# Increased CD11b+ Cells and Interleukin-1 (IL-1) Alpha Levels During Cardiomyopathy Induced by Chronic Adrenergic Activation

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**ABSTRACT:** **Background:** Systemic CD11b+ cells have been associated with several cardiac diseases, such as chronic heart failure.

**Objectives:** To assess the levels of circulating CD11b+ cells and pro-inflammatory cytokines in cardiomyopathy induced by chronic adrenergic stimulation.

**Methods:** Male Lewis rats were injected with low doses of isoproterenol (isoprel) for 3 months. Cardiac parameters were tested by echocardiography. The percentage of CD11b+ cells was tested by flow cytometry. The levels of inflammatory cytokines in the sera were determined by an inflammation array, and the expression levels of cardiac interleukin-1 (IL-1) receptors were analyzed by real-time polymerase chain reactions. Cardiac fibrosis and inflammation were determined by histological analysis.

**Results:** Chronic isoprel administration resulted in increased heart rate, cardiac hypertrophy, elevated cardiac peri-vascular fibrosis, reduced fractional shortening, and increased heart weight per body weight ratio compared to control animals. This clinical presentation was associated with accumulation of CD11b+ cells in the spleen with no concomitant cardiac inflammation. Cardiac dysfunction was also associated with elevated sera levels of IL-1 alpha and over expression of cardiac IL-1 receptor type 2.

**Conclusions:** CD11b+ systemic levels and IL-1 signaling are associated with cardiomyopathy induced by chronic adrenergic stimulation. Further studies are needed to define the role of systemic immunomodulation in this cardiomyopathy.

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**KEY WORDS:** isoprel, left ventricular (LV) dysfunction, CD11b+ cells, interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β)

Chronic sympathetic stimulation may induce cardiac pump dysfunction, mostly through reduced systolic function and left ventricular (LV) dilatation [1,2]. Different mechanisms have been suggested for adrenergic-induced cardiomyopathy, including myocardial necrosis [3], reductions in myocardial collagen cross-linking [4], disruption of calcium handling balance [5], and increased cardiomyocyte apoptosis [6]. These effects were shown to be reversible on withdrawal of the excessive adrenergic stimulus [7] and blockade of the renin-angiotensin system [8].

Bone marrow-derived CD11b+ cells were previously linked to cardiac function following myocardial infarction [9]. In chronic heart failure patients, CD11b+ cells were shown to be upregulated on peripheral monocytes and polymorphonuclear cells in association with heart failure symptoms [10]. In this study, elevated monocyte levels strongly correlated with a reduced event-free survival. Recent studies have found that CD11b+ cells were also linked to the development of arrhythmia [11]. CD11b integrin-mediated polymorphonuclear infiltration of the atria correlated with the formation of fibrosis, which promotes the initiation and propagation of atrial fibrillation.

In the present study, we sought to assess the levels of CD11b+ cells as well as inflammatory cytokines in the circulation of low-grade chronic isoproterenol-induced cardiomyopathy in a rat model. The data obtained may enable us to gain understanding regarding the potential involvement of paracrine effectors in cardiomyopathy induced by chronic adrenergic activation and to identify potential biomarkers.

## PATIENTS AND METHODS

### ETHICS STATEMENT

All experimental protocols carried out on the animals in this study were approved by the Tel Aviv Sourasky Medical Center institutional review board (Helsinki Committee), which constitutes the Institutional Animal Care and Use Committee (IACUC).

### IN VIVO MODEL

Twenty 3 month old male Lewis rats (10/arm) were treated five times per week with intraperitoneal (IP) injections of isoprel 0.1 mg/Kg (Isuprel® Abbott, Abbott Park, IL, USA) or with vehicle only (saline) for 12 weeks. Cardiac function and heart rates were then evaluated by transthoracic echocardiography (Acuson, Sequoia, Siemens, Washington, DC, USA) following anesthetics with ketamine (40 mg/kg) and xylazine (8 mg/kg) [12].

### EX VIVO ANALYSIS

Following termination, hearts and spleens were removed and sera withdrawn. Isolated LV tissues were fixed with 4% paraformaldehyde, sliced into transverse sections and paraffinyzed.

