Brazilian Journal of Medical and Biological Research (2013) 46: 601-606, http://dx.doi.org/10.1590/1414-431X20132894
ISSN 1414-431X Short Communication

An interleukin-33/ST2 signaling deficiency reduces overt pain-like behaviors in mice

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Abstract

Interleukin (IL)-33, the most recent member of the IL family of cytokines, signals through the ST2 receptor. IL-33/ST2 signaling mediates antigen challenge-induced mechanical hyperalgesia in the joints and cutaneous tissues of immunized mice. The present study asked whether IL-33/ST2 signaling is relevant to overt pain-like behaviors in mice. Acetic acid and phenyl-*p*-benzoquinone induced significant writhing responses in wild-type (WT) mice; this overt nociceptive behavior was reduced in ST2-deficient mice. In an antigen-challenge model, ST2-deficient immunized mice had reduced induced flinch and licking overt pain-like behaviors. In the formalin test, ST2-deficient mice also presented reduced flinch and licking responses, compared with WT mice. Naive WT and ST2-deficient mice presented similar responses in the rota-rod, hot plate, and electronic von Frey tests, indicating no impairment of motor function or alteration in basal nociceptive responses. The results demonstrate that IL-33/ST2 signaling is important in the development of overt pain-like behaviors.

Key words: Interleukin-33; ST2; Pain; Nociception; Inflammation

Introduction

Overt nociception/overt pain-like behavior models are widely used to assess the activity of novel candidate analgesic drugs and their mechanisms of action. These tests involve the injection of stimuli with irritating characteristics, which rapidly promotes behaviors such as abdominal writhing, flinching, or licking of the injected paw (1-6). The stimuli are generally chemical [e.g., phenyl-*p*-benzoquinone (PBQ), acetic acid, formalin] (1-3), but may also be biological (e.g., zymosan) (5) or even an antigen (4).

Abdominal writhing induced by acetic acid or PBQ is dependent on the release of inflammatory mediators, such as cytokines and prostanoids (2,5). In the PBQ model, writhing depends on the cytokines interleukin (IL)-18, interferon gamma (IFN-γ), and endothelin-1 (ET-1) (2). The mechanism for mediating the acetic acid-induced writhing response (2) depends on activation of peritoneal macrophages and mast cells that then release cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-8, as well as eicosanoids and sympathomimetic amines (5). Nevertheless, despite differences in the peripheral mediators involved in PBQ- and acetic acid-induced writhing responses, both depend on spinal activation of mitogen-activated protein kinases, phosphatidylinositol 3-kinase and microglia (1).

In the formalin test, the subcutaneous injection of formalin into the mouse hind-paw induces a nociceptive response that consists of two phases. Phase 1 (0-5 min after formalin injection) is the neurogenic phase and is generally attributed to a direct effect of the stimulus on nociceptors, whereas phase 2 (10-30 min after formalin injection) involves the subsequent development of inflammation, which is mediated by cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, and prostaglandins (PGs) (6-9). Overt pain-like behavior can also be induced by antigen challenge in immunized animals (4). For instance,

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Received December 18, 2012. Accepted May 9, 2013. First published online July 16, 2013.

intraplantar injection of ovalbumin (OVA) induces significantly more paw licking in immunized mice than in non-immunized mice, by an ET-1-dependent mechanism (4).

IL-33 is the most recent addition to the IL-1 cytokine family that includes IL-1β and IL-18. IL-33 exerts its biological activity by interacting with a heteromeric receptor composed of ST2, the IL-33 specific subunit of the receptor, and the IL-1 receptor accessory protein, which is shared with IL-18 (10,11). IL-33 is a pleiotropic cytokine implicated in various inflammatory conditions and diseases (11). IL-33 plays a role in Th1, Th2, and Th17 adaptive responses, innate inflammation, and as an endogenous danger signal (11).

