REFERENCES

1. VanGuilder HD, Vrana KE, Freeman WM, *Biotechniques*. 2008;44:619–626.
2. Eisenberg E, Levanon EY, *Trends Genet*. 2013;29:569–574.
3. Suzuki T, Higgins PJ, Crawford DR, *Biotechniques*. 2000;29:332–337.
4. Tricarico C, Pinzani P, Bianchi S, et al. *Anal Biochem*. 2002;309:293–300.
5. Zainuddin A, Chua KH, Abdul Rahim N, et al. *BMC Mol Biol*. 2010;11:59.
6. Schmittgen TD, Zakrajsek BA, *J Biochem Biophys Methods*. 2000;46:69–81.
7. Kheirelseid EA, Chang KH, Newell J, et al. *BMC Mol Biol*. 2010;11:12.
8. Purmonen S, Manninen T, Pennanen P, et al. *Oncol Rep*. 2008;19:1627–1634.
9. Ashcroft KJ, Syed F, Bayat A, *PLoS ONE*. 2013;8:e75600.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Data S1 Supplemenatary materials & methods
Data S2 Supplementary references
Table S1 Clinical Characteristics of subjects
Table S2 Primer-specific amplification efficiencies
Table S3 Primer sequences and size of qRT-PCR products
Table S4 Ranking of internal control genes
Figure S1 Agarose gel electrophoresis of qRT-PCR products from 10 selected internal control genes
Figure S2 Dissociation curve analysis of primer-specific amplification
Figure S3 Relative mRNA expression of RPLP0 and PPIA genes in human dermal fibroblasts

DOI: 10.1111/exd.13098

Accepted: 30 May 2016

Topical treatment with a two-component gel releasing nitric oxide cures C57BL/6 mice from cutaneous leishmaniasis caused by *Leishmania major*

1 | BACKGROUND

The incidence of the parasitic disease leishmaniasis is 1.5–2 million new cases per year predominantly in (sub)tropical regions of the world.[1] Most of the endemic regions belong to developing countries, and the disease is thus a burden especially for the poor and those with coinfections, for example with HIV.[1,2] Due to these findings, there is a strong need for a vaccine and efficient therapeutics against leishmaniasis.

Treatment against cutaneous leishmaniasis (CL) is based on systemic or local approaches dependent on the *Leishmania (sub)species* involved, the type of lesions and the immune status of the patient.[3] For systemic treatment, pentavalent antimony, liposomal amphotericin B and miltefosine are most commonly used. Local treatment may be considered in cases with few lesions, in those caused by *L. major or L. tropica*, and in some cases from the New World that exhibit a low tendency to visceralize and/or induce mucocutaneous disease, and for those who cannot receive systemic treatment, for example during pregnancy.[4] Topical treatment modalities include intra-lesional antimony, local excision of single lesions, photodynamic therapy, cryosurgery, thermotherapy and others, but there are limited data about the safety and efficacy of some of these modalities.

2 | QUESTION ADDRESSED

In prior single-case reports, successful local treatment of CL with topical nitric oxide (NO) donors was reported.[5–7] In addition, in a controlled clinical trial a NO-releasing patch revealed cure in ~40% of cases.[8] In humans and mice, NO release by activated lesional macrophages (mediated by IFN-γ release from Th1/Tc1 cells) is crucial for intracellular parasite clearance.[9,S1–S3] NO induces oligonucleosomal fragmentation of *Leishmania* DNA, and NO donors have significant leishmanicidal activity in vitro.[S4] In addition to this direct antileishmanial effect, NO exerts self-regulating and immunoregulatory effects in adoptive and innate immunity.[S5,S6] However, the stability of NO donor formulations is problematic for use in everyday practice. In the presence of oxygen and water, NO is partially oxidized into nitrogen dioxide. Further chemical reactions lead to formation of mainly nitrite and smaller amounts of nitrate ions.[S7] To control the rate of NO release, one may admix a sodium nitrite aqueous gel with a second gel containing a reductant like ascorbic acid immediately before use.[S8] An acidified nitrite solution containing 50 mmol/L nitrite releases 1.100 mmol NO/min, leading to a transcutaneous penetration of 0.5–1 nmol NO/min × cm^2 if used in a cream.[S9,S10] In this study, we tested the efficacy of a NO donor gel formulation for treating murine experimental leishmaniasis.