*The first and second authors contributed equally to this study

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The blocks were then sectioned into 5 µm slices, which were then stained with Masson's trichrome staining to assess the levels of interstitial fibrosis and inflammation. The slides were also stained with anti-rat CD68 to determine the occurrence of cardiac macrophages. Spleens were mechanically chopped followed by 5 minutes incubation with erythrocyte lysis buffer (Qiagen, Germany). Splenocytes were then centrifuged and filtered through 70 µm mesh filters to obtain single cell suspensions. The cells were then stained with anti-CD11b-PE conjugated antibodies (eBioscience, Thermo Fisher Scientific, USA), or with their corresponding isotype controls, for 1 hour at 4°C in the dark and analyzed by flow cytometry (BD FACSCanto™ II, BD Biosciences, USA).

### LEVELS OF CIRCULATORY CYTOKINES

Sera levels of cytokines were assessed by Rat Inflammation Array Q1 kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions and analyzed by the specific Q-analyzer software (RayBiotech, Norcross, GA, USA).

### REAL-TIME POLYMERASE CHAIN REACTION (PCR) ANALYSIS

RNA extracts (500 ng) were transcribed using Verso™ RT-PCR Kits (ABgene, USA). A quantitative PCR was performed with the Sybr Green PCR kit (Invitrogen, Israel). The primers used for real-time analysis included *serca* (5'ATTG TTCGAAGTCTGCTTCTGT3'/ 5'CATAGGTTGATCCA GTTATGGTA3'), *IL-1 receptor type I (IL1RI)* (5'TTGTCTCA TTGTGCCTCTGC3'/ 5'AAGAGGACAGCTGCGAATGT3') and *IL-1 receptor type 2 (IL1R2)* (5'GTGATCATTTCT CCCCTGGA3'/ 5'CACGATGGTGTTGGAAGATG3'). The relative mRNA expression of the target genes was normalized to the expression of the GAPDH reference gene.

### STATISTICAL ANALYSIS

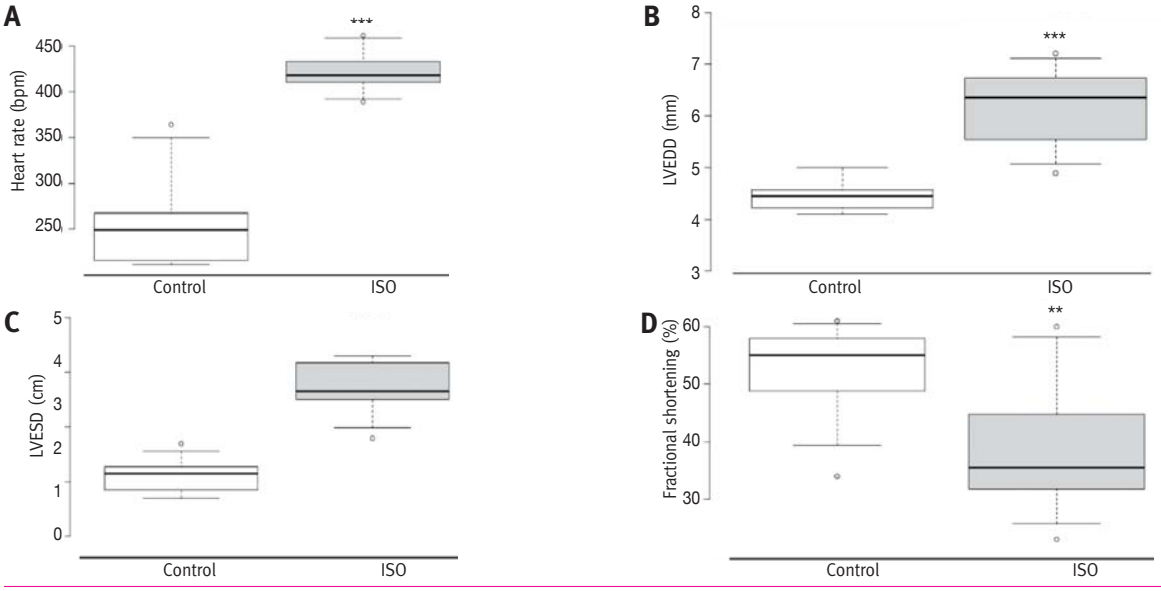
Groups were compared by two-tailed Student's *t*-test (IBM SPSS statistics software, version 23, IBM Corp, Armonk, New York, USA) unless specified otherwise. Significance was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$. Results are expressed as mean ± standard deviation (SD).

