Overexpression of CD39 in Mouse Airways Promotes Bacteria-Induced Inflammation

Emilie Théâtre, Kim Frederix, William Guilmain, Céline Delierneux, Christelle Lecut, Lucien Bettendorff, Vincent Bours and Cécile Oury

J Immunol published online 16 July 2012
http://www.jimmunol.org/content/early/2012/07/16/jimmunol.1102600

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/07/16/jimmunol.1102600.0.DC1

Why The JI? Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Overexpression of CD39 in Mouse Airways Promotes Bacteria-Induced Inflammation

Emilie Théâtre,*1 Kim Frederix,*1 William Guilmain,* Céline Delierneux,* Christelle Lecut,* Lucien Bettendorff, † Vincent Bours,* and Cécile Oury*

In airways, the ecto-nucleoside triphosphate diphosphohydrolase CD39 plays a central role in the regulation of physiological mucosal nucleotide concentrations and likely contributes to the control of inflammation because accelerated ATP metabolism occurs in chronic inflammatory lung diseases. We sought to determine whether constant elevated CD39 activity in lung epithelia is sufficient to cause inflammation and whether this affects the response to acute LPS or *Pseudomonas aeruginosa* exposure. We generated transgenic mice overexpressing human CD39 under the control of the airway-specific Clara cell 10-kDa protein gene promoter. Transgenic mice did not develop any spontaneous lung inflammation. However, intratracheal instillation of LPS resulted in accelerated recruitment of neutrophils to the airways of transgenic mice. Macrophage clearance was delayed, and the amounts of CD8+ T and B cells were augmented. Increased levels of keratinocyte chemoattractant, IL-6, and RANTES were produced in transgenic lungs. Similarly, higher numbers of neutrophils and macrophages were found in the lungs of transgenic mice infected with *P. aeruginosa*, which correlated with improved bacteria clearance. The transgenic phenotype was partially and differentially restored by coinhibition of P2X2 or P2X7 receptor antagonists or of caffeine with LPS. Thus, a chronic increase of epithelial CD39 expression and activity promotes airway inflammation in response to bacterial challenge by enhancing P1 and P2 receptor activation.

The Journal of Immunology, 2012, 189: 000–000.

A

irway epithelia constitute an essential protective barrier against lung infection, coordinating luminal and interstitial responses to inhaled pathogens through signals provided by epithelial, inflammatory, and immune cells. Extracellular nucleotides provide an elaborated cell communication system in mammalian tissues including the airways (1). The major source of extracellular nucleotides in normal airways is the epithelium, secreting ATP under resting conditions and in response to various mechanical stimulations, including membrane stretch, shear stress, hypotonicity-induced swelling, and after physical interaction with air contaminants or microbes (2). Extracellular ATP and adenosine contribute to mucociliary clearance (3), a process critical for maintain}

---

*Interdisciplinary Cluster of Applied Genoproteomics-Inflammation, Immunity, Unit of Human Genetics, Laboratory of Thrombosis and Haemostasis, University of Liège, Liège 4000, Belgium; †Interdisciplinary Cluster of Applied Genoproteomics-Neurosciences, Unit of Bioenergetics and Cerebral Excitability, University of Liège, Liège 4000, Belgium

1E.T. and K.F. contributed equally to this work.

Received for publication September 8, 2011. Accepted for publication June 12, 2012. This work was supported by the Belgian National Fund for Scientific Research (Projects 3.4599.07 and 3.4584.09), the Belgian Science Policy (Interuniversity Attraction Pole [IAP] 6/18), and the Fonds Spéciaux Pour la Recherche (Project I-05/02) of the University of Liège, Liège, Belgium; and †Interdisciplinary Cluster of Applied Genoproteomics-Inflammation, Immunity, Unit of Human Genetics, Laboratory of Thrombosis and Haemostasis, B34, Avenue de l’Hôpital 1, B-4000 Liège, Belgium. E-mail address: cecile.oury@ulg.ac.be

The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; GIGA, Interdisciplinary Cluster of Applied Genoproteomics; hCD39, human CD39; KC, keratinocyte chemoattractant; mCC10, mouse Clara cell 10-kDa protein; αMβ2eATP, αMβ2-methylene ATP, WT, wild-type.
To study the consequences of a chronic increase of surface CD39 activity in lung inflammation, we generated transgenic mice over-expressing human CD39 (hCD39) in airway epithelia and characterized their response to a bacterial challenge. Some of the results of these studies have been previously reported in the form of an abstract (18).

