Cyclosporine A can induce DNA double strand breaks: implications for bone marrow transplantation regimens particularly for individuals with defective DNA repair.

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Summary.

Several human disorders mutated in core components of the major DNA double strand break (DSB) repair pathway, non-homologous end joining (NHEJ), have been described. Cell lines from these patients are characterised by sensitivity to DSB-inducing agents. LIG4 syndrome patients specifically, for unknown reasons, respond particularly badly following treatment for malignancy or bone marrow transplantation (BMT). We report the first systematic evaluation of the response of LIG4 syndrome to compounds routinely employed for BMT conditioning. We found human pre-B lymphocytes, a key target population for BMT conditioning, when deficient for DNA ligase IV, unexpectedly exhibit significant sensitivity to cyclosporine A (CSA) the principal prophylaxis for Graft versus Host Disease (GvHD). Furthermore, we found that CSA treatment alone or in combination with busulphan and fludarabine resulted in increased levels of DSBs specifically in LIG4 syndrome cells compared to wild type or Artemis-deficient cells. Our study shows that CSA can induce DSBs and that LIG4 syndrome patient’s fail to adequately repair this damage. These DSBs likely arise as a consequence of DNA replication in the presence of CSA. This work has implications for BMT and GvHD management in general and specifically for LIG4 syndrome.

Keywords: Cyclosporine A, DSBs, LIG4 syndrome
Introduction.

Bone marrow transplantation (BMT) remains one of the only curative options available to treat progressive anaemia, combined immunodeficiency and/or lymphoma development in the context of DNA damage response defective disorders such as Fanconi anaemia and Nijmegen breakage syndrome(1, 2). Non-myeeloablative hematopoietic stem cell transplantation (NHSCT) using reduced doses of DNA damaging agents has improved the success rate for transplantation in this context(1, 3). Several human disorders have now been described that are defective in nonhomologous end-joining (NHEJ), the principal pathway by which human cells repair DNA double strand breaks (DSBs)(4, 5). These include LIG4 syndrome, which is caused by hypomorphic mutations in DNA ligase IV, Artemis-dependent severe combined immunodeficiency (ART-SCID), caused by mutations in the Artemis endonuclease and XRCC4-like factor (XLF)/Cernunnos-dependent SCID, caused by mutations in a XLF/Cernunnos (5-8). Increased cellular and clinical radiosensitivity is a feature of these conditions due to their failure to repair ionising radiation induced DSBs(6). Because of the central role played by NHEJ in V(D)J recombination all of these disorders initially present with moderate-to-severe combined immunodeficiency necessitating clinical intervention often culminating in BMT(9). But, anecdotal (unpublished) and evidence from the literature suggests that LIG4 syndrome patients fair particularly poorly following BMT (Table 1). Indeed, there is only one detailed report of a successful BMT in LIG4 syndrome using NHSCT with a modified conditioning regimen(10). This is in marked contrast to that of ART-SCID where NHSCT has been used successfully in these patients even before the underlying causative genetic defect was identified(9, 11, 12). The reason for the difference in
response in these two patient groups that display similar levels of radiosensitivity is unclear. To date, there has not been a report of BMT in XRCC4-like factor (XLF)/Cernunnos-dependent SCID.

The agents commonly employed as part of the conditioning regimen prior to NHSCT include busulphan (Myellan), a bifunctional DNA cross-linking agent, fludarabine (Fludara), a nucleoside analogue and methotrexate (Amethopterin), a dihydrofolate reductase inhibitor. All of these compounds can induce DSBs either directly or indirectly. Cyclosporine A (CSA) is a widely used prophylaxis for Graft-versus-Host Disease (GvHD), which represents a significant post-transplantation complication(13, 14). CSA binds to the cyclophilin class of proteins that can then function as an inhibitor of calcineurin, a serine-threonine phosphatase (also called PP2B). CSA’s utility as an immunosuppressive agent is thought to derive from its ability to prevent nuclear localisation of transcription factor NFAT (nuclear transcription factor in activated T cells)(15). NFAT is involved in the transcription of various cytokines (eg. IL-2) that are required to activate T cells. Inhibition of NFAT nuclear localization by CSA results in profound systemic immunosuppression.

