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# No prolongation of skin allograft survival by immunoproteasome inhibition in mice

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ABSTRACT

The immunoproteasome, a distinct class of proteasomes, which is inducible under inflammatory conditions and constitutively expressed in monocytes and lymphocytes, is known to shape the antigenic repertoire presented on major histocompatibility complex (MHC) class I molecules. Moreover, inhibition of the immunoproteasome subunit LMP7 ameliorates clinical symptoms of autoimmune diseases *in vivo* and was shown to suppress the development of T helper cell (Th) 1 and Th17 cells and to promote regulatory T-cell (Treg) generation independently of its function in antigen processing. Since Th1 and Th17 cells are detrimental and Treg cells are critical for transplant acceptance, we investigated the influence of the LMP7-selective inhibitor ONX 0914 in a mixed lymphocyte reaction (MLR) *in vitro* as well as on allograft rejection in a MHC-disparate (C57BL/6 to BALB/c) and a multiple minor histocompatibility antigen (miHA)-disparate (B10.Br to C3H) model of skin transplantation *in vivo*. Although we observed reduced allo-specific IL-17 production of T cells *in vitro*, we found that selective inhibition of LMP7 had neither an influence on allograft survival in an MHC-mismatch model nor in a multiple minor mismatch skin transplantation model. We conclude that inhibition of the immunoproteasome is not effective in prolonging skin allograft survival in skin allotransplantation.

## 1. Introduction

The 26S proteasome is a multicatalytic protease in the nucleus and cytoplasm of all eukaryotic cells responsible for the ATP dependent degradation of the bulk (80 90%) of cellular proteins with critical functions in multiple biological processes (Goldberg et al., 2002). In cells of hematopoietic origin, or in response to interferon (IFN) γ, the constitutively expressed catalytically active β subunits are replaced by their inducible counterparts low molecular mass polypeptide (LMP)2 (β1i), multicatalytic endopeptidase complex subunit (MECL) 1 (β2i), and LMP7 (β5i) during neosynthesis, thereby building the so called immunoproteasome (Groettrup et al., 2001). The incorporation of the inducible subunits leads to minor structural changes within the proteasome, to a marked change in the cleavage preference, and to an enhanced production of T cell epitopes (Groettrup et al., 2001; Basler et al., 2013; Groettrup et al., 2010; Huber et al., 2012). Immunoproteasomes do not only play an important role in generating major histocompatibility complex (MHC) class I ligands for T cell activation in the periphery but also in shaping the naive T cell repertoire in the thymus and regulating immune responses (Basler et al., 2013; Groettrup et al., 2010).

$CD4^+$ T cells have long been known to play a central role in mediating transplant rejection (Jiang et al., 2004). Acute allograft rejection is a T cell dependent phenomenon and may be triggered by different types of T helper cells. T helper (Th)1 cell responses initiate allograft rejection by promoting proliferation of alloreactive $CD8^+$ T cells or by inducing a delayed type hypersensitivity (DTH) reaction mediated by macrophages. Additionally, Th1 cells promote transplant rejection by activating B cells to produce allo specific antibodies or directly through Fas/Fas ligand (Fas L) induced cytotoxicity (Jiang et al., 2004). In recent years, it turned out that not only Th1 but also Th17 cells mediate acute allograft rejection by recruiting neutrophils and monocytes into the graft which then contributes to transplant inflammation (Itoh et al., 2011; Gorbacheva et al., 2010; Miura et al., 2003). Importantly, it has

*Abbreviations:* LMP, low molecular mass polypeptide; MECL, multicatalytic endopeptidase complex; miHA, minor histocompatibility antigen; MHC, major histocompatibility complex; CsA, cyclosporine A; MLR, mixed lymphocyte reaction

