The inflammatory response in myocarditis and acute myocardial infarction
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Citation for published version (APA):

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Chapter 4

Colchicine aggravates coxsackievirus B3 infection in mice

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* International Journal of Cardiology 2016; in press
SUMMARY

Background. There is a clinical need for immunosuppressive therapy that can treat myocarditis patients in the presence of an active viral infection. In this study we therefore investigated the effects of colchicine, an immunosuppressive drug which has been used successfully as treatment for pericarditis patients, in a mouse model of coxsackievirus B3(CVB3)-induced myocarditis.

Methods. Four groups of C3H mice were included: Control mice (n=8), mice infected with CVB3 (1x10^5 PFU, n=10), mice with colchicine administration (2 mg/kg i.p, n=5) and mice with combined CVB3 infection and colchicine administration (n=10). After three days, the heart, pancreas and spleen were harvested and evaluated using (immuno)histochemical analysis and CVB3 qPCR.

Results. Mice were terminated at day 3 post-virus infection as colchicine treatment rapidly resulted in severe illness and mortality in CVB3-infected mice. Colchicine significantly decreased the number of macrophages in the heart in CVB3-infected mice (p<0.01) but significantly increased the number of neutrophils (p<0.01). In the pancreas, colchicine caused complete destruction of the acini in the CVB3-infected mice and also significantly decreased macrophage (p<0.01) and increased neutrophil numbers (p<0.01). In the spleen, colchicine treatment of CVB3-infected mice induced massive apoptosis in the white pulp and significantly inhibited the virus-induced increase of megakaryocytes in the spleen (p<0.001). Finally, we observed that colchicine significantly increased CVB3 levels in both the pancreas and the heart.

Conclusion. Colchicine treatment in CVB3-induced myocarditis has a detrimental effect as it causes complete destruction of the exocrine pancreas and enhances viral load in both heart and pancreas.
INTRODUCTION

Myocarditis is a difficult clinical entity due to its heterogeneity in aetiology, clinical presentation and outcome as well as the lack of specific treatment options. Current myocarditis treatment generally consists of standard heart failure therapeutics. However, these are useful in symptom repression and preventing on-going decline of cardiac function, they do not treat the underlying disease itself. As such, other treatment options are needed.

Several clinical trials have investigated immunosuppressive drugs, such as prednisone, azathioprine and cyclosporine as treatment for myocarditis. The results of several of these trials have suggested that patients with active viral infection do not respond favourably to immunosuppression, presumably because immunosuppression hinders or prevents viral clearance. Current guidelines state that immunosuppressive therapy may be considered in myocarditis patients only after ruling out active infection on endomyocardial biopsies via PCR. As viral infection is the most prevalent cause of myocarditis, immunosuppressive treatment that does not negatively affect viral clearance and therefore does not require virus-negativity would be of great benefit to the management of myocarditis patients.

An immunosuppressive drug that increasingly is becoming of interest for cardiovascular disease in general is colchicine. Colchicine has been used for over 2000 years to treat gout. Recent publications have shown effectiveness of colchicine for treating pericarditis and it is rapidly becoming standard therapy for this condition. As pericarditis can coincide with myocarditis, colchicine may be an interesting potential treatment option for myocarditis patients as well. There is some evidence that colchicine might be beneficial in patients even while active viral infection exists. In a recent case series of 5 patients with Epstein-Barr virus/Cytomegalovirus co-infection leading to viral myocarditis, the use of colchicine as adjunct to conventional therapy resulted in increased left ventricular ejection fraction. Also, a large part of acute pericarditis is thought to be infectious, especially of viral etiology. In several clinical trials studying the effects of colchicine treatment on acute as well as recurrent pericarditis, significant benefit over conventional treatment and no serious adverse effects of colchicine treatment were reported, while in these studies patients with viral infections were not excluded. These results further suggest that colchicine may be suitable for treatment of viral inflammation. We therefore examined the effects of colchicine administration in a mouse model of acute coxsackievirus B3 (CVB3)-induced myocarditis.
MATERIALS & METHODS