IL-33/ST2 signaling is involved in pain (12). IL-33 mediates methylated bovine serum albumin (mBSA)-induced cutaneous and articular mechanical hyperalgesia in immunized mice via activation of the TNF-α → IL-1β → IFN-γ → ET-1 → PGE_2 signaling cascade (12). Furthermore, IL-33/ST2 signaling contributes to carrageenan-induced innate inflammatory pain, triggering the production of TNF-α, CXCL1, IL-1β, ET-1, and PGE_2 (13). These data suggest that the role of IL-33 in pain is wider than just its involvement in innate and Th1/Th17-dependent mechanical hyperalgesia (12,13) because the molecules produced in response to IL-33 also mediate nociceptive responses in other pain models, as well as promoting overt pain behaviors (2,4,5,7). Thus, in this study, we used ST2-deficient mice to investigate the role of IL-33/ST2 signaling in abdominal writhing induced by acetic acid and PBQ, and in formalin- and OVA challenge-induced paw flinch and licking responses in naive and immunized animals, respectively.

Material and Methods

Reagents

Acetic acid and formalin were obtained from Mallinckrodt Baker S.A. (Mexico), PBQ and OVA were from Sigma-Aldrich (USA), and DMSO was from Merck (Germany). The doses of these stimuli were chosen based on pilot studies and previous data from our laboratory (1,2,6).

Animals

All experiments were performed on sex-matched BALB/c wild-type ($ST2^{+/+}$) and BALB/c background ST2-deficient ($ST2^{-/-}$) mice (14), weighing 20-25 g. The experiments were conducted between 9:00 am and 5:00 pm. The mice were bred at the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil. Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines and with the approval of the Ethics Committee of the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil. All experiments were double-blinded.

Writhing response tests

The PBQ- and acetic acid-induced writhing model experiments were performed as described previously, using the same doses (1,2). PBQ (630 μg diluted in 10 mL 2% DMSO in saline), acetic acid [0.6% (v/v) diluted in saline], or vehicle was injected (10 mL/kg) into the peritoneal cavity of each mouse. Next, each mouse was placed in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhing responses (contraction of the abdominal muscles together with stretching of hind limbs or rotation of the trunk) occurring between 0 and 20 min after stimulus injection. The intensity of the writhing response was expressed as the cumulative number of movements occurring in 2-min bins over 20 min.

Formalin test

The number of paw flinches and time spent licking the paw were counted between 0 and 30 min after intraplantar injection of formalin [2.5% (v/v) diluted in 25 μL saline] or vehicle as described previously (6). The period was divided into 5-min bins, which clearly demonstrated the presence of the first (0-5 min) and second (10-30 min) phases that are characteristic of the model (6-9).

Measurement of motor coordination/function

Mouse motor coordination/function was evaluated using the rota-rod test. The apparatus consisted of a 2.5-cm diameter bar that was subdivided into 6 compartments by 25-cm diameter disks (model 7600; Ugo Basile, Italy). Mice were placed on the bar while it was rotating at constant speeds of 10 or 15 rotations per min (rpm), and the duration that they were able to remain on it was determined. The cutoff time used was 180 s.

Hot plate test

Mice were placed in a 10-cm diameter glass cylinder on a hot plate (Hot Plate HP-2002, Insight Equipamentos, Brazil) maintained at 55°C. The reaction time was scored when the animal jumped, flinched or licked its paws. A maximum latency (cutoff) was set at 30 s to avoid tissue damage (6).

OVA immunization procedure and challenge

Mice were immunized with a single subcutaneous dose of 50 μg OVA plus 5 mg $Al(OH)_3$, diluted in 200 μL sterile saline. After 14 days, mice were challenged with 1 μg/paw OVA or vehicle (saline). The number of paw flinches and time spent licking the paw was determined over 30 min (4). The dose of the antigen challenge was determined by preliminary studies in our laboratory.

Statistical analysis

Results are reported as means ± SE of 7 mice per group per experiment, and represent two separate experiments. Statistical differences between groups

were determined by the two-tailed Student *t*-test for unpaired samples (Figures 1 and 3) or one-way ANOVA followed by the Bonferroni *t*-test (Figure 2). All statistical analyses were performed using the GraphPad Prism 5 software (USA). The significance level was set at $P<0.05$.