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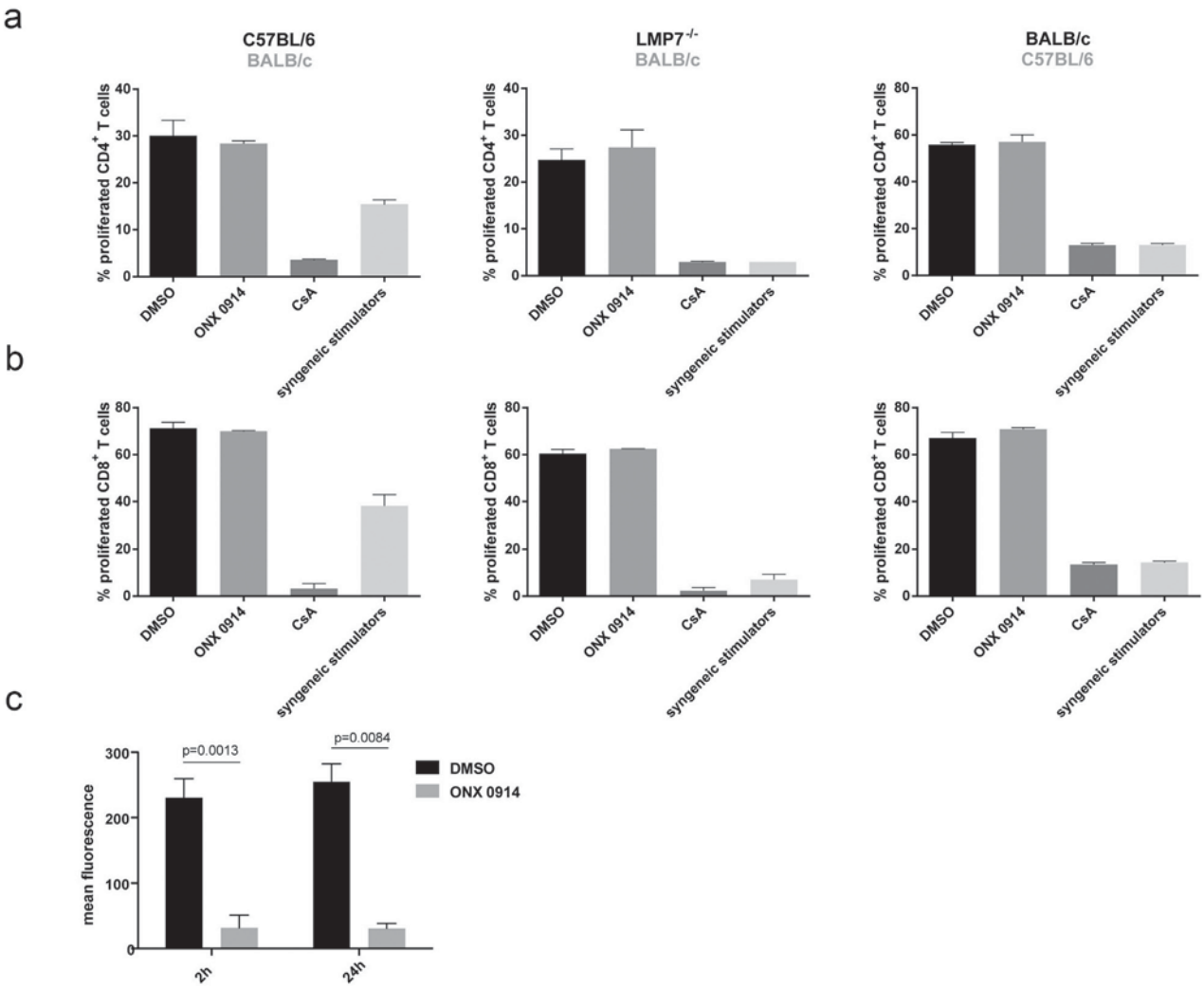


**Fig. 1.** Influence of LMP7 inhibition on allogeneic T-cell proliferation in a mixed lymphocyte reaction. CFSE labeled T cells magnetically isolated from bulk splenocytes of C57BL/6 (H-$2^b$), LMP7 $^{-/-}$ (H-$2^b$), and BALB/c (H-$2^d$) mice (indicated in black above the panels) were treated with DMSO, ONX 0914 (250 nM), or CsA (1 μM) and cultured in the presence of allogeneic irradiated stimulator splenocytes (indicated in gray above the panels) for 96 h. Syngeneic stimulator splenocytes were used as negative control. Graphs show proliferation of (a) $CD4^+$ and (b) $CD8^+$ T cells as measured by CFSE dilution. Data are presented as mean ± SD from duplicates and represent one out of three independent experiments. Data were analyzed by student's *t*-test (two-tailed). (c) CFSE labeled T cells magnetically isolated from bulk splenocytes of C57BL/6 mice were treated with DMSO or ONX 0914 (250 nM) and cultured in the presence of allogeneic irradiated stimulators (BALB/c). 2 h and 24 h later, the proteasomal chymotrypsin-like activity in the cells was determined by the hydrolysis of the cell permeable substrate Meo-Suc-GLF-AMC. Depicted is the mean ± SEM of the mean fluorescence of cells derived from three different mice measured in sextuplicates. The highest fluorescence value was set to 100%. Data were analyzed by paired student's *t*-test (two-tailed).

been demonstrated that regulatory T cells (Tregs) induce and maintain tolerance to the allograft in experimental and clinical transplantation (Wood et al., 2012).

