Background

Respiratory infections by *Streptococcus (S.) pneumoniae* remain a major cause of community-acquired pneumonia and sepsis (Pneumoniae, n.d.; Musher & Thorner, 2014; Angus & van der Poll, 2013). The entry of pneumococci in the lower airways triggers a local innate host defense response, characterized by release of cytokines and chemokines leading to polymorphonuclear cell (PMN) and monocyte recruitment in order to expel the bacteria (van der Poll & Opal, 2009; Koppe et al., 2012). An exaggerated inflammatory response resulting from inadequate elimination of pneumococci, however, may hamper lung function (Thompson et al., 2017; van der Poll et al., 2017). Drugs that can reduce overwhelming lung inflammation during pneumonia may serve as adjuvant treatment in patients with community-acquired pneumonia (Muller-Redetzky et al., 2015).

Several pattern recognition receptors contribute to the initiation of an inflammatory response to pneumococci, such as Toll-like receptor 2 (TLR2), TLR4, TLR9, triggering receptor expressed on myeloid cells (TREM)-1 and inflammasomes (van der Poll & Opal, 2009; Koppe...
et al., 2012; Rabes et al., 2016; Hommes et al., 2014). Bruton’s tyrosine kinase (Btk) is a versatile signaling protein downstream several immune receptors (Weber et al., 2017), including TLR2, TLR4 and TLR9 (Liljeroos et al., 2007; Liu et al., 2011; Doyle et al., 2007), the Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome (Ito et al., 2015) and TREM-1 (Ormsby et al., 2011). In macrophages, Btk regulates the secretion of inflammatory cytokines upon TLR, NLRP3 and TREM-1 stimulation (Weber et al., 2017; Liljeroos et al., 2007; Liu et al., 2011; Doyle et al., 2007; Ito et al., 2015; Ormsby et al., 2011). Furthermore, in PMN, Btk is involved in adherence to and crawling along the endothelium and migration into tissues (Yago et al., 2010; Mueller et al., 2010; Volmering et al., 2016). The fact that expression of Btk is confined to immune cells, including B cells and innate immune cells such as macrophages, monocytes and PMN (Weber et al., 2017), makes Btk an interesting target for intervention of the exaggerated inflammatory response during pneumococcal pneumonia.

Ibrutinib is an irreversible inhibitor of Btk, which inactivates its kinase domain and thereby the capacity of Btk to phosphorylate and activate downstream signaling proteins (Honigberg et al., 2010). Ibrutinib is currently used in patients for treatment of B cell malignancies (Hendriks et al., 2014) and chronic graft versus host disease (Miklos et al., 2017). Furthermore, prolonged treatment with ibrutinib has been found effective in chronic autoimmune disease models mediated by B cells (Crofford et al., 2016). Little is known, however, about the efficacy of ibrutinib in acute inflammatory responses in vivo mediated by myeloid cells (Florence et al., 2018).

In the current study we investigated the potential of ibrutinib to inhibit acute inflammatory processes in the lung in mouse models of acute lung inflammation elicited by lipoteichoic acid (LTA), a pro-inflammatory component of the gram-positive cell wall, and antibiotic-treated pneumococcal pneumonia.

**Methods**

**Animals**

Btk−/− and CD19-BTK mice were generated as previously described (Hendriks et al., 1996; Maas et al., 1999). C57Bl/6 male mice were bought from Charles River, Maastricht, Netherlands. All mouse lines were backcrossed at least eight times to C57Bl/6 background. All animals were specific pathogen-free and housed in the Animal Research Institute Amsterdam facility under standard care. All experiments were carried out in accordance with the Dutch Experiment on Animals Act and were approved by the local animal welfare committee of the Academic Medical Center.

**Results**

Ibrutinib inhibits LTA and S. pneumoniae-induced macrophage and PMN activation in vitro

Ibrutinib is an irreversible inhibitor of the kinase activity of Btk and able to inhibit autophosphorylation of Btk on tyrosine Y223 (Honigberg et al., 2010). Ibrutinib completely abrogated autophosphorylation of Btk Y223 in the murine macrophage cell line RAW264.7 stimulated with LTA (Fig. 1a) and reduced tumor necrosis factor (TNF) secretion upon stimulation with S. pneumoniae or different concentrations of LTA (Fig. 1b–c). Ibrutinib also significantly reduced LTA or S. pneumoniae-induced CD11b expression on PMN (Fig. 1d).

