Multiple-Site Activation of the Cysteiny1 Leukotriene Receptor 2 Is Required for Exacerbation of Ischemia/Reperfusion Injury

Nathan C. Ni, Laurel L. Ballantyne, Jeffrey D. Mewburn, Colin D. Funk

Objective—Transgenic overexpression of the human cysteiny1 leukotriene receptor 2 (CysLT, R) in murine endothelium exacerbates vascular permeability and ischemia/reperfusion injury. Here, we explore the underlying mechanisms of CysLT, R activation-mediated inflammation and delineate the relative contributions of endogenous murine CysLT, R and the transgene-derived receptor.

Approach and Results—We created a novel mouse with only endothelial-expressed CysLT, R (endothelium-targeted overexpression mice [EC]/CysLT,R-knockout mice [KO]) by crossing EC with KO to dissect the role of endothelial CysLT, R in tissue injury. Surprisingly, we discovered that damage in EC/KO mice was not elevated (24% versus 47% EC) after ischemia/reperfusion. We examined vascular permeability and leukocyte recruitment/rolling responses in the cremaster vasculature after cysteiny1 leukotriene (cysLT) stimulation. Mice possessing transgenic endothelial CysLT, R overexpression, whether EC or EC/KO, when stimulated with cysLTs, exhibited vascular hyperpermeability, declining leukocyte flux, and a transient increase in slow-rolling leukocyte fraction. Mice lacking endogenous CysLT, R (both KO [20±3 cells/min] EC/KO [24±3]) showed lower-rolling leukocyte flux versus wild-type (38±6) and EC (35±6) mice under unstimulated conditions. EC/KO mice differed from EC counterparts in that vascular hyperpermeability was not present in the absence of exogenous cysLTs.

Conclusions—These results indicate that endothelial and nonendothelial CysLT,R niches have separate roles in mediating inflammatory responses. Endothelial receptor activation results in increased vascular permeability and leukocyte slow-rolling, facilitating leukocyte transmigration. Nonendothelial receptors, likely located on resident/circulating leukocytes, facilitate endothelial receptor activation and leukocyte transit. Activation of both receptor populations is required for injury exacerbation. (Arterioscler Thromb Vasc Biol. 2014;34:321-330.)

Key Words: permeability ■ cysteiny1 leukotriene receptor 2 ■ inflammation ■ leukocytes ■ myocardial infarction

Myocardial infarction is one of the leading killers in the Western world today, and ever increasing obesity rates in the industrialized world make cardiovascular disease a growing threat to public health.1 Myocardial infarction is characterized by a blockage of the coronary circulation, usually caused by atherosclerotic plaque rupture, leading to cessation of oxygen and nutrient delivery to the myocardium. Timely restoration of flow to the affected area, reperfusion, is paramount. However, this treatment method is also a double-edged sword; although necessary to salvage cardiac function, it also evokes reperfusion damage, a combination of oxidative stress and inflammation that results in additional myocardial injury.2

Leukotrienes are potent proinflammatory lipid mediators synthesized from arachidonic acid via the actions of 5-lipoxygenase and 5-lipoxygenase–activating protein. Cysteiny1 leukotrienes (cysLTs) are a subfamily of leukotriene molecules, thus termed because of a common cysteine moiety in their respective structures. The cysLTs, leukotriene C4, D4, and E4 (LTC4, D4, and E4, respectively), exert their effects by binding to sequenced G-protein–coupled receptors: cysteiny1 leukotriene receptor 1 (CysLT1R), cysteiny1 leukotriene receptor 2 (CysLT2R), and a novel CysLT, receptor (GPR99).3 CysLT,R binds LTD4 preferentially to LTC4, whereas CysLT,R binds LTD4 and LTC4 with equal affinity. Both receptors bind LTE4, the most stable and abundant cysLT, with weak affinity.4 In contrast, GPR99 displays preferential binding affinity for LTE4 with weak affinity for LTC4 and LTD4.5

The involvement of CysLT,R in airway inflammation has been studied extensively. Indeed, CysLT,R pharmacological antagonists are popular antiasthma treatment options.

