Base excision repair regulates PD-L1 expression in cancer cells

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Abstract
Programmed death-ligand 1 (PD-L1) is a key factor influencing cancer immunotherapy; however, the regulation of PD-L1 expression in cancer cells remains unclear, particularly regarding DNA damage, repair and its signalling. Herein, we demonstrate that oxidative DNA damage induced by exogenously applied hydrogen peroxide (H2O2) upregulates PD-L1 expression in cancer cells. Further, depletion of the base excision repair (BER) enzyme DNA glycosylase augments PD-L1 upregulation in response to H2O2. PD-L1 upregulation in BER-depleted cells requires ATR/Chk1 kinase activities, demonstrating that PD-L1 upregulation is mediated by DNA damage signalling. Further analysis of The Cancer Genome Atlas revealed that the expression of PD-L1 is negatively correlated with that of the BER/single-strand break repair (SSBR) and tumours with low BER/SSBR gene expression show high microsatellite instability and neoantigen production. Hence, these results suggest that PD-L1 expression is regulated in cancer cells via the DNA damage signalling and neoantigen–interferon-γ pathway under oxidative stress.

Highlights
- Exogenous oxidative DNA damage upregulates PD-L1 expression in cancer cells.
- BER deficiency augments PD-L1 upregulation following oxidative DNA damage.
- Tumour samples with BER/SSBR mutations show high microsatellite instability, neoantigen and PD-L1 expression.
- PD-L1 and BER/SSBR gene expressions are negatively correlated in clinical specimens.

Introduction
Immune checkpoint therapy has recently emerged as a promising next-generation cancer treatment. One such therapy in particular, that is, anti-programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) antibody therapy has been widely applied to treat several types of cancer [1, 2]. Anti-PD-1/PD-L1 antibody restores antitumor immune responses by disrupting the interactions between PD-1 and its ligand, PD-L1, thereby providing effective antitumor effects by augmenting the body’s own immune activity against the tumour. However, patient responses to this treatment are highly variable; anti-PD-1/ PD-L1 antibodies alone produce dramatic response rates for high responders (~5% of patients), whereas approximately 40% of patients show cancer progression despite treatment [3–6]. Therefore, the cause of distinct responsivity against

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microenvironment. These responses result in the release of levels of mutations and neoantigen loads in tumours, treatment must be elucidated to improve therapeutic efficacy. Alternatively, other therapies must be developed which may be combined with the anti-PD-L1/anti-PD-L1 antibody, such as radiotherapy and other chemotherapies, to achieve favourable outcomes. Accumulating ongoing clinical studies demonstrate that the combination of local irradiation with anti-PD-L1/anti-PD-L1 checkpoint blockade treatment is a feasible and synergistic treatment for cancer, showing improved patient outcomes when compared with immune checkpoint inhibitor therapy alone [7–9]. Because the presence of PD-L1 influences the efficacy of anti-PD-L1/anti-PD-L1 therapy, an understanding of the molecular mechanisms underlying the regulation of PD-L1 expression in tumours is critically important [10–12].

In terms of PD-L1 expression in tumours, interferon (IFN) is considered a critical factor inducing PD-L1 upregulation. Recent studies have revealed that type I IFN (α and β) and type II IFN (γ) cause PD-L1 upregulation in all cells, including cancer cells; however, it is believed that IFNγ is the strongest inducer among the three types of IFNs [13]. IFNγ binds to the IFNγ receptor and stimulates downstream signalling (i.e. JAK1/2, STAT1/2/3 and IRF1), inducing PD-L1 expression [13, 14]. Evidence also suggests that cancer cells with multiple gene mutations (high mutational loads) show greater PD-L1 expression in tumour tissue [15]. Notably, high response rates of cancers with microsatellite instability (MSI), a hallmark of genome instability, to anti-PD-1 therapy have been reported [16, 17]. MSI-positive tumours presenting neoantigens promotes the release of IFNγ from tumour-infiltrating lymphocytes (TILs), and the released IFNγ enhances PD-L1 expression in tumours and immune cells [15]. Therefore, anti-PD-1/anti-PD-L1 therapy is considered useful for tumours exhibiting high MSI. This notion is strongly supported by the observation that mismatch repair (MMR)-deficient patients exhibited a higher rate of progression-free survival following anti-PD-1/anti-PD-L1 therapy; therefore, MMR status is a potent predictive marker of patient response to anti-PD-L1 therapy [16, 17]. High PD-L1 expression was also observed in DNA polymerase epsilon-mutated cancers [18]. More recently, the defect of a chromatin remodelling factor, ARID1A, correlates with high MSI and mutation load across multiple human cancer types due to the attenuation of MMR activity, which eventually phenocopies the MMR-defective tumours in terms of the increase in neoantigen–IFNγ/anti-PD-L1 pathway [19]. Alternatively, homologous recombination (HR)-deficient tumours were also shown to exhibit greater neoantigen loads, TILs and PD-1/anti-PD-L1 expression in tumours and immune cells [20]. Thus, the evidence suggests that genomic instability causes high levels of mutations and neoantigen loads in tumours, resulting in activation of immune responses in the tumour microenvironment. These responses result in the release of IFNγ from immune cells, stimulating PD-L1 expression in the tumour-associated surrounding cells.