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism software (San Diego, CA). Data are given as means and standard deviation (SD) in tables and bar graphs. Differences between groups were analyzed using Mann–Whitney U-test; comparisons between more than two groups were first performed using Kruskal–Wallis 1-way analysis of variance test. A value of $P<0.05$ was considered statistically significant.

**Cell stimulation and analysis of inflammatory responses**

Cell stimulation, cell counting, flow cytometry, immunohistochemistry, pathology, measurement of organ damage, protein measurements, western blotting procedures and reagents can be found in the Additional file 1: supplemental methods.
Ibrutinib reduces LTA-induced myeloid cell responses during acute lung inflammation

To determine whether ibrutinib was capable of diminishing LTA-induced lung inflammation, we treated mice orally with ibrutinib or vehicle and analyzed inflammatory responses 6 and 21 h after intranasal LTA administration. The strategy for in vivo ibrutinib treatment was based on calcium flux analyses of spleen B cells isolated from ibrutinib or vehicle-treated mice (Additional file 2: Figure S1). Ibrutinib or vehicle treatment was commenced 3 h prior to LTA instillation and repeated 12 h after the first treatment for analysis of lung inflammation at 21 h. After 6 h of LTA inflammation, no difference in pulmonary total cell counts in BALF was detected between groups. At 21 h, however, ibrutinib treatment caused a significant reduction in total BALF cell counts (p = 0.04) (Fig. 2a). Flow cytometric analysis of cell types in BALF showed that at 21 h more alveolar macrophages (AM) were retrieved from the ibrutinib treated group as compared to the vehicle treated group (p = 0.01), whereas monocyte and PMN numbers were similar (Fig. 2b-d and Additional file 3: Figure S2A). At 21 h of LTA inflammation, AM and monocyte counts were similar between groups, but PMN numbers were reduced by ibrutinib treatment (p = 0.04) (Fig. 2b-d). Analysis of PMN numbers in lung tissue by immunohistochemistry showed no differences between groups at 6 and 21 h after LTA inoculation (Fig. 2e-f).

To assess whether ibrutinib altered activation of AM and PMN in the lung, flow cytometric analysis of CD11c and CD11b, respectively, was done. At 6 h, no differences were found in CD11c and CD11b expression, but at 21 h expression of CD11c on AM was significantly reduced in ibrutinib treated mice (p = 0.001) (Fig. 2g and Additional file 4: Figure S3A), whereas CD11b expression on PMN was increased (p = 0.01) (Fig. 2g and Additional file 4: Figure S3A). To determine whether ibrutinib affected levels of PMN derived factors in BALF, we measured myeloperoxidase (MPO) and elastase. Ibrutinib treatment lowered MPO (p = 0.02 and p = 0.0003 respectively) and elastase (p = 0.04 and p = 0.0002 respectively) concentrations at 6 and 21 h (Fig. 2h). A clear effect of ibrutinib was also found on cytokine levels in BALF. Ibrutinib treatment significantly lowered TNF levels at 6 and 21 h (p = 0.002 and p = 0.0002 respectively) and interleukin (IL)-6 levels at 21 h (p = 0.0002) (Fig. 2i). Levels of the chemokines CXCL1 and CXCL2, however, were not altered (Additional file 5: Table S1).

To determine if vascular leakage was influenced by ibrutinib treatment, we measured BALF total protein and IgM levels. Both total protein and IgM levels were not different at 6 h, but were significantly reduced in the ibrutinib treated group at 21 h (p = 0.002 and p = 0.0001 respectively) (Fig. 2j). Ibrutinib treatment, however, did not influence lung pathology (Fig. 2k-l). Together these results indicate that ibrutinib is capable of inhibiting several critical myeloid cell responses during LTA-induced acute lung inflammation.

