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The role of sex differences in autophagy in the heart during coxsackievirus B3 induced myocarditis

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Abstract

Under normal conditions, autophagy maintains cardiomyocyte health and integrity through turnover of organelles. During stress, oxygen and nutrient deprivation or microbial infection, autophagy prolongs cardiomyocyte survival. Sex differences in induction of cell death may to some extent explain the disparity between the sexes in many human diseases. However, sex differences in gene expression, which regulate cell death and autophagy were so far not taken in consideration to explain the sex bias of viral myocarditis. Coxsackievirus B3 (CVB3) induced myocarditis is a sex-biased disease, with females being substantially less susceptible than males and sex hormones largely determine this bias. CVB3 was shown to induce and subvert the autophagosome for its optimal viral RNA replication. Gene expression analysis on mouse and human, healthy and CVB3 infected, cardiac samples of both sexes, suggests sex differences in autophagy related gene expression. This review discusses the aspects of sex bias in autophagy induction in cardiomyocytes.

Keywords

coxsackievirus B3 (CVB3); autophagy; sex bias; myocarditis

AUTOPHAGY

Autophagy (or autophagocytosis) was identified almost half a century ago as an intracellular pathway that degrades mitochondria and cytoplasmic material [1,2]. In the current view, autophagy occurs as a cellular response to stress, metabolic starvation and amino acid deprivation, in response to misfolded proteins, or infection with intracellular pathogens. Three distinct mechanisms have been identified to date: macroautophagy and microautophagy, which occur throughout the orders of living organisms, and mammalian-restricted chaperone-mediated autophagy (CMA). Common to these diverse pathways is the use of evolutionarily conserved autophagy-related genes (ATG).

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During macroautophagy, double membrane vesicles form around damaged cell organelles or unused proteins, which are then degraded. The starvation-induced degradative autophagy pathway involves the hyperphosphorylation of Atg13 through the protein kinases target of rapamycin (Tor), and phosphoinositide-3-kinase (PI3K), as well as ubiquitin-like conjugation reactions, which induce the lipidation of microtubule-associated protein light chain 3 (LC3), also known as Atg8, and the expansion of autophagic membranes [3,4]. The completed vesicle, termed autophagosome, is dissipated via the subsequent fusion of the autophagosome with lysosome [5]. The regulation of autophagy by autophagy-related proteins and additional proteins is reviewed in detail by Mehrpour et al. [6].

In contrast, microautophagy requires the inclusion of cytoplasmic material into the lysosome by membrane protrusion or invagination [7,8]. During chaperone-mediated autophagy, which is the most selective mechanism, cytosolic chaperones deliver proteins to the surface of lysosomes. These substrate proteins unfold to allow their crossing of the lysosomal membrane, where they are subjected to degradation [9]. In the remaining review, the focus is only on macroautophagy and is simply referred to as autophagy.

AUTOPHAGY IN CARDIOMYOCYTES

The precise role of autophagy in the heart is incompletely understood. As a postmitotic cell, the cardiomyocyte utilizes basal levels of autophagy for general cellular maintenance and organelle homeostasis [10,11]. An upregulation of autophagy to maintain energy accessibility and to support cell remodeling in the heart is essentially stress dependent. When cardiac stress is sustained for extended periods of time, cardiomyocytic remodeling has been shown to occur through alterations of cytoskeletal or mitochondrial architecture [12,13], which was later proposed in part to be mediated via the autophagy pathway [14]. Autophagy is further detected in cardiomyocytes in ischemic hearts [15], as well as in failing cardiomyopathic hearts [16,17], and autophagy inhibition was shown to augment the development of cardiac hypertrophy [18,19]. Cardiac hypertrophy is associated with increased protein synthesis and cells during hypertrophy show structural alteration and dysfunction of intracellular organelles. Although the functional significance of autophagy during cardiac hypertrophy and in the remodeling heart is not fully understood, autophagy may promote protein turnover and the removal of damaged proteins or organelles, which could otherwise pose a threat to normal cardiac function [20]. In adult mice, temporally-controlled cardiac-specific deficiency of Atg5 was shown to cause cardiac hypertrophy, left ventricular dilatation and contractile dysfunction, accompanied by increased levels of ubiquitination [20]. Although it was demonstrated that a specific ATG5 gene deletion in cardiomyocytes during early cardiogenesis does not lead to phenotypic alterations under basal conditions, only one week of treatment with pressure overload was shown to cause development of cardiac dysfunction and left ventricular dilatation [20]. This suggests that the upregulation of autophagy in a diseased heart could be an adaptive protective response against hemodynamic or neurohormonal stresses.