Animal procedures
This study was approved by the VUmc animal ethics and welfare committee, and conforms to the Guide for care and use of laboratory animals published by the US National Institutes of Health. Thirty-three male C3H mice (Harlan, Horst, The Netherlands) were divided into four groups: uninfected control mice (Control group; n=8, 10-12 weeks old), CVB3-infected mice (CVB3 group; n=10, 6 weeks old), colchicine-treated uninfected mice (colchicine group; n=5, 6 weeks old) and CVB3-infected colchicine-treated mice (CVB3+colchicine group; n=10, 6 weeks old). The CVB3 and CVB3+colchicine groups received an intraperitoneal (i.p.) injection containing 1x10⁵ plaque forming units of CVB3 (Nancy strain, ATCC, Manassas, VA) on day 0, while the control and colchicine groups received an i.p. saline injection. On day 1, the colchicine and CVB3+colchicine groups received an i.p. colchicine injection (2 mg/kg, Sigma-Aldrich, St Louis, MO), while the control and CVB3 groups received an i.p. saline injection. Mice were terminated on day 3 and the heart, lungs, spleen, kidney, liver and pancreas were excised. Of the hearts, a cross-sectional ventricular tissue sample was frozen in -80°C for viral genome analysis. The remaining heart tissue and other organs were fixed in 4% formaldehyde and embedded in paraffin for histochemical and immunohistochemical analyses.

Histological staining
Formaldehyde-fixed paraffin-embedded tissue sections (4μm thick) were deparaffinised and rehydrated using xylene and ethanol respectively. For histological tissue analysis a haematoxylin-eosin stain was used. This stain was performed by submerging deparaffinised sections in Meyer’s haematoxylin for 5 minutes followed by washing in tap water and counterstaining with eosin for 30 seconds. Afterwards, the slides were washed again in tap water, dehydrated and covered.

Immunohistochemistry
Deparaffinised and rehydrated formaldehyde-fixed tissue sections (4μm thick) were submerged in methanol containing 0.3% H₂O₂ to block endogenous peroxidase. Antigen retrieval was achieved by either boiling in citrate buffer (pH 6) in a microwave oven for (for CD45, macrophage, γH2AX- and VP1 staining) or by incubation with activated pepsin at 37°C (for Ly-6G and CD61 staining). Details on the used antibodies, sera and reagents can be found in Supplemental table 1. In short: for macrophage, Ly-6G and γH2AX stainings, the sections were pre-treated with normal sera. Lymphocytes were visualized with rat-α-mouse-CD45 (BD Biosciences, Breda, the Netherlands), macrophages with rabbit-α-mouse-macrophage (Accurate Chemical & Scientific Corporation, Westbury, NY), neutrophils with rat α-mouse-Ly-6G (BD Biosciences), double-stranded
DNA breaks with rabbit-α-γH2AX (Cell Signaling Technology, Danvers, MA), megakaryocytes with rabbit-α-CD61 (GeneTex Inc., Irvine, CA) and enteroviral protein with mouse-α-enterovirus VP1 (Leica Biosystems, Son, The Netherlands). Bound primary antibodies were labelled with horseradish peroxidase (HRP), which was visualized with diaminobenzidine (Dako, Glostrup, Denmark). For VP1, the Mouse-On-Mouse (M.O.M.) ImmunodetectionKit™ (Vector Laboratories, Burlingame, CA) was used. The slides were counterstained with haematoxylin, dehydrated and covered.

**Quantitative analysis of tissue slides**

All quantitative analyses were performed blinded using a light microscope at 400x magnification, apart from VP1-stained sections and the surface areas which were analysed on scanned slides.