Materials and Methods

Generation of transgenic mice

A construct containing the mouse Clara cell 10-kDa protein (mCC10) promoter upstream of the hCD39 cDNA was prepared in the pCDNA3 vector (Invitrogen, Carlsbad, CA). For hCD39 cDNA, total RNA extracted from HUVEC was reverse transcribed with the Transcription First Strand kit (Roche Biochemicals, Mannheim, Germany). The following primers were used for PCR: 5′-GAAAACAAAAGCTGCTACT-3′ and 5′-CAGTAAAGGCCAAGGAAGC-3′, and TOPO TA cloning (Invitrogen) was performed. For the mCC10 promoter, we took advantage of the high density of Clara cells in the mouse respiratory epithelium and used the CC10 promoter to target the expression of CD39 to the airways. The approach used has previously been described by Zhu et al. (19). The mCC10 promoter was amplified by PCR using the following primers: 5′-GGTAAGGCCTGGGATGGCTACAA-3′ and 5′-GGGATGTTGGGTGGTGTGGC-3′. These primers were designed to incorporate HindIII and BamHI restriction sites. The DNA fragment encompassing the transgene and the pCDNA3 polyadenylation signal was amplified by PCR, purified, and resuspended in the microinjection buffer (0.5 mM Tris-HCl and 25 mM EDTA [pH 7.4]). Transgenic mice were generated by zygote pronuclear injection. Data from two independent experiments on four mice per founder line. *p < 0.05 versus WT. **p < 0.01 versus vehicle (control). Data represent mean ± SEM from three independent experiments on four mice per founder line. *p = 0.004 versus WT.

Immunohistochemistry

Frozen lung sections of 4 μm were incubated with the monoclonal anti-hCD39 Ab (Ancell, Bayport, MN) followed by Envision+ System Labeled Polymer-HRP Anti-Mouse Ab (DakoCytomation, Glostrup, Denmark). Anti-CD45 staining (rat anti-mouse CD45; BD Pharmingen, San Jose, CA) was performed on paraformaldehyde-fixed paraffin-embedded sections according to classical procedures.

LPS and P. aeruginosa instillation

C57BL/6J wild-type (WT) or transgenic mice (8–12 wk old) were anesthetized with isoflurane (Forene; Abbott, Ottignies, Belgium). Ultra Pure Escherichia coli LPSs (InvivoGen, San Diego, CA) were administered by intratracheal instillation (5 μg/mouse in 50 μl sterile saline). Saline was instilled as control. In some experiments, LPS and α,β-methylene ATP (α,βMeATP) (200 μM; Sigma-Aldrich, Bornem, Belgium), NF449 (0.1 μM; Tocris Biosciences, Bristol, U.K.), or caffeine (20 mg/kg; Sigma-Aldrich) were instilled simultaneously. Acute
infection with P. aeruginosa (PAK laboratory strain) was performed by intratracheal instillation of $1 \times 10^7$ bacteria (CFU) in a volume of 50 µl PBS was instilled as control. Bacteria load was determined by counting the number of viable bacteria in lung homogenates at different times post-infection. Serial dilutions of homogenates were plated on Luria-Bertani agar for 24 h at 37°C.

**Bronchialveolar lavage, cell count, cytokine level quantification, and alveolar permeability**

Bronchialveolar lavage (BAL) was performed according to classical procedures. Differential cell counts were determined on cytospins (Cytospin, Westwood, MA) using morphological criteria after Giemsa-Wright staining (Diff-Quick stain set; Medion Diagnostics, Düdingen, Switzerland). Levels of cytokines in BAL fluid (BALF) supernatants were quantified using Bio-Plex Cytokine Bead Array analyses according to the manufacturer’s instructions (Bio-Rad, Nazareth Eke, Belgium; and BD Biosciences, Erembodegem, Belgium).