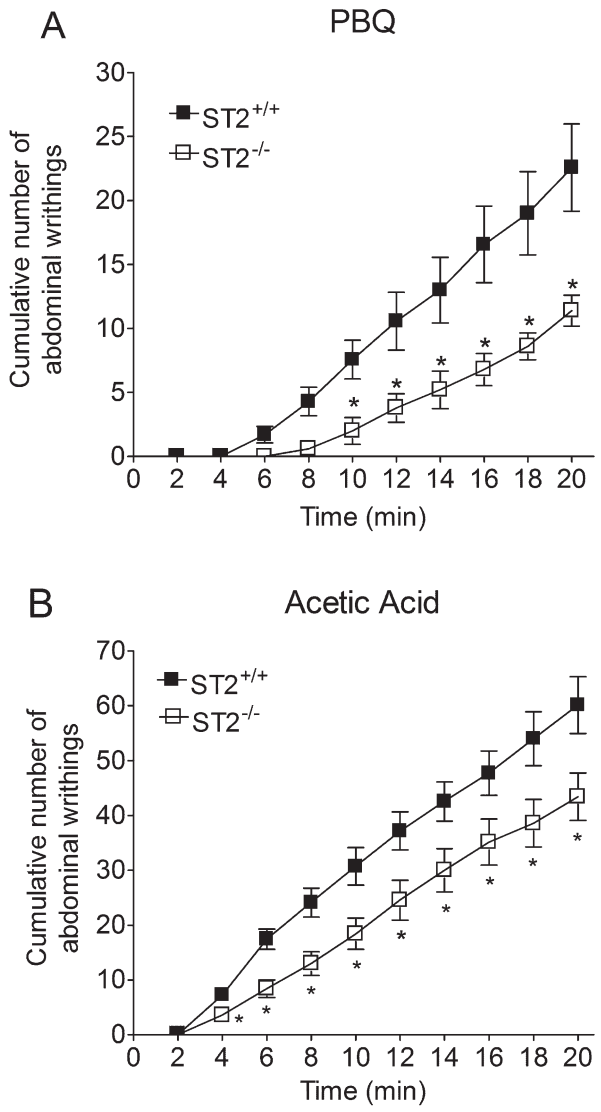

Figure 1. Role of IL-33/ST2 receptor in phenyl-*p*-benzoquinone (PBQ)- and acetic acid-induced writhing responses. $ST2^{+/+}$ and $ST2^{-/-}$ mice received an intraperitoneal injection (10 mL/kg) of *A*, PBQ (630 µg diluted in 10 mL 2% DMSO in saline) or *B*, 0.6% acetic acid diluted in saline. The cumulative number of writhing responses was evaluated between 0 and 20 min and reported at 2-min intervals. n = 7 per group per experiment, representative of two separate experiments. *$P<0.05$, compared to the $ST2^{+/+}$ group (Student *t*-test).

Results

Role of IL-33/ST2 in PBQ- and acetic acid-induced writhing responses

The role of IL-33/ST2 was evaluated in PBQ- and acetic acid-induced overt pain-like behavior. $ST2^{-/-}$ mice showed reduced PBQ-induced writhing responses compared with $ST2^{+/+}$ mice (Figure 1A). The differences were significant at each interval between 10 and 20 min. Acetic acid-induced writhing responses were also diminished in $ST2^{-/-}$ mice, compared with $ST2^{+/+}$ mice (Figure 1B), and the differences were significant between 4 and 20 min. The vehicles, saline and 2% DMSO in saline, did not induce writhing responses in any of the mice (data not shown).

Role of IL-33/ST2 in OVA-induced paw flinching and licking

$ST2^{+/+}$ and $ST2^{-/-}$ mice challenged with 1 µg/paw OVA 14 days after immunization with the antigen displayed paw flinches and licking behavior. Both the paw flinches (Figure 2A) and time spent licking the paw