Alternatively, recent studies suggest that exogenous cellular stress also upregulates PD-L1 expression in cancer cells [21–23]. We recently demonstrated that PD-L1 expression in cancer cells is upregulated in response to DNA double-strand breaks (DSBs), which are the most critical type of genotoxic stress [22]. This upregulation requires ATM/ATR/Chk1 kinase activities, suggesting that DNA damage response is a trigger for PD-L1 upregulation in cancer cells. Ionising radiation (IR) generates DSBs, which activate DNA damage signals, such as ATM, ATR and Chk1. Consistent with our findings, use of a specific ATR inhibitor (ATRi) significantly prevents PD-L1 upregulation, resulting in reduced numbers of tumour-infiltrating T regulatory cells and increased CD8+ T cell activity in mouse models [24]. In contrast to IR, oxidative stress induces base damage and single-strand breaks (SSBs), but does not directly induce DSBs. Reactive oxygen species (ROS) produced under oxidative stress are a major source of DNA damage, and cancer cells in particular are characterised by persistent oxidative stress and high levels of ROS [25, 26]. Human tumour cells show enhanced production of hydrogen peroxide (H2O2) compared with non-transformed cell lines [25]. Persistent oxidative stress in cancer cells promotes cancer growth and metastasis of the cell clones, leading to further deleterious mutations [26–28]. The chronic inflammation associated with carcinomas stimulates ROS production, causing additional genetic instability [29]. Such high oxidative stress creates selective pressure favouring cells with enhanced growth, invasion and metastasis characteristics [26, 30]. Under these oxidative tumour conditions, oxidative DNA damage is chronically generated, and >100 types of oxidative base lesions are reported, for example, 8-hydroxyguanine (8-oxoG), thymine glycol (Tg) and formamidopyrimidine (Fapy) [31]. Therefore, it is thought that multiple DNA glycosylases and thymine glycol (Tg) and formamidopyrimidine (Fapy) [31]. Therefore, it is thought that multiple DNA glycosylases and thymine glycol (Tg) and formamidopyrimidine (Fapy) [31]. Therefore, it is thought that multiple DNA glycosylases deal specifically with each type of base lesion. In base excision repair (BER), cells have appropriate DNA glycosylases to remove each oxidative base lesion and leave a SSB, subsequently initiating SSB repair (SSBR) [32]. Notably, excessive base damage under high levels of ROS production in cancer cells upregulates DNA damage signalling [33]. This signalling may be augmented by the generation of genotoxic replication fork collapse during cell cycle progression under a BER/SSBR-defective background. More than hundred single-nucleotide polymorphisms in BER genes are identified, and these polymorphisms may cause deleterious effects in specific human cancers [34, 35]; however, it is unclear whether loss of BER/SSBR activity influences PD-L1 expression, which may cause an immune-suppressive tumour environment. Such tumours may be preferable for the application of anti-PD-1/anti-PD-L1...
immunotherapy. In the present study, we investigated the involvement of the BER/SSBR pathway in the context of oxidative DNA damage in PD-L1 expression.

**Results**

**PD-L1 expression is upregulated by oxidative stress in cancer cells**

Recently, we demonstrated that DNA damage signals from DSBs upregulate PD-L1 expression in cancer cells [22]. In the present study, we first examined whether oxidative DNA damage affects PD-L1 expression in cancer cells. Exogenously applied \( \text{H}_2\text{O}_2 \) was used in the present study to induce oxidative DNA damage. To examine PD-L1 expression on the surface of cancer cells, PD-L1 levels were analysed via flow cytometry after \( \text{H}_2\text{O}_2 \) treatment. Notably, PD-L1 expression on the cell surface was upregulated in U2OS, H1299 and MCF7 in response to \( \text{H}_2\text{O}_2 \) treatment (Fig. 1a–d), as were the levels of PD-L1 protein and messenger RNA (mRNA) expression (Fig. 1e, f). Similar to DSBs from X-rays or etoposide, phosphorylation of STAT1 and STAT3 proteins and IRF1 expression were upregulated in response to \( \text{H}_2\text{O}_2 \) treatment (Fig. 1g) [22]. The levels of DNA damage was monitored by \( \gamma\text{H}_2\text{AX} \) foci after \( \text{H}_2\text{O}_2 \) treatment (Supplementary Fig. 1).

Taken together, these results suggest that oxidative DNA damage may induce PD-L1 upregulation via DNA damage signalling in cancer cells.

**Depletion of BER factors enhances PD-L1 expression after oxidative stress**

The pathway of BER is initiated by the removal of damaged bases by DNA glycosylases (e.g. NTH1, OGG1, NEIL1 or MUTY), followed by DNA nick generation by endonucleases (e.g. APEX1). Following the removal of the damaged base, SSBR proteins repair the nick by either the XRCC1/POL\( \beta \)/LIG3-dependent short patch or the FEN1/
Depletion of base excision repair (BER) genes enhances programmed death-ligand 1 (PD-L1) upregulation after oxidative DNA damage. a Relative ionising radiation (IR)-induced PD-L1 intensity compared with control small interfering RNA (siRNA) in U2OS cells was examined via immunoblotting 48 h after X-irradiation. Raw images of the immunoblotting are shown in Supplementary Fig. 2a–h. b–e Depletion of NTH1 enhances PD-L1 upregulation after application of hydrogen peroxide (H$_2$O$_2$). PD-L1 cell-surface expression was examined via flow cytometry, as shown in representative histograms (b) and graphs representing U2OS (c), H1299 (d) and MCF7 (e) cells. Cancer cells were harvested 48 h after the application of 50 μM H$_2$O$_2$.