**Flow cytometry**

Cells isolated from BALF were stained with FITC-conjugated CD45R/B220 (RA3-6B2), FITC-conjugated CD8a (Ly-2), allophycocyanin-conjugated CD3e (145-2C11), and PE-conjugated CD4 (GK1.5). These Abs were purchased from BD Pharmingen (San Jose, CA). Analyses were performed on a FACS Canto flow cytometer (BD Biosciences).

**Luciferase assay and HPLC analysis**

Supernatants of BALF were used to determine extracellular ATP levels in the airways of the mice with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). For the analyses of adenyl purines, proteins from BALF supernatants were precipitated with 12% trichloroacetic acid. Derivatization of purines was performed with 1 M chloroacetaldehyde, and the resulting fluorescent etheno-species were analyzed by reversed-phase HPLC as previously described (2, 21).

**Statistical analyses**

One-way ANOVA tests with Bonferroni adjustment were used for multiple comparisons. Statistical significance was set at $p < 0.05$.

**Results**

**Generation of transgenic mice overexpressing the hCD39 in the airway epithelium**

On three independent zygote injections of the mCC10-hCD39 transgene, 77 offspring were obtained, and 24 animals were found to be transgenic by PCR screening. Three founder lines were used throughout our study. The transgenic mice had no apparent physiologic abnormalities. Real-time RT-PCR analyses using primer pairs that selectively amplify hCD39 cDNA showed the presence of hCD39 transcripts in the lungs of the three independent lines of transgenic mice (Fig. 1A). The levels of the endogenous pulmonary mouse CD39, CD73, and adenosine deaminase transcripts remained similar to that of WT mice (Supplemental Fig. 1). No transgene expression was found in any of the other organs tested (data not shown). The expression of hCD39 protein was demonstrated by immunoblotting of mouse lung extracts (data not shown). Immunohistochemistry of lung sections revealed selective hCD39 staining in the transgenic epithelia (Fig. 1B). The ATP levels were reduced in BALF of the

---

**FIGURE 2.** Increased inflammatory cell recruitment in hCD39 TG lungs in response to LPS challenge (5 µg/mouse via intratracheal instillation). (A) Lung immunohistochemistry. Anti-CD45 staining on lung sections 2, 6, and 14 h after LPS instillation shows increased leukocyte infiltration for hCD39 mice compared with WT. Original magnification ×400. (B) Differential cell counts in BALF after LPS challenge show increased recruitment and delayed clearance of inflammatory cells in hCD39 mice. Time courses after LPS for neutrophils (top panel) and macrophages (middle panel) are shown. Data represent mean ± SEM for $n = 6$ mice/group. Control (C) represents averaged data for vehicle instillation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus WT. The bottom panel shows flow cytometric analyses of lymphocyte CD4+, CD8+, and CD3+ B220+ subpopulations in the BALF of WT and hCD39 mice 120 h following LPS instillation. **$p < 0.01$ versus WT.
three transgenic lines as compared with WT animals (Fig. 1C), indicative of increased CD39 enzymatic activity. Accordingly, HPLC analyses revealed elevated AMP (data not shown) and adenosine levels in transgenic BALF (Fig. 1D). All three lines showed similar phenotypes.

Increased leukocyte infiltration in the lungs of hCD39 transgenic mice after acute LPS exposure

Under basal conditions, inflammatory cell counts in BALF of WT and transgenic mice were identical, and only macrophages were present (data not shown). Lung histology was found to be normal. MUC5AC mRNA expression levels remained low (Supplemental Fig. 2A), and mucus could not be detected following periodic acid-Schiff and alcian blue colorations (Supplemental Fig. 2B).