*Pancreas:* Morphological normal pancreatic acini and Langerhans islets were counted on HE-stained slides. The numbers of lymphocytes (CD45), macrophages, neutrophils (Ly-6G) and γH2aX-positive cells were counted. Only positive extravascular cells within the exocrine or endocrine tissue of the pancreas were counted. CD45 is also present on non-lymphocytic cells, but it is known that CD45 can be used as a general lymphocyte marker based on its morphology.\(^\text{13}\)

*Heart:* The numbers of extravascular lymphocytes, macrophages and neutrophils within the myocardium were counted.

*Spleen:* Megakaryocytes were counted based on their characteristic morphology and CD61-positivity. Cell numbers were expressed per mm\(^2\) of red and white pulp combined.

The surface areas of heart and pancreatic tissue were measured using a PathScan Enabler IV digital slide scanner (Meyer Instruments, Houston, TX). Surface measurements were performed using QuickPHOTO MICRO software v3.0 (Promicra, Prague, Czech Republic). Spleen sections and VP1-stained pancreatic sections were scanned using a Pannoramic DESK (3D histech, Budapest, Hungary). Red and white pulp surface areas were measured using Pannoramic Viewer v1.15.3 (3D histech). VP1-stained sections were analysed with ImageJ software (Bethesda, MD) using colour thresholding to determine the percentage of positively stained tissue.

**Coxsackievirus qPCR**

For detection of CVB3 in the mouse cardiac tissue samples, tissue samples were homogenized and genomic material was isolated using the Magna Pure LC Total Nucleic Acid Isolation Kit (Roche, Penzberg, Germany), following manufacturer’s instructions. Genomic DNA (gDNA) and RNA were isolated separately. RNA was converted to cDNA and pooled with the isolated genomic DNA prior to enteroviral qPCR. Conversion to cDNA and the enteroviral qPCR in the mouse hearts was performed as described previously.\(^\text{14}\) To compensate for
variations in the amount of genomic material put in the PCR-reactions, DNA concentrations in the pooled gDNA/cDNA samples were measured using a NanoDrop1000 spectrophotometer v3.7.1 (Thermo Fischer Scientific, Waltham, MA).

**Statistical Analysis**
All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). As the data were not normally distributed the Mann–Whitney U-test was used to compare groups. The differences in survival were analysed with the Mantel-Cox test. A p-value smaller than 0.05 was considered statistically significant. Values in text are given as median(25th percentile–75th percentile).

**RESULTS**

**Colchicine causes terminal illness in mice after CVB3 infection**
Three days after viral infection (two days after colchicine administration) 5 out of 10 mice in the CVB3+colchicine group were found dead (figure 1), and the remaining 5 mice showed signs of severe discomfort (closed eyes, pilo-erection, arched back, lack of activity). Because of this, all the mice of the different experimental groups were terminated 3 days post-infection. Mice from the other three experimental groups showed no signs of discomfort at this point, although the mice in the colchicine group experienced a slight weight loss (9.4(7.4–9.6)% loss compared to start weight).

![Figure 1. Survival](image)

*Figure 1. Survival*
Kaplan-Meyer survival curve of control mice (Control, n=8), Coxsackievirus B3-infected mice (CVB3, n=10), colchicine-treated mice (Colchicine, n=5) and Coxsackievirus B3-infected mice with colchicine treatment (CVB3+colchicine, n=10). p.i.: post-infection. *: p<0.05 vs. Control. #: p<0.05 vs. CVB3.
Figure 2. Inflammatory cell numbers in cardiac tissue.
Number of lymphocytes(A), macrophages(B) and neutrophils(C) per mm² ventricular myocardium of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. *: p<0.05, **: p<0.01, ***: p<0.001.