Ibrutinib reduces inflammatory myeloid cell responses in the lung during antibiotic-treated pneumococcal pneumonia

Next, we investigated the effect of ibrutinib on an already ongoing inflammatory response in the lung in the established model of pneumococcal pneumonia (Hoogendijk et al., 2012; Rijneveld et al., 2003). Mice were infected intranasally with highly virulent serotype 3 S. pneumoniae strains.
bacteria and treated 24 h later with ceftriaxone. Concomitant with ceftriaxone and 12 h later, vehicle or ibrutinib was given and mice were sacrificed 48 h after bacterial inoculation. Mice sacrificed at 24 h after bacterial inoculation without additional treatment served as control (n = 4), to assess inflammatory parameters at the start of treatment. Ceftriaxone treatment significantly reduced CFU in BALF, spleen and blood compared to the 24 h control group (vehicle or ibrutinib vs control all p < 0.01) (Fig. 3a). Ibrutinib treatment did not alter bacterial numbers compared to vehicle treatment.

Analysis of BALF cells (Additional file 3: Figure S2B) showed that the number of cells in vehicle treated mice at 48 h increased as compared to the 24 h controls (p = 0.02), indicative of ongoing inflammation despite bacterial eradication. Total cell numbers in the ibrutinib treatment group, however, were markedly reduced as compared to the vehicle treatment group (p < 0.03) and similar to the untreated group (Fig. 3b). In vehicle treated mice, PMN numbers increased as compared to the 24 h time point (p = 0.04). Ibrutinib treated mice had lower PMN numbers compared to the vehicle treatment group.
Ibrutinib treatment largely reduced the influx of monocytes as compared to vehicle treatment \( (p = 0.03) \), but monocyte numbers were slightly higher as compared to the 24 h controls \( (p = 0.008) \) (Fig. 3d). AM numbers were increased at 48 h as compared to 24 h \( (p < 0.01 \) for both treatment groups vs controls), but no difference was found between ibrutinib and vehicle treatment (Fig. 3e).

Flow cytometry analysis of CD11c on AM and CD11b on PMN was employed to assess activation of these cell types in the lung. The vehicle treatment group showed a significant induction in CD11c expression on AM compared to 24 h time point \( (p = 0.004) \). Ibrutinib treatment, however, abrogated the increase in CD11c expression on AM \( (p = 0.0002) \) (Fig. 3f and Additional file 4: Figure S3B). PMN CD11b expression was significantly induced in both vehicle and ibrutinib treated mice as compared to the 24 h
controls ($p < 0.05$ for both treatment groups vs controls). CD11b expression did not differ between vehicle and ibrutinib treated mice (Fig. 3g and Additional file 4: Figure S3B). Analysis of PMN derived factor concentrations showed that both MPO and elastase levels in BALF were strongly reduced by ibrutinib treatment ($p = 0.01$ and $p = 0.02$ respectively)(Fig. 3h).

In line with bacterial eradication, ceftriaxone abrogated TNF release in BALF (Additional file 7: Table S2). Ibrutinib treatment did not influence cytokine and chemokine release in BALF as compared to vehicle treatment (Additional file 7: Table S2).

Analysis of plasma leakage into the lung as represented by BALF protein and IgM levels showed no significant changes, but ibrutinib treated mice did show a trend towards decreased protein and IgM levels compared to vehicle treated mice ($p = 0.06$ and $p = 0.18$, respectively) (Fig. 3i). Lung pathology slightly increased from 24 h to 48 h after infection ($p = 0.01$ for both vehicle or ibrutinib vs control)(Fig. 3j and Additional file 6: Figure S4B), but was not different between the ibrutinib treatment group and the vehicle treatment group. These results reveal that ibrutinib is able to reduce specific aspects of augmenting acute lung inflammation during antibiotic-treated pneumococcal pneumonia.

**Ibrutinib reduces systemic cell activation during antibiotic-treated pneumococcal pneumonia**

Since $S. pneumoniae$ infected mice were bacteremic at the time ibrutinib treatment was commenced (Fig. 3a), we also analyzed the effect of ibrutinib on systemic inflammatory responses during antibiotic-treated pneumococcal pneumonia. Measurement of blood total leukocyte and monocyte numbers at 24 h after infection and in vehicle or ibrutinib treated mice at 48 h after infection showed no differences between groups (Fig. 4a). The number of blood PMN in both treatment groups was decreased as compared to the 24 h time point (both $p < 0.01$)(Fig. 4a and Additional file 3: Figure S2C).

