Effect of combined sublethal X-ray irradiation and cyclosporine A treatment in NOD scid gamma (NSG) mice

Lia WALCHER1), Claudia MÜLLER1), Nadja HILGER1,2), Anna KRETSCHMER1), Lilly STAHL1), Simone WIGGE3), Jens RENGELSHAUSEN3), Anne M. MÜLLER1) and Stephan FRICKE1)

1)Fraunhofer Institute for Cell Therapy and Immunology, Perlickstrasse 1, 04103 Leipzig, Germany
2)Institute for Clinical Immunology, University of Leipzig, Johannisallee 30, 04103 Leipzig, Germany
3)Grünenthal GmbH, Zieglerstrasse 6, 52078 Aachen, Germany

Abstract: Cyclosporine A (CsA) is used in hematopoietic stem cell transplantations (HSCT) to prevent graft-versus-host disease (GvHD). GvHD is the most severe side effect of allogeneic HSCT and efficient therapies are lacking. Mouse models are an essential tool for assessing potential new therapeutic strategies. Our aim is to mimic a clinical setting as close as possible using CsA treatment after sublethal irradiation in NSG mice and thereby evaluate the feasibility of this mouse model for GvHD studies. The effect of CsA (7.5 mg/kg body weight) on sublethally X-ray irradiated (2 Gy) and non-irradiated NSG mice was tested. CsA was administered orally every twelve hours for nine days. Animals irradiated and treated with CsA showed a shorter survival (n=3/10) than irradiated animals treated with NaCl (n=10/10). Furthermore, combined therapy resulted in severe weight loss (82 ± 6% of initial weight, n=7, day 8), with weight recovery after the CsA application was ceased. A high number of apoptotic events in the liver was observed in these mice (0.431 ± 0.371 apoptotic cells/cm², n=2, compared to 0.027 ± 0.034 apoptotic cells/cm², n=5, in the non-irradiated group). Other adverse effects, including a decrease in white blood cell counts were non-CsA-specific manifestations of irradiation. The combination of CsA treatment with irradiation has a hepatotoxic and lethal effect on NSG mice, whereas the treatment without irradiation is tolerated. Therefore, when using in vivo models of GvHD in NSG mice, a combined treatment with CsA and X-ray irradiation should be avoided or carefully evaluated.

Key words: cyclosporine A, GvHD, irradiation, mouse model, NSG

Introduction

Cyclosporine A (CsA) is one of the most frequently used drugs for immunosuppression and has widely been used in both experimental and clinical settings since 1978 [9, 16]. It is a lipophilic and cyclic endecapeptide [31] that was isolated from Tolypocladium inflatum [3, 16]. CsA acts as a calcineurin inhibitor that inhibits T-cell proliferation [27]. The metabolism is primarily hepatic [17, 34, 44] with a half-life of around twelve hours when orally administered [24]. The enzymes mainly involved are CYP3A4 and CYP3A5, both members of the cyto-
chrome P450 family [5, 18, 34]. Due to the variable expression of these isoenzymes, the bioavailability of CsA is inconstant [29, 41]. Therefore, products currently used in the clinic are emulsion-based such as Sandimmun®, leading to a higher oral bioavailability with less variability when the drugs are orally administered [25].

The elimination of CsA metabolites occurs via the bile; only a small proportion of the parent drug is excreted in the urine [8, 54]. CsA is used in allogeneic hematopoietic stem cell transplantation (HSCT) to prevent graft-versus-host disease (GVHD) [35, 52, 56]. It acts by inhibiting the formation of pro-inflammatory cytokines as well as the activation and proliferation of lymphocytes [50]. GVHD is one of the major serious immunological complications of allogeneic HSCT [6].

A key component prior to HSCT in patients is irradiation [1, 26, 36]. To our knowledge, there are no studies or data on the detailed effects of CsA in NOD.Cg-Prkdc<sup>scid</sup> II<sup>2rg<sup>em1Sug</sup></sup>/JicTac (NOG) or sublethally X-ray irradiated NSG mice so far. Gregoire-Gauthier et al. previously demonstrated the use of CsA in a mouse model of GVHD [22, 23]. Different from our study, human peripheral blood mononuclear cells (PBMCs) were applied before CsA administration, creating so-called xenogeneic NSG mice [22, 23]. Exclusive effects of CsA and X-ray irradiation on NSG mice could not be assessed using this model. This and other previous applications of CsA in mouse models are summarized in Table 1. The aim of our study was to determine potentially toxic effects of per os (p.o.) application of CsA in NSG mice with or without sublethal X-ray irradiation.