Bortezomib, a dipeptide boronate, is the first in class proteasome inhibitor approved for the treatment of multiple myeloma (Richardson et al., 2005). In addition, bortezomib was evaluated in clinical studies for solid tumors, including non small cell lung cancer (Li et al., 2010), and has demonstrated therapeutic efficacy in preventing chronic rejection of kidney grafts (Raghavan et al., 2010; Vogelbacher et al., 2010; Walsh et al., 2010). Most well characterized proteasome inhibitors mediate equivalent inhibition of both proteasome chymotrypsin like activities (β5 and LMP7) and have considerable toxicities that limit their clinical utility in chronic inflammatory diseases (Richardson et al., 2006). Immunoproteasomes are highly expressed in cells of hematopoietic origin implying that inhibitors specifically targeting catalytically active immunosubunits might be a powerful tool for the treatment of inflammatory disorders while sparing other tissues. Their superiority in terms of drug resistance and toxicity may render immunoproteasome selective inhibitors as promising candidates for the treatment of allograft rejection. ONX 0914 (formerly designated PR 957) is an irreversible proteasome inhibitor that selectively targets the LMP7 subunit of immunoproteasomes being 20 to 40 fold more selective for the LMP7 subunit than for the next most sensitive subunits, β5 or LMP2 (Muchamuel et al., 2009). The molecular reason for the LMP7 selectivity of ONX 0914 has recently been elucidated by crystallographic studies (Huber et al., 2012). The S1 pocket of LMP7 is more spacious compared to that of β5. Thereby, the morpholine derivative adduct formation between the active site threonine and the pharmacophore of ONX 0914 would require a dislocation of Met45 in β5 which would in turn result in energetically unfavorable major structural changes within the protein. Treatment with ONX 0914 was shown to attenuate several inflammatory diseases in mouse models at doses of less than one tenth of the maximum tolerated dose, a therapeutic window that is not achievable with nonselective inhibitors (Groettrup et al., 2010; Muchamuel et al., 2009; Basler et al., 2010; Basler et al., 2014; Basler et al., 2015; Mundt et al., 2016a). Additionally, under polarizing conditions *in vitro*, ONX 0914 suppressed the development of Th1 and Th17 cells and promoted Treg cell development without affecting the differentiation of Th2 cells (Muchamuel et al., 2009; Kalim et al., 2012). Nota bene, these consequences of immunoproteasome