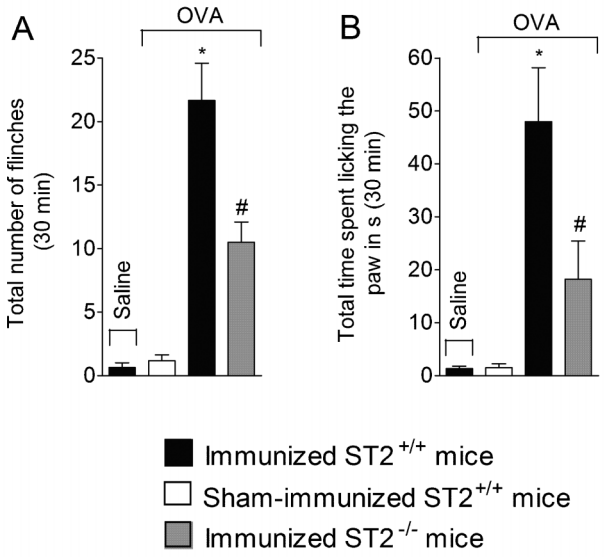

Figure 2. Role of IL-33/ST2 receptor in ovalbumin (OVA) challenge-induced overt pain-like behavior in immunized mice. $ST2^{+/+}$ and $ST2^{-/-}$ mice were immunized with a single dose of 50 µg OVA plus 5 mg $Al(OH)_3$ diluted in saline. After 14 days, mice were challenged with 1 µg/paw OVA and the number of flinches (*A*), and time spent licking the paw (*B*) were quantified for 30 min. n = 7 per group per experiment, representative of two separate experiments. *$P<0.05$, immunized $ST2^{+/+}$ mice receiving OVA compared to sham-immunized $ST2^{+/+}$ mice receiving OVA and immunized $ST2^{+/+}$ mice receiving saline. #$P<0.05$, immunized $ST2^{-/-}$ mice receiving OVA compared to immunized $ST2^{+/+}$ mice receiving OVA (one-way ANOVA followed by the Bonferroni *t*-test).

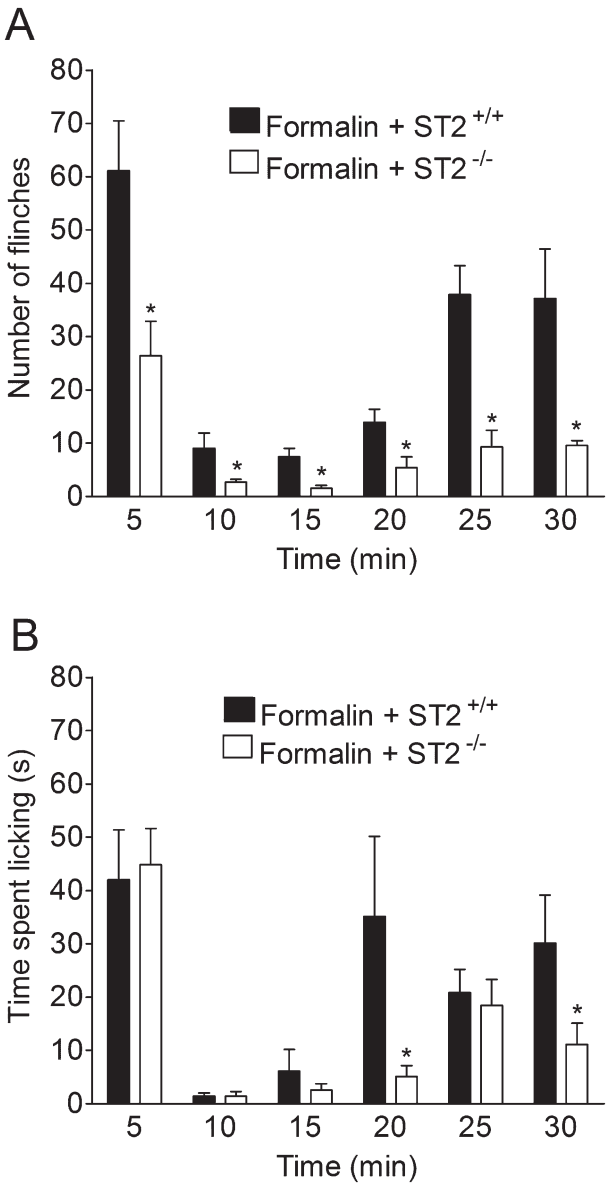

Figure 3. Role of IL-33/ST2 receptor in formalin-induced paw flinching (*A*) and time spent licking (*B*). $ST2^{+/+}$ and $ST2^{-/-}$ mice received an intraplantar injection of 25 µL 2.5% formalin diluted in saline. The overt pain-like behavior was determined as the number of flinches and time spent licking the paw for 30 min at 5-min intervals. n = 7 per group per experiment, representative of two separate experiments. *P<0.05, compared to the $ST2^{+/+}$ group (Student *t*-test).