3 | EXPERIMENTAL DESIGN

We used a two-component gel consisting of ascorbic acid 5% and sodium nitrite 5% (composition described in Table S1).[S11] The components are mixed just before application to skin. In this study, we assessed the treatment efficacy of this NO donor gel on the disease course of *L. major*-infected C57BL/6 mice. This mouse strain is considered to exhibit all main features of human CL.[9]

4 | RESULTS AND CONCLUSIONS

First, mice were infected with 2×10^5 infectious-stage parasites into ear dermis (Fig. 1a). To assess the treatment efficacy in mice infected with physiologically relevant low doses of parasite mimicking natural transmission of parasite into human hosts, we also performed infections with as low as 1000 parasites (Fig. 1b). In both settings, starting at the time when lesions became visible, 200 mg of mixed gel was applied twice per week for several weeks. Notably, as soon as treatment was started, progressive ear lesion development stopped and a significant difference between lesions sizes of treated and untreated ears became obvious. Interestingly, in high-dose infections, lesions remained significantly smaller despite end of treatment in week 4, whereas in low-dose infections, lesions' sizes started growing after stopping treatment indicating that parasite replication restarted again. Despite transient hyperaemia and redness, no side effects were obvious.

To better mimic the clinical situation in which a patient presents with an already-existing lesion, we also assessed the treatment efficacy of NO donor gel treatment commencing at the peak of the lesions in week 4 (high-dose infection) and week 6 (low-dose infection), respectively. Even when the treatment was initiated for established lesions, the NO donor gel (Fig. 1a,b), but not its inactive single components (Fig. S1), significantly and strongly reduced lesion volumes within 1–2 weeks post-treatment initiation and promoted faster lesion resolution as compared to untreated controls.

We next determined parasite loads in CL lesions with and without treatment at different time points. In line with our hypothesis, NO donor gel treatment for >3 weeks induced a strong and clear-cut reduction in lesional parasite loads (Fig. 1c). To our surprise, NO donor gel treatment also led to a reduction of parasite numbers in spleens of infected mice indicating that topical treatment inhibited subsequent parasite visceralization (Fig. 1d).

Finally, the cytokine response in supernatants from lymphocytes taken from draining lymph nodes of NO donor gel-treated mice was compared to that of those without treatment. Interestingly, in all treatment groups, we detected elevated levels of IL-12p40 and IFN-γ and unaltered amounts of IL-4 (Fig. 1e). Early treatment with NO donor gel appeared to also influence IL-10 production, whereas treatment of established lesions did not. To assess the dependence of treatment on the ability to induce these proinflammatory cytokines, we assessed the efficacy of topical NO donor gel in the therapeutic setting using IFN-$\gamma^{-/-}$, IL-12p40$^{-/-}$ and iNOS$^{-/-}$ C57BL/6 mice (Fig. S2). Of note, all