Because of the poor clinical outcome of LIG4 syndrome patients to BMT we set-out to investigate whether the failure to repair DSBs induced by the agents used during NHSCT could have a disproportionately adverse impact in the context of DNA ligase IV deficiency. Using the MTT assay, we evaluated the sensitivity of human pre-B lymphocytes either proficient or engineered by gene targeting to be deficient for DNA ligase IV to the components employed clinically as part of the conditioning regimen prior to BMT and for GvHD prophylaxis. These included busulphan,
fludarabine, methotrexate and cyclosporine A. Furthermore, using an indirect immunofluorescence assay based on 53BP1 foci formation as a sensitive marker for DSBs, we evaluated the impact of BMT conditioning regimens on DSB formation and repair in NHEJ-defective human cells.

**Materials and Methods.**

*Cell lines.*

Wild type (WT; Nalm 6) and DNA ligase IV knockout (LigIV-/-; N114-P2) pre-B human lymphocyte cells have been described previously(16). These cells were cultured in RPMI-1640 supplemented with L-glutamine and 15% foetal calf serum. Primary human skin fibroblasts were grown in MEM supplemented with L-glutamine and 15% foetal calf serum. The wild type (WT; 1BR.3), LIG4 syndrome (LIG4; 411BR) and Artemis-SCID (ART-SCID; CJ179) have been described elsewhere(6,17). The LIG4 syndrome fibroblast was derived from a 9 yrs old patient with three homozygous alterations in DNA Ligase IV (8C>T(A3V), 26C>T(T9I) and 833G>A(R278H))(6). The ART-SCID fibroblast (CJ179) was derived from an immunodeficient child and fails to express a detectable *Artemis* transcript due to a genomic deletion(17).

*Drugs.*

All drugs/chemicals were obtained from Sigma-Aldrich UK Ltd (Poole, UK) and made up fresh prior to each survival. Busulphan was dissolved in dimethylsulphoxide (DMSO) and made up to 5mM in phosphate buffered saline (PBS; ≤2% v/v DMSO).
fludarabine and cyclosporine A were dissolved in DMSO. Methyl methane sulphonate was diluted in complete medium.

**Antibodies.**

Anti-53BP1 (BL181) antibody was obtained from Universal Biologicals (Cambridge, UK). Anti-bromodeoxyuridine (Bu20A) antibody was obtained from Autogen BioClear (Whilshire, UK).

**Immunofluorescence based DNA Double strand break repair assay.**

Immunofluorescence based detection of 53BP1 foci formation using an anti-53BP1 specific antibody was used as an indirect highly sensitive assay for monitoring DNA double strand break (DSB) formation and repair, as previously characterised(17). 53BP1 is one of many proteins recruited to a DSB. Following treatment of primary fibroblasts with various agents, single DSB’s are visualised indirectly as discrete microscopically detectable 53BP1 foci (Fig 3a). Cells were also treated with 50μM bromodeoxyuridine (BrdU) to label S-phase cells that were specifically visualised following immunofluoresence using an anti-bromodeoxyuridine antibody. Standard immunofluorescence procedures for primary skin fibroblast have been described in detail elsewhere(17). Images were captured using a Zeiss-Axioplan microscope using Simple-PCI software.

**MTT assay.**

For MTT survival analysis cells were treated in RPMI-1640 without phenol red (Fischer Scientific UK LTD) in 24 well plates and grown for 5 days. The drug(s) was not removed. Methylthiazolyldiphenyl-tetrazolium bromide (MTT), dissolved in
RPMI-1640 without phenol red, was added to each well (0.5mg/ml) and incubated for 3-6hrs to allow the insoluble formazan crystals to form. Acidic iso-propanol (40mM HCl in absolute iso-propanol) was added to each well to dissolve the formazan crystals. Following removal of cell debris by centrifugation, absorbance of the converted dye was measured at 570nm with background subtraction at 650nm.