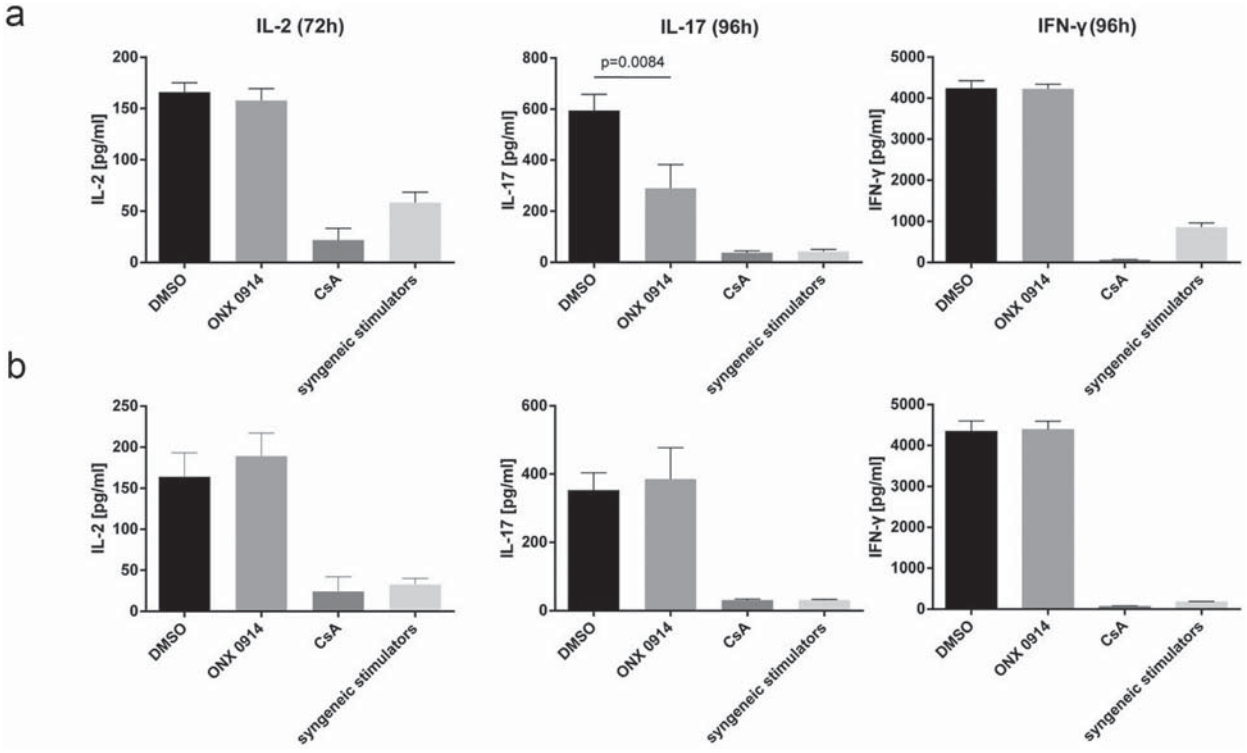


**Fig. 2.** Influence of ONX 0914 on allo-specific cytokine production in a mixed lymphocyte reaction. T cells magnetically isolated from bulk splenocytes of (a) C57BL/6 (H-$2^b$) and (b) LMP7 $^{-/-}$ (H-$2^b$) mice were treated with DMSO, ONX 0914 (250 nM), or CsA (1 μM) and cultured in the presence of allogeneic irradiated stimulator splenocytes from BALB/c (H-$2^d$) mice for 72 96 h, as indicated. Syngeneic stimulator splenocytes (syngeneic stimulators) were used as negative control. Data show mean ± SD of IL-2, IFN-γ, and IL-17 concentrations in the supernatant from triplicates and represent one out of two independent experiments as determined by ELISA. Data analyzed by student's *t*-test (two-tailed).

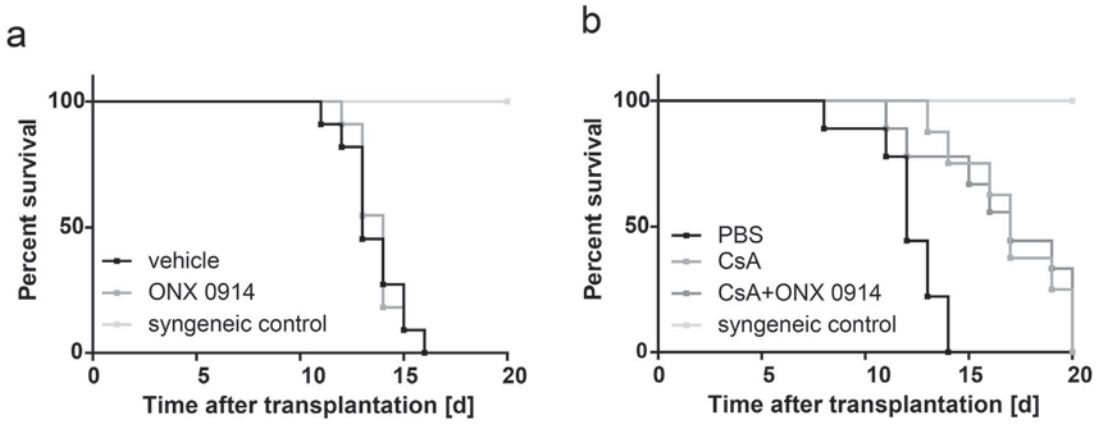


**Fig. 3.** Influence of LMP7 inhibition on MHC-mismatched skin allograft rejection. BALB/c recipient mice were transplanted with fully-thickness tail skin of C57BL/6 mice and (a) treated with vehicle (s.c.) or ONX 0914 (10 mg/kg, s.c.) every second day or (b) treated daily with PBS (i.p.) or CsA (5 mg/kg; i.p.) ± ONX 0914 (10 mg/kg, s.c.) every second day from day 1 of transplantation onwards. Graft survival was monitored daily after removal of the bandage. Graphs show survival curves and represent pooled data from (a) three independent experiments (n = 11 per group and n = 4 syngeneic controls) or (b) two independent experiments (n = 8-9 per group and n = 4 syngeneic controls). With p(PBS *vs.* CsA) = 0.0007 and p(PBS *vs.* CsA + ONX 0914) = 0.0024. Data were analyzed by Log-rank (Mantel-Cox) Test.