POLδε/LIG1-dependent long patch [32]. To address the question of whether the repair factors in BER and SSBR are involved in PD-L1 upregulation, we examined PD-L1 expression in cells having small interfering RNA (siRNA)-depleted BER/SSBR genes. Notably, most BER or SSBR siRNAs augmented PD-L1 upregulation compared with that in control cells after X-ray irradiation (Fig. 2a and Supplementary Fig. 2a–h). X-irradiation, which also generate a substantial number of base damage and SSBs, was used because the X-ray method was more suitable for the analysis of siRNA screens than H$_2$O$_2$. Next, we focused on BER genes, particularly NTH1, OGG1 and NEIL1, because these proteins are involved in repair of both endogenously and exogenously generated oxidised bases, such as Tg, Fapy and 8-oxoG, in the BER pathway. Flow cytometry analysis revealed that NTH1 depletion caused greater PD-L1 upregulation in response to H$_2$O$_2$ treatment than control siRNA in U2OS, H1299 and MCF7 cells (Fig. 2b–e). Similar upregulation was observed by using a distinct siRNA oligonucleotide, indicating that the greater PD-L1 upregulation observed was not caused by siRNA off-target effects (Fig. 2f and Supplementary Fig. 3a). In control cells, approximately >25–50 μM of H$_2$O$_2$ was required for PD-L1 upregulation (Fig. 1e, 2g). Notably, we found that NTH1 depletion caused PD-L1 upregulation even after 10 μM H$_2$O$_2$ treatment (Fig. 2g), which does not induce DSBs (Supplementary Fig. 1), demonstrating that BER deficiency enhances oxidative DNA damage-dependent PD-L1 upregulation without causing direct DSB induction (Fig. 2g). Because multiple types of oxidative base damage are generated in response to oxidative stress, we examined PD-L1 expression in cells with multiple combinations of depleted DNA glycosylases. Depletion of single NTH1 or OGG1 significantly augmented PD-L1 upregulation after H$_2$O$_2$ treatment (Fig. 2h and Supplementary Fig. 3b). Double knockdown of NTH1 + OGG1 and OGG1 + NEIL1 and triple knockdown of NTH1 + OGG1 + NEIL1 also exhibited greater PD-L1 upregulation than single NTH1- or OGG1-depleted cells, although the combination of double or triple siRNA did not show synergistic effects (Fig. 2h and Supplementary Fig. 3b). Together, these data demonstrate that BER deficiency augments PD-L1 expression via oxidative DNA damage in cancer cells. Notably, the involvement of the BER pathway strongly suggests that
oxidative DNA damage-dependent signalling is important for PD-L1 upregulation in response to exogenous oxidative stress.

**PD-L1 upregulation requires ATR/Chk1 signalling in exponentially growing cells after oxidative DNA damage**

Next, to address the question of whether PD-L1 upregulation after H$_2$O$_2$ treatment is dependent on DNA damage signalling, we examined PD-L1 expression in the presence of ATRi. The H$_2$O$_2$-dependent upregulation of PD-L1 on the surface of control and NTH1-depleted cells was suppressed by ATR or Chk1 inhibition (Fig. 3a–c). The ATR-Chk1-dependent upregulation of PD-L1 was confirmed through immunoblotting analysis (Fig. 3d). Thus, these results suggest that ATR-Chk1-dependent DNA damage signalling is required for PD-L1 upregulation in control and BER-defective cells.

It is generally understood that base damage and SSBs do not cause G1/S checkpoint arrest, because these types of DNA damage and signalling do not sufficiently activate the machinery of the G1/S cell cycle checkpoint. In contrast, it is well known that cells accumulate in the G2 phase following introduction of base damage and SSBs because base damage and SSB generate genotoxic replication fork collapse including DSBs, which activate ATR/Chk1 signalling, in the S phase [32, 36]. Thus, we hypothesised that PD-L1 upregulation in response to H$_2$O$_2$ treatment may require cell cycle progression from G1 to S/G2 cells. To address this, we treated cells with H$_2$O$_2$ when the cells were confluent and examined PD-L1 expression (Fig. 3e and Supplementary Fig. 4a, b). Notably, confluent cells showed no increase in PD-L1 expression after H$_2$O$_2$ treatment (Fig. 3f; confluent cells exhibited significantly less accumulation of the G2 phase after H$_2$O$_2$ treatment or X-ray irradiation than exponentially growing cells, confirming that cells did not progress from G1 to S/G2 under confluence (Supplementary Fig. 4a–c)). Consistent with this notion, a substantial increase in DSBs formation was observed in the S phase after H$_2$O$_2$ treatment, although not as many DSBs were generated as many with X-rays in G1 cells (Supplementary...
Furthermore, we found that phosphorylation of STAT1/3 and IRF1 expression were not largely activated in confluent cells (Fig. 3g). Taken together, these results suggest that oxidative DNA damage-dependent PD-L1 upregulation requires ATR/Chk1 signalling, which is likely caused by genotoxic DNA replication stress during cell cycle progression.