Results.

We specifically chose human pre-B lymphocytes for analysis as these represent an important target population for conditioning regimens. We treated wild type (WT) and DNA ligase IV knockout (Lig IV\(^{-/-}\)) human pre-B lymphocytes with busulphan, fludarabine and methotrexate either alone (Fig 1a-c) or in various combinations (Fig 1d) and determined survival after 5 days using MTT analysis. We failed to find any significant sensitivity in the absence of DNA ligase IV (Lig IV\(^{-/-}\)) to any of these agents individually (Fig 1a-c). Furthermore, no additional significant sensitivity was observed in DNA ligase IV\(^{-/-}\) pre-B lymphocytes when they were treated with all of these agents simultaneously (Fig 1d). This data suggests that complete loss of DNA ligase IV does not selectively significantly sensitize human pre-B lymphocytes to busulphan, fludarabine or methotrexate. Furthermore, when used in combination, these compounds do not act to synergistically sensitize human pre-B lymphocytes deficient for DNA ligase IV.

Cyclosporine A is currently a cornerstone for the treatment and/or prevention of GvHD(14). Unexpectedly, we found that DNA ligase IV knockout (Lig IV\(^{-/-}\)) human pre-B lymphocytes were selectively sensitive to killing by CSA (Fig 2a).
Similarly, when CSA was used in combination with busulphan and fludarabine, we found that the DNA ligase IV⁻/⁻ pre-B lymphocytes were more sensitive than their wild type (WT) counterparts (Fig 2b). Since DNA ligase IV functions as a core component in one of the principal DNA double strand break repair pathways, NHEJ, we investigated the possibility that this selective sensitivity of DNA ligase IV⁻/⁻ cells towards CSA could be the consequence of CSA-induced DSB formation. It has not been previously reported that CSA can, either directly or indirectly, induce DSBs. Following DSB formation in mammalian cells, one of the earliest detectable responses is the phosphorylation (ATM and DNA-PK dependent) of the histone H2A variant H2AX (termed γ-H2AX). It has been shown that DSB formation and repair can be very sensitively monitored in cells by indirect immunofluorescence microscopy using antibodies specific for γ-H2AX(17). It has been shown that each discrete microscopically detectable γ-H2AX focus likely represents a single DSB(18-20). To monitor DSB formation we used primary skin fibroblasts that are more amenable to this type of microscopic analysis compared to lymphocytes. 53BP1 is one of many proteins recruited very rapidly to a DSB. Microscopically detectable 53BP1 foci are formed, co-localise and are repaired with the same kinetics as those of γ-H2AX(17, 21). For practical reasons (to allow co-staining with anti-BrdU) we utilised 53BP1 foci formation as a surrogate marker for DSBs instead of monitoring γ-H2AX foci formation (Fig 3a). We used exponentially growing primary fibroblasts from a clinically unaffected (wild type; WT), a radiosensitive DSB-repair defective LIG4 syndrome patient (LIG4) and Artemis-defective SCID patient (ART-SCID) that we previously characterised(6, 17). Interestingly, we found an increased amount of DSBs (53BP1 foci) in cells that had traversed S-phase (BrdU positive) following a 24 hrs treatment with CSA specifically in the LIG4 syndrome cells compared to WT and
ART-SCID (Fig 3a and b). 53BP1 foci were not observed in cells that did not stain for BrdU, under these conditions. Staining for S-phase cells using anti-bromodeoxyuridine (BrdU) showed that all of the cell lines had comparable levels of S-phase cells (20-23%) and were therefore growing at similar rates. This indicates that CSA treatment results in DSB formation in cells that have traversed S-phase and that these breaks persist in LIG4 syndrome cells specifically.