**Table 1**
Survival of C57BL/6 skin allografts in BALB/c recipients treated with ONX 0914.

| Donor | Recipient | Treatment | Graft survival time (d) | Median graft survival time (d) ± SD |
|---|---|---|---|---|
| C57BL/6 | BALB/c | vehicle s.c.<br>ONX 0914s.c. | 11, 12, 13, 13, 13, 13, 14, 14, 15, 15, 16<br>12, 13, 13, 13, 13, 14, 14, 14, 14, 15, 16 | 13 ± 1.3<br>14 ± 1.0 |

inhibition occurred in the absence of antigen presenting cells and were independent of the function of the immunoproteasome in antigen processing.

These findings suggest the immunoproteasome as a promising target for therapeutic intervention in several inflammatory disorders and encouraged us to investigate whether selective inhibition of LMP7 is able to prolong graft survival in two skin transplantation models. However, despite influencing allo specific Th17 cell responses *in vitro*, treatment of recipient mice with ONX 0914 had no influence on the allogeneic rejection response *in vivo*.

**Table 2**
Survival of C57BL/6 skin allografts in BALB/c recipients treated with ONX 0914 in combination with low dose cyclosporine A.

| Donor | Recipient | Treatment | Graft survival time (d) | Median graft survival time (d) ± SD |
|---|---|---|---|---|
| C57BL/6 | BALB/c | PBS i.p.<br>CsA i.p.<br>CsA i.p.<br>ONX<br>0914s.c. | 8, 11, 12, 12, 12, 13, 14, 14<br>13, 14, 16, 17, 17,19, 20, 20<br>11, 12, 15, 16, 17, 19, 20, 20, 20 | 12 ± 1.8<br>17 ± 2.5<br>17 ± 3.2 |

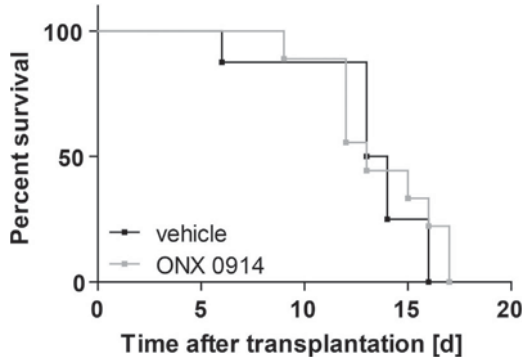


**Fig. 4.** Influence of LMP7 inhibition on multiple miHA mismatched skin allograft rejection. C3H recipient mice were transplanted with fully-thickness tail skin of B10.Br donor mice and treated with vehicle (s.c.) or ONX 0914 (10 mg/kg, s.c.) every second day from day 1 of transplantation onwards. Graft survival was monitored daily after removal of the bandage. Graphs show survival curves and represent pooled data from two independent experiments (n = 8-9 per group). Data were analyzed by Log-rank (Mantel-Cox) Test.

## 2. Materials and methods

### 2.1. Mice

C57BL/6 (H $2^b$) mice, BALB/c (H $2^d$) mice, C3H (H $2^k$), and B10.Br (H $2^k$) were originally purchased from Charles River, Germany. LMP7 gene targeted mice (H $2^b$) (Fehling, 1994) were provided by John Monaco (University of Cincinnati, Cincinnati, OH). Mice were kept in a specific pathogen free facility and used at 8 10 weeks of age. Mouse experiments followed the Principles of Laboratory animal care and the German Law on the Protection of Animals. The animal experiments were approved by the Review Board of Regierungspräsidium Freiburg.

### 2.2. Immunoproteasome inhibition

For *in vitro* experiments, the LMP7 selective inhibitor ONX 0914 (formerly PR 957, contributed by Christopher J. Kirk, Kezar Life Science, South San Francisco, CA) was dissolved at a concentration of 10 mM in DMSO and stored at −80 °C. For *in vivo* proteasome inhibition, ONX 0914 was formulated in an aqueous solution of 10% (w/v) sulfobutylether β cyclodextrin (Captisol®) and 10 mM sodium citrate (pH 6) referred to as vehicle and administered to mice as an s.c. bolus (100 µl) dose of 10 mg/kg.

### 2.3. Cyclosporine A (CsA) treatment

For *in vitro* experiments, CsA (Sigma) was dissolved at a concentration of 50 mM in EtOH and stored at −20 °C. For *in vivo* treatment, CsA (Sandimmune®, Novartis) was formulated in PBS and administered to mice as an i.p. bolus (100 µl) dose of 5 mg/kg.