Colchicine increases neutrophils and decreases macrophages in the heart after CVB3 infection
The hearts were examined to determine whether colchicine had affected CVB3-induced myocarditis. In control mice a low number of lymphocytes was present in the myocardium (0.2(0.1-0.3) cells/mm²)(figure 2A). Compared to the control group, the CVB3 (4.1(3.1-5.2) cells/mm²), colchicine (3.2(2.2-4.0) cells/mm²) and CVB3+colchicine (2.9(1.3-4.0) cells/mm²) groups showed a statistically significant increase in lymphocytes. The control and CVB3 groups had a similar low number of macrophages (0.5(0.2-0.7) and 0.6(0.4-1.0) cells/mm² respectively; figure 2B). In the colchicine group significantly increased macrophage numbers (1.2(0.7-2.1) cells/mm²) were observed compared to the control group, while in the CVB3+colchicine group macrophage numbers (0.2(0.1-0.3) cells/mm²) were significantly decreased compared to the other three groups.
Neutrophil numbers were similar between the control (1.1(0.5-2.1) cells/mm²), CVB3 (0.9(0.5-2.1) cells/mm²) and colchicine groups (0.9(0.3-1.4) cells/mm²)(figure 2C). However, in the CVB3+colchicine group the number of neutrophils was significantly increased (4.1(1.7-5.6) cells/mm²) compared to the other three groups.

**Colchicine causes severe damage to pancreatic acini after CVB3 infection**

We subsequently examined HE-stained slides of several organs. No morphological cell damage was seen in the heart, liver, kidney or lungs in any of the groups. However, in the pancreas we found considerable damage of the pancreatic acini (figure 3A-D). Compared to the control group (439(392-474) acini/mm²), the number of intact pancreatic acini was significantly decreased in the CVB3 group (116(78-216) acini/mm²), but also in the colchicine group (228(208-251) acini/mm²)(figure 3E). In the CVB3+colchicine group, intact acini were virtually absent (0.2(0.1-1.1) acini/mm²) and significantly lower compared to all other groups. Next, we stained the pancreases for γH2Ax (figure 3F), which is part of the DNA-repair mechanism and used as a biomarker of double-strand DNA breaks. It is known that γH2Ax staining can identify damaged cells in advance of morphological signs of cell damage, and remains present until cell death when DNA repair is ineffective. The γH2Ax-positive nuclei were found diffusely in both areas of still intact acini and morphologically damaged areas. Compared to the control group (0.0(0.0-0.1) nuclei/mm²), the number of γH2Ax-positive nuclei in the CVB3 (80.3(58.2-122.0) nuclei/mm²), colchicine (14.7(11.1-16.0) nuclei/mm²) and CVB3+colchicine groups (17.0(15.0-20.4) nuclei/mm²) were significantly increased. A significantly decreased number of γH2Ax-positive nuclei were present in the pancreases of mice in the CVB3+colchicine group compared to the CVB3 group (figure 3G), albeit in the CVB3+colchicine group morphological normal acini were almost completely diminished already what could explain the lack of γH2Ax staining in this particular group.

The Langerhans islets were not morphologically affected by either CVB3, colchicine or CVB3+colchicine (figure 3A-D). In line herewith, only a few sporadic γH2Ax-positive nuclei were found in the CVB3 group (0 to 1 positive nuclei per islet), but none in the other groups. However, compared to the control group (1.1(0.8-1.5) islets/mm²), the CVB3 and CVB3+colchicine groups showed a significant decrease in the number of Langerhans islets (0.6(0.2-0.9) and 0.2(0.1-0.4) islets/mm² respectively; figure 3H). The number of Langerhans islets in the colchicine group (1.1(0.7-2.7) islets/mm²) differed significantly only compared to the CVB3+colchicine group.
Figure 3. Pancreatic damage
A-D. Haematoxylin–Eosin staining of a pancreatic tissue sample from a representative control mouse (A), Coxsackievirus B3-infected mouse (B), colchicine-treated mouse (C) and Coxsackievirus B3-infected mouse with colchicine treatment (D), displaying intact pancreatic acini (examples marked with arrows) and Langerhans islets (L.I). Scale bar: 100 μm. E. Number of intact pancreatic acini per mm² of pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. F. γH2AX-positive nuclei (arrows) in the pancreas of a CVB3-infected mouse. Scale bar: 50 μm. G. Number of γH2AX-positive nuclei per mm² pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. H. Number of Langerhans islets per mm² of pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. *: p<0.05, **: p<0.01, ***: p<0.001.