## RESULTS

### ELEVATED HEART RATE, CARDIAC HYPERTROPHY, AND PERI-VASCULAR FIBROSIS

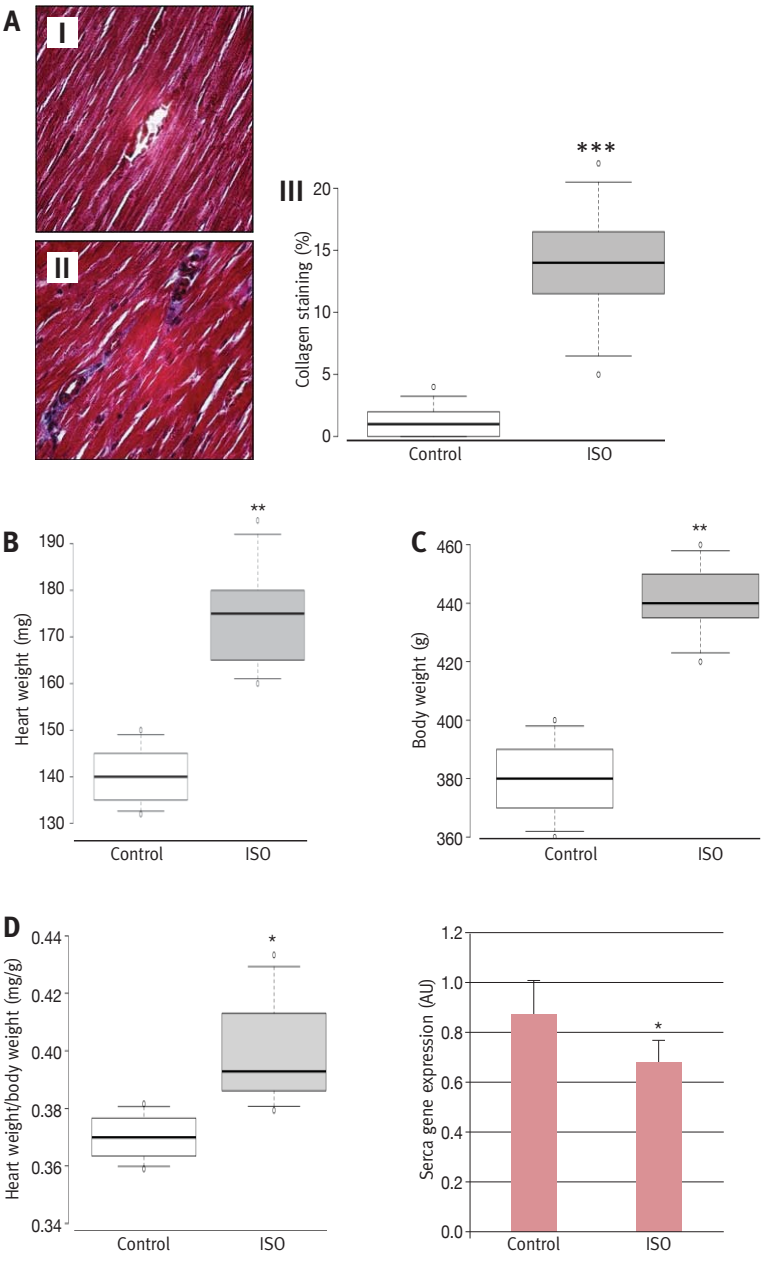
To characterize cardiac structure and function following 12 weeks of exposure to isoprel, rats were given an echocardiography scan. As expected, the isoprel-treated animals exhibited a significantly elevated heart rate: 423 ± 25 beats per minute (bpm) for the isoprel treated group vs. 252 ± 50 bpm for the control group, $P < 0.001$. Furthermore, a significant increase was observed in both left ventricular end diastolic diameter (LVEDD) (6.2 ± 0.8 mm compared to 4.5 ± 0.3 mm in the isoprel group vs. controls, respectively, $P < 0.001$) and in left ventricular end systolic diameter (LVESD) (3.7 ± 0.5 mm compared to 2.1 ± 0.3 mm in isoprel vs. control, respectively, $P < 0.001$), indicating a significant global LV dilation. A reduced fractional shortening (38.8 ± 11.9% in the isoprel group vs. 52.6 ± 8.2% in controls, $P = 0.008$) was observed [Figure 1 A-D]. The histologic analysis