AUTOPHAGY IN CARDIOMYOCYTES DURING VIRAL INFECTION

Autophagy is also a cellular mechanism to clear intracellular pathogens, and therefore being beneficial to the host [21,22]. Various microorganisms have developed molecular strategies to escape or to counteract autophagy for their own replication advantage and survival. Historically, poliovirus, which belongs to the same Picornaviridae family as CVB3, was the first RNA virus showing characteristic morphological changes by electron microscopy, indicating autophagy [23]. On another hand, the RNA-containing viruses Human Immunodeficiency Virus (HIV)-1 and influenza A virus, as well as the DNA viruses Herpes Simplex Virus (HSV)-1 and Cytomegalovirus (CMV), which was shown to play a role in the development of atherosclerosis, were shown to suppress autophagy by inhibition of autophagosome maturation through the physical interaction of viral proteins with autophagosomal proteins [24–27].

The replication of CVB3, which is a major cause of viral myocarditis, relies on the rearrangement of intracellular membranes into double-membrane vesicles [28,29]. CVB3 was reported to hitchhike the autophagic pathway to gain a replication advantage on the surface of autophagosomes (Figure 1) [28]. In human HeLa or HEK 293T cell lines infected with CVB3, ultrastructural analysis revealed accumulations of morphological fairly uniform double-membrane vesicles, and the LC3-II/LC3-I ratio showed correspondingly a characteristic increase [28], which indicates that increased basal autophagy of cardiomyocytes could promote viral replication. Confirming this observation, in the presence of the PI3K-inhibitor 3-Methyladenine (3-MA), the expression of the capsid viral protein 1 (VP1) was shown to decrease, but rapamycin-treatment and starving conditions increased viral titers [28]. Moreover, increased levels of intracellular VP1, and increased viral release were observed upon knockdown of the lysosome-associated membrane protein 2 (LAMP-2) [28]. LAMP-2 deletion in mice was previously shown to induce a massive accumulation of autophagic vacuoles in various tissues, especially in cardiac myocytes, which in turn lead to reduced heart contractility [30].

Although autophagosome formation is induced during CVB3 infection the maturation of autophagosome and autophagic protein degradation was shown to be inhibited [28,31]. For example, during CVB3 infection the expression of the autophagy-mediated degradation marker p62 was shown not to be altered, indicating the absence of protein degradation by the lysosome and a lack of viral clearance [28]. In vivo experiments with transgenic mice expressing LC3 conjugated with green fluorescent protein (GFP) revealed a steady augmentation of LC3-II levels in pancreatic acinar cells, and protein degradation was inhibited [31]. Furthermore, the large GFP-LC3 containing structures were shown in infected cells, but the fact that GFP-LC3 was confined to localized puncta, which did not co-localize with LAMP-2, indicated rather a blockade of autophagic flux. In addition to characteristically sized autophagosomes, small vesicles resembling autophagosomes – similar to the vesicles described during infection with poliovirus - were observed in high amounts in CVB3 infected cells [31].

In summary, it is apparent that CVB3 induces productive autophagy to promote its own replication and utilize it as a source for metabolites. However, the subversion of the

autophagy machinery by inhibition of autolysosome maturation and therefore autophagic degradation by CVB3 virus, may contribute also to the pathogenesis of viral myocarditis beyond impacting cardiomyocyte viability.