We then performed intratracheal instillation of a unique moderate dose of bacterial LPS. Under these conditions, ATP levels in transgenic BALF remained lower than in WT BALF for up to 5 d postinstillation, whereas adenosine levels were constantly increased (Fig. 1D). Histological examination of lung sections and anti-CD45 staining showed leukocyte infiltration (Fig. 2A), alveolar edema, and congestion, which all were more pronounced in transgenic lungs. Accordingly, the amounts of neutrophils in the BALF of transgenic mice were significantly increased after 2, 6, and 12 h following LPS as compared with WT mice (Fig. 2B). Although macrophage numbers were not different between WT and transgenic animals until 3 d, macrophage amounts remained significantly higher in transgenic BALF 5 d after LPS treatment. At this time, ~25% lymphocytes were present in BALF, among which the amounts of CD8+ T and B220+ B cells were significantly increased in transgenic versus WT BALF (Fig. 2B). CD4+ T cell amounts were normal. Inflammation was resolved by day 14 both for hCD39 and WT mice (Fig. 2B).

Elevated LPS-induced alveolar permeability and inflammatory cytokine production in the lungs of transgenic mice

In agreement with a proinflammatory phenotype for LPS-treated hCD39 mice, alveolar permeability was significantly increased in hCD39 as compared with WT mice (Fig. 3A). To further analyze this phenotype, we then measured cytokine levels in BALF. LPS administration induced transient production of the cytokines tested: keratinocyte chemoattractant (KC), IL-6, TNF-α, IL-1β, INF-γ, CCL-2, RANTES, and G-CSF (data not shown). Among these cytokines, levels of KC, RANTES, and IL-6 were significantly elevated in transgenic versus WT BALF (Fig. 3B). KC and IL-6 levels reached higher values 2 h after LPS treatment; IL-6 and RANTES levels were more elevated at 14 h. As previously described (17), mouse CD39 mRNA transcripts were increased upon LPS treatment, both in WT and transgenic lungs (Supplemental Fig. 3).

Increased P2 receptor activity in hCD39 transgenic lungs

The proinflammatory response of transgenic mice may result from increased ATP degradation, adenosine accumulation, or both. Moreover, increased CD39 activity may also improve P2 receptor signaling by limiting receptor desensitization by released ATP (22, 23). To address this question, we studied the effects of P2 receptor antagonists on LPS-induced inflammation in WT and transgenic mice. In transgenic mice, the broad-spectrum P2 receptor antagonist suramin (data not shown) and the selective P2X1 and P2X7 receptor antagonists NF449 (24) and A438079 (25) reversed the early increase of neutrophil recruitment to levels found in WT BALF (Fig. 4A). Neutrophil counts measured 48 h after LPS instillation were also significantly reduced by NF449 and A438079. These antagonists did not affect the response of WT mice. In contrast, neither NF449 (data not shown) nor A438079 (Fig. 4A) affected the macrophage recruitment measured after 48 h. The delay of macrophage clearance observed in transgenic mice at 5 d following LPS was restored by A438079. At 5 d after LPS instillation, lymphocyte recruitment to transgenic lungs was significantly inhibited by treatment with A438079. The stable ATP analog α,βMeATP acts preferentially as an agonist for P2X3 and P2X7 receptor subtypes, but it can also target other P2X subtypes expressed on airway epithelia (26). In vivo, this compound has demonstrated P2X7 receptor desensitizing properties (27). To further investigate the role of P2X
receptors in the phenotype of CD39 transgenic mice, α,βMeATP was coinstilled with LPS. Similarly as treatment with NF449, this agonist prevented the recruitment of neutrophils specifically to transgenic lungs 2 h after simultaneous instillation of LPS and P2 receptor antagonists, as indicated. (B) Effects of instillation of α,βMeATP as a desensitizing agonist. Bars represent mean ± SEM; n = 5 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 versus WT, 4p < 0.001, 6p < 0.01, 8p < 0.05 versus control (C).

Increased leukocyte infiltration in the lungs of hCD39 transgenic mice upon acute infection with P. aeruginosa