(Figure 2B) induced by OVA challenge were reduced significantly in $ST2^{-/-}$ mice, compared with $ST2^{+/+}$ mice. Vehicle (saline), in immunized mice, and OVA challenge in the sham-immunized group did not induce significant flinching or licking behaviors. $ST2^{-/-}$ mice showed the same responses as $ST2^{+/+}$ mice in the sham-immunized group challenged with OVA and the immunized group challenged with saline (data not shown).

Role of IL-33/ST2 in formalin-induced paw flinching and licking

The paw flinch responses of $ST2^{-/-}$ mice were reduced at all time points compared with those of $ST2^{+/+}$ mice (Figure 3A), and the time spent licking the injected paw was reduced in $ST2^{-/-}$ compared with $ST2^{+/+}$ mice during the 15-20-min and 25-30-min intervals (Figure 3B). Injection of vehicle (saline) did not induce flinch or licking responses in either $ST2^{+/+}$ or $ST2^{-/-}$ mice (data not shown).

Effect of IL-33/ST2 signaling deficiency on motor coordination/function and thermal nociceptive threshold

Rota-rod and hot plate tests were used to evaluate the role of IL-33/ST2 signaling on motor coordination/function and thermal nociceptive threshold. No significant differences (P>0.05) were observed in the time that $ST2^{-/-}$ mice (179.80 ± 0.20 or 162.40 ± 11.03 s) and $ST2^{+/+}$ mice (178.70 ± 1.30 or 175.70 ± 4.30 s) remained on the rota-rod at 10 or 15 rpm, respectively. In the hot plate test, no significant difference (P>0.05) was observed in the reaction time of $ST2^{-/-}$ mice (10.45 ± 0.50 s) and $ST2^{+/+}$ mice (11.45 ± 1.04 s; data not shown).

Discussion

IL-33 is a pleiotropic cytokine involved in adaptive and innate immune responses (11). The role of IL-33/ST2 signaling in pain was first demonstrated using a Th1/Th17 immunization protocol. Treatment with soluble ST2 reduced antigen challenge-induced mechanical cutaneous and articular hyperalgesia in mice by preventing the production of inflammatory molecules, including the cytokines TNF-α, IL-1β and IFN-γ, ET-1, and PGE_2 (12). It is interesting to note that IL-18 also mediates hyperalgesia induced by antigen challenge in immunized mice by triggering the production of IFN-γ, ET-1, and PGE_2 (15) and is an important cytokine in the PBQ-induced writhing response (2). We reason that IL-33/ST2 signaling could also contribute to overt pain-like responses, as already observed in relation to IL-18.

Furthermore, since IL-33 and IL-18 receptors share a common beta chain, the IL-1 receptor accessory protein (11), and both receptors mediate hyperalgesia in antigen-induced inflammation via IFN-γ, ET-1 and PGE_2, a similar profile of responses to their activation might be expected. However, IL-18 mediates the PBQ- but not the acetic acid-induced writhing response (2), whereas IL-33/ST2 mediates the nociceptive response in both models. IL-33/ST2 triggers production of TNF-α and IL-1β, which are important cytokines in the acetic acid-induced writhing response (5). This mechanism of IL-33, compared with that of IL-18, explains why IL-33/ST2 mediates both the acetic acid- and

PBQ-induced writhing responses. Thus, it seems likely that the role of IL-33/ST2 in pain may be broader than that of IL-18. Furthermore, consistent with a role of IL-33 in abdominal pain, IL-33 peritoneal fluid and serum levels are elevated in 75 and 23% of patients with endometriosis, respectively, primarily in deeply infiltrating endometriosis presenting painful symptoms, such as dysmenorrhea (16).

In the OVA challenge in immunized mice, the results indicate a role for IL-33/ST2 in Th2 inflammation-induced overt pain-like behavior, increasing the possible role of IL-33/ST2 in nociception beyond Th1/Th17 and innate responses, as demonstrated previously (12,13). In the OVA model, mast cell degranulation induces ET-1/ET_A receptor-dependent overt pain-like behavior (4). Likewise, in the model of Th1/Th17 inflammation in immunized mice challenged with mBSA, and in innate inflammation induced by carrageenin, IL-33/ST2 also induces ET-1-dependent mechanical hyperalgesia (12,13). Thus, it seems reasonable to expect that IL-33/ST2 could trigger an ET-1-dependent nociceptive response in Th2 inflammation models, but this phenomenon remains to be explored further.