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### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>BayCysLT&lt;sub&gt;i&lt;/sub&gt;</td>
<td>3-[[3-(carboxycyclohexyl)amino][carbonyl]-4-[3-[(4-phenoxybutoxy)phenyl][propoxy]-benzoic acid</td>
</tr>
<tr>
<td>Bay-u9773</td>
<td>4-[[1R,2E,4E,6Z,9Z]-1-[(1S)-4-carboxy-1-hydroxybutyl]-2,4,6,9-pentadecatetraenyl][thio]-benzoic acid</td>
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<tr>
<td>CysLT&lt;sub&gt;T&lt;/sub&gt;</td>
<td>cysteinyl leukotriene</td>
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<tr>
<td>CysLT&lt;sub&gt;T,R&lt;/sub&gt;</td>
<td>cysteinyl leukotriene E receptor</td>
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<td>CysLT&lt;sub&gt;T,R&lt;/sub&gt;</td>
<td>cysteinyl leukotriene receptor 1</td>
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<td>CysLT&lt;sub&gt;T,R&lt;/sub&gt;</td>
<td>cysteinyl leukotriene receptor 2</td>
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<tr>
<td>EC</td>
<td>hEC-CysLT&lt;sub&gt;R&lt;/sub&gt; mouse model</td>
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<tr>
<td>EC/KO</td>
<td>hEC-CysLT&lt;sub&gt;R&lt;/sub&gt; x CysLT&lt;sub&gt;R&lt;/sub&gt;-knockout mouse model</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GPR99</td>
<td>G-protein–coupled receptor 99</td>
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<tr>
<td>HAMI3379</td>
<td>3-[[3-(carboxycyclohexyl)amino][carbonyl]-4-[3-[(4-cyclohexyloxy)butoxy]phenyl][propoxy]-benzoic acid</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>KO</td>
<td>cysteinyl leukotriene receptor 2-knockout mouse model</td>
</tr>
<tr>
<td>LAD</td>
<td>left anterior descending coronary artery</td>
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<tr>
<td>LT</td>
<td>leukotriene</td>
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<td>RFU</td>
<td>relative fluorescent units</td>
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<tr>
<td>TEM</td>
<td>Tie2-expressing monocytes</td>
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<tr>
<td>WT</td>
<td>wild-type mouse model</td>
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CysLT<sub>T,R</sub> in humans, is expressed in umbilical vein endothelial cells, macrophages, platelets, the cardiac Purkinje system, and coronary endothelial cells. CysLT<sub>T,R</sub> research had been previously hampered by a lack of selective pharmacological agents, the majority of work instead using the nonselective cysLT antagonist/partial agonist Bay-u9773 or genetic models of CysLT<sub>R</sub> expression modulation. However, recent studies have characterized 2 novel CysLT<sub>R</sub>-selective antagonists: HAMI3379 and BayCysLT2, which are proving to be useful as tools in eicosanoid research.

There is evidence linking cysLTs to cardiovascular disease and inflammation. Expression of components of the leukotriene synthesis pathway is found in human atherosclerotic lesions. Furthermore, genetic polymorphisms in 5-lipoxygenase pathway genes have been correlated to increased myocardial infarction and stroke risk in some populations but not all. CysLT<sub>R</sub> can mediate vascular permeability, susceptibility to gastrointestinal tract inflammation, and increased vulnerability to ischemic injury in the heart and brain. CysLT<sub>R</sub> expression is increased after hypoxic/ischemic stress. Previous work from our laboratory has shown that CysLT<sub>R</sub>-selective antagonism can abrogate myocardial ischemia/reperfusion injury in transgenic mice overexpressing the human CysLT<sub>R</sub> receptor in vascular endothelium (hEC-CysLT<sub>R</sub>). However, we were unable to delineate the relative roles of the transgenic endothelial CysLT<sub>R</sub> versus endogenous murine CysLT<sub>R</sub> present on various cell types.

To study this, we have created a novel mouse model by crossing CysLT<sub>R</sub>-knockout mice (KO) with hEC-CysLT<sub>R</sub> mice (EC), resulting in a model with selective endothelial CysLT<sub>R</sub> expression. Using these novel mice, we examine how CysLT<sub>R</sub> mediates postischemic myocardial injury and vascular inflammatory responses.

### Materials and Methods

A summary of the murine genotypes used in this study can be found in the Table. Detailed Materials and Methods are available in the online-only Supplement.

### Results

**Transgenic CysLT<sub>R</sub> Is Found in Both Tissues and Circulating Cells**

*Pie*2 is known to be expressed in certain circulating leukocytes in addition to endothelial cells and, thus, we compared transgenic CYSLTR2 mRNA expression levels in tissues and circulating cells from mice possessing the CYSLTR2 transgene. Comparable amounts of CYSLTR2 expression was noted between EC and EC/KO mice in lung tissues. Less CYSLTR2 expression was found in peripheral blood leukocytes and bone marrow cells in both EC and EC/KO mice relative to lung expression. We also confirmed the absence of transgene expression in WT mice, as well as the absence of endogenous murine Cysltr2 expression in EC/KO mice (Figure I in the online-only Data Supplement). These results indicate that *Pie*2-expressing nonendothelial cells do not comprise the bulk of transgenic CYSLTR2 present in our overexpression mouse models. In addition, there is no difference in transgenic CYSLTR2 expression between EC and EC/KO mice. This indicates that any phenotypic differences noted between EC and EC/KO mice are likely the result of removal of the endogenous murine CysLT<sub>R</sub>.

**Endothelium-Targeted CysLT<sub>R</sub> Overexpression Does Not Exacerbate Infarction Damage in the Absence of Endogenous CysLT<sub>R</sub>**

Our previous findings have established that transgenic CysLT<sub>R</sub> overexpression in the endothelium, in the presence of endogenous murine CysLT<sub>R</sub> expression, results in significantly increased infarction volume after myocardial ischemia/reperfusion in mice. However, transgenic mice without endogenous CysLT<sub>R</sub> expression (EC/KO) did not show this phenomenon (Figure 1). Indeed, both WT (23.2±2.2%, n=13) and EC/KO (24.0±3.0%, n=14) groups showed significantly lower infarction volumes than EC mice (47.2±1.9%, n=12; P<0.001 against both groups). Volume at risk did not vary between any of the experimental groups (Figure 1B). We also found that CysLT<sub>R</sub>-knockout mice (KO; 17.8±2.2%, n=12) did not show a difference in infarction volume relative to WT mice.