Colchicine increases neutrophils and decreases macrophages in the pancreas after CVB3 infection
To analyse the inflammatory response in the pancreas, extravascular lymphocytes, macrophages and neutrophils were quantified in the combined acini and Langerhans islets of the pancreas. In line with the cellular damage,
inflammatory cells were only found in the acini areas, not in the Langerhans islets (not shown). Lymphocytes were virtually absent in the pancreas of control mice (0.1(0.0-0.2) cells/mm²; figure 4A). Compared to the control group, the CVB3 (2.0(0.7-5.8) cells/mm²), colchicine (3.3(2.5-11.5) cells/mm²) and CVB3+colchicine groups (5.0(2.6-9.0) cells/mm²) all showed a significant increase in the number of lymphocytes, which did not differ significantly between each other.

Macrophage numbers in the CVB3, Colchicine and CVB3+colchicine groups (10.2(8.1-15.7), 5.8(1.9-11.9) and 1.3(0.8-4.7) cells/mm² respectively) were significantly increased compared to the control group (0.1(0.0-0.3) cells/mm²)(figure 4B), although in the CVB3+colchicine group they were significantly decreased compared to the CVB3 group. Similarly, in the CVB3, Colchicine and CVB3+colchicine groups significantly increased neutrophils numbers were observed (7.4(6.4-13.2), 3.5(0.9-17.3) and 22.3(15.2-24.6) cells/mm² respectively; figure 4C) compared to the control group (0.1(0.0-0.2) cells/mm²).
The highest number of neutrophils was found in the CVB3+colchicine group which was significantly increased compared to the CVB3 group.

Figure 5. Spleen histology
A-D. Haematoxylin-Eosin staining of a splenic tissue sample from a representative control mouse(A), Coxsackievirus B3-infected mouse(B), colchicine-treated mouse(C) and Coxsackievirus B3-infected mouse with colchicine treatment(D), displaying red and white pulp. Scalebar: 100 μm. D. Apoptosis in white pulp (example 4x magnified). E. Area of white pulp as percentage of the total splenic tissue area of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. F. CD61-staining of the spleen of a CVB3-infected mouse, displaying a megakaryocyte (brown). Scalebar: 25 μm. G. Number of megakaryocytes per mm² spleen of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. For one mouse in the control group and one mouse in the Colchicine group, the remaining splenic tissue was insufficient for megakaryocyte analysis. Horizontal lines in dot plots: median. *: p<0.05, **: p<0.01, ***: p<0.001.
Colchicine causes massive apoptosis in the white pulp of the spleen after CVB3 infection, and decreases the number of megakaryocytes

Morphological changes were also observed in the spleen of CVB3+colchicine treated mice. Compared to the control group (figure 5A), the CVB3 group (figure 5B) and the colchicine group (figure 5C), the white pulp of the CVB3+colchicine group displayed massive apoptosis (figure 5D). The relative surface areas of red and white pulp did not differ between the groups (figure 5E). We also observed changes in megakaryocytes numbers (figure 5F) in the red pulp. In both the colchicine and CVB3+colchicine groups the megakaryocytes numbers were significantly decreased (0.3(0.2-0.7) and 2.5(2.2-4.5) cells/mm² respectively) compared to both the control group (4.0(3.3-8.7) cells/mm²) and CVB3 group (7.4(5.7-8.5 cells/mm²; figure 5G). In addition, for the colchicine group this was also significantly decreased compared to the CVB3+colchicine group.

Colchicine increases the virus load in the heart and pancreas after CVB3 infection

Finally, we studied whether colchicine affected the viral load of the heart and the pancreas. In the frozen tissues samples obtained from the hearts, the levels of CVB3 RNA were determined by qPCR. Compared to the CVB3 group, the CVB3+colchicine group had a significantly increased quantity of viral RNA in the heart (159(27-2738) and 27517(22451–51889) arbitrary units respectively, figure 6A). Heart samples from the control group and colchicine group were negative for CVB3 RNA (not shown).