To determine whether the proinflammatory phenotype of the hCD39 transgenic mice could be reproduced during infection with live bacteria, we instilled the mice with P. aeruginosa and counted inflammatory cells in their BALF after 24 h. In agreement with our LPS data, CD39 overexpression augmented inflammatory cell recruitment into the lungs (Fig. 7A). In transgenic lungs, both neutrophil and macrophage counts were increased in conditions in which only neutrophil recruitment occurred in WT lungs. Similarly as with LPS, bacteria-induced KC and RANTES secretion was increased in hCD39 BALF as compared with WT BALF (Fig. 7B), whereas TNF-α, IL-1β, and CCL-2 levels did not differ between WT and hCD39 mice (data not shown). Kinetics of KC and RANTES production displayed striking differences. Although KC levels were significantly higher in hCD39 BALF as compared with WT BALF at 2 h postinfection, production of this chemokine.
declined more rapidly for transgenic mice. Also, RANTES production occurred earlier for transgenic mice and resolved when reaching peak values in WT. Such resolution differences were not observed in response to LPS challenge. Other differences with LPS concerned bacteria-induced IL-6 production that was identical in hCD39 and WT mice and IFN-γ levels that were significantly higher in hCD39 BALF. These results prompted us to analyze the ability of hCD39 mice to clear bacteria. Interestingly, transgenic mice could better eliminate bacteria than WT mice, as determined by counting live bacteria in lungs (Figs. 7C, 8).

**Discussion**

By generating transgenic mice overexpressing hCD39 in airway epithelia, we created a model of chronic ATP removal and elevation of adenosine levels in the airways. These mice did not develop spontaneous lung inflammation, indicating that increased CD39 activity is not sufficient to cause inflammation. Nevertheless, the transgenic mice developed an enhanced inflammatory response upon intratracheal instillation of a moderate dose of LPS or lung infection with *P. aeruginosa*. Thus, a chronic increase of CD39-mediated nucleotide metabolism on the airway surface, as observed in chronic pulmonary inflammatory diseases (6), promotes the inflammatory response to bacterial challenge. Inflammation was initiated earlier than in WT mice, resulting in improvement of bacteria clearance. Transgenic mice displayed accelerated neutrophil recruitment into the lungs at early time points following LPS instillation. At later time points, delayed clearance of macrophages was observed, as well as increased lymphocyte recruitment. Accordingly, production of the chemokines KC and RANTES was increased (LPS) or occurred earlier (*P. aeruginosa*) in transgenic lungs as compared with WT lungs. In response to live bacteria, KC and RANTES production ended sooner for transgenic mice, which coincided with strongly reduced bacteria load, whereas neutrophil and macrophage counts were still higher. At this time, IFN-γ levels were increased in transgenic lungs, which may be in agreement with its important immunomodulatory role during infection (28). In light of the recently proposed proinflammatory role for ATP in the airways (8, 10, 29) and because CD39 deficiency in mice leads to increased LPS-induced lung inflammation (17), the observed transgenic mouse phenotype was unexpected. The use of P2X1 and P2X7 receptor antagonists (NF449 and A438079) enabled us to reconcile these apparent discrepancies. Indeed, these antagonists decreased the LPS-induced immune cell recruitment in transgenic mice, whereas they were mainly inactive in WT animals, indicating that P2 receptor function is facilitated by CD39 overexpression. In animals with smoke-induced lung injury, Cicko et al. (12) have observed a specific upregulation of the P2Y2 receptor on blood and lung neutrophils and macrophages. Lucattelli et al. (11) have shown that cigarette smoke-induced inflammation was associated with an upregulation of the P2X7 receptor on blood and airway neutrophils, alveolar macrophages, and in whole lung tissue. Up-regulation of P2X7 receptors on BAL macrophages and blood eosinophils has also been observed in patients with chronic asthma (30). In our study, P2X1 and P2X7 mRNA levels were found to be
unchanged in transgenic lungs and were not upregulated following LPS treatment (Supplemental Fig. 4). Upregulation of P2X1 and P2X7 receptor expression can therefore not explain the observed increased activity of these receptors in transgenic airways. In contrast, levels of P2Y2R transcripts were increased by \( \sim 5 \)-fold in transgenic lungs. Thus, even if we were not able to show specific contribution of P2Y2 receptors in our transgenic mouse phenotype due to lack of selective antagonists for this receptor, these receptors may be involved. Our data with the selective P2X7 receptor antagonist A438079 depict an important role of this receptor in neutrophil and lymphocyte recruitment into the lungs as well as in macrophage survival, which may contribute to antibacterial immunity under conditions of CD39 overexpression. In agreement with its effect on neutrophil infiltration, A438079 also potently inhibited early KC production in transgenic lungs. These data are in line with recent studies using antagonists and P2X7-deficient mice in models of lung fibrosis induced by airway-administered bleomycin (31), OVA-hydrated potassium aluminium sulfate (alum) and/or host dust mite model of asthmatic airway inflammation (30), and cigarette smoke-induced lung inflammation and emphysema (12). P2X7 receptors are involved in caspase-1-mediated IL-1\( \beta \) and IL-18 maturation and secretion by LPS-primed monocytes/macrophages (32); however, we could not detect higher IL-1\( \beta \) levels in the lungs of transgenic mice compared with WT. P2X7 receptors also contribute to IL-6 secretion and to dendritic cell (33) and lymphocyte activation and migration (34–36). In addition, our findings with the selective P2X7 antagonist NF449 reveal a novel role for P2X1 receptors in LPS-induced neutrophil and lymphocyte recruitment into the lungs. This observation is compatible with the recent findings on a role for P2X1 receptors in neutrophil chemotaxis (37), acting in cooperation with P2Y2 and adenosine A3 receptors (38, 39). P2X1 receptors have recently been found to be expressed on T cells and to participate in their activation in vitro (40). Our in vivo data suggest for the first time, to our knowledge, that P2X1 receptors may also contribute to airway adaptive immunity by controlling lymphocyte influx into the lungs. In our study, we observed an increase of CD8\(^+\) T lymphocytes in the lungs of LPS-treated transgenic mice, whereas CD4\(^+\) T lymphocyte counts were normal. Interestingly, CD8\(^+\) T cells predominate over CD4\(^+\) T cells in the airways and lung parenchyma of patients with COPD (41).