Since autophagy was shown to play a role in viral RNA sensing via toll-like receptor 3 (TLR3) signaling, dysregulation of the autophagy pathway in CVB3-infected cardiomyocytes may interfere with TLR3-mediated antiviral response, resulting in compromised viral clearance and increased myocardial damage during viremia, which is necessary for the antiviral interferon pathway [32]. Indeed, TLR3-deficient mice show vulnerability to CVB3 infection and develop acute myocarditis [33].

The picornavirus capability of autophagosome induction is not only important for type I IFN production, but might also have function regarding myocarditis induction. Autophagosomal epitope processing could be an effective primary target for CVB3-induced autoimmunity. CD4⁺ T cells reactive to cardiac myosin- α were shown to induce autoimmune myocarditis after being adoptively transferred into uninfected recipients in a mouse model [34,35]. Classically, CD4⁺ T cells recognize exogenous antigens which are loaded onto major histocompatibility complex class II (MHC II) molecules through the endosome pathway [36]. It is now well recognized that autophagy of self-proteins provides an effective pathway for endosome loading of MHC II molecules from autophagosomes [37]. In this manner, myosin or other heart protein-specific autoimmune CD4⁺ T cells should be able to directly interact with the cardiomyocyte leading to myocyte death or dysfunction. The mechanism by which CVB3 upregulates autophagosome formation, but at the same time restricts autophagic degradation, is unknown.

INTERPLAY OF AUTOPHAGY AND CELL DEATH IN CARDIOMYOCYTES

An increase of polyubiquitinated proteins due to insufficient induction of autophagy in cardiomyocytes may increase endoplasmic reticulum stress and apoptosis. Thus, the balance between autophagy, apoptosis and necrosis is a key for therapeutic intervention in heart diseases [38]. In fact, features of all three processes, autophagy, apoptosis and necroptosis, were concurrently observed in failing hearts [39]. Autophagy is known to be a pro-survival response against apoptosis. The dysregulation of autophagy may decrease the viability of virus-infected cardiomyocytes because it cannot protect the host from virus-induced apoptosis.

It was shown that the type I and II angiotensin II receptors (AT1 and AT2) regulate cardiomyocyte autophagy activity [40]. AT1 was shown to trigger autophagy in neonatal cardiomyocytes as well as subsequent autophagic cell death and AT2 counteract the process [40]. The alteration by AT1 and AT2 may have a contrary effect on virus-infected cardiomyocytes as they will rather propagate viral replication thus triggering autophagic cell death.

An important protein that regulates not only autophagy and apoptosis, but also necroptosis is the cellular FLICE-inhibitory protein (c-FLIP) [41–45], which was also shown to play a role in cardioprotection, and is therefore currently considered to be a target for gene therapeutic treatment of heart failure after myocardial infarction [46]. c-FLIP is an enzymatically