Of the pancreas, no frozen tissue was obtained. Therefore, in the pancreas the viral load was quantified using an immunohistochemical staining against the CVB3 protein VP1. VP1-stainings in the control and colchicine groups only showed minor a-specific background staining (not shown). In the CVB3 group (figure 6B) and CVB3+colchicine group (figure 6C) strong VP1-staining was found in the pancreatic acini. In the CVB3+colchicine group some VP1 staining was also seen in the periphery of the Langerhans islets (figure 6D). The percentages of the pancreas positive for VP1 in both the CVB3 group (21.9(13.5-30.9)% of surface area) and CVB3+colchicine group (49.7(38.8-56.9)% of surface area) were significantly larger compared with the control and colchicine groups. In addition, the VP1-positive area in the CVB3+colchicine group was significantly larger compared to the CVB3 group (figure 6E).
**Figure 6. Viral load in heart and pancreas**
A. Coxsackievirus B3 DNA/RNA levels in cardiac tissue of Coxsackievirus B3-infected mice and Coxsackievirus-infected mice treated with colchicine. B-C. Coxsackievirus B3 protein VP1 staining (brown) on pancreatic tissue of a representative Coxsackievirus B3-infected mouse(B), and Coxsackievirus B3-infected mouse with colchicine treatment(C). Scale bar: 200µm. D. Coxsackievirus B3 protein VP1 staining (brown) on a Langerhans islet of a Coxsackievirus B3-infected mouse with colchicine treatment. Scale bar: 100 µm. E. Percentage of spleen tissue area with positive VP1-staining of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. ***: p<0.001.

**DISCUSSION**

We have studied colchicine therapy in mice with acute CVB3-induced myocarditis. We found that colchicine treatment induced severe illness and increased mortality within three days in mice with CVB3-induced myocarditis. Colchicine also caused an increase in neutrophil infiltration but a decrease in macrophage infiltration in both pancreas and heart, coinciding with a significantly increased viral load in both organs. Additionally, CVB3 alone caused a partial degeneration of the pancreatic acini, while for CVB3 and colchicine combined these were almost completely destroyed. Finally, colchicine induced massive apoptosis in the white pulp of the spleen of CVB3-infected mice. These results indicate that colchicine treatment may cause serious adverse effects in case of an active viral infection.

In the past decade, several clinical trials have shown both in patients with acute pericarditis and recurrent pericarditis that colchicine reduced the
recurrence rate of pericarditis and reduced symptom persistence (reviewed by Imazio8). There is evidence suggesting that recurrent pericarditis is mainly immune-mediated, whereas acute pericarditis often has an infectious cause, which is thought to be predominantly viral in developed countries.16 Therefore it has been suggested that corticosteroids given in the index pericarditis attack can favour the occurrence of relapses because of their detrimental effect on viral replication.17 However, in none of the colchicine trials, serious adverse effects related to the colchicine treatment were observed. In contrast, in the present study colchicine induced a large increase in viral load both in the heart and the pancreas, which seems at odds with results from the acute pericarditis trials. Although the inclusion criteria in these trials encompassed patients with a viral aetiology, there was no mention of viral diagnostics having been performed8 and consequently no proof was presented of active viral infection in these patients, nor of putative effects of colchicine thereon. Even more, if patients with pericarditis of viral aetiology were included in these trials, given the high prevalence, it would have been unknown how long after putative infection colchicine therapy was started. In our study colchicine therapy was started one day after infection. It is theoretically possible that this early time point prevented the initiation of a proper immune response leading to uncontrolled viral spread and replication and high viral loads, whereas it is unlikely that colchicine therapy was started this early after infection in the patient studies.