A likely mechanism for DSB formation during S phase is the collision of replications forks with single stranded DNA breaks (SSBs). SSBs occur spontaneously at high levels in mammalian cells but are usually repaired rapidly by the base excision repair pathway (BER)(22-25). CSA has been proposed to inhibit DNA damage-induced over-expression of DNA polymerase β, which plays an important role in BER(24, 26). To examine whether CSA might enhance the persistence or formation of DSBs following replication, we examined the impact of CSA on DSBs arising following treatment with methyl methane sulphonate (MMS), an agent that can induce SSBs in S-phase (Fig 3c). Following exposure to 1mM MMS for 1hr and incubation for a further 24hrs, a small increase in DSBs (5-8 53BP1 foci) was observed in WT, LIG4 syndrome and ART-SCID primary fibroblasts that had traversed S-phase (BrdU positive (+ive)). Non-BrdU labelled cells showed no increase in DSB formation suggesting that DSB formation is replication dependent. This is consistent with the notion that DSBs can arise following replication of SSBs but that under normal conditions they are rapidly repaired. Strikingly, when CSA (5μM) was included with MMS a significant increase in DSBs was observed specifically in LIG4 syndrome fibroblasts (23-30 53BP1 foci) compared to WT or ART-SCID (5-10 53BP1 foci). This suggests that CSA-induced DSBs can form,
likely indirectly, from SSBs during S-phase. Furthermore, these DSBs specifically persist in LIG4 syndrome cells compared to ART-SCID (Fig 3c).

Finally, we examined whether the increased sensitivity of DNA Ligase IV-/- cells to combined treatment with CSA, busulphan and fludarabine, conditions which mimic NHSCT conditioning and GvHD prophylaxis, was a consequence of increased DSB formation (Fig 4). Using WT and LIG4 syndrome primary fibroblasts, following treatment with modest concentrations of busulphan (5μM) together with fludarabine (1μM), no significant difference in DSBs was observed between these cell lines (Fig 4 Bus+FluD). This was distinct to treatment with CSA (5μM) alone where an increased level of DSBs were observed in LIG4 syndrome cells derived from S-phase specifically (Fig 4 CSA and Fig 3b). Strikingly, when these cell lines were treated for 24 hrs with CSA (5μM) simultaneously with busulphan (5μM) and fludarabine (1μM), a significant increased level of DSBs were now observed specifically in the LIG4 syndrome cells compared to WT (Fig 4 Bus+FluD+CSA). This data suggests that CSA can induce DSBs alone but particularly in combination with busulphan and fludarabine and that these DSBs remain at an elevated level in LIG4 syndrome-derived patient cells.

Collectively our data identifies CSA as an important source of DSBs especially when used in combination with busulphan and fludarabine, similarly to conditions used for NHSCT conditioning and GvHD prophylaxis. Furthermore, DNA ligase IV-/- pre-B lymphocytes and LIG4 syndrome primary fibroblasts are particularly sensitive to CSA-induced DSB formation.
Discussion.