### Materials and Methods

#### Animals

Male NSG (NOD.Cg-Prkdc<sup>scid</sup> II<sup>2rg<sup>em1Wjl</sup></sup>/SzJ) mice were purchased from Charles River, Sulzfeld Germany. The handling and accommodation followed the guideline of the University of Leipzig animal care committee and the regional board of animal care responsible for Leipzig (Landesdirektion Leipzig). Survival and weight were assessed and reported; blood cell counts and surface markers were measured weekly [20, 30].

For data evaluation, animals were divided into two subgroups: an abort group comprising those animals, with an early death (before day 13) and a final group comprising those animals, which survived until the end of the experiment. Since non-irradiated mice treated with CsA did not show signs of weight loss or early death, an abort group was artificially created by taking out five animals on day 9 of the experiment. This was done to enable the analysis of short-term effects of CsA in the non-irradiated mice as well.

#### X-ray irradiation and application of CsA

NSG mice were sublethally irradiated (2 Gy) four hours prior to the first oral CsA application. Irradiation was performed with SARRP (Xstrahl LTD, Camberley, UK). For irradiation, the mice were anesthetized with isoflurane in a mobile anesthesia machine (VetTech Solutions ITD, Congleton, UK) provided for this purpose and then transferred into the irradiation apparatus. After irradiation, the mice were returned to the cages and observed until complete consciousness was regained.

CsA (Sandimmun Optoral®, 100 mg/ml, 22.5 µl, Novartis Pharma GmbH) was added to sterile NaCl solution.

### Table 1. Summary of CsA application in mouse models described in literature

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Dose and route of application</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>C.B-17 SCID, C.B-17 SCID/beige mice</td>
<td>10 mg/kg/day or 20 mg/kg/day subcutaneously (s.c.) for 16 to 21 days</td>
<td>[40]</td>
</tr>
<tr>
<td>SCID</td>
<td>10 mg/kg/day intraperitoneally (i.p.) for two days followed by 15 mg/kg every other day i.p. for three weeks</td>
<td>[21]</td>
</tr>
<tr>
<td>Xenogeneic NSG</td>
<td>0.375 mg/mouse/day i.p. from day 0 to +23, 2–3 Gy irradiation</td>
<td>[22, 23]</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt; II&lt;sup&gt;2rg&lt;sup&gt;em1Sug&lt;/sup&gt;&lt;/sup&gt;/JicTac (NOG)</td>
<td>30 mg/kg s.c., five times per week until day 15, 3 times per week until day 64; or 5 mg/kg vs. 30 mg/kg 3 times per week throughout the entire study</td>
<td>[49]</td>
</tr>
<tr>
<td>NSG</td>
<td>Oral and i.p. for prophylaxis of GVHD, dose or duration of application is not indicated</td>
<td>[19]</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>30 mg/kg/day i.p.</td>
<td>[32]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>20 mg/kg/day i.p. until remission of acute GVHD</td>
<td>[55]</td>
</tr>
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(B. Braun Melsungen AG, Melsungen Germany, 0.9%, 2.9775 ml). The current body weight (BW) of the animals was measured prior to each application in order to determine the correct application volume (7.5 mg/kg BW). The substances were administered orally every twelve hours for nine days using a gavage, which was rinsed with autoclaved drinking water before and between the individual applications. The timing of application and the application route were selected according to the manufacturer’s instructions, which suggest an oral application, and dividing the daily dose into two separate applications. The application period was limited to nine days, because irradiated animals showed a high mortality and a poor health condition. For a better reproducibility, also non-irradiated animals received CsA for nine days as a control group.

Flow cytometry
For a detailed analysis of immune cell surface markers, blood samples were measured weekly using flow cytometry. For compensation of fluorochromes and calibration, compensation beads (CompBeads, BD Biosciences, Heidelberg, Germany) were used according to the manufacturer’s recommendations. After adding heparin (10 µl per sample, Rationpharm® GmbH, Ulm, Germany), blood cells were diluted in 100 µl PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na 2 HPO 4, 2 mM KH 2 PO 4) and incubated with antibodies (CD11b- aPc-cy7, Ly6c/6g-Pe, both BD Biosciences, Heidelberg, Germany) according to the manufacturer’s protocol. Erythrocytes were lysed with 1 ml lysis solution (BD Biosciences, Heidelberg, Germany). Cell pellets were resuspended in 100 µl PBS [20].