To assess activation of circulating monocyte and PMN, we analyzed expression of CD11b. On monocytes, expression of CD11b was increased in vehicle and ibrutinib treated mice as compared to 24 h controls ($p = 0.004$ for both vehicle or ibrutinib vs control) (Fig. 4b and Additional file 4: Figure S3C). Ibrutinib treatment resulted in a trend towards decreased CD11b expression as compared to vehicle treatment ($p = 0.08$). On PMN, CD11b expression was significantly increased in vehicle treated mice as compared to 24 h controls ($p = 0.004$), but ibrutinib decreased CD11b expression as compared to vehicle ($p = 0.04$) (Fig. 4b and Additional file 4: Figure S3C). To analyze PMN activation...
further, we determined plasma MPO and elastase levels (Fig. 4c). MPO did not show significant changes between 24 and 48 h or between treatment groups (Fig. 4c). Plasma elastase concentrations were increased in vehicle treated mice at 48 h as compared to the 24 h controls \((p=0.02)\). Ibrutinib treatment strongly reduced elastase concentrations as compared to vehicle treatment \((p=0.02)\) (Fig. 4c).

To assess whether ibrutinib influenced organ damage, we measured plasma levels of lactate dehydrogenase (LDH) as a marker of general cell death, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) as markers of liver damage and urea as marker for kidney injury. Only plasma urea levels increased at 48 h in both treatment groups \((p=0.006\) for both treatment groups vs control) as compared to the 24 h control group (Fig. 4d). Ibrutinib treatment did not significantly reduce LDH, ALAT, ASAT or urea as compared to vehicle treatment, although a trend to lower plasma LDH levels was noted \((p=0.1)\). Assessment of the effect of ibrutinib on plasma cytokines was inconclusive, as ceftriaxone treatment decreased the levels of TNF, IL-6, CCL2 and interferon-\(\gamma\) (IFNy) below the limit of detection (Additional file 7: Table S2).

Together, these data suggest that ibrutinib reduces activation of circulating neutrophils during ceftriaxone-treated pneumococcal pneumonia.

Discussion

Lung inflammation is crucial for host defense against invading pathogens which have entered the lower airways. Overwhelming lung inflammation, however, is unwanted as this may hamper lung function and has detrimental effects on the host (Angus & van der Poll, 2013; van der Poll et al., 2017; Muller-Redetzky et al., 2015). In the present study, we investigated the effect of the Btk inhibitor ibrutinib on acute lung inflammatory responses induced by LTA and by highly virulent serotype 3 S. pneumoniae in a clinically relevant setting, i.e. in the context of postponed treatment supplementing antibiotic therapy. In a prophylactic approach, ibrutinib treatment diminished several aspects of LTA-induced lung inflammation, including cytokine release, AM activation and PMN influx into the bronchoalveolar space, which most likely caused the decrease in MPO and elastase levels, and plasma leakage into the lung. Postponed treatment with ibrutinib also reduced inflammatory myeloid cell responses in the lung and circulation during ongoing pneumococcal pneumonia without affecting bacterial clearance. In this model, established lung inflammation was present at the start of treatment and further progressed despite bacterial eradication by ceftriaxone. In this setting, ibrutinib reduced AM activation, reduced systemic PMN activation and substantially diminished monocyte and PMN migration into the bronchoalveolar space. Also during pneumococcal pneumonia, the decreased PMN influx most likely caused the decreased MPO and elastase levels. The effect of ibrutinib on acute inflammatory responses during ceftriaxone-treated pneumococcal pneumonia was not as pronounced as in LTA-induced lung inflammation likely because ibrutinib treatment was started 24 h after induction of infection, thus well after the initiation of inflammatory processes in the lung and activation of Btk expressing cells. Furthermore, Ibrutinib treatment did not alter the number of PMN in the lung parenchyma and total lung pathology evoked by LTA or S. pneumoniae and had no effect on levels of plasma markers for organ injury in the later model, presumably because the majority of resident lung I cells do not express Btk but contribute significantly to these aspects of lung inflammation (Dudek et al., 2016). Nevertheless, these findings indicate that ibrutinib has the capacity to ameliorate several aspects of the acute inflammatory response in the lung during ongoing pneumococcal pneumonia. Whether ibrutinib has potential as adjuvant treatment to antibiotics in patients who are at risk of developing acute respiratory distress syndrome or sepsis requires further investigation.

