockade. Response and overall survival for CNS tumours by (a) synonymous variants, (b) non-synonymous variants, (c) SNVs/Mb, (d) total indels, (e) total mutations/ Mb, (f) neoantigens, and (g) MS-indels.

939

940 **Extended Data Fig.4: Tumour immune microenvironment, survival and response to PD-**
 941 **1 blockade.** Response and overall survival by (a) CD3, (b) CD4, and (c) CD8-positive T-cell
 942 infiltration in the pre-ICI tumour specimens.

943

944 **Extended Data Fig.5: Total CD8 cells and response to PD-1 blockade. (a)** Responders had
 945 higher levels of CD8 T-cell infiltration as compared to non-responders in all tumours. Green-
 946 gastrointestinal, blue- CNS tumours. **(b)** Representative images from non-CNS solid tumours,
 947 all of which demonstrated high CD8 and **(c)** PD-L1 expression.

Extended Data Fig.6: CNS tumours: Immune microenvironment, survival and response to PD-1 blockade. Response and overall survival in CNS tumours by (a) PD-L1, (b) CD8, (c) CD3, (d) CD4, and (e) CD68 expression.

952

953 **Extended Data Fig.7: Genomic and immune markers of response in tumours exhibiting**
 954 **flare.** Responders without flare, non-responders and flare stratified by **(a)** SNVs/mb, **(b)** MS-
 955 indels, **(c)** PD-L1 and **(d)** CD8 expression.

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958 **Extended Data Fig.8:** Representative images (20X) from patient P31 demonstrating **(a)** CD8
959 and **(b)** PD-L1 expression in pre-ICI and flare samples.

Extended Data Fig.9: Immune activation in patients experiencing tumour flare. Flow cytometry dot-plots from two additional patients (P39, P40) comparing pre-ICI and flare samples, showing (a, b) TIGIT and 4-1BB expressing CD8⁺ T-cells, (c, d) Ki67 expressing CD8⁺ T-cells, and (e, f) TIGIT and 4-1BB expressing CD4⁺ T-cells.

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Extended Data Table S2:

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Binary logistic regression analysis for response to ICI in RRD cancers

Variables	Odds ratio	95% CI	p-value
Age	1.19	0.8, 1.6	0.26
Male vs female gender	0.14	0, 6.1	0.30
CNS vs non-CNS solid tumours	1.15	0.0, -	0.99
Nivolumab vs Pembrolizumab	0.01	0, 3.6	0.12
High SNV/Mb (>median)	0.03	0, 0.5	<u>0.01</u>
High MS-indel (>median)	0.08	0, 2.1	0.13

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Extended Data Table S3:

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Cox regression analysis for OS following ICI in patients with RRD cancers

Variables	Odds ratio	95% CI	p-value
Age	0.88	0.7, 1.1	0.33
Male vs female gender	1.20	0.2, 6.1	0.83
CNS vs non-CNS solid tumours	0	0.0, -	0.96
Nivolumab vs Pembrolizumab	1.20	0.1, 19.8	0.89
High SNV/Mb (>median)	5.57	0.9, 32.4	<u>0.05</u>
High MS-indel (>median)	3.15	0.6, 16.4	0.17

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Figures

Figure 1

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Supplementary Files

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- [ExtendedDataTableS1FINAL.pdf](#)