### 2.4. Skin allograft transplantation

Recipient BALB/c or C3H mice were anesthetized with a mixture of ketamine (120 mg/kg; i.p.) and xylazine (10 mg/kg, i.p.) in isotonic sterile saline solution and additionally received carprofen (5 mg/kg, s.c.) to reduce pain on the day of transplantation and 24 h later. The dorsal skin was shaved and a 1 $cm^2$ graft bed was prepared on the lateral back. Donor C57BL/6 or B10.Br mice were sacrificed and 1 $cm^2$ full thickness tail skin grafts were prepared and transplanted to the beds on the back of the recipient mice. After removal of the bandage, graft survival was monitored daily and rejection was defined by complete destruction of the skin graft, as assessed by visual inspection.

### 2.5. Mixed lymphocyte reaction

Influence of ONX 0914 on allo specific T cell proliferation was assessed by mixed lymphocyte reaction. MACS (Mouse Pan Isolation Kit II, Miltenyi) sorted splenic T cells originated from naïve C57BL/6 (H $2^b$), BALB/c (H $2^d$), or LMP7$^{-/-}$ (H $2^b$) mice were used as responders, while residual splenocytes ( T cells) derived from C57BL/6 or BALB/c mice were used as stimulators. Responder T cells were carboxy fluorescein succinimidyl ester (CFSE) labeled as previously described (Moebius et al., 2010) and pulsed with DMSO or 250 nM ONX 0914 in IMDM 10% FCS at 37 °C for 2 h or continuously treated with 1 µM CsA, respectively. $1 \times 10^6$ responder T cells were cultured with $1 \times 10^5$ irradiated (2000 rad) stimulators in IMDM 10% FCS in a 96 well plate. The cultures were incubated at 37 °C in 5% $CO_2$ for 72 96 h. Cell culture supernatants were analyzed for IL 2, IL 17, and IFN γ by ELISA according to the manufacturer's protocol (eBioscience) and allo specific proliferation of $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry.

### 2.6. Flow cytometry

Flow cytometry was performed as previously described (Mundt et al., 2015). Shortly, cells were stained with antibodies to CD4 (eBioscience, clone GK1.5), and CD8 (eBioscience, clone 53 6.7) in 50 µl FACS buffer (2% FBS, 2 mM EDTA, 2 mM $NaN_3$ in PBS) at 4 °C for 20 min, washed two times and acquired with the Accuri™ C6 flow cytometer (BD Biosciences).

### 2.7. Proteasome activity assay

An MLR was setup as described above. 2 h and 24 h later, the cell permeable substrate MeoSuc GLF AMC (Harding et al., 1995) (Bachem)

**Table 3**
Survival of B10.Br allografts in C3H recipients treated with ONX 0914.

| Donor | Recipient | Treatment | Graft survival time (d) | Median graft survival time (d) ± SD |
|---|---|---|---|---|
| B10.Br | C3H | vehicle s.c.<br>ONX 0914s.c. | 6, 13, 13, 13,14, 14, 16, 16<br>9, 12, 12, 12, 13, 13, 15, 16, 17, 17 | 13.5 ± 2.9<br>13 ± 2.6 |

(10 mM in DMSO) was added at 40 μM (in PBS + 25 mM HEPES) to the cells and incubated for 1 h at 37 °C. The fluorescence intensity in the wells containing the cells was measured at an excitation wavelength of 360 nm and emission wavelength of 465 nm (Infinite M200 pro, TEC AN). The fluorescence intensity in wells with stimulators alone was subtracted from values obtained with MLR.

### 2.8. Statistical analysis

The statistical significance was determined using the Student *t* test, two way ANOVA or Log rank (Mantel Cox) test with two tailed P value. All statistical analyses were performed using GraphPad Prism Software (GraphPad, San Diego, CA). Statistical significance was achieved when $p < 0.05$. If not indicated otherwise, differences are not significant.