**PD-L1 expression in tumours is negatively correlated with mRNA expression of BER genes**

The findings of the tissue culture analysis prompted us to investigate the correlation between the expression of the PD-L1 and BER/SSBR genes in tumours. Therefore, we examined the mRNA expression levels using The Cancer Genome Atlas (TCGA), which is publicly available genomic dataset used to analyse gene expression, including its mutation status, in clinical specimens [37]. Notably, we found that the expression of most BER/SSBR genes exhibits a negative correlation with PD-L1 expression (Fig. 4a, Table 1 and Supplementary Fig. 5a–c). In agreement with the results of the tissue culture analysis, the STAT1, STAT3 and IRF1 genes, which are suggested to be required for PD-L1 expression, exhibit a positive correlation (Supplementary Fig. 5d–f) [14]. Hence, our TCGA analysis strongly supports the notion that the BER/SSBR pathway is involved in the regulation of PD-L1 expression in tumours.

**Tumours having mutations in any of the BER genes exhibit high PD-L1 expression in clinical specimens**

Next, to analyse whether mutations in BER/SSBR genes effect PD-L1 expression in clinical specimens, we examined the expression of PD-L1 mRNA in tumour samples with or without mutations in the genes involved in BER/SSBR (a list of BER genes in the TCGA analysis is shown in Supplementary Table 5). Notably, we found that tumours having mutations in any of the BER genes exhibit statistically significant increases in PD-L1 expression (Fig. 5a). The number of damaged bases and SSBs is estimated at approximately ~2,000–10,000 per cell per day in normal tissues [38, 39]. Notably, it has been suggested that a greater number of base damage/SSBs is generated in tumours because of high ROS production [40, 41]. Therefore, BER/SSBR deficiency leads to point mutations or small insertions/deletions if endogenous DNA damage is misrepaired [31]. Because a high number of point mutations and small deletions/insertions may be the cause of neoantigen production, we examined the neoantigen levels in tumours having mutations in any of the BER/SSBR genes. Notably, we discovered that tumours having mutations in the BER genes show substantially greater neoantigen production than wild-type tumours (Fig. 5b). These findings suggest that the greater PD-L1 expression in tumours having BER mutations is caused by higher neoantigen production through the IFNγ-STAT1/3-IRF1 pathway. Next, to examine whether high PD-L1 expression in tumours having mutations in the BER/SSBR genes is solely dependent on the increase in neoantigens, we compared the PD-L1 expression between the BER wild type and mutant in tumours having low or high neoantigens. Notably, we found that tumours having BER mutations exhibited statistically greater PD-L1 expression in a low neoantigen background (Fig. 5c). This result strongly supports the notion obtained by in vitro analysis that high levels of PD-L1 expression in tumours having BER/SSBR mutations are not completely dependent on the neoantigen pathway, that is, the DNA damage signalling pathway is another factor influencing PD-L1 expression in tumours having BER mutations. In addition, tumours having BER/SSBR mutations with high neoantigen production also exhibited further increases in PD-L1 expression compared with the BER/SSBR wild type with high neoantigen production (Fig. 5c). Taken together, our dataset analysis provides the novel notion that BER/SSBR suppresses PD-L1 expression in tumours by both the DNA damage signalling and neoantigen–IFNγ pathways.

**DNA damage signalling upregulates PD-L1 irrespective of MSI and CIN status in cancer cells**

Next, to investigate whether BER/SSBR deficiency affects MSI or chromosomal instability (CIN), which are related to the frequency of mutational loads and PD-L1 expression via neoantigen–IFNγ pathway in tumours, MSI status in clinical specimens was examined using the TCGA data set. Previous studies demonstrated that MMR deficiency causes high levels of MSI [42–44]. Tumours harbouring mutations in MMR genes exhibited high neoantigen and mutational loads as well as high PD-L1 expression and prominent efficacy in anti-PD-1 therapy [15–17]. Consistent with the previous report, our dataset analysis showed high MSI in tumours having mutations in MMR genes (Fig. 6a). Notably, tumours having mutations in BER/SSBR genes also exhibit high levels of MSI that are comparable to the levels found in MMR-mutated tumours of BER/SSBR wild-type group (Fig. 6a). Surprisingly, 85% of tumours having mutations in both MMR and BER genes exhibit MSI, strongly suggesting that MMR and BER/SSBR suppresses MSI independently (Fig. 6a). Next, to address whether exogenous oxidative DNA damage-dependent PD-L1 upregulation is affected by MSI/CIN status (Fig. 6b), PD-L1 expression in colorectal cancer cell lines was examined after H2O2 treatment or X-ray irradiation. Notably, all colorectal cancer cell lines showed an increase in cell-surface PD-L1 expression after H2O2 treatment or X-ray irradiation, suggesting that PD-L1 expression is upregulated by DNA.
Programmed death-ligand 1 (PD-L1) expression in tumours is negatively correlated with base excision repair (BER) messenger RNA (mRNA) expression. a–f Analysis of PD-L1 mRNA levels in relation to those of BER genes. Scatter plots are shown between PD-L1 and NTH1 (a), OGG1 (b), NEIL1 (c), MUTY (d), APEX1 (e) and XRCC1 (f). mRNA levels from the The Cancer Genome Atlas-breast invasive carcinoma (TCGA-BRCA) study. PD-L1 values over $4 \times 10^5$ are excluded from the graphs. Values are shown in Table 1. g–l Volcano plots showing Spearman’s correlation and estimated significance of the correlation between PD-L1 and BER genes. NTH1 (g), OGG1 (h), NEIL1 (i), MUTY (j), APEX1 (k) and XRCC1 (l) mRNA levels from RNA-sequencing (RNA-seq) data across several TCGA cancer studies, calculated using TIMER (Tumour Immune Estimation Resource) and adjusted for tumour purity. Each dot represents a cancer type in TCGA. Studies with $p$ value < $1 \times 10^{-5}$ were labelled. Correlations with other genes are shown in Supplementary Fig. 5.
Table 1 PD-L1 expression in tumours is negatively correlated with mRNA expression of BER genes