inactive paralogue of apoptosis-inducing caspase-8, and participates in the tumor necrosis factor (TNF) signaling pathway [47]. It occurs naturally in a long (c-FLIP_L), and the alternatively spliced short isoform (c-FLIP_S) [48]. Intriguingly, several viruses have usurped FLIP (v-FLIP), but always only the short form [49]. Although all forms of FLIP can heterodimerize with caspase-8, the C-terminal caspase-8 activation loop is only present in c-FLIP_L [50,51]. By contrast, c-FLIP_S and v-FLIP reduce caspase-8 activity. In addition, the short forms of c-FLIP also suppress ground state autophagy by preventing binding and processing of microtubule-associated protein light chain 3 (LC3) by autophagy related gene 3 (ATG3) [43]. FLIP over-expression represses cell death by autophagy, as induced by rapamycin, an mammalian Target of rapamycin (mTor) inhibitor [43]. In contrast, c-FLIP_S was also shown to possess anti-apoptotic properties during viral infection by enhancing autophagosome formation in an effort to clear virus and to extend cell survival [52]. This observation is supported by the demonstration that nitrosylation of FLIP inhibits Fas-ligand induced activation of NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) under physiological condition [53], and endogenous nitric oxide production was shown to promote autophagy [54]. The exact role of FLIP in autophagy, especially during viral infections, is still not fully understood. Our preliminary data obtained during infection with CVB3 suggest that c-FLIP_S supports autophagy and therefore enhances CVB3 replication (Buskiewicz, unpublished). Furthermore, we found evidence that c-FLIP_S, but not c-FLIP_L, interferes with the antiviral retinoic acid inducible gene I (RIG-I) helicase signaling pathway, which was shown to protect the heart during CVB3 infection [55]. Recent evidence indicates that the autophagy related proteins Atg5-Atg12 will interfere with RIG-I pathway mediated type I interferon (IFN) signaling through direct interaction with RIG-I helicases and the mitochondrial antiviral-signaling protein (MAVS) via their caspase recruitment domains (CARD) [56]. Thus, in contrast, it appears that a member of the autophagic mechanism blocks innate immune responses, contrary to anti-pathogenic properties, and hence promoting viral replication. If FLIP protein and its isoforms can differentially balance between autophagy, apoptosis, necropotosis and innate immunity in response to viral infections, this would be a new and highly novel area for further investigations.

AUTOPHAGY IN HEART DISEASE

Sex differences in susceptibility to heart diseases

The relevance of gender for cardiovascular disease and normal heart function has been extensively investigated [57,58]. Women live longer than men and experience fewer atherosclerotic cardiovascular events [59]. Although the majority of autoimmune diseases, for example rheumatoid arthritis, systemic lupus erythematosus or Grave's disease occur predominately in females, CVB3-associated myocarditis prevails in males [60]. Men are twice as likely to contract severe myocarditis as women, except during the last trimester of pregnancy, when women can also develop severe and extensive heart damage [61,62]. As in humans, male and pregnant female mice develop significantly more cardiac injury and have greater concentrations of virus in their hearts than virgin females [60]. Males develop a CD4⁺ Th1 inflammatory response, whereas females develop a CD4⁺ Th2 response, and only males activate heart-antigen reactive autoimmune CD8⁺ effector T cells leading to

myocarditis [63]. Various mechanisms have been suggested to explain the sex-biased susceptibility differences, and several factors, such as TLRs, as well as regulatory and $\gamma\delta^+$ T-cells, were identified to contribute to this sex bias in myocarditis susceptibility [63,64]. The gender-influence of myocarditis susceptibility is particularly dependent on sex-associated hormones, as androgens such as testosterone and progesterone enhance disease, while estrogen inhibits it [60].

Sex-biased differences in autophagy and the development of heart disease

Sex-based differences in mechanisms of protection in cardiac injury have been previously shown to be associated with estrogen-dependent inhibition of apoptosis under base conditions [65,66]. Most of the observations focused on the changes of Bcl2 family, including both pro-apoptotic and anti-apoptotic proteins. It was shown that sex affects autophagy but the nature of the sex effect can differ depending upon cell type, tissue, organism and type of microbial infection [67–70]. Most studies now show that autophagy limits myocyte death during acute ischemia and reperfusion injury, and consequently improves cardiac function [71–73]. It was demonstrated in Syrian hamsters that the autophagy-markers Beclin-1 and LC3-II were increased in females compared to males [69], and other studies demonstrated autophagy stimulation by 17β -estradiol and progesterone in bovine mammary epithelial cells [68]. It was furthermore proposed that estrogen receptor- α facilitates cardioprotection in hearts of male and female TNF receptor (TNFR) knockout mice, in which oxidative stress and autophagy was induced [74]. Augmented signaling of signal transducer and activator of transcription 3 (STAT3) in females, possibly concomitant with the induction of autophagy, was suggested to be an origin of sex differences in protection in mouse myocarditis model [74,75]. On the contrary, augmented autophagosome formation, lower mitochondrial respiration and an increase in cell death was shown in nutrient-deprived neurons of males, versus similarly treated neurons from females [76]. These investigators did not observe a similar difference in fibroblasts, indicating that sex differences in autophagy may be tissue specific. In cancer induced cardiac atrophy in mice, autophagy was increased three-fold in males than females [77]. In this model, estrogen signaling through its receptor conferred partial protection to the females implying a suppressive effect on autophagy. The potential conclusion therefore is that sex bias matters in autophagy, but that the sex effect may not be the same under all circumstances. In certain tissues or certain situations males might develop better autophagy than females, while in other cases females may give the better autophagy response.