NHSCT using reduced doses of DNA damaging agents has significantly improved the outcome of BMT in individuals compromised for DNA repair(1, 12). But, LIG4 syndrome patients, particularly compared to ART-SCID, generally have a poorer outcome(5, 9, 11, 12, 27, 28). Multiple factors could be responsible for this, especially the general health of the patients prior to conditioning. Nevertheless, since compounds that can form DSBs are used during NHSCT we sought to determine whether DNA ligase IV deficiency could adversely hyper-sensitize individuals to such treatments. Here, we report the first detailed evaluation of the response of DNA ligase IV defective cells to the compounds used for BMT and GvHD prophylaxis. We found that human DNA ligase IV-/- pre-B lymphocytes are not significantly selectively sensitive to busulphan, fludarabine or methotrexate alone or when treated with these agents simultaneously. Unexpectedly, we found that DNA ligase IV-/- pre-B human lymphocytes were selectively sensitive to cyclosporine A, probably the most widely used prophylaxis for GvHD. Furthermore, when CSA was used in combination with busulphan and fludarabine, these cells exhibited significant hypersensitivity. Crucially, we found that CSA treatment resulted in DSB formation even in non-heamatopoetic human cells (primary skin fibroblasts). We suggest that these DSBs arise likely indirectly from single strand breaks occurring during DNA replication. Importantly, we showed that these CSA-induced DSBs are formed at increased levels in DNA Ligase IV-deficient background following co-treatment with busulphan and fludarabine. Finally we showed that these DSBs are repaired with slower kinetics in the absence of DNA ligase IV compared to Artemis-deficiency.
Our data unexpectedly identified CSA as an agent capable of causing DSB formation in human cells specifically following replication. Interestingly, CSA treatment, either alone or in combination with other BMT drugs, did not result in an increased level of DSBs in ART-SCID fibroblasts compared to those of LIG syndrome. The roles of Artemis and DNA ligase IV in NHEJ are distinct(17, 29). LIG4 syndrome cells show slow kinetics of repair of all DSBs whilst ART-SCID cells rejoin the majority of DSBs with normal kinetics but are defective in a specific subset of DSBs. Increased levels of residual CSA-induced DSBs seen in the LIG4 syndrome cells are likely a result of their general inability to repair all types of DSBs compared to ART-SCID cells. This provides a provocative potential explanation as to why ART-SCID individuals routinely successfully undergo NHSCT compared to LIG4 syndrome. Our findings also add weight to the increasing evidence that NHEJ can function to repair replication associated DSBs, which has previously been proposed to be carried out by homologous recombination(30).

CSA is used as prophylaxis for GvHD, but GvHD treatment protocols vary significantly(14). A previous study among the European Group for Blood and Marrow Transplantation (EBMT) found that CSA treatment protocols and target doses vary widely amongst European transplantation centres(31). Whilst the average initial daily dose of CSA was 3mg/kg (i.v) the range was 1-20mg/kg (i.v). Target CSA blood concentrations ranged from 250-400μg/l, which is equivalent to 0.19μM-0.3μM CSA. Whilst these low μM concentrations represent an approximately 10 fold difference to those used in our experiments (2-5μM), crucially, our cellular work utilised a short-term exposure to a single dose of CSA. The cumulative effect on DSB formation from repeated or chronic exposure to low μM concentrations of CSA could be
physiologically relevant. Interestingly, the LIG4 syndrome patient that successfully underwent NHSCT recently reported by Gruhn et al, received a CSA dosage at the lower end of the European range (3mg/kg/day)(31). Our data suggests that caution should be exercised regarding CSA treatment regimens specifically in the context of LIG4 syndrome.

An important general implication of our findings that CSA can form DSBs relates to malignancy. Long-term use of calcineurin inhibitors such as CSA in solid organ and bone marrow transplantation is associated with increased risk of cancer(32-34). Whilst immunosuppression appears to be the principal predisposing factor others have argued that compounds such as CSA can also inhibit the repair of DNA damage(33, 35-37). A precedent for the role of immunosuppressive agent-induced DNA damage likely impacting on cancer predisposition has recently been described regarding azathioprine(38, 39). Un-repaired DSBs can result in deletions and/or act as a platform for translocations with obvious implications for malignant transformation(40-42). If prolonged CSA treatment induces persistent DSBs this could compromise genomic stability not just in the context of DNA ligase IV deficiency. Whether this could play any role in BMT-derived secondary cancers is unclear, although worthy of further investigation(32, 43).
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DNA ligase IV\(^{-/}\) human pre-B lymphocytes are not selectively sensitive to killing by busulphan, fludarabine or methotrexate, alone or in combination.