Upon LPS challenge, the stable ATP analog \( \alpha,\beta\text{MeATP} \), a P2X-desensitizing agonist, demonstrated potent anti-inflammatory prop-
properties both in WT and transgenic mice that were characterized by an almost full inhibition of inflammatory cell recruitment. This agonist is more potent than the two other P2X antagonists used in our study, suggesting that it affects additional or multiple P2 receptor subtypes. Further investigations are required to identify the precise target(s) of this compound.

A contribution of adenosine in the transgenic airway phenotype was assessed by use of caffeine, a broad-spectrum inhibitor of adenosine receptors. Adenosine has been involved in leukocyte migration and inflammatory cell death in several in vitro studies (42). More importantly, chronic adenosine elevations in the airways were found to promote a proinflammatory role (43–46). Notably, adenosine deaminase-deficient mice, showing lung adenosine accumulation, spontaneously develop lung inflammation and damage (46). In our study, costimulation of caffeine with LPS suggested that elevated adenosine levels participated in increasing neutrophil influx into the lungs of transgenic mice, possibly through A3 receptors. Adenosine receptors were also involved in macrophage survival during resolution of inflammation, but this function did not differ between WT and transgenic mice. Finally, caffeine increased lymphocyte influx in mice of both genotypes, which would support immunosuppressive function of adenosine. The increase of lymphocyte influx in transgenic lungs can therefore not be explained by elevated adenosine levels and adenosine receptor activation and would rather depend on P2 receptors. Because adenosine promotes the production of IL-6 by many cell types through engagement of the A3R, contributing to inflammation and fibrosis (47), it is possible that the increase of IL-6 levels in BALF of LPS-treated transgenic mice would be mediated at least partially by adenosine. Detailed assessment of differential contribution of adenosine receptor subtypes to our transgenic phenotype would require the availability of selective antagonists or their genetic ablation in an hCD39-overexpressing background.

Thus, we propose a model in which CD39 upregulation in chronic lung epithelia improves host immunity by locally removing desensitizing excess extracellular ATP and promoting P2X1 and P2X7 receptor-mediated immune cell recruitment. See text for details. Gray arrows, not demonstrated in our study.

**FIGURE 8.** Chronic CD39 overexpression in lung epithelia improves host immunity by locally removing desensitizing excess extracellular ATP and promoting P2X1 and P2X7 receptor-mediated immune cell recruitment. See text for details. Gray arrows, not demonstrated in our study.