<table>
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<tr>
<th>Study</th>
<th>Gene</th>
<th>Correlation</th>
<th>Approximation formula</th>
<th>$R^2$ value</th>
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<td>MUTY</td>
<td>Negative</td>
<td>$y = -0.1289x + 62,651$</td>
<td>0.00666</td>
</tr>
<tr>
<td></td>
<td>XRCC1</td>
<td>Negative</td>
<td>$y = -0.052x + 68,517$</td>
<td>0.010200</td>
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<tr>
<td></td>
<td>LIG3</td>
<td>Negative</td>
<td>$y = -0.4651x + 86,154$</td>
<td>0.0357</td>
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<tr>
<td></td>
<td>POLB</td>
<td>Negative</td>
<td>$y = -0.0323x + 54,917$</td>
<td>0.0015</td>
</tr>
<tr>
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<td>FEN1</td>
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<td>$y = -0.0059x + 52,684$</td>
<td>0.0006</td>
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<td>Negative</td>
<td>$y = -0.08x + 130,731$</td>
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Table 1 (continued)

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<tr>
<th>Study</th>
<th>Gene</th>
<th>Correlation</th>
<th>Approximation formula</th>
<th>$R^2$ value</th>
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<tr>
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<td>OGG1</td>
<td>Positive</td>
<td>$y = 0.8718x + 90,145$</td>
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<tr>
<td></td>
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<td>Negative</td>
<td>$y = -0.0174x + 150,280$</td>
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<tr>
<td></td>
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<td>$y = -1.5622x + 140,024$</td>
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<td>STAD</td>
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<td>$y = 0.0639x + 50,293$</td>
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<td>$y = -0.7292x + 98,723$</td>
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<td>$y = -0.8543x + 87,406$</td>
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**PD-L1 programmed death-ligand 1, mRNA messenger RNA, BER base excision repair, COAD colon adenocarcinoma, BRCA breast invasive carcinoma, UCEC uterine corpus endometrial carcinoma, SKCM skin cutaneous melanoma, LUAD lung adenocarcinoma, HNSC head and neck squamous cell carcinoma, STAD stomach adenocarcinoma**

damage signalling irrespective of MSI or CIN status (Fig. 6c). Hence, our in vitro analysis demonstrates that exogenous DNA damage including DNA base damage, SSBs and DSBs leads to further PD-L1 upregulation irrespective of MSI or CIN status.

**Discussion**

In the present study, we demonstrated that oxidative DNA damage upregulates PD-L1 expression in cancer cells. In particular, the importance of BER/SSBR activity is pronounced following exposure to exogenous oxidative stress produced by ROS, such as H$_2$O$_2$, because depletion of DNA glycosylases involved in the BER pathway, such as NTH1 or OGG1, caused greater PD-L1 upregulation after H$_2$O$_2$ treatment. Furthermore, the upregulation of PD-L1 in control and NTH1-depleted cells is mediated via the ATR/Chk1 pathway, as PD-L1 is upregulated in response to signalling from DSBs induced by X-rays or etoposide [22]. In addition, TCGA data set analysis indicates that the expression of PD-L1 is negatively correlated with that of the BER/SSBR genes in clinical specimens, and tumour samples harbouring mutations in any of the BER/SSBR genes exhibit high neoantigen expression compared with samples without mutations in these genes. Notably, tumour samples
Fig. 5 Tumours having mutations in base excision repair (BER) genes exhibit higher programmed death-ligand 1 (PD-L1) expression. a Significant increases in PD-L1 expression were observed in association with mutations in any of the BER genes. The Cancer Genome Atlas-colon adenocarcinoma (TCGA-COAD) (colon), breast invasive carcinoma (BRCA) (breast) and uterine corpus endometrial carcinoma (UCEC) (uterine) studies were analysed regarding their mutation in 20 BER genes included in this study. b Levels of neoantigens are shown in relation to the BER mutation status. c PD-L1 expression levels are shown according to their BER mutation status and neoantigen level. Analyses of other tumour types are shown in Supplementary Fig. 6.