Wild type (WT) and DNA ligase IV\(^{-/}\) human pre-B lymphocytes (LigIV\(^{-/}\)) were chronically exposed to increasing concentrations of busulphan (a), fludarabine (b) and methotrexate (c) in culture for 5 days. Survival was determined using the MTT assay. Drugs were not removed during the course of the experiment.

d). Wild type (WT) and DNA ligase IV\(^{-/}\) human pre-B lymphocytes (LigIV\(^{-/}\)) were treated simultaneously with different combinations of busulphan (Bus, 5\(\mu\)M), fludarabine (FluD, 0.05\(\mu\)M) and methotrexate (MTX, 0.025\(\mu\)M) to investigate the potential for these agents to kill these cell synergistically. Cells were incubated for 5 days prior to analysis by MTT. Drugs were not removed.

DNA Ligase IV\(^{-/}\) pre-B lymphocytes are selectively sensitive to CSA.
a). The survival of Wild type (WT) and DNA ligase IV<sup>−/−</sup> human pre-B lymphocytes (LigIV<sup>−/−</sup>) to increasing concentrations of CSA was determined by MTT assay 5 days post-treatment. CSA was not removed.

b). Wild type (WT) and DNA ligase IV<sup>−/−</sup> human pre-B lymphocytes (LigIV<sup>−/−</sup>) were treated simultaneously with busulphan (5μM), fludarabine (1μM) and CSA (2μM) and survival determined after 5 days culture using the MTT assay. Drugs were not removed.

**Figure 3.**

CSA induces more DSBs in LIG4 syndrome cells compared to ART-SCID and these DSBs likely arise from single strand breaks during DNA replication.

a). Wild type (WT) and DNA ligase IV syndrome (LIG4) primary skin fibroblasts were treated with CSA (5μM) for 24 hrs. DSB formation was monitored indirectly by examining the formation of 53BP1 foci at the site of DSBs by indirect immunofluorescence using an antibody against 53BP1. DSBs were visualised as discrete microscopically detectable 53BP1 foci. Cells were counterstained with a DNA specific stain (DAPI) to identify the nucleus.

b). Wild type (WT), DNA ligase IV syndrome (LIG4) and Artemis-defective SCID (ART-SCID) primary skin fibroblasts were untreated (UNT) or treated with CSA (5μM). 53BP1 foci formation in bromodeoxyuridine positive cells (BrdU +ive) was determined 24 hrs post-treatment. All cell lines were also initially treated with 50μM
bromodeoxyuridine to label S-phase cells that were specifically identified as the bromodeoxyuridine positive staining (BrdU +ive) by immunofluorescence. CSA and BrdU were not removed during the course of the experiment.

c). Wild type (WT), DNA ligase IV syndrome (LIG4) and Artemis-defective SCID (ART-SCID) primary skin fibroblasts were untreated (UNT) treated with methylmethane sulphonate (MMS) and/or CSA (5μM) and examined for 53BP1 foci formation in bromodeoxyuridine positive cells (BrdU +ive) 24 hrs post-treatment. All cell lines were also initially treated with 50μM bromodeoxyuridine. MMS treatment (1mM) was for 1hr. Neither CSA nor BrdU were removed during the course of the experiment.

**Figure 4.**

Co-treatment of LIG4 syndrome cells with CSA, busulphan and fludarabine results in increased levels of DSBs compared to WT cells. There was no evidence of DSB formation in non-BrdU labelled cells.

a). 53BP1 foci were monitored in cells that had traversed S-phase 24 hrs following treatment with different combinations of busulphan (Bus, 5μM), fludarabine (FluD, 1μM) and CSA (5μM) in wild type (WT) and DNA ligase IV syndrome (LIG4) primary skin fibroblasts. All cell lines were initially treated with 50μM bromodeoxyuridine as above and drugs were not removed. BrdU +ive = bromodeoxyuridine positive (S-phase) cells.
Tables 1.

A summary of the outcome of clinical management of BMT on ART-SCID and LIG4 syndrome patients.

EDX, Endoxan (cyclophosphamide), VOD, veno-occlusive disease, MUD, matched unrelated donor, MTX, methotrexate, EBV-NHL, Epstein Barr virus positive non-Hodgkin lymphoma, NHSCT, nonmyeloablative hematopoietic stem cell transplantation.