**Figure 1.** Echocardiography analysis suggesting left ventricular dysfunction and hypertrophy following chronic treatment with isoprel **[A]** heart rate **[B]** left ventricular end diastolic diameter **[C]** left ventricular end systolic diameter **[D]** fractional shortening




n=10/arm, ISO = Isoprel, $^{**}P < 0.01$, $^{***}P < 0.001$

**Figure 2.** Cardiac pathology, left ventricular dysfunction and chronic heart failure induced by isoprel
**[A]** Masson's trichrome staining for collagen deposition:
**I.** Representative capture of left ventricular section derived from control animal
**II.** Representative capture of isoprel-treated left ventricular section
**III.** Box plot representing the median percentage of collagen deposition ± SD (n=10/group)
**[B and C]** Box plots showing the median differences in heart weight and body weight between the two groups (± SD)
**[D]** Box plot representing the differences in the heart weight/body weight ratios (mg/g)
**[E]** Bar graph illustration of the relative expression of serca in the left ventricle (mean ± SD)




SD = standard deviation, n=10/arm
$P < 0.05$, $P < 0.01$, $P < 0.001$

showed a significant interstitial fibrosis, mainly peri-vascular, in the isoprel group (13.8 ± 4.6% vs. 1.4 ± 1.3%) in the isoprel group compared to controls, $P \leq 0.001$ [Figure 2A I-III]. A significant increase in heart weight (175 ± 13.7 mg in the isoprel group vs. 140.4 ± 7.3 in the control group, $P = 0.002$) [Figure 2B] as well as in the animals' body weight (441 ± 15.2 g in the isoprel group vs. 380 ± 15.8 g in controls, $P < 0.001$) [Figure 2C] and animals' weight per body weight ratio (0.41 ± 0.01 in the isoprel group vs. 0.37 ± 0.009 in controls, $P = 0.02$) [Figure 2D] was observed. Interestingly, real time PCR data indicated a 25% downregulation in cardiac serca following treatment with isoprel: 1.006 ± 0.069 compared to 0.756 ± 0.174 in the isoprel group vs. controls, respectively (arbitrary units, $P = 0.029$) [Figure 2E], in line with previous reports regarding downregulation of this $Ca^{2+}$ channel in cardiac dysfunction [13].

### ELEVATED SYSTEMIC LEVELS OF CD11B+ CELLS

To assess whether chronic exposure to isoprel could affect the systemic levels of the CD11b+ sub-population, we performed flow cytometry and fluorescence-activated cell sorting (FACS) analyses on splenocytes derived from animals in the isoprel group or controls using the CD11b antibody that identifies monocytes, macrophages, and dendritic cells [14]. Interestingly, the flow cytometry data indicated a marked elevation ($P < 0.001$) in the percentage of CD11b+ cells in the isoprel group (53.3 ± 5.8%) relative to controls (31.7 ± 5.2%). The elevated expression of CD11b was more evident in those animals that exhibited factors of safety (FS) values lower than 35%: 57.4 ± 4.4% (n=5) compared to the ones exhibiting a more preserved FS: 49.2 ± 3.7% (n=5, $P = 0.01$), indicating that LV dysfunction may be associated with a higher number of circulating macrophages [Figure 3A]. A representative flow cytometry capture of CD11b staining of LV cells derived from controls or isoprel treated animals is shown in Figure 3B. Interestingly, Pearson's correlation analysis demonstrated a fair association between the percentage of systemic CD11b+ cells and the number of interstitial fibrotic areas in the cardiac sections. The linear trend line with its adjusted $R^2$ of 0.73 is shown in Figure 3C ($P < 0.001$). While the CD11b+ population was expanded in the isoprel treated animals, we did not detect macrophages infiltration of the myocardium, as evaluated by CD68 immuno-staining (data not shown).

### INCREASED SECRETION OF INTERLEUKIN 1 ALPHA (IL-1 ALPHA)

We next assessed whether the observed rise in CD11b+ was accompanied by a parallel elevation of inflammatory cytokines in the sera. To this end we applied a cytokine array assay. We observed an eightfold elevation of IL-1 alpha in the sera of the isoprel treated animals compared to their control counterparts (403 ± 119 pg/ml in the isoprel group vs. 52 ± 15 pg/ml in controls, $P < 0.001$). Furthermore, IL-1 alpha levels were significantly higher in animals exhibiting FS lower than 35%