A limitation of this study is that we are unable to provide a mechanism through which colchicine treatment resulted in enhanced viral load in the heart and pancreas. The primary mechanism of action of colchicine is tubulin disruption. Through this mechanism colchicine was shown to inhibit leukocyte recruitment, adhesion and transmigration.18, 19 Additionally, colchicine was shown to inhibit several cellular leukocyte functions, such as activation of the inflammasome and IL1β release in macrophages20 and superoxide production in neutrophils21 in response to inflammatory microcrystals as well as to down-regulate tumor necrosis factor receptors on macrophages.22 The results in the present study were not uniformly in line with these findings. Although the number of macrophages was indeed decreased in the affected organs of colchicine-treated CVB3-infected mice compared to untreated CVB3-infected mice, conversely the number of infiltrated neutrophils was increased. At this moment we cannot adequately explain this seeming discrepancy with the literature, although the effects of colchicine on neutrophil extravasation in the setting of an acute viral infection were, to the best of our knowledge, never studied before. Moreover, it is unknown whether the functioning of leukocytes, those infiltrated as well as those in the blood, was affected in the colchicine-treated mice. It may well be that the dramatic increase in viral load in the affected organs of colchicine-treated CVB3-infected mice is more the result of its inhibiting effect on systemic leukocyte function than of the number of cells infiltrating the affected organs. This theory is supported by the relative low number of infiltrated
leukocytes in the hearts of colchicine-treated CVB3-infected mice despite the high viral load.

It is also unidentified whether the disastrous effects of colchicine in our model were virus-specific, or whether colchicine would have similar effects in infections of other common cardiotropic viruses such as parvovirus B19 and influenza viruses. Gultekin and Kucukates treated five patients with low dose colchicine (0.5 mg twice daily) for myocarditis associated with EBV/CMV co-infection (without pericarditis) and found no severe complications in these patients. However, effects of colchicine treatment on virus titres in these patients were not reported.

Although it is known that myocarditis can coincide with pericarditis, in only two of the colchicine trials, i.e. the ICAP trial and the CORP-2 trial, patients with evidence of myocardial involvement, as indicated by elevated serum troponin levels, were excluded. While in the other trials myocarditis was not listed as an exclusion criterium, nor was myocarditis depicted in the patient characteristics. Therefore it is unclear whether patients with myocarditis were included in these studies.

In the spleen, we observed that colchicine induced massive apoptosis of the white pulp in the spleen in CVB3-infected mice. Apoptosis of both B and T lymphocytes in the spleen also early after virus infection has been reported before in mice, the extent of which has been related to the viral dose. As colchicine treatment induced such a high viral load, this may explain why the splenic apoptosis we observed in the colchicine-treated CVB3-infected mice was much more apparent than in the untreated CVB3-infected mice. Moreover, the splenic apoptosis likely was not a direct effect of colchicine as in uninfected colchicine-treated mice no apparent splenic apoptosis was noted. Noteworthy, colchicine also had an effect on megakaryocyte numbers in the spleen. Colchicine significantly reduced the number of megakaryocytes in the red pulp, both in uninfected and CVB3-infected mice, to well below their level in control mice. Whether this is a result of a toxic effect of colchicine on megakaryocytes or that colchicine prevents megakaryocytic entry into the spleen is not clear. To the best of our knowledge a reducing effect of colchicine on the number of splenic megakaryocytes in vivo has never been described before.

CVB3 infection and damage of the pancreas in mice has been described before. In accordance with our study it was shown that CVB3 primarily infects the acini and not the Langerhans islets. Colchicine treatment increased the damage exclusively in the acini, not in the islets, suggesting this additional effect is likely explained by the induced increase in viral titres. The number of intact acini in uninfected colchicine-treated mice was also decreased compared to controls, suggesting a cytotoxic effect of colchicine in these areas as well. The increase of inflammatory cells in the heart due to colchicine alone further suggests cytotoxicity. Colchicine was administered before in a similar dose (2 mg/kg) in mouse models without reported adverse effects, although whether
the pancreas was studied is unclear. In a study into the role of microtubules in acute pancreatitis in rats Ueda et al. used a high dose of colchicine (10 mg/kg), without noting any histopathological damage.29 Notwithstanding this, different sensitivity to colchicine-induced toxicity between different species or between different strains cannot be excluded. To the best of our knowledge, colchicine was not yet studied in the C3H mouse strain that we used.