### Table. CysLT<sub>R</sub> Expression Profile in Featured Mouse Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Endothelial Expression</th>
<th>Nonendothelial Expression</th>
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<tbody>
<tr>
<td>WT</td>
<td>mCysLT&lt;sub&gt;R&lt;/sub&gt;++</td>
<td>mCysLT&lt;sub&gt;R&lt;/sub&gt;++</td>
</tr>
<tr>
<td>EC</td>
<td>mCysLT&lt;sub&gt;R&lt;/sub&gt;+</td>
<td>mCysLT&lt;sub&gt;R&lt;/sub&gt;+</td>
</tr>
<tr>
<td>KO</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>EC/KO</td>
<td>hCysLT&lt;sub&gt;R&lt;/sub&gt;+++</td>
<td>None</td>
</tr>
</tbody>
</table>

Expression levels (denoted by + signs) of endogenous murine (mCysLT<sub>R</sub> (cysteinyl leukotriene receptor 2)) and transgenic (hCysLT<sub>R</sub>) human CysLT<sub>R</sub> in endothelial and nonendothelial cells in various murine genotypes. EC indicates endothelium-targeted CysLT<sub>R</sub> overexpressing mouse model; KO, CysLT<sub>R</sub>-knockout mouse model; and WT, wild-type mouse model.
had a tendency of lower infarction volumes than WT and EC/KO groups (Figure 1C; \( P=0.096 \) versus WT, \( P=0.11 \) versus EC/KO). These findings were unexpected because endogenous CysLT2R expression is significantly less than transgene expression.21 These results indicate that CysLT2R-mediated exacerbation of myocardial ischemia/reperfusion injury is not solely mediated by endothelium-targeted CysLT2R.

Vascular Permeability Responses in EC/KO Mice Differ From Those in EC Mice

Given that CysLT2R-mediated myocardial injury seems not to be exclusively mediated by the endothelium-overexpressed CysLT2R transgene, we sought to investigate how and to what extent knockout of endogenous CysLT2R would affect endothelial/vascular permeability. This is especially pertinent because based on previous work11,14 CysLT2R-mediated endothelial permeability facilitates tissue injury by permitting leukocyte extravasation, inflammation, and edema. We examined FITC-albumin extravasation from postcapillary venules in the cremaster muscle both before and after exogenous leukotriene stimulation. Minimal extravascular fluorescence accumulation before leukotriene stimulation was observed in WT, KO, and EC/KO mice. However, EC mice showed significantly higher absolute fluorescence accumulation (Figure 2B), and rate of fluorescence accumulation (Figure 2C) as previously determined, which we surmised, was because of surgery-induced cysLT release from resident tissue macrophages/mast cells activating high numbers of endothelial cell-expressed CysLT2R.12

Administration of cysLTs resulted in an increase in absolute FITC-albumin extravasation in all experimental groups (Figure 3A). EC and EC/KO groups demonstrated increased total leakage and rate of leakage versus the WT group post-cysLTs. However, cysLT-challenged KO mice showed significantly less FITC-albumin extravasation compared with WT mice (Figure 3B and 3C). Interestingly, EC/KO mice, despite possessing identical transgenic overexpression of CysLT2R in the endothelium as EC mice, only showed elevated extravascular FITC-albumin accumulation versus WT mice after cysLT stimulation. This is in contrast to the increased FITC-albumin extravasation observed in EC mice compared with WT mice, both before and after cysLT stimulation.

Genetic Knockout of Endogenous CysLT2R Reduces Basal Rolling Leukocyte Flux in Cremaster Vasculature

The endothelium plays a large role in regulating leukocyte capture and transmigration during inflammatory responses,26 and we have previously reported increased leukocyte adhesion molecule mRNA expression in EC mice after ischemia/reperfusion injury.11,14 To examine whether CysLT2R expression modulation affects leukocyte recruitment and behavior, we examined leukocyte flux under basal and cysLT-stimulated conditions in the cremaster vasculature. Rolling leukocyte flux (the number of rolling leukocytes passing through the vasculature/min) was higher in mice expressing endogenous CysLT2R (WT: 38±6 cells/min, \( n=12 \); EC: 35±6, \( n=16 \)) than mice lacking endogenous receptor (KO: 20±3, \( n=16 \); EC/KO: 24±3, \( n=14 \)) and mice under basal conditions (Figure 4). Mice that possessed transgenic endothelium-targeted CysLT2R overexpression (EC and EC/KO) did not show significantly altered basal rolling leukocyte counts versus nontransgenic counterparts (WT and KO). Complete blood counts for all genotypes were undertaken, and no significant differences in baseline leukocyte numbers or differential counts were detected (data not shown).