Opposite to the inhibition of various inflammatory responses in the lung, ibrutinib enhanced certain inflammatory parameters, including AM numbers and CD11b expression on PMN after LTA administration. Although total PMN numbers in the bronchoalveolar space after LTA administration were decreased upon ibrutinib treatment, their increased CD11b expression suggests that highly activated blood PMN were still capable to migrate into the airways. Moreover, the higher number of AM in BALF of ibrutinib treated mice early after LTA administration may be explained by decreased adherence of ibrutinib treated AM to the lung epithelium, as observed previously in mice with impaired integrin expression (Anas et al., 2016). Further experiments are required to determine whether ibrutinib alters integrin expression on AM and to assess whether the observed modest changes in CD11b and CD11c expression produced by ibrutinib are biologically significant.

The beneficial effect of ibrutinib on acute inflammatory responses in the lung may result from direct interference of receptor signaling in AM and PMN. TLR2 is essential for responsiveness of AM to LTA and S. pneumoniae (Knapp et al., 2004; Knapp et al., 2008) and required for PMN responses to LTA (Lotz et al., 2004). Furthermore, TLR2 is an important receptor for development of both LTA-induced and S. pneumoniae-induced lung inflammation (Knapp et al., 2004; Knapp et al., 2008). Previously, it has been shown that Btk regulates LTA-induced TLR2 responses in macrophages
In line with these findings, we found that Btk controls LTA and *S. pneumoniae*-induced macrophage and PMN activation in vitro and in vivo. The amelioration of inflammatory responses in the lung during pneumococcal pneumonia by ibrutinib may also result from inhibition of other Btk-dependent receptors that regulated *S. pneumoniae*-induced lung inflammation (Hommes et al., 2014; Branger et al., 2004; Albiger et al., 2007; van Lieshout et al., 2018), including TLR4, TLR9, TREM-1 and NLRP3 (Weber et al., 2017; Ito et al., 2015; Ormsby et al., 2011). Additionally, the amelioration of acute lung inflammatory responses by ibrutinib may result from direct inhibition of Btk downstream PSGL-1, CD44 and G-protein receptors in PMN (Yago et al., 2010; Mueller et al., 2010; Volmering et al., 2016), which are essential for PMN migration (Hyun & Hong, 2017). Of note, besides Btk, ibrutinib may target other Tec and Src family kinases (Honigberg et al., 2010), but none of these have been described to affect LTA or *S. pneumoniae*-induced lung inflammation to our knowledge.

Besides directly affecting PMN responses, the anti-inflammatory effect of ibrutinib in the lung compartment may also result from decreased vascular cell activation caused by reduced TNF production. TNF is important for upregulation of adhesion molecules on vascular endothelial cells, and subsequent PMN adhesion and migration (Hyun & Hong, 2017). The finding that ibrutinib reduced vascular leakage during LTA-induced lung inflammation supports the hypothesis that ibrutinib may also act indirectly on cells, as endothelial cells do not express Btk. However, it is unlikely that the amelioration of acute inflammatory responses in the lung by ibrutinib resulted solely from reduced TNF production, since we previously found that anti-TNF therapy impaired bacterial clearance during ceftriaxone-treated pneumococcal pneumonia and aggravated pathology (Rijneveld et al., 2003).

The results of the present study are in line with previously published work showing that inhibition of Btk protected against acute lung injury evoked by polymicrobial sepsis (Zhou et al., 2014), the combination of lipopolysaccharide and immune complexes (Krupa et al., 2014) or the combination of trauma and hemorrhagic shock (Liu et al., 2017). Recently, it was described that intranasal administration of ibrutinib ameliorated survival and decreased lung pathology, PMN influx, neutrophil extracellular trap formation, plasma leakage and cytokine production after influenza infection (Florence et al., 2018). Although these findings suggest that ibrutinib has potency to ameliorate specific aspects of lung inflammation, further investigations have to be done to assess the effectiveness, persistence and safety of this irreversible Btk inhibitor in acute myeloid cell-mediated inflammation.