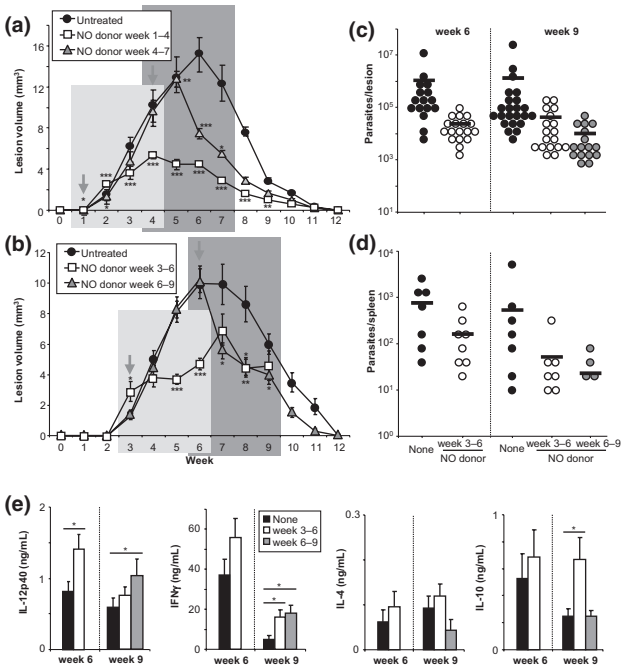

FIGURE 1 Local treatment with NO donor improves disease outcome of *Leishmania major*-infected individuals. Groups of 5 C57BL/6 mice were infected intradermally with 2×10^5 (a) or 10^3 (b–d) infectious-stage metacyclic promastigotes of *L. major*. Mice were treated early on between weeks 3 and 6, or—similar to clinical situations—between weeks 6 and 9 with fully developed lesions. Treatment was performed 2×/week by application of 200 mg of gel on each infected ear. (a,b) Lesion sizes were determined weekly in 3 dimensions using a calliper (mean±SEM, n≥10). (c,d) Mice were harvested in week 6 or week 9, and parasite loads were determined by limiting dilution assays (means are represented by bars, and dots show parasite numbers/each individual ear, n≥6). (e) Antigen-specific cytokine release of draining lymph node cells was determined in 48-hr supernatants by ELISA (n≥8, mean±SEM). Statistical differences to controls are indicated as $^*P<.05$, $^{**}P<.005$ and $^{***}P<.002$

of these strains are known to be highly susceptible to infection despite the genetically resistant background, as these cytokines are essential for the host defense against *L. major*.[9] Interestingly, in IL-12p40 knockout mice, NO donor gel treatment between weeks 4 and 6 induced partial protection, but overall, NO donor gel was unable to prevent progressive disease in all knockout strains studied. Thus, in situations with absent mechanisms normally responsible for parasite elimination, NO donor gel treatment may not be effective enough to induce cure of lesions.

5 | CONCLUSION

In summary, application of NO donor gel prevented the development of full-blown lesions and induced reliably quicker lesion resolution in both a prophylactic and a therapeutic setting. This was paralleled by decreased numbers of viable parasites in both lesions and viscera indicating a direct parasite-eliminating effect

of the NO donor gel. Interestingly, the NO donor also shifted the local immune response towards Th1 additionally amplifying anti-*Leishmania* immunity.[S11,S12] This was corroborated by the finding that in IL-12-, IFN-γ- and NO-deficient mice, treatment was ineffective. Thus, it appears that topical NO donor gel application may be highly suitable especially for patients with an (at least partially) competent immune system. Future studies in humans will have to verify the efficacy of this well-tolerated topical treatment against cutaneous leishmaniasis.

ACKNOWLEDGEMENTS

This work was supported by grants from the DFG to EvS.

AUTHOR CONTRIBUTION

BL, AG and EvS performed research; AG and EvS designed the study; FB, BL and EvS analysed the data; and FB, AG and EvS wrote the manuscript.

CONFLICT OF INTERESTS

The authors have declared no conflicting interests.

Keywords
leishmaniasis, nitric oxide, Th1/2, treatment

Florian Butsch
Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

Beate Lorenz
Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

Alfred Goldinger
Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

Hospital Pharmacy, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

Esther von Stebut
Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

Correspondence
Esther von Stebut,
Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany.
E-mail: vonstebu@mail.uni-mainz.de

REFERENCES

1. Murray HW, Berman JD, Davies CR, et al. *Lancet*. 2005;366:1561–1577.
2. Ngouateu OB, Weller K, Bröhl K, et al. *Exp Dermatol*. 2015;24:302–304.
3. Boecken G, Sunderkötter C, Bogdan C, et al. *J Dtsch Dermatol Ges*. 2011;9:1–51.
4. World Health Organisation. WHO Technical Report Series. Geneva, WHO, 2010. http://whqlibdoc.who.int/trs/WHO_TRS:_949_eng.pdf. Accessed 26 July 2016.
5. López-Jaramillo P, Ruano C, Rivera J, Terán E, et al. *Lancet*. 1998;351:1176–1177.
6. Davidson RN, Yardley V, Croft SL, et al. *Trans R Soc Trop Med Hyg*. 2000;94:319–322.
7. Zeina B, Banfield C, al-Assad S. *Clin Exp Dermatol*. 1997;22:244–245.
8. López-Jaramillo P, Rincón MY, García RG. *Am J Trop Med Hyg*. 2010;83:97–101.
9. Sacks D, Noben-Trauth N. *Nat Rev Immunol*. 2002;2:845–858.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Data S1 Supplementary References
Figure S1 NO donor gel is only effective when mixing the two inactive components
Figure S2 The effect of topical NO donor gel was partially dependent on Th1/Tc1-dependent immune mechanisms
Table S1 Two-component-NO-donor gel (1:1 Gel 1 and Gel 2)