Endothelial-Targeted CysLT2R Overexpression Results in Diminishing Leukocyte Flux in Cremaster Vasculature After Leukotriene Stimulation

Next, we investigated the effects of cysLT stimulation on rolling leukocyte flux in murine cremaster postcapillary venules. WT mice (\( n=6 \)) showed an increase in rolling leukocyte flux after 5 minutes post-cysLT stimulation (+13±5 cells/min; \( P=0.07 \) versus start), and this was maintained until the end of the experiment at 15 minutes (+12±5; \( P=0.07 \) versus start). CysLT-stimulated cremaster preparations of KO mice (\( n=6 \)) did not show significant changes in rolling leukocytes throughout the duration of the experiment. However, cysLT stimulation of the cremaster vasculature in mice possessing transgenic endothelial CysLT2R overexpression resulted in a decrease in rolling leukocytes. Rolling leukocyte count per minute dropped in EC mice (\( n=9 \)) by 5 minutes (−8±5) and had significantly decreased by the 15-minute time point (−13±6; \( P=0.04 \) versus start). Rolling leukocyte flux in EC/KO mice (\( n=7 \)) was not changed at 5 minutes (−2±2) but showed a significant decline by 15 minutes (−10±3; \( P=0.002 \) versus start). Changes in rolling leukocyte flux in EC and EC/KO mice were statistically
significant versus WT mice (Figure 5). These results indicate that endothelial CysLT$_2$R overexpression significantly alters rolling leukocyte flux counts postleukotriene stimulation.

Cysteinyl Leukotriene Stimulation Results in Increased Leukocyte Slow-Rolling Fraction in Cremaster Vasculature in Mice Expressing Endothelial CysLT$_2$R

Before firm adhesion and transmigration, leukocytes undergo slow rolling, a phenomenon characterized by leukocyte velocity decreasing to <10 μm/s. Slow rolling is mediated by E-selectin/CD18 binding and is triggered by proinflammatory cytokines.$^{27,28}$ We examined rolling leukocyte velocity in cremaster venules in the absence and presence of cysteinyl leukotriene stimulation. Rolling leukocyte velocity distribution in the unstimulated cremaster muscle did not differ significantly among the 4 genotypes. However, WT (n=6 mice, 58% slow rolling leukocytes → 79%), EC (n=8 mice, 65% → 78%), and EC/KO (n=7 mice, 59% → 70%) groups all presented a significant shift in rolling leukocyte velocity distribution toward increased slow-rolling (sub 10 μm/s) at 5 minutes postcysLT stimulation (Figure 6). KO mice (n=6 mice, 61 → 64%) did not display this trend. The presence of this phenomenon in EC/KO mice indicates that it is the endothelial CysLT$_2$R niche that drives promotion of slow-rolling leukocytes. Interestingly, although WT mice showed a significantly increased proportion of slow-rolling leukocytes through all 3 poststimulation time points, both EC and EC/KO mice saw slow-rolling cell fraction return to prestimulation levels by 10 minutes. Median rolling leukocyte velocity in the unstimulated cremaster muscle did not differ significantly between the 4 genotypes. However, median rolling leukocyte velocity in WT, EC, and EC/KO mice all decreased significantly at 5 minutes postleukotriene stimulation. This phenomenon persisted in WT and EC mice at 10 and 15 minutes (albeit nonsignificantly in the latter group) but was transient in EC/KO mice. In addition, median velocity in EC mice was significantly lower than EC/KO mice at 10 minutes poststimulation.

**Figure 2.** Vascular permeability postsurgical intervention (basal) is elevated in EC mice, but not in endothelium-targeted human cysteinyl leukotriene receptor 2 (CysLT$_2$R) overexpressing CysLT$_2$R-knockout (EC/KO) mice. A, Intravital microscopy recordings of vascular leakage in cremaster postcapillary venules. Representative images of cremaster preparations from mice possessing (from left to right) normal (wild type [WT]), endothelium-elevated (EC), no (KO), or endothelium-only (EC/KO) CysLT$_2$R expression are depicted. Top shows fluorescence recordings at the start of the experiment (t=0); bottom shows fluorescence recordings 1 minute later. Scale bar, 50 μm. B, Absolute change and (C) rate of change in extravascular fluorescence were elevated in EC mice. *P<0.05; values given as mean±SEM, n=6 to 7.
This indicates that nonendothelial CysLT$_2$R activation may also play a role in mediating/sustaining leukocyte slow-rolling.

**Discussion**

**Endothelial CysLT$_2$R Is Not the Sole Mediator of CysLT$_2$R-Mediated Ischemia/Reperfusion Injury Exacerbation**

Endothelial expression of functionally relevant CysLT$_2$Rs has been well established for almost a decade now, and endothelial-targeted overexpression of CysLT$_2$R results in exacerbation of vascular permeability, edema, upregulation of cell adhesion molecules, and myocardial infarction damage. The ability of CysLT$_2$R pharmacological antagonism to abolish these phenomena lent support to the idea that endothelial-expressed CysLT$_2$R was the principal mediator of these effects. Given the disparity in levels between endogenous murine CysLT$_2$R expression and the overexpressed endothelial-targeted hCysLT$_2$R transgene, it was also logical to think that these biological responses were endothelium driven.

However, our experiments using a novel endothelium-limited CysLT$_2$R-overexpressing mouse model indicate that CysLT$_2$R-mediated exacerbation of inflammation is only partially driven by endothelial cell–expressed receptors, and that other CysLT$_2$R receptor niches may play a vital role in initiating and mediating the inflammatory response. Despite possessing the same overexpression of endothelial hCysLT$_2$R as the EC model, EC/KO mice have significantly less severe myocardial damage after ischemia/reperfusion. Because transgenic CysLT$_2$R account for the majority of endothelial CysLT$_2$R in EC mice, it was not expected that removal of the murine endothelial receptor would affect CysLT$_2$R-mediated mechanisms involving the endothelium, indicating that the endothelium is not the sole mediator of increased I/R injury in EC mice.
We also note for the first time that KO mice show a tendency for reduced I/R damage versus WT counterparts. The reason that the difference between damage in KO and WT mice is relatively small compared with that between WT and EC mice is that basal CysLT2R expression in murine cardiac vasculature is low. However, basal human CysLT2R expression is higher, and leukotriene production and leukotriene receptor expression are elevated in pathological states, indicating that our EC model more closely represents human CysLT2R expression levels. Indeed, our experimental data (Figure II in the online-only Data Supplement) indicate that human CYSLTR2 expression is higher than that found in our EC mice.