compared to the ones exhibiting higher FS values (491 ± 105 pg/ml vs. 316 ± 35 pg/ml in the former compared to the latter, n=5/sub-group, $P = 0.02$) as shown in Figure 4A. Pearson's chi-square test indicated a significant correlation between IL-1 alpha levels ≥ 400 pg/ml and FS ≤ 35% ($P = 0.02$), suggesting that chronic systemic inflammation takes place in clinical conditions involving chronic adrenergic activation. Nevertheless, it is worthwhile to note that IL-1 beta and tumor necrosis factor (TNF) alpha-two cytokines, which are also known to be secreted by macrophages upon inflammatory stimuli, did not demonstrate any differential levels between isoprel and control groups. Among other cytokines tested, IL-2 and interferon gamma, which are mainly secreted by T cell lymphocytes, exhibited elevated sera levels in the isoprel group compared to controls ($P < 0.001$), although with no apparent association with systolic function, as reflected by FS ($P > 0.05$) [Figure 4B and 4C].

### OVER-EXPRESSION OF CARDIAC IL-1 RECEPTOR TYPE II

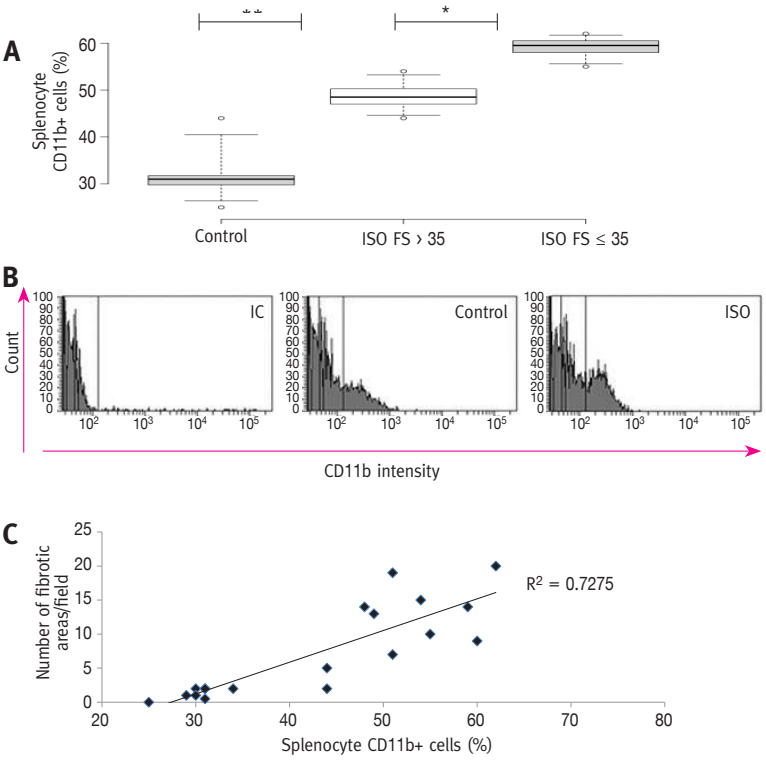
Because of the findings pointing to expansion of the CD11b+ cells, which is accompanied by elevated levels of systemic IL-1 alpha in association with depressed cardiac function, we sought to assess whether the expression levels of IL-1 receptors in the heart were modified upon adrenergic stimulation. Surprisingly, real-time PCR analysis did not show differential expression of IL-1 receptor type 1 (IL1R1), which is known to transduce signal transduction triggered by IL-1 [Figure 4D]. Nevertheless, we documented a 13-fold over-expression of IL-1 receptor type 2 (IL1R2) [Figure 4E], which is considered a decoy receptor that blocks IL-1 signal transduction [15,16], thus attenuating IL-1-mediated inflammatory processes.