In the formalin test, ST2 deficiency resulted in a decreased response in both phases, mainly in the flinch response assay, but with significant inhibition seen also in the second phase of the licking response. In the first phase of the formalin test, there is participation by mast cell-derived mediators, such as histamine (8), and, considering that IL-33 activates the constitutively expressed ST2 receptors in mast cells (10,11), it is possible that involvement of IL-33/ST2 in the first phase of the formalin test might be related to the activation of mast cell-derived mediators. The reduction in formalin-induced nociception was more evident in the second phase, with inhibition of both flinching and licking responses. In that phase, there is production of cytokines, such as TNF-α and IL-1β, and inhibition of the activity of those cytokines reduces nociceptive behavior (7). In antigen- and carrageenin-induced hyperalgesia IL-33/ST2, signaling mediates the production of hyperalgesic TNF-α and IL-1β (12,13). Thus, it seems reasonable to suggest that IL-33/ST2 could induce overt pain-like behavior in the second phase of the formalin test by triggering the production of nociceptive cytokines, such as TNF-α and IL-1β.

During the review process of this manuscript, it was reported that the intraplantar or intrathecal administration of IL-33 induces overt pain-like behavior. Moreover, intraplantar or intrathecal administration of IL-33 increases, and treatment with soluble ST2 (a decoy receptor for IL-33) reduces, the overt pain-like behavior induced by formalin (17). Those results are consistent with the present data and the rationale that IL-33/ST2 signaling itself can trigger overt pain-like behavior.

Furthermore, IL-33 is constitutively expressed in normal human tissues, and its level is abundant in endothelial and epithelial cells *in vivo*, indicating that IL-33 can be released promptly. IL-33 has a role as a nuclear factor and is considered to be a ''danger'' signal, similar to HMGB1 and IL-1α, functioning as an alarm to the immune system when endothelial and/or epithelial cells are subject to damage (18). This concept is in line with the involvement of IL-33/ST2 in acute nociceptive events such as the writhing response, in which the stimulus is injected into the highly vascularized peritoneal cavity, and the paw flinch and licking responses, with the possible release of IL-33 by epithelial cells and keratinocytes and activation of mast cells, fibroblasts, and macrophages (11,12,15,16,18,19). Furthermore, ST2 is expressed by neurons, and IL-33 is expressed by neurons and astrocytes in the spinal cord of mice in a model of encephalomyelitis (20), suggesting that IL-33 could be produced by astrocytes and neurons and act on ST2 receptors expressed by neurons in the spinal cord, which is consistent with the overt pain-like behavior induced by the intrathecal injection of IL-33 (17). Nevertheless, the cellular sources and targets of IL-33 remain to be determined in pain models to establish whether it has direct and/or indirect effects on nociceptive neurons.

Notably, differences were not observed between naive $ST2^{-/-}$ and $ST2^{+/+}$ mice in the rota-rod, hot plate (data not shown), or electronic pressure meter tests (12,13). These data indicate that the motor coordination/function in $ST2^{-/-}$ mice is preserved, and that there is no alteration of basal nociceptive responses to thermal or mechanical stimuli.

In conclusion, the present data indicate that, in addition to promoting mechanical hyperalgesia (12,13), IL-33/ST2 signaling is important in overt pain-like behavior triggered by a variety of phlogistic agents including acetic acid, PBQ, formalin, and OVA challenge in immunized mice. These results increase the relevance of IL-33/ST2 signaling in nociception and suggest that the potential of IL-33 targeting therapies to control inflammatory pain deserves to be investigated further.

Acknowledgments

Research supported by SETI/Fundação Araucária (State of Paraná), FAPESP, CNPq and CAPES (Brazil), and Arthritis Research Campaign (UK), Chief Scientist Office (Scotland), and the Medical Research Council (UK). M.S.N. Hohmann received a Brazilian fellowship from Departamento de Ciência e Tecnologia da Secretaria de Ciência, Tecnologia e Insumos Estratégicos, Ministério da Saúde (Decit/SCTIE/MS) through CNPq and the support of Fundação Araucária.

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