Explaining our results required closer examination of the various proinflammatory mechanisms stemming from CysLT,R activation. We have shown that basal rolling leukocyte flux is significantly higher in mice that possess endogenous nonendothelial CysLT,R (WT and EC groups) compared with mice that do not (KO and EC/KO groups), regardless of presence or absence of transgenic endothelial CysLT,R. Although leukocyte recruitment to injured tissue is a key component of tissue repair, exacerbated leukocyte recruitment and improper resolution of the inflammatory response can result in additional injury. We have previously shown that transgenic overexpression of endothelial CysLT,R results in vascular hyperpermeability, and our EC/KO model was in agreement with this finding, showing significantly elevated extravascular FITC-albumin accumulation after cysLT stimulation. The extent and magnitude of the vascular permeability response in EC/KO mice was comparable with that seen in EC mice, indicating that nonendothelial CysLT,R does not play a significant role in CysLT,R-mediated vascular hyperpermeability.

The presence of rolling leukocytes does not automatically translate into leukocyte transmigration. Indeed, the presence of a patrolling vascular leukocyte reservoir consisting primarily of rolling or crawling monocytes that do not extravasate until after exposure to irritants and injury has been characterized in the mesenteric and coronary vasculature. However, we saw a significant decrease in rolling leukocyte flux as well as a transient but significant increase in slow-rolling leukocyte proportion in EC and EC/KO mice after cysLT stimulation. These findings, in combination with the aforementioned cysLT-induced hyperpermeability response in EC and EC/KO mice, support a mechanism whereby CysLT,R activation results in increased rolling leukocyte flux, decreased rolling leukocyte velocity, and vascular hyperpermeability, resulting in the elevated edema and leukocyte extravasation into the injured myocardium, which have been shown to be hallmarks of CysLT,R-mediated exacerbation of I/R injury.

However, although vessels in EC mice showed leakage in the absence of exogenous cysLT stimulation, vessels in EC/KO mice did not. Moos et al attributed the phenomenon in EC/KO mice to small amounts of leukotrienes liberated during the
surgical exposure of the cremaster muscle taking advantage of an increased abundance of endothelial CysLT$_2$R. If this is the case, why do these liberated leukotrienes not exert a similar effect on vessels in EC/KO mice? The answer may be that leukotrienes released during tissue injury do not directly activate endothelial CysLT$_2$R. Rather, trauma results in localized leukotriene production/release and the subsequent activation of nonendothelial CysLT$_2$Rs. This in turn results in cysLT production in close proximity to endothelial cells, a vital feature given the short half-lives of LTC$_4$ and LTD$_4$, resulting in the activation of endothelial CysLT$_2$R and autocrine cysLT production mechanisms. Sufficient leukotriene presence (ie, exogenous application) would be able to bypass the nonendothelial step and directly activate endothelial receptors, explaining how EC/KO mice can still present hyperpermeability after exogenous stimulation. EC/KO mice did not show any significant change in leukocyte rolling velocity distribution. **P<0.01; ***P<0.001 vs untreated.

A limitation of the study is using the cremaster vasculature to extrapolate findings to the coronary circulation, given the known physiological and anatomic heterogeneity among microvascular beds. A novel and nonobtrusive method of real-time cardiac intravital microscopy imaging has just been realized, affording an opportunity to examine the similarities and differences in response to cysLT stimulation directly when this technique becomes more widely available. Furthermore, although the Tie2 promoter, used in our transgenic mice, specifies endothelial-targeted gene expression, it may also lead to expression in a small subset of monocytes, termed Tie2-expressing monocytes.

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Figure 6. Rolling leukocyte velocity distributions in mice before and after cysteinyl leukotriene (cysLT) stimulation. Histograms of rolling leukocyte velocities in murine cremaster vasculature before and after application of 5 μmol/L cysLTs. Velocities of individual leukocytes from 6 to 9 mice (roughly equal numbers of leukocytes were tracked in each mouse) were calculated, and the results were compiled (n values indicate total number of cells tracked). WT (A–D) mice showed persistent and significant increase in slow-rolling (≤10 μm/s) leukocyte fraction after cysLT application. EC (E–H) and EC/KO (M–P) mice showed a significant increase in slow-rolling leukocyte fraction at 5 minutes that disappeared by the 10-minute mark. KO (I–L) mice did not show any significant change in leukocyte rolling velocity distribution. **P<0.01; ***P<0.001 vs untreated.
These monocytes are highly angiogenic, vital to tumor neoangiogenesis, and are mobilized after ischemia for the revascularization of ischemic tissue. Indeed, the majority of Tie2+ monocytes in mice are resident cluster of differentiation (CD)11b+Gr-1^low/neg monocytes, thought to be instrumental to inflammatory response resolution, therefore, it will be interesting to investigate the role of CysLT2R activation/overexpression in this cell population in future studies.