Organ analysis and histology (TUNEL assay)
The liver weight was determined for the organ analysis. For the histological analysis, the samples were fixed in 4% formaldehyde (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 24 h and subsequently dehydrated using a dewatering apparatus (Leica TP 1020 Biosystems, Eisfeld, Germany). After dewatering, the samples were embedded in paraffin (Thermo Scientific, Darmstadt, Germany) and cut into 3 µm thick sections [30]. The detection of apoptotic cells was performed using the TdT-mediated dUTP-biotin nick end labelling (TUNEL, ApopTag Merck) for light microscopy as described by Loo [38]. Accurate cell numbers in TUNEL slides were determined by image thresholding using ImageJ software (v1.46r, NIH, USA). Per mouse, five randomly chosen visual fields of one histological slide of the liver were microscopically recorded in 10-fold magnification. Data was acquired per visual field and group [30].

Measurement of laboratory parameters in plasma
To analyze the clinical laboratory parameters, serum levels of alkaline phosphatase (ALP), cholinesterase (CHE), alanine aminotransferase (ALAT), glutamate dehydrogenase (GLDH) and bilirubin were measured using AU480® Chemistry Analyzer (Beckman Coulter, Krefeld, Germany) as described earlier [30]. In brief, the plasma from blood of different time points was collected by centrifugation at 300 × g for 10 min and stored at −20°C. Measurement was carried out according to the instructions given by the manufacturer. Some values could not be obtained either due to hemolysis or lack of material.

Data acquisition and statistical tests
BD FACSCantoTMII Flow Cytometry System with BD FACSDIVA™ Software 6.1.3 software (BD Biosciences Heidelberg, Germany) and scil Vet abc™ Hematology Analyzer (scil animal care co. GmbH, Viernheim, Germany) were used to acquire the data. Limit values stated in the following comply with scil Vet standards. Graphs were created using GraphPad Prism 5 (v5.02, GraphPad Software Inc., La Jolla, CA, USA). The results are represented as mean ± SD. Data was checked for Gaussian distribution by D’Agostino and Pearson omnibus normality test using GraphPad Prism. P values were determined by analysis of variances (ANOVA) for data that passed the normality test (α=0.05) or by Kruskal-Wallis test for non-normally distributed data using GraphPad Prism. Log-Rank test and pair-wise multiple comparison of means (Holm-Sidak method) were used for the analysis of Kaplan-Meier survival curves. A P value <0.05 indicates significant differences Asterisks in figures are used as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results
CsA treatment after X-ray irradiation causes severe weight loss
The animals, which were sublethally irradiated (2 Gy, X-ray) and treated with CsA (7.5 mg/kg BW, n=10), had a shorter survival (30%, time of death between days 6
and 13) in comparison to irradiated animals receiving 0.9% NaCl (100%, n=3, day 62, Fig. 1A) and in comparison to non-irradiated animals (100%, n=10, Fig. 1B). The log-rank-test showed no significant difference between the survival of the groups (P=0.051). Due to the poor health status of the mice (low weight, death of five animals), CsA administration was stopped on day 9. Thereafter, one additional mouse died on day 13. Furthermore, the irradiated and CsA-treated mice experienced severe weight loss (Fig. 1C), with significantly lower weights compared to the other groups (P=0.008). The surviving animals recovered well after the CsA application was ceased and started gaining weight from day 11 onwards (Fig. 1C, 96 ± 6%, n=4), reaching their initial weight on day 35. In the control group, which was also irradiated but received only 0.9% NaCl, a similar but reduced weight loss (96 ± 3%, day 9, n=3) and subsequent recovery was observed. Animals treated with CsA but without irradiation also experienced weight loss, however, not as severe as the irradiated mice (Fig. 1D, 94 ± 3% on day 5, n=5). These mice also fully recovered to their initial weight on day 12. Together, these results indicate that there is an additive negative effect of CsA and X-ray irradiation in NSG mice.
X-ray irradiation depletes white blood cells; additional CsA application may lead to a decrease in monocyte/macrophage/neutrophil population

The irradiated groups showed a decrease in white blood cells (WBCs) until day 10 (Fig. 2A). In comparison, the non-irradiated groups showed a stable WBC count within this time frame (Fig. 2B). In addition, WBCs were analyzed by flow cytometry. Figs. 2C and D illustrate the flow cytometric analysis of Ly6C/6G and CD11b, representing the monocyte/macrophage/neutrophil cell population, at three different time points (day−1 or −2 = days before administration, day 9 or 10, and day 57; for detailed scatter plots see Supplementary Fig. 1).
there was also a drop in the monocyte/macrophage/neutrophil population in all groups on day 9. Notably, there is a strong deviation amongst the samples, and those samples with low values also had a low overall number of events in the leukocyte gate (<1,000). These findings indicate that X-ray irradiation generally affects white blood cells, whereas application of CsA might lead to a specific depletion of the monocyte/macrophage/neutrophil population of immune cells in some animals.