**Conclusions**

The results of the current study reveal that ibrutinib inhibits myeloid cell responses during lung inflammation evoked in mice by LTA and antibiotic-treated pneumococcal pneumonia. Our findings suggest that ibrutinib has the potential to inhibit several aspects of ongoing lung inflammation in acute infectious settings by diminishing myeloid cell activation and migration.

**Additional files**

**Additional file 1:** Supplemental methods (DOC 71 kb)

**Additional file 2:** Figure S1. Ibrutinib inhibits calcium flux of splenic B cells in vivo. Maximum ratio of bound/free indo-1 AM during anti-IgM induced calcium flux experiments on isolated splenic B cells from CD19-BTK and Btk−/− mice treated with vehicle or ibrutinib 3 and 12 h previously (n = 4 for the vehicle group, n = 2 for the other groups). CD19-BTK mice, Btk−/− mice with transgenic expression of human Btk under the CD19 promoter, were used for their robust calcium flux (Kil et al, 2012). Btk−/− mice were included as a control. Calcium flux was inhibited completely at 3 h after ibrutinib treatment since at this time point the calcium flux was similar to Btk−/− splenic B cells treated with ibrutinib. 12 h after ibrutinib treatment calcium flux was partially restored. Data are represented means with SD or representative histograms are shown. (DOC 273 kb)

**Additional file 3:** Figure S2. Flow cytometric gating strategy to determine the percentage of cell subsets in BALF and blood. (a) Gating strategy to determine the percentage of alveolar macrophages (AMs), monocytes (monos) and polymorphonuclear cells (PMN) in BALF after intranasal LTA administration. (b) Gating strategy to determine the percentage of AMs, monos and PMN in BALF after intranasal *S. pneumoniae* administration. (c) Gating strategy to determine the percentage of monos and PMN in blood. (DOC 1368 kb)

**Additional file 4:** Figure S3. Representative histograms of markers for cell activation. (a) Representative histograms of CD11c expression on alveolar macrophages (AMs) and CD11b expression on polymorphonuclear cells (PMN) in BALF after intranasal LTA administration. (b) Representative histograms of CD11c expression on alveolar macrophages (AMs) and CD11b expression on polymorphonuclear cells (PMN) in BALF after intranasal *S. pneumoniae* administration. (c) Representative histograms of CD11b expression on monocytes (mono) and polymorphonuclear cells (PMN) in blood after intranasal *S. pneumoniae* administration. (DOC 257 kb)

**Additional file 5:** Table S1. BALF chemokine levels 6 and 21 h after intranasal LTA administration in vehicle and ibrutinib treated mice. (DOC 40 kb)

**Additional file 6:** Table S2. BALF chemokine levels 6 and 21 h after intranasal LTA administration in vehicle and ibrutinib treated mice. (DOC 40 kb)

**Additional file 7:** Table S3. BALF and plasma cytokine and chemokine levels in a clinical model of *S. pneumoniae* infection. (DOC 41 kb)

**Abbreviations**

ALAT: Alanine aminotransferase; AM: Alveolar macrophages; ASAT: Aspartate aminotransferase; BTK: B-cell TK; CD: Cluster of differentiation; CII: C–C motif ligand; CFU: Colony forming units; CXCL: C–X–C motif ligand; IFNγ: Interferon-γ; IL: Interleukin; LDH: Lactate dehydrogenase; LTA: Lipoteichoic acid; MPO: Myeloperoxidase; NLRP3: Nod-like receptor family pyrin domain containing 3; PMN: Polymorphonuclear cell; S.
pneumoniae: Streptococcus pneumoniae; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TREM: Triggering receptor expressed on myeloid cells

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Authors' contributions
Contribution: A.P.N.A.d.P., A.F.d.V., and T.v.d.P. designed the study. R.W.H. generated Btk−/− and CD19−/− mice. A.P.N.A.d.P., Z.L., R.d.B. acquired all data. A.P.N.A.d.P., S.F., O.J.d.B. analyzed all pathology. All authors were involved in interpretation of the data. A.P.N.A.d.P. and A.F.d.V. drafted the manuscript and all authors reviewed and revised it critically for important intellectual content. All authors have final approval of this version to be submitted.

Ethics approval
All experiments were carried out in accordance with the Dutch Experiment on Animals Act and were approved by the local animal welfare committee of the Academic Medical Center.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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