We certainly cannot rule out the possibility that the activation of CysLT2R on Tie2+ myeloid cells is involved in the mechanisms explored herein. However, our data indicate that these cells do not play a significant role because overexpression of CysLT2R on Tie2+ myeloid cells is common to both EC and EC/KO mice, and thus would not explain the differences presented between the 2 genotypes.

Various Tissue-Specific CysLT2R Niches Play Divergent yet Synergistic Roles in Mediating CysLT2R-Mediated Ischemia/Reperfusion Injury Exacerbation

We previously proposed a mechanism whereby CysLT2R activation after ischemia/reperfusion injury resulted in elevated leukocyte extravasation and increased inflammation, resulting in increased tissue injury. Our present findings indicate that leukotriene release after injury/ischemia activates CysLT2R niches, likely circulating leukocytes, and that this facilitates leukocyte recruitment to the site of injury and activation of endothelial CysLT2R (possibly via localized cysLT production). Endothelial CysLT2R activation results in vascular hyperpermeability, resulting in edema, and an increase in the proportion of slow-rolling leukocytes, promoting...
leukocyte extravasation. Activation of both endothelial and nonendothelial CysLT,Rs likely yields a synergistic effect. Insufficient endothelial CysLT,R activation would result in higher numbers of rolling leukocytes that do not extravasate en masse (seen in WT mice), whereas absence of nonendothelial activation would result in limited-rolling leukocyte flux (seen in EC/KO mice), and thus decreased leukocyte migration to the surrounding tissue, regardless of vascular hyperpermeability (Figure 8).

In conclusion, we have used a novel endothelium-limited CysLT,R-overexpressing mouse model to delineate the differing roles that endothelial and nonendothelial CysLT,R niches play in mediating postinjury inflammatory responses. We show that nonendothelial CysLT,R niches are vital to leukocyte recruitment and endothelial CysLT,R activation, whereas endothelial CysLT,R activation mediates extent of vascular permeability. Although further work is required to clarify the precise molecular mechanisms underlying these phenotypes (ie, cytokine release profile or induction of angiogenesis), as well as any involvement by other cell types (ie, adipose tissue) in close proximity to the vasculature, the revelation that endothelial CysLT,R activation alone does not govern CysLT,R-mediated proinflammatory responses indicates that more attention should be devoted to the role of nonendothelial CysLT,R expression in cardiovascular injury.

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Disclosures

None.

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Significance

This study, to our knowledge, is the first to explore the contributions of human cysteinyl leukotriene receptor 2 (CysLT2R) expression niches to inflammatory response modulation in vivo. Leukotrienes have been linked to cardiorespiratory disease for decades, but the major focus of the work has focused on asthma. Recent work has uncovered that CysLT2R activation results in exacerbation of postischemia/reperfusion injury, but the underlying mechanisms remain unclear. We present here evidence that CysLT2R-mediated exacerbation of ischemia/reperfusion injury is not solely driven by endothelial receptor activation. Instead, activation of nonendothelial CysLT2Rs, most likely located in circulating leukocytes, facilitates the activation of endothelial receptor niches, which in turn results in vascular hyperpermeability and leukocyte extravasation. These findings aid in our understanding of leukotriene-mediated inflammatory responses and also provide a potential therapeutic target for regulating not only postmyocardial infarction inflammation but also other leukotriene-mediated inflammatory mechanisms.
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Materials and Methods

Mouse Models

All mice used in this study have a C57Bl/6 genetic background. hEC-CysLT2R (EC) transgenic mice were previously described 1. Briefly, these mice possess 7 copies of the human CYSLTR2 coding region placed under the control of the Tie2 promoter/enhancer for endothelial-targeted CysLT2R overexpression. LacZ-CysLT2R knockout (KO) mice contain a LacZ cassette under control of the Cysltr2 promoter with Cysltr2 disruption. X-Gal staining can be used as a reporter for native sites of CysLT2R expression 2. These two strains were crossed to generate a whole-body (including endogenous endothelial expression) CysLT2R knockout mouse that possesses endothelium-targeted overexpression of the human CysLT2R. This mouse is referred to as EC/KO. Details of the endogenous and transgenic CysLT2R expression profile of each genotype utilized can be found in Table 1.