Based on the literature that autophagy may impact cardiovascular diseases and exhibit a sex bias, it is relevant to consider whether autophagy is differentially regulated or induced in male and female mouse hearts during CVB3 infection, and if autophagy has a beneficial or detrimental impact on viral myocarditis. The question is whether myocytes of males have higher autophagy baseline activity, or through which mechanism CVB3 upregulates, and the host downregulates autophagosome-formation upon infection.

Microarray and proteomic data identified sex-associated gene expression in healthy human male versus female hearts [78]. Besides allosomal genes, sex-biased gene expression was also detected on autosomal chromosomes. Of particular interest are α -carbonic anhydrase

genes, which were found to be down-regulated in both humans and mice [79,78]. Although not directly related to autophagy induction, this cytolytic protein is found to localize at the very early stages during stress to autophagosomes and lysosomes [80], and expression in the heart contributes to cardiac hypertrophy and heart failure [81]. We have analyzed the Affymetrix datasets (HgU133 Plus 2.0 array) of human myocardial samples from healthy organ donors (n = 6 men and women, age between 40-70, respectively) and failing hearts of CVB3-infected men before transplantation (MAS5.0 data were obtained from Genomics of Cardiovascular Development, Adaptation, and Remodeling, NHLBI Program for Genomic Applications, Harvard Medical School. URL: <http://www.cardiogenomics.org>). Our analysis of the deposited human array confirmed the sex-biased expression of carbonic anhydrase 3 [79], and further identified that apolipoprotein 6L and apolipoprotein J/clusterin were significantly down regulated in human male hearts, as compared to females (Table 1). In atherosclerotic cells apolipoprotein L6 promotes apoptosis and induces atherosclerotic lesions by blockade of beclin1-dependent autophagy [82]. Apolipoprotein J/clusterin, on another hand, has been hypothesized to be cytoprotective and be induced during autoimmune myocarditis and numerous other inflammatory injuries [83]. The comparison of protein expression in the hearts from male patients suffering from CVB3 myocarditis indicates that apolipoprotein L6 is significantly upregulated in failing myocarditic hearts (Table 1). Another significantly upregulated autophagy-related protein in the failing human male heart was Toll like receptor 8 (TLR8) (Table 1). Interestingly, in intestinal epithelial cells the production of TLR-mediated interleukin-8 requires autophagy [84], and impaired TLR8 signaling was detected in multiple sclerosis [85]. Furthermore, the inhibition of HIV-1 through vitamin D-mediated autophagy was shown to be induced by TLR8 ligands [86]. We demonstrated previously in the mouse model a significant sex-dependent difference of TLR2 and TLR4 expression at an early stage of CVB3-infection (i.e. three days) on the levels of cardiac mRNA and lymphoid cell protein [64].

The identified differences in human genes between sexes in the healthy individuals provide the necessary basis for a better understanding of the physiological differences in cardiomyocyte physiology. However, since there are no samples of CVB3-infected female human hearts available, the human microarray analysis cannot explain and/or predict which genes will change their expression profile during viral infection. Furthermore, the human samples represent the late stages of myocarditis, where gene expression changes rather represent the response of myocytes to adaptive immune response. To address the question how gene expression changes at the very early stages after CVB3 infection in the heart of both sexes, we have analyzed the recently acquired and published microarray data obtained from CVB3-infected cardiac tissues from male and female mice [64], with special emphasis focus on autophagy and its correlation to the innate immune response to CVB3 infection.