Base excision repair regulates PD-L1 expression in cancer cells
harbouring BER/SSBR mutations exhibit greater PD-L1 expression regardless of the number of neoantigens. Finally, we showed that DNA damage signalling upregulates PD-L1 irrespective of MSI and CIN status in cancer cells. Together, these results suggest that PD-L1 expression is augmented by BER deficiency through the DNA damage signalling and neoantigen–IFNγ pathways. This is the first report demonstrating that BER is a critical factor regulating PD-L1 expression in cancer cells, and BER/SSBR gene status will be an important information to predict the efficacy of anti-PD-1/PD-L1 therapy.

Oxidative stress damages DNA bases via ROS-mediated reactions, causing oxidative lesions such as 8-oxoG, Tg and Fapy, and generates SSBs. In the tumour environment, oxidative stress is caused by either intrinsic (oncogenes activation, loss of functional p53, aberrant metabolism and mitochondrial dysfunction) or extrinsic (inflammatory cytokines, hypoxic environment and imbalance of nutrients) factors [26]. Because X-rays/γ-rays are also a common oxidative DNA damage inducers via ROS production, DNA damage signalling from oxidative stress should be considered in radiotherapy. Following the removal of damaged bases by DNA glycosylases, a nick is generated that is recognised by SSBR proteins, and the damage is repaired through the SSBR pathway [32]. Thus, defects in BER/SSBR cause accumulation of damaged bases or SSBs. It is thought that this DNA damage is not critical unless cells are actively dividing; however, once the cell cycle progresses into the S phase, the DNA replication machinery encounters the damaged bases and SSBs, which are then converted to cytotoxic DNA damage, leading to replication fork collapse [45, 46]. For example, when replication stalls at damaged bases, DSBs are generated during the process of DNA repair [46]. Alternatively, one-ended DSBs (also called single-ended DSBs) arise when replication forks encounter unrepaired SSBs [46]. Hence, BER/SSBR-defective cells cause cytotoxic replication fork collapse under exponentially growing condition. Notably, PD-L1 upregulation is observed when cells are exponentially growing; this suggests that replication fork collapse during the S phase plays an important role in signalling pathways that upregulate PD-L1 expression. In addition, we speculate that further cell cycle progression following mitosis may also be important to augment DNA damage signalling for PD-L1 upregulation because unrepaired DNA damage generated during DNA replication fork collapse may cause abnormal mitotic segregation, including micronuclei production. Such damaged cells may cause further accumulation of DNA damage in the next cycle of DNA replication in the S phase, inducing substantial DNA damage signalling in the S/G2 phase. This idea is supported by a latest report showing that cytotoxic DNA damage in the G1 phase of the next cell cycle following replication fork collapse is accumulated in HR-defective cells [47]. DSBs generated during replication fork collapse are preferentially repaired by HR [48]. In the process of HR, DSB ends are processed by DNA nucleases such as EXO1. RPA is recruited to the resected ssDNA region, and RPA recruits ATRIP followed by ATR
 activation. ATR is activated by autophosphorylation and further phosphorylates Chk1, causing the activation of Chk1 and its downstream pathway. Although DNA replication does not extensively activate ATR/Chk1 signalling in normal cells without exogenous DNA damage, this signalling is activated by replication fork collapse via the mechanisms described above, particularly when the SSBR or BER pathways are downregulated. Thus, we propose that defects in BER and SSBR increase PD-L1 upregulation via ATR/Chk1 signalling at DNA replication fork collapse in response to oxidative DNA damage.

Accumulating studies have demonstrated that BER/SSBR defects yield high mutation frequency in vitro and in vivo analysis; however, the levels of mutational loads (i.e. neoantigens and MSI in cancer cells) in the context of BER/SSBR gene status have not been well considered. In human cells, multiple DNA glycosylases are designed to remove damaged purine and pyrimidine bases, and it has been estimated that approximately 2,000–10,000 oxidative DNA lesions, including damaged bases and SSBs, occur per cell per day [38, 39]. Notably, a greater number of oxidative DNA lesions are suggested to occur in cancer cells [40, 41]. These oxidative DNA lesions are normally repaired by BER/SSBR in the interphase of the cell cycle. However, failures in DNA glycosylase-dependent base removal leave damaged bases, which can result in a base mispair during DNA replication. Therefore, multiple point mutations may accumulate in cancer cells, particularly if cells are BER/SSBR deficient. Consistent with this notion, our TCGA analysis revealed that tumours harbouring mutations in BER/SSBR genes exhibit a high frequency of MSI. Surprisingly, the percentage of MSI in BER/SSBR-mutated tumours is comparable to that in MMR-mutated tumours; more importantly, approximately 85% of tumours having mutations in both BER/SSBR and MMR genes display MSI. The additive increase in MSI frequency suggests independent contributions of BER/SSBR and MMR to the repair of damaged bases; MMR contributes to re-incorporation of the correct base if the wrong base is paired with a damaged base during DNA replication in the S phase, whereas BER principally removes damaged bases and incorporates correct bases during the interphase. Notably, BER/SSBR-mutated tumours contain high levels of neoantigen expression and a correspondingly high frequency of MSI. Because neoantigens lead to T cell activation, neoantigen-associated T cell activation may be highly induced in BER/SSBR-defective tumours. Such a high frequency of MSI may be related to the incidence of BER/SSBR-associated adenomatous polyposis and colorectal cancer [49]. Thus, our findings uncover the involvement of BER in PD-L1 expression in cancer cells. These findings highlight a possibility that BER can be used as a potent marker to predict the efficacy of anti-PD-1/PD-L1 therapy, not only in MMR in colorectal cancer but also in other types of cancer.