Isolation of Bone Marrow Cells and Peripheral Blood Leukocytes

Bone marrow cells were obtained by flushing murine femurs and tibiae with phosphate buffered saline (PBS). Effluent was collected and centrifuged, and the pellet was used for RNA extraction. Peripheral blood leukocytes were obtained by collecting blood from mice via cardiac puncture using syringes containing 0.1 ml heparin (Sandoz Canada Inc, Boucherville, QC, Canada), and then subjecting heparinized blood to Ficoll-Paque (GE Healthcare, Mississauga, ON, Canada) gradient centrifugation. Leukocytes were thus collected, washed with PBS, and pelleted for RNA extraction.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from murine and human tissues were obtained using guanidinium thiocyanate-phenol-chloroform extraction 3. In brief, cells or tissue were manually homogenized while immersed in TRI reagent (Sigma-Aldrich, St. Louis, MO). Chloroform (Thermo Fisher Scientific, Ottawa, ON, Canada) was then added, and the suspension was separated into three phases via centrifugation (10 min, 13,000g, 4°C). The clear upper aqueous layer was isolated, and RNA was precipitated using isopropranol, pelleted, and resuspended in diethyl pyrocarbonate-treated double-distilled H2O (Invitrogen, Carlsbad, CA). RNA quality and quantity were assessed using an Aglient Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) and Nanodrop N-1000 spectrophotometer (Nanodrop, Wilmington, DE), respectively. Total RNA was reverse-transcribed to cDNA using the iScript kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Quantitative real-time PCR was performed using a thermal cycler (Applied Biosystems model 7500) with SYBR Green PCR master mix (Bio-Rad Laboratories, Mississauga, ON, Canada). Gapdh or GAPDH was used as a control housekeeping gene. Human CYSLTR2 (forward: 5’ TGA AAC CAT CCA TCT CCG TAT 3’; reverse: 5’ TGT GCA GTT CCT GCT GTT GTT AT 3’), murine Cysltr2 (forward: 5’ CAT GTG TAT GCC TGA TGT CTA CCA 3’; reverse: 5’ CAT TTG CCG TAC CCA GTC TCA 3’), and murine Tie2 primers (forward: 5’ CGG ACT GAC TAC GAG CTG TG 3’; reverse: 5’ CTT GGA GGA GGG AGT CCG AT 3’) were acquired from Eurofins MWG Operon (Huntsville, AL). Data were calculated using the 2^{-∆∆CT} method and are presented as fold-induction of transcripts for target genes normalized to Gapdh (forward: 5’ CAT GGC CTT CCG TGT TCC TA 3’; reverse: 5’ ATG CCT GCT TCA CCA 3’).
CCT TCT 3') or GAPDH (forward: 5' CAC CAT CTT CCA GGA GCG AG 3'; reverse: 5' AAA TGA GCC CCA GCC TTC TC 3'), with respect to controls.

**Myocardial Ischemia/Reperfusion**

Myocardial infarction was induced via non-permanent left anterior descending coronary arterial ligation, as previously outlined 4. Briefly, analgesia was administered (20 mg/kg tramadol (Ultram; Chiron AS, Trondheim, Norway)) at least 1 h prior to surgery in mice 14-18 weeks of age. Mice were then anesthetized with 5% isoflurane, intubated, and constantly ventilated (150 breaths/min) with 1 to 5% isoflurane throughout the procedure. An incision was made at the fourth intercostal space, with 50 μl of 50% lidocaine/50% bupivacaine injected subcutaneously along the incision line as an analgesic. The intercostal muscles were cut in order to expose the heart. The pericardium was pulled apart, and 6-0 silk suture (Ethicon, Somerville, NJ) passed underneath the LAD and surrounding myocardium. Ischemia was induced for 30 minutes by tightening the suture against a piece of PE-10 tubing placed on top of the LAD and confirmed by visible paling of the affected myocardium and/or visibly altered ventricular contraction rhythm. Removal of the tubing and loosening of the ligature allowed reperfusion. The surgical site was closed and mice were extubated as soon as they exhibited signs of consciousness, followed by subcutaneous administration of 0.5 to 1.0 ml of warm lactated Ringer's solution (Baxter, Mississauga, ON), and returned to their cages once fully mobile. The entire procedure was performed on a heated pad. All surgical procedures and treatment regimens were approved by the University Animal Care Committee at Queen's University and adhered to the guidelines of the Canadian Council of Animal Care.

**Analysis of Infarct Volume**

Infarct volume was analyzed 48 hours post surgery as previously described 4. The heart was excised and retrogradely perfused via the aorta with phthalocyanine blue ink dye (Liquitex, Cincinnati, OH) following suture re-tightening in order to demarcate the non-risk region. The heart was then rinsed in ice-cold PBS, blotted dry, frozen in plastic wrap at -20°C for 15 minutes, and cut transversely into six 1.0 mm sections. Sections were immersed in 2,3,5-triphenyltetrazolium chloride (1%; Sigma-Aldrich) for 15 min at 37°C to demarcate viable and necrotic tissue. Stained sections were photographed on both sides using a digital camera (Q-Color5; Olympus, Tokyo, Japan). The infarct area (pale white), the area at risk (brick red), the non-risk area (blue), and the total left ventricular area were calculated for both sides of each section using ImageJ software (National Institutes of Health, Bethesda, MD). As described previously 3,4, infarct size was calculated as the ratio of the infarct volume to the volume at risk. Animals with risk volume in the 35 to 70% range of total left ventricle volume were used as inclusion criteria in the study.

**Intravital Microscopy of the Cremaster Vasculature**

Vascular permeability and leukocyte recruitment were examined using intravital confocal microscopy. Male mice 12-16 weeks of age were anaesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg). A catheter was placed in the right jugular vein for experiments that required intravenous FITC-albumin injection. The cremaster muscle was then exposed as described previously 5. Post-capillary venules (20-50 μm) were visualized using a Quorum WaveFX-X1 spinning disk confocal system (Quorum...
Technologies Inc., Guelph, ON, Canada). Recordings were taken with a Hamamatsu EM-CCD camera (model 09100-13; Hamamatsu Photonics, Hamamatsu, Japan) using MetaMorph software (Molecular Devices, Sunnyvale, CA).