CsA combined with X-ray irradiation increases liver toxicity in NSG mice

As CsA is a hepatotoxic agent, apoptotic cells in the liver were detected by histological TUNEL staining. The TUNEL staining showed that animals of the irradiated and CsA-treated group, which died between days 6 and 13, had a significantly higher apoptotic cell count (0.431 ± 0.371 cells/cm², n=10 histological visual fields of n=2 animals) in comparison to the animals of the CsA-treated group, which survived until the end of the experiment, (0.021 ± 0.015 cells/cm², n=15 histological visual fields).
of n=3 animals) as well as the control group (0.044 ± 0.057 cells/cm², n=5 in the final group) nor in the control group (0.044 ± 0.057 cells/cm², n=3), (Fig. 3D). Still, sporadic patches of apoptotic cells could be seen in mice receiving CsA but no irradiation (Fig. 3C). In conclusion, increased apoptosis of liver cells in NSG mice was observed when mice were irradiated prior to CsA application.

In order to further investigate the toxic effects of CsA and irradiation on the liver, the liver weights and serum parameters ALP, CHE, ALAT, GLDH and bilirubin were determined. Interestingly, no abnormalities were observed here and all parameters were consistent despite CsA administration and irradiation (Supplementary Fig. 2). Therefore, considering hepatotoxicity, treatment with CsA together with irradiation has a direct effect exclusively on liver cell apoptosis in this model.

Discussion

GvHD is the most severe complication after allogeneic HSCT [6]. Due to a lack of efficient therapies, it is necessary to develop suitable animal models to research new treatment strategies [47]. In this experiment, immune-deficient NSG mice were treated with 7.5 mg/kg BW CsA every twelve hours with or without prior sublethal irradiation of 2 Gy. Our aim was to determine the potential toxic effects of CsA application in combination with irradiation on NSG mice in order to evaluate the potential of this model for a subsequent transplantation of human PBMCs and T-cells for GvHD studies. NSG mice were chosen as a basis to gain first information for future experiments of testing CsA in humanized NSG mice. Such a model can be a useful tool for testing novel drug candidates.

In general, CsA is well-tolerated in various mouse models such as BALB/c [55], C57BL/6 [46], NOG [49] or NOD-SCID mice [32]. Nevertheless, adverse effects of CsA could be shown in previous studies in humans and rodents and include organ damage to the liver and kidney [4, 10, 15], hypertension [45] and several other toxicities [14]. Specifically, irradiation and treatment with CsA in NSG mice was previously reported in a xenogeneic model, where human PBMCs were injected between sublethal irradiation and intraperitoneal administration of CsA [22, 23]. In this setting, there was a mortality of 40%, although this could likely be either a result of GvHD or of the irradiation and CsA treatment.

We observed that the combination of CsA treatment with irradiation was toxic to the animals, causing weight loss, hepatotoxicity, and the death of the animals. In the log-rank-test, the difference in survival of treated versus control groups was not significant. This is likely due to the fact that CsA application was stopped on day 9. In fact, the shown experiments were initially intended to be part of a dose-response study with 7.5 mg/kg BW CsA as the lowest dose and a duration of application for 16 days. These parameters were selected based on other studies reported in literature (see Table 1). Surprisingly, there were already strong adverse effects with this dose after nine days when combined with irradiation, which is why the application was stopped. For the same reason, higher doses were not tested. When the CsA application was terminated early, a partial recovery of the mice was observed. The surviving animals showed no long-term effects of the treatment, as concluded by healthy weight, white blood cell levels, and absence of liver cell apoptosis. Treatment with CsA alone or irradiation alone on the other hand had a less harmful effect on the animals, neither one of the two being lethal. Still, irradiation of 2 Gy caused some adverse effects in the animals such as weight loss and decrease of white blood cells. The myeloablative and myelosuppressive effects of irradiation are well-described in literature [39, 53]. Moreover, the sensitivity of NSG mice towards X-ray irradiation has previously been shown; NSG mice react to sublethal total body irradiation with myelodysplasia, blood protein reductions and body composition changes [33]. In comparison, CsA treatment without prior irradiation only had minor effects, namely a slight weight decrease and sporadic events of liver cell apoptosis. Likewise, Lim et al. showed that CsA treatment of primary mouse hepatocytes did not lead to a significant increase in apoptosis rate [37].