Recently, Vendetti et al. [24] demonstrated that inhibition of ATR kinase activity by a specific inhibitor, AZD6738, attenuates radiation-induced PD-L1 upregulation in tumour cells. Notably, the blockage of PD-L1 upregulation by AZD6738 substantially decreased the number of tumour-infiltrating T regulatory cells and increased CD8+ T cell activity after IR in mouse models. This strongly supports our model that ATR/Chk1 signalling is critically important for PD-L1 upregulation in tumour cells, and the expression of PD-L1 may affect immune cell activity in the tumour environment. In our previous study, we demonstrated that DSBs produced by X-ray irradiation and the subsequent DNA damage signalling upregulated PD-L1 expression in cancer cells [22]. The present study further expands our understanding of the link between DNA damage responses and PD-L1 expression in cancer cells. From this study, we understand that PD-L1 expression in cancer cells is affected by not only DSB repair but also BER/SSBR gene status; notably, we propose that in any case, the central signalling factor is ATR/Chk1 signalling and it is activated through DSB induction or DNA replication fork collapse in the S phase (Fig. 7). On the other hand, with regard to BER/SSBR, high neoantigen production resulting from high mutational loads, including MSI, is associated with PD-L1 expression (Fig. 7). Taken together, we propose that it is critically important to consider the gene

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Fig. 7 Model for programmed death-ligand 1 (PD-L1) upregulation in base excision repair (BER)-defective tumours. Under oxidative stress conditions, base damage and/or single-strand breaks (SSBs) are produced in tumours in response to oxidative DNA damage, but they are rapidly repaired if BER/SSBR is functional. In BER/SSBR-defective cells, PD-L1 is upregulated through either the neoantigen–interferon-γ (IFNγ) pathway, which is caused by a higher DNA mutation load, or via the ATR/Chk1-STAT pathway induced by genotoxic stress in cycling cells. The latter ATR/Chk1 signalling is augmented particularly when cells are exposed to exogenous oxidative stress.
status of BER/SSBR, as well as the DSB repair genes, when anti-PD-1/PD-L1 therapy is applied under oxidative stress in a tumour microenvironment, particularly in combination with radio/chemotherapy.

**Materials and methods**

**Cell culture, irradiation and drug treatment**

Cancer cells (U2OS, H1299, HCT116 p53+/+ and p53−/−, RKO, WiDr and MCF7) were cultured in Eagle’s minimum essential medium with 10% foetal calf serum. X-ray irradiation was performed at 100 kVp and 20 mA with copper (0.5 mm)–aluminium (1.0 mm) filters (Faxitron Bioptics, Tucson, AZ, USA). The dose rate was set at 0.5 Gy/min. Thirty per cent H2O2 (Wako, Japan) was applied at 25, 50, or 100 µM (8.5, 17 or 34 × 10−4%). For H2O2 treatment, cells were treated for 60 min in the culture medium in a 5% CO2 incubator at 37 °C. Thirty per cent H2O2 was kept in 4 °C. For each experiment, a solution with the indicated concentration of H2O2 was prepared using DNA/RNA-, protein- and pathogen-free water prior to use. H2O2 treatment against cells was performed in the dark and under protein- and pathogen-free conditions. Afterwards, cells were washed three times with phosphate-buffered saline (PBS), fresh culture medium was added and cells were further incubated until harvest. Ten micromoles of ATRi (VE821; Axon Medchem) or Chk1 inhibitor (Chk1i) (100 nM UCN-01: Calbiochem or 50 nM MK8776: AdooQ Bioscience) were added when the media were refreshed after H2O2 treatment until cells were harvested. For positive control, cells were treated with 1 ng/mL of IFNγ for 24 h. The reagents used are listed in Supplementary Table 1.

**siRNA knockdown**

siRNA transfection was performed using HiPerFect (Qiagen). Cells were trypsinised and siRNA was added to the suspended cells. After 24 h, cells were re-suspended via trypsinisation and re-transfected with siRNA. Cells were incubated for 24 h after the second transfection before DNA damage induction. The sequences of the siRNA oligonucleotides used are listed in Supplementary Table 2. In figures, siRNA s1 was used unless stated.

**Immunoblotting and immunofluorescence**

Immunoblotting was performed as described previously [50]. Briefly, cells were washed with 1× PBS, and total cell lysate was harvested by adding 1× sample buffer (50 mM Tris, 2% sodium dodecyl sulphate, 6% glycerol, 74 mM 3-mercaptop-1,2-propanediol and 0.02% bromophenol blue). The harvested cell lysate was boiled at 95 °C for 5 min and sonicated to digest the DNA. Sample was run in the 4–15% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) and transferred to nitrocellulose membrane. After 30 min of blocking with 5% milk in TBST, the membrane was incubated with the primary antibodies overnight at room temperature. Following incubation with secondary antibodies, the ECL chemiluminescence signal was detected using a LAS-600 Bioimaging Analyser System. Immunofluorescence study was performed as described previously [50]. The antibodies used are listed in Supplementary Tables 3 and 4.