## 3. Results

### 3.1. LMP7 inhibition has no influence on allo specific T cell proliferation but reduces allo specific IL 17A production of T cells in vitro

In order to investigate the impact of the immunoproteasome inhibition on allogeneic immune responses, we analyzed the effect of ONX 0914 on T cell proliferation in a mixed lymphocyte reaction (MLR) *in vitro*. To this aim, we treated magnetically sorted splenic CFSE labeled responder T cells with ONX 0914 and cultured them in the presence of irradiated allogeneic stimulator splenocytes. We found that LMP7 inhibition had no influence on the percentage of proliferated $CD4^+$ or $CD8^+$ T cells after 96 h of allogenic stimulation (Fig. 1a and b) whereas cyclosporine A (CsA) effectively blocked allo specific proliferation. Activity assays with a cell permeable fluorogenic substrate for the chymotrypsin like activity demonstrated that ONX 0914 was active in the MLR (Fig. 1c). Furthermore, we measured allo specific IL 2, IL 17, and IFN γ release of T cells by ELISA. While ONX 0914 treatment did not reduce IL 2 or IFN γ production it resulted in decreased IL 17A levels in the supernatant of the MLR (Fig. 2a). This effect was LMP7 specific since a reduction of IL 17A was not detected when T cells from $LMP7^{-/-}$ mice were treated with ONX 0914 (Fig. 2b).

### 3.2. ONX 0914 treatment does not prolong graft survival in an MHC mismatched skin allograft transplantation model

LMP7 inhibition was shown to suppress the differentiation of Th1 and Th17 cells *in vitro* and *in vivo* (Muchamuel et al., 2009; Basler et al., 2014; Kalim et al., 2012; Mundt et al., 2016b; Zilberberg et al., 2015). Consequently, the decreased allo specific Th17 differentiation of ONX 0914 treated cells *in vitro* prompted us to investigate the influence of LMP7 inhibition on allogeneic immune responses *in vivo* using a murine skin allograft transplantation model. For this purpose, C57BL/6 tail skin grafts were transplanted onto the back of BALB/c mice. The recipient mice were treated with ONX 0914, at a dose previously shown to be effective in preventing autoimmunity, or vehicle (Captisol®), respectively, and the graft was monitored daily for signs of rejection. Both vehicle and ONX 0914 treated mice rejected their allograft within 11 16 days with a median graft survival time (MGST) of 13 and 14 days, respectively (Fig. 3a, Table 1), whereas syngeneic transplants were not rejected. Hence, no influence of LMP7 inhibition on the graft survival could be observed.

Next, we investigated whether ONX 0914 treatment in combination with a low dose CsA treatment could act synergistically in prolonging skin graft survival. CsA inhibits allo reactive Th1 responses, thus a combination of drugs could result in such a synergism. We compared skin allograft survival of BALB/c mice treated with 5 mg/kg CsA only or in combination with 10 mg/kg ONX 0914 (Fig. 3b, Table 2). We detected a delayed rejection in mice treated with low dose CsA (MGST: 17 days) compared to the PBS treated control group (MGST: 12 days). However, additional ONX 0914 treatment did not further prolong allograft survival (MGST: 17 days). Hence, our data suggest that inhibiting the immunoproteasome is not effective in preventing acute T cell mediated allograft rejection in an MHC disparate skin transplantation model.

### 3.3. LMP7 inhibition does not prolong graft survival in a multiple miHA mismatch skin allograft transplantation model

Primary skin grafts are known to be very sensitive to rejection compared to other organs and the effect of ONX 0914 might be too subtle to detect it in a transplantation model with a high degree of genetic mismatch as it is the case for the C57BL/6 to BALB/c strain combination (Fig. 3a). Hence, we decided to investigate the influence of LMP7 inhibition in a less sensitive multiple minor histocompatibility antigen (miHA) mismatch MHC matched transplantation model between B10.Br (H $2^k$) donor and C3H (H $2^k$) recipient mice. B10.Br tail skin grafts were transplanted onto the back of C3H mice. The recipient mice were treated with 10 mg/kg ONX 0914 or vehicle, respectively, and the graft was monitored daily for signs of rejection. However, we could not detect any influence on graft survival in this model (Fig. 4, Table 3). Vehicle treated mice displayed a MGST of 13.5 days while ONX 0914 treatment resulted in a MGST of 13 days.

## 4. Discussion

Allograft rejection is a major threat to clinical organ transplantation and only due to immunosuppression over 90% of most organ transplants survive (Winsett et al., 2001). Immunosuppressive drugs, however, affect not only the cells of the immune system, but have adverse side effects on other cells or tissues and long term administration of these compounds can cause nephrotoxicity, susceptibility to infection, and onset of diabetes (Schweer et al., 2014; Nankivell et al., 2004). This ultimately evokes the search for new and safe immunosuppressive drugs.