## DISCUSSION

Tachycardia induced cardiomyopathy (TIC) is defined as ventricular systolic/diastolic dysfunction resulting from a prolonged increased heart rate that is reversible upon control of the arrhythmia, until some deleterious no-return point. Adverse remodeling induced by abnormal calcium handling and reduced cellular energy storing has been proposed as the underlying mechanisms [17,18]. It has been reported that daily administration of high doses of catecholamines, ranging from 5 to 25 mg/kg TIC, which could result in acute myocarditis in mice as early as 7 days after treatment starts [19]. These findings prompted us to investigate the inflammatory status in long-term non-acute adrenergic activation. To this end, we set up an animal model for cardiomyopathy induced by long-term chronic exposure to low doses of catecholamines. Since peripheral levels of CD11b cells were shown to be correlated to heart failure and arrhythmia [9-11], we sought to assess whether this type of cardiomyopathy is associated with modified systemic levels of CD11b+ cells or pro-inflammatory cytokines. Three months after treatment with low doses of isoprel, we documented cardiac hypertrophy, LV dysfunction, and increased body weight pointing to chronic heart failure. These cardiac changes were associated with elevation of CD11b+ cell levels and increased systemic levels of IL-1 alpha with no apparent signs of myocardial inflammation. Our data demonstrated over-expression of the inhibitory IL1R2 without concomitant apparent cardiac inflammation. We thus assume that in the clinical presentation of chronic low-grade adrenergic stimulation, the LV may develop protection mechanisms from inflammatory reactions. Interestingly, the data presented herein suggest that systemic expression levels of CD11b+ cells following exposure to chronic adrenergic stimulation may point to the degree of pathological interstitial fibrosis in the heart.

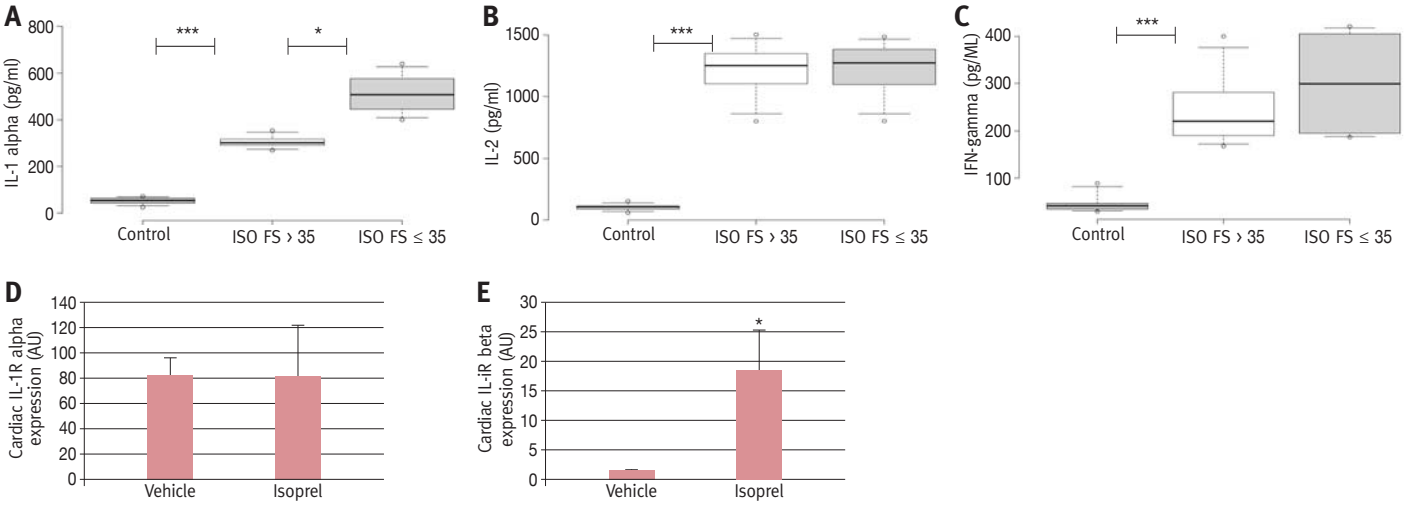
Although detected at relatively low levels in the circulation, IL-1 cytokines induce potent inflammatory signals. The biological activity of IL-1 cytokines is controlled at the level of their production and maturation, receptor binding, and post-receptor signaling. Both IL-1 alpha and IL-1 beta are strong

**Figure 3.** Expansion of the CD11b+ cells in the spleen following treatment with isoprel **[A]** Box plot showing the median percentage of CD11b+ cells in the spleen in controls (n=10) vs. isoprel-treated animals exhibiting FS ‹ 35% (n=5) or FS › 35% (n=5) **[B]** Reparative FACS histograms showing the percentage of CD11b+ cells in splenocytes derived from control or isoprel-treated animals stained with isotype control or with CD11b PE-conjugated antibodies. **[C]** Correlation trendline between the percentage of splenic CD11b+ cells and the number of cardiac areas of interstitial fibrosis