**Quantification of mRNA expression levels using real-time PCR**

Total RNA was extracted from cells using a NucleoSpin RNA Clean-up System (Macherey-Nagel) at the indicated time points after exposure to H2O2. A PrimeScript RT Reagent Kit (Perfect Real Time) (TaKaRa) was used to reverse transcribe complementary DNA (cDNA) from the total RNA, according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using StepOne-Plus (Life Technologies). Reactions (20 µL each) were prepared in duplicate in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems). Each reaction contained 0.5 µM of each primer, 0.2 µM probe, 10 µL of Taqman Universal PCR Master Mix (Applied Biosystems) and cDNA as a template. The expression levels were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated using the 2−ΔΔCt method. qPCR settings were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The primers and probes used for qPCR are listed below:

- **PD-L1 forward**: 5′-GGAGATTAGATCCGTAGGAAAACC-3′
- **PD-L1 reverse**: 5′-AACGGAGATGAGATGTCAGTGCTA-3′
- **PD-L1 probe**: 5′-AGATGGCTCCCAGAATTACCAGTGAGTCC-3′
- **GAPDH forward**: 5′-CTCCTCTGACTTCAACAGCGA-3′
- **GAPDH reverse**: 5′-CCAAATTCGTTGTCTACATCAAGGA-3′
- **GAPDH probe**: 5′-ATGCCAGCCCCAGCGCTCAAAAGGT-3′

**Flow cytometry analysis of cell-surface PD-L1**

Cancer cells were incubated for 48 h after exposure to X-ray irradiation or H2O2 and then harvested for flow cytometry analysis. Adherent cells were harvested by shake-off in 1
mM EDTA/PBS without trypsinisation. Harvested cells were washed with 1 mM EDTA/PBS and then stained with anti-PD-L1 antibodies for 20 min on ice. Dead cells detected using propidium iodide (Sigma-Aldrich) were excluded from the analysis. Flow cytometry analysis was performed using an Attune NxT Flow Cytometer (Thermo Fisher Scientific). The mean fluorescence intensity (MFI) (PD-L1 - isotype) is obtained by subtracting the MFI of the isotype control from the MFI of PD-L1.

**Dataset analysis of clinical specimens by using TCGA**

Normalised RNA sequence and mutation status data provided by TCGA project were downloaded from the Genomic Data Commons Data Portal. Studies of TCGA-breed invasive carcinoma (BRCA), colon adenocarcinoma (COAD) and uterine corpus endometrial carcinoma (UCEC) were highlighted in the analysis. The neoantigen data were obtained from The Cancer Immunome Atlas (http://tcia.at/). The upper quartile of clonal neoantigens was used to separate tumours with high and low neoantigen burden (>75 percentile = high; <75 percentile = low) [51]. Samples with or without mutations, including indels and point mutations, in 20 BER genes (NTH1, OGG1, APEX1, APEX2, PARP1, PARP2, XRCCL1, Lig1, Lig3, Fen1, Neil1, Neil2, Neil3, Polb, Pnkp, Aplf, Ung, Smug1, Mpg, and Muty) were compared using Student’s t test, and box plots were created using the GraphPad Prism 7.0 software. The analysis results of other tumours (head and neck squamous cell carcinoma: HNSC; lung adenocarcinoma: LUAD; skin cutaneous melanoma: SKCM and stomach adenocarcinoma: STAD) are shown in Table 1. Correlation plots across TCGA cancer types were made with correlation calculation results adjusted for tumour purity using TIMER (Tumour Immune Estimation Resource) accessed at https://cistrome.shinyapps.io/timer/ [52]. MMR genes (Mlh1, Mlh3, Msh2, Msh3, Msh6, Pms1, Pms2, Pold1 and Pole) in this study were used for MSI analysis [53]. The total number of samples used in this study are as follows: COAD (480), BRCA (1109), UCEC (607), LUAD (1086), SKCM (470), HNSC (502) and STAD (375) (see also Supplementary Table 5). Samples harbouring indels or point mutations in either PD-L1 or IRF-1 were excluded from the analysis shown in Fig. 4a–f, Fig. 5a–c, Fig. 6a, Supplementary Fig. 6a–c and Table 1. In the volcano plots produced using TIMER (Fig. 4g–l and Supplementary Fig. 5a–f), exclusion was not applied.

**Statistical analysis**

Statistical significance was determined via Student’s two-tailed t tests using the GraphPad Prism 7.0 software. *P < 0.05, **p < 0.01, ***p < 0.001.

**Data availability**

All relevant data are available from the corresponding author(s) upon reasonable request.

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**Author contributions** A.S. designed the experiments and wrote the paper with T.B.M.P. The experiments including immunoblotting, qPCR and flow cytometry were performed by T.B.M.P., H.S., Y.H. and A.S. The dataset of TCGA was developed by T.Y. The TCGA analysis was performed by T.B.M.P. and Y.H. under the supervision of T.Y. Acquired data was analysed and interpreted by T.B.M.P., H.S., T.Y. and A.S. The manuscript was reviewed by T.O., K.D.H. and T.N. Administrative, technical or material support was provided by T.O., S. G. and T.N. The study was supervised by A.S.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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**References**