In the present study, we investigated the influence of ONX 0914, an LMP7 selective inhibitor, on skin allograft rejection. With the discovery of IL 17 producing $CD4^+$ T cells in 2005 a role of Th17 mediated allograft rejection was suggested by a vast body of data (Itoh et al., 2011; Gorbacheva et al., 2010; Min et al., 2009). Th17/Treg ratios were found to be significantly higher during allograft rejection, suggesting that Th17 to Treg imbalance plays a role in the development of allograft rejection (Min et al., 2009). Thus, reversing this imbalance by LMP7 inhibition (Kalim et al., 2012) seemed to be a promising approach to interfere with allograft rejection. However, although we observed reduced IL 17A production in an MLR *in vitro* (Fig. 2), the influence of LMP7 inhibition was not sufficient to interfere with acute allogeneic responses during transplant rejection *in vivo*. Treatment of recipient mice with ONX 0914 did not prolong graft survival in a murine MHC mismatched skin allograft transplantation model (Fig. 3a) neither alone nor in combination with a low dose CsA treatment (Fig. 3b).

A reason for the insufficient suppression of allogeneic immune responses by ONX 0914 treatment might be explained by the fact, that the fully MHC disparate skin allograft transplantation is very sensitive to rejection eliciting strong allogeneic immune responses. Moreover, it has been shown that T cell derived IL 17 is critical for spontaneous rejection of miHA but not MHC disparate skin grafts (Vokaer et al., 2010). These facts suggest that the effect of ONX 0914 on allogeneic immune responses might not be strong enough to prolong graft survival in this model. Therefore, we investigated the influence of immunoproteasome inhibition in a less sensitive skin transplantation model between multiple miHA disparate mice (C3H and B10.Br). However, ONX 0914 treatment also failed to protract graft rejection in this model (Fig. 4).

Hence, immunoproteasome inhibition does not appear as a suitable approach to treat acute T cell mediated skin allograft rejection. Whether this approach could be effective in other models of graft rejection would have to be a subject of future investigations.

Interestingly, Zilberberg et al. reported LMP7 inhibition to be effective in ameliorating graft versus host disease (GvHD) in a miHA disparate murine blood and marrow transplant (BMT) model by decreasing endogenous miHA presentation and consequently reducing allogeneic stimulation and cytokine production (Zilberberg et al., 2015). In contrast to our study, Zilberberg et al. used a daily or even 2 x daily treatment regimen, which might have a stronger effect than our treatment strategy. We have chosen to treat the mice three times a week with 10 mg/kg, a dose, which was proven to be effective in autoimmune models like for example EAE. This dose is still LMP7 selective and well below the maximum tolerated dose (MTD) of 30 mg/kg in mice (Muchamuel et al., 2009; Basler et al., 2010; Basler et al., 2014). However, the *in vivo* effect of ONX 0914 on GVHD reported in the study of Zilberberg et al. is rather moderate with all mice still succumbing to GVHD but with slightly delayed kinetics.

More recently, E. Sula Karreci et al. could show that a newly described LMP7 specific immunoproteasome inhibitor (designated DPLG3) prolonged survival of cardiac allografts in mice and allowed long term survival when combined with single dose CTLA4 Ig treatment (Sula Karreci et al., 2016). Importantly, DPLG3 is a non covalent N,C capped dipeptide inhibitor and hence has a different mode of action compared to ONX 0914. Although DPLG3 is reported to be a very potent inhibitor with a low $IC_{50}$ and a high selectivity index, the authors used a rather high dose for their *in vivo* experiments. On the other hand, they failed to show that this treatment regimen is still LMP7 selective or to determine the MTD for this compound. Moreover, even with a dose of 25 mg/kg the inhibitor by itself did not prolong cardiac allograft survival. Only in combination with CTLA4 Ig, which itself significantly prolonged the survival time by about 50 days, DPLG3 was able to inhibit allograft rejection.

Collectively, LMP7 inhibition seems to have a minor to moderate potential in reducing acute allogeneic T cell responses at least when given in combination with other drugs of current maintenance regimens like *e.g.* calcineurin inhibitors, mTOR inhibitors, anti proliferative agents (*e.g.* mycophenolic acid), costimulation blockers, and corticosteroids.

Taken together, immunoproteasome inhibition does not appear to be a suitable approach to treat acute T cell mediated skin allograft rejection.

## Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

## Authorship

S.M. designed and performed experiments and wrote the manuscript. B.S. provided advice and help for skin allograft transplantation and corrected and refined the manuscript. M.B. performed experiments, supervised the project and corrected and refined the manuscript. M.G. supervised the project and corrected and refined the manuscript.


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