References

19. Perico N, Ostermann D, Bontempeill M et al. Colchicine interferes with L-selectin and leukocyte function-associated antigen-1 expression on human T lymphocytes and inhibits T cell activation. *J


**Supplementary table 1. Antibodies, sera and blocking reagents in order of application.**

<table>
<thead>
<tr>
<th>Stain</th>
<th>Product</th>
<th>Source</th>
<th>Dilution</th>
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<td>Macrophage</td>
<td>Normal Swine Serum, MONX10964</td>
<td>Monosan</td>
<td>1:20</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-mouse macrophage, AIAD31240</td>
<td>Accurate</td>
<td>1:100</td>
<td>60 min.</td>
</tr>
<tr>
<td></td>
<td>Swine anti-rabbit HRP, P0217</td>
<td>Dako</td>
<td>1:100</td>
<td>30 min.</td>
</tr>
<tr>
<td>Ly-6G (neutrophils)</td>
<td>Normal rabbit serum, X0902</td>
<td>Dako</td>
<td>1:50</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Rat anti-mouse Ly-6G, BD551459</td>
<td>BD Pharamingen</td>
<td>1:200</td>
<td>60 min.</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-rat biotinylated, E0468</td>
<td>Dako</td>
<td>1:300</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>Streptavidin HRP, P0397</td>
<td>Dako</td>
<td>1:100</td>
<td>60 min.</td>
</tr>
<tr>
<td>CD61 (megakaryocytes)</td>
<td>Rabbit anti-CD61, GTX50435</td>
<td>GenTex Inc.</td>
<td>1:100</td>
<td>60 min.</td>
</tr>
<tr>
<td>Ly-6G (neutrophils)</td>
<td>Swine anti-rabbit HRP, P0217</td>
<td>Dako</td>
<td>1:100</td>
<td>30 min.</td>
</tr>
<tr>
<td>Enterovirus VP1</td>
<td>Avidin, X0590</td>
<td>Dako</td>
<td>1:1</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Biotin, X0590</td>
<td>Dako</td>
<td>1:1</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>M.O.M. Mouse Ig blocking reagent, BMK 2202</td>
<td>Vector</td>
<td>2 drops/2.5 ml*</td>
<td>60 min.</td>
</tr>
<tr>
<td></td>
<td>M.O.M. Diluent, BMK 2202</td>
<td>Vector</td>
<td>1:12.5*</td>
<td>5 min.</td>
</tr>
<tr>
<td></td>
<td>NCL-ENTERO</td>
<td>Leica</td>
<td>1:1000†</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>M.O.M. Biotinylated anti-mouse IgG reagent, BMK 2202</td>
<td>Vector</td>
<td>1:250†</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Streptavidin HRP, P0397</td>
<td>Dako</td>
<td>1:200</td>
<td>60 min.</td>
</tr>
<tr>
<td>γH2AX</td>
<td>Normal Swine Serum, MONX10964</td>
<td>Monosan</td>
<td>1:20</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>Phospho-Histone H2A.X Rabbit mAb, 9718</td>
<td>Cell Signaling tech.</td>
<td>1:250</td>
<td>60 min.</td>
</tr>
<tr>
<td></td>
<td>Swine anti-rabbit HRP, P0217</td>
<td>Dako</td>
<td>1:300</td>
<td>30 min.</td>
</tr>
</tbody>
</table>

*Antibodies and sera were diluted in Normal Antibody Diluent (ImmunoLogic, Duiven, the Netherlands) unless mentioned otherwise. * = Diluted in PBS. † = Diluted in M.O.M. Diluent. RT= room temperature unless mentioned otherwise.