FS = factors of safety, FACS = fluorescence-activated cell sorting
$P ‹ 0.05$, $P ‹ 0.01$

**Figure 4.** Elevated systemic levels of pro-inflammatory cytokines and IL-1R-beta in the isoprel group. Box plots showing the median levels ± SD of IL-1 alpha **[A]**, IL-2 **[B]** and interferon gamma **[C]** in the vehicle-control group (n=10) relative to the isoprel-treated animals exhibiting FS ‹ 35% or FS › 35% (n=5/ group). **[D]** Bar graph showing the expression of IL-1R alpha relative to controls (mean ± SD) and **[E]** the expression of IL-1R beta relative to controls (mean ± SD)




IL = interleukin, SD = standard deviation, n=6/group, AU = arbitrary units, FS = factors of safety

*P* = 0.046, *P* ‹ 0.05, *P* ‹ 0.01, *P* ‹ 0.001

pro-inflammatory agents that perform many of the same functions and bind to the IL-1 receptor [20]. In stimulated macrophages, IL-1 alpha is synthesized de novo and can be actively secreted or passively released from apoptotic cells [21]. IL-1 beta, however, is synthesized as a leaderless precursor that must be cleaved by inflammasome-activated caspase-1 [22]. Likewise, two types of receptors for the pro-inflammatory IL-1 are known. IL1R1I, which is responsible for transducing signals triggered by IL-1 [23], as well as IL-1R2, which lacks the cytoplasmic signaling domain, thus serves as a decoy receptor that blocks IL-1 signal transduction [24]. Indeed, numerous studies have demonstrated that IL1R2 acts exclusively to counter IL-1 activity [15,16] and suggested that tissue-specific production of IL-1R2 can mediate IL-1 antagonism in the microenvironment of the injured tissue without systemic consequences [16].

Similar to our results, it has been documented that psychological stress as well as stress-related diseases are associated with elevated plasma levels of IL-1, noting that stress is associated with an increase in IL-1 production [25]. A few reports also pointed to splenic accumulation of CD11b+ cells concomitantly with IL-1 secretion [25]. We thus suggest that chronic long-term "low level" adrenergic activation could result in sustained systemic inflammation, mainly, IL-1 alpha driven, with no apparent inflammation in the injured heart.

### STUDY LIMITATION

In the current study we did not show direct evidence regarding the cause-effect relationship between cardiac phenotype and peripheral activation of CD11b+ cells as well as IL-1 alpha production. Moreover, in the current model, cardiac function and systemic levels of pro-inflammatory cytokines were examined right after a 12 week treatment regimen with isoprel. However, we have not tested the inflammatory status with specific focus on the expression levels of peripheral CD11b cells and IL-1 alpha after isoprel withdrawal. These interesting issues will be addressed in our future in vivo studies in which we will try to determine the feasibility and the time course for disease reversibility in animals treated with isoprel or without an IL-1 alpha signaling blocker.

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## Capsule

### A clue to a drug's neurotoxicity?

The drug BIA 10-2474 (BIAL, Portugal) inhibits fatty acid amide hydrolase (FAAH), a lipase that degrades a specific endocannabinoid. On the basis of this activity, BIA 10-2474 was being developed as a potential treatment for anxiety and pain. In a phase 1 trial of the drug, one subject died, and four others suffered brain damage. As an initial step in investigating whether inhibition of off-target proteins by BIA 10-2474 might contribute to its clinical neurotoxicity, van Esbroeck et al. used activity-based proteomic assays to identify proteins targeted by the drug. Studying human cells and brain samples from subjects not associated with the trial, they found that BIA 10-2474 targeted several different lipases in addition to FAAH. It also substantially altered lipid metabolism in cultured neurons.

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Eitan Israeli

## Capsule

### An antisensible approach to target *KRAS*

Mutations that cause activation of the *KRAS* oncogene are common in human cancer, including treatment-resistant tumor types such as lung and pancreatic cancer. *KRAS* is notoriously difficult to target with small molecules. To overcome this issue, Ross et al. turned to genetic technology to develop an antisense oligonucleotide-based therapy for inhibiting *KRAS*. The antisense oligonucleotide was chemically modified to allow systemic delivery through subcutaneous injection, avoiding the need for a specialized delivery vehicle. The authors tested the efficacy of this therapy in multiple mouse models of non-small cell lung cancer and evaluated its safety in primates, demonstrating its potential suitability for translation to humans.

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