In order to assess vascular permeability, fluorescein isothiocyanate (FITC)-labeled albumin (25 mg/kg body weight) was injected via the jugular catheter, and fluorescence intensity in the preparation was recorded for 1 minute. At this point, cysLTs (5 μmol/L each LTC4 and LTD4) were superfused onto the visualized vessel – following which fluorescence readings were recorded for a further 14 minutes.

To evaluate leukocyte flux in the cremaster vasculature, mice were prepared as above without catheterization. Bright-field images were recorded for 2 min prior to cysLT administration, following which bright-field segments were recorded at 5, 10, and 15 minutes post-cysLTs for 2 min. No difference in vessel diameter was noted between experimental groups (data not shown).

**Analysis of Vascular Permeability**

Vascular permeability was assessed by measuring FITC-albumin extravasation from the vasculature into the surrounding tissue. Fluorescence intensity, ranging from 0-65536 arbitrary units as defined by the software auto scale function, was measured at five 1 mm² sites surrounding the post-capillary venule using MetaMorph software. The absolute fluorescence intensity was recorded every 5 frames, with measurement areas manually adjusted to account for field-of-view drift. The first derivative of the absolute fluorescence intensity was calculated to determine the fluorescence intensity rate of change (a measurement previously termed LIFT – leakage intensity factor for tissues).

**Analysis of Vascular Leukocytes**

Bright-field recordings of post-capillary venules (20-50 μm) in the cremaster muscle were taken pre/post-leukotriene stimulation. Two min sequences were recorded prior to leukotriene stimulation and at 5, 10, and 15 min following stimulation. Rolling leukocyte flux, defined as the number of rolling leukocytes/min in the observed vessel, was visually analyzed by three observers blinded to the study group using VirtualDub version 1.9.9. Leukocyte velocity of 15-30 randomly selected cells was measured by tracking individual leukocytes/mouse/time point using Image-Pro Plus (Media Cybernetics Inc., Rockville, MD). Median leukocyte velocity was used as a representative indicator of flow speed, as average velocity would be improperly skewed by loosely adherent fast rolling leukocytes. Rolling leukocyte speed distribution was analyzed and presented using histograms.

**Complete Blood Count**

Blood was extracted from mice using a heparinized 25G needle via cardiac puncture immediately following CO2-asphyxiation and was stored in 1.5 ml eppendorf tubes. Samples were kept on ice until analysis using a scil Vet abc machine (scil Vet Novations; Barrie, ON, Canada).

**Statistical Analysis**
For each experimental group, the mean and standard error were calculated. To compare groups at a single timepoint, we performed a two-tailed unpaired Student’s t-test. To compare timepoints within a single group, we performed a two-tailed paired Student’s t-test. To compare groups over multiple time-points, we performed one way analysis of variance with post hoc Newman-Keuls t-tests. Comparison of rolling leukocyte velocity between groups was done using a nonparametric Kruskal-Wallis one-way ANOVA to determine significant differences, followed by Dunn's test for multiple comparisons. All statistical analysis was carried out using Prism 5.0 (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered a statistically significant difference.
**Tables**

**Table 1: CysLT$_2$R expression profile in featured mouse genotypes.** Expression levels (denoted by + signs) of endogenous murine and transgenic human CysLT$_2$R in endothelial and non-endothelial cells in various murine genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Endothelial Expression</th>
<th>Non-Endothelial Expression</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>mCysLT$_2$R +</td>
<td>mCysLT$_2$R +</td>
</tr>
<tr>
<td>EC</td>
<td>mCysLT$_2$R +</td>
<td>mCysLT$_2$R +</td>
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<td></td>
<td>hCysLT$_2$R +++</td>
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<tr>
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<td>None</td>
<td>None</td>
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<tr>
<td>EC/KO</td>
<td>hCysLT$_2$R +++</td>
<td>None</td>
</tr>
</tbody>
</table>
References


7. Jung U, Ley K. Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. *Journal of immunology*. 1999;162:6755-6762
Supplementary Figure I: relative expression of (A) murine Cysltr2, (B) human CYSLTR2, and (C) murine Tie2 in lung tissue (containing vascular endothelium), bone marrow (BM) cells, and peripheral blood cells (PBL) in WT, EC, and EC/KO mice. No significant differences in Cysltr2 expression was noted between WT and EC mice, and minimal Cysltr2 was expressed in EC/KO mice (A). Likewise, no significant differences in CYSLTR2 expression was noted between EC and EC/KO mice, and the transgene was not found in WT mice (B). Transgenic CYSLTR2 expression in lung tissue was elevated relative to BM cells and PBLs in both EC and EC/KO mice. Murine Tie2 expression was markedly higher in lung tissue relative to BM and PBLs in both EC and EC/KO mice, indicating transgene targeting of predominantly endothelial cells over Tie2-expressing leukocytes (C). n = 3.
Supplementary Figure II: relative expression of CYSLTR2 in cardiovascular tissues from humans and transgenic endothelial cell-targeted CYSLTR2 overexpressing mice. Human CYSLTR2 expression in aortae and atriae from both humans (black) and EC mice (white) were examined via qPCR. Human samples showed significantly elevated CYSLTR2 gene expression relative to murine tissue counterparts. CYSLTR2 expression was normalized to either human GAPDH or murine Gapdh, corresponding to the sample origin. * = p < 0.05, ** = p < 0.01. n = 3-4.