This study shows that the combination of multiple oral doses of 7.5 mg/kg BW CsA and sublethal irradiation of 2 Gy was lethal for NSG mice, whereas treatment with CsA alone or irradiation alone is well-tolerated by the animals. The lethal effect may specifically be attributed to the induction of apoptosis in the liver as also described.
in literature [37]. Other indications of liver toxicity such as changes in liver weight or serum parameters, however, were not observed. It seems that despite the increased apoptosis rate, the liver is still enzymatically functional. It may be possible that the number of non-apoptotic hepatocytes is sufficient to compensate for the apoptotic hepatocytes, but since this is just an assumption, direct evidence is necessary to validate this hypothesis. In a study on effects of CsA in a rat model, congruent observations of steady liver enzymes were made [2]. The measured enzyme activities can be assumed to be reliable, since serum samples have a good stability at −20°C [2, 12]. Furthermore, a decrease in Ly6C/6G and CD11b double positive monocytes/macrophages/neutrophils is a possible effect of CsA and irradiation. Since this conclusion is based on the data of only one animal, it should be carefully interpreted. The observed decrease in Ly6C/6G- and CD11b-positive cells on day 9 in non-irradiated animals could more likely be an artefact caused by a low cell number rather than an actual effect of CsA or NaCl. Still, an influence of the treatment on monocytes/macrophages/neutrophils is plausible since an effect of CsA on the functionality and viability of monocytes is reported in literature [13, 28].

According to our data and to experiments described in literature [15, 37], the direct cause of decreased survival by the combination treatment is most likely hepatotoxicity. Weight loss is a general systemic symptom of a poor health condition and can therefore also be attributed to the combination treatment. Other direct causes of weight loss and decreased survival cannot be ruled out and therefore it should be emphasized that hepatotoxicity is one but possibly not the sole cause for the toxic effects of CsA and irradiation. Another possible reason for mortality after CsA treatment and irradiation is damages to the kidney: CsA is well known to cause nephrotoxicity via the generation of reactive oxygen species [51]. This is further enhanced by radiation-induced nephropathy [7, 11] and might therefore add to the lethality of this treatment.

The results of this study provide useful information for future experiments using human cells for transplantation in NSG mice. When using in vivo models of GvHD in NSG mice, a combined treatment with oral CsA and X-ray irradiation should be avoided or carefully evaluated. Increasing the number of grafted cells is a possible strategy to induce GvHD without the need for irradiation. An alternative solution to model simultaneous immunosuppression and irradiation in NSG mice is varying the immunosuppressive agent, the route of application of CsA, or the irradiation source. To our knowledge other studies on NSG mice using different immunosuppressive therapies or different sources of irradiation have not yet been published. Nevertheless, experiments using different mouse models can be considered, even though results cannot be directly transferred to NSG mice. Panoskaltsis-Mortari et al. analyzed the effects of another frequently used regimen for conditioning in B10.BR (H2b) mice: cyclophosphamide combined with total body irradiation. They showed that this treatment was tolerated but had side effects including weight loss and pulmonary fibrosis [42, 43]. Different modes of irradiation have been evaluated by Schwarte and Hoffmann, concluding that mice have a higher survival after photon irradiation than after gamma irradiation [48]. These irradiation procedures should be investigated in parallel with CsA application regarding toxicity. Another approach to modify the presented mouse model to achieve a higher survival rate is to change the doses of CsA (e.g., higher concentrations but fewer applications) or the irradiation dose (although other GvHD mouse models reported in literature use even higher doses than 2 Gy [47]).

In summary, we presented the effects of oral CsA administration with or without sublethal X-ray irradiation (2 Gy) on NSG mice. Our results evaluate a suitable immunosuppressive strategy and protocol for further transplantation experiments using human cells. In NSG mice, oral administration of CsA without prior irradiation is favorable. If irradiation is indispensable, different immunosuppressive strategies and adaption of the protocols should be considered.

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