As described previously, the RNA for microarray analysis was obtained from male and female C57Bl/6 mice, which were uninfected or infected with 10^2 PFU of CVB3 and evaluated for myocarditis and cardiac virus titers at 3 and 6 days post infection [64]. In our previous studies myocarditic inflammation was not observed in either male or female mice 3 days post infection, but by day 6, both male and female mice showed signs of cardiac inflammation, with male mice having a higher myocarditis score than female mice (mean cardiac score 0.54 ± 0.11 for females and 1.75 ± 0.11 for males, $p < 0.001$) [64]. We have

further analyzed the RNA for the presence of viral genome, and could confirm that at both days 3 and 6 after infection CVB3 RNA was present in the heart of both female and male mice. Therefore we considered that the data obtained from day 3 samples represent the innate immune response in gene expression of cardiomyocytes, versus gene expression of day 6 should also show changes of gene expression in response to myocarditis induction. Three representative hearts from each group were chosen, based first on histology score to ensure infection, and then based on RNA quality and amount of RNA recovered. Samples were individually run on the Affymetrix Mouse Gene 1.0st Array Chip. Individual results were averaged by group and analyzed by the University of Vermont Bioinformatics group as described previously [64].

Interestingly, we have observed a differential basal expression of ATG5 between males and females, where females possess higher expression levels (Table 2). Remarkably, the expression of ATG5 seems to be suppressed following infection, but only significantly in female mice (Table 2). Surprisingly, pathway analysis of the same data indicates that the differences in ATG5 gene expression between the sexes correlate with the gene expression of RIG-I pathway. RIG-I pathway was shown to be suppressed by Atg5–Atg12 conjugate and block innate antiviral immune responses, thereby contributing to RNA virus replication in host cells. [56]. We have observed a high upregulation of both RIG-I-like helicases RIG-I and MDA5 (Melanoma Differentiation-Associated protein 5) in female and male mice, with the greater prevalence in females compared to males. The highest expression is observed for RIG-I helicase at day 3 after infection (~ 11 fold increase) rather than for MDA5, although it was previously demonstrated that MDA5 is the primary helicase recognizing picornaviruses [87]. Unlike MDA5, RIG-I is proteolytically cleaved by the picornaviral proteinase 3C^{pro} [88], which suggests a role of RIG-I during the earliest stages of viral entry and replication. Furthermore, newer studies indicate a redundancy of viral recognition by both RIG-I and MDA5 for various viruses such as West Nile and Dengue virus, as well as for a vaccine strain of measles virus [89-91]. The reverse correlation between ATG5 and RIG-I helicase expression suggests that the innate immune response may be inhibited by the autophagic pathway, which may contribute to decreased type I IFN secretion and viral replication. The decreased level of ATG5 in females during CVB3 infection would then suggest that at the early stages of infection female mice are protected having lower ATG5 gene expression to promote viral replication and higher RIG-I helicase expression to stimulate type I interferon secretion. An alternative possibility is that the augmented RIG-I expression in infected females suppresses autophagy by sequestering ATG5 protein from the autophagosome. So far it is not known whether RIG-I signaling may suppress or block autophagy, and why the Atg5–Atg12 complex targets this pathway. It is an interesting aspect for further investigation, since autophagy occurs also under physiological conditions and because so far the autophagic process cannot be inhibited by a specific reagent without influencing other signaling pathways.

A further gene which was differentially regulated between the sexes in heart tissue was c-FLIP. Although c-FLIP_S was shown to suppress RIG-I pathway, the long form was shown in our studies to protect the heart in mouse model from myocarditis (Buskewicz unpublished) [92,93]. The expression pattern of c-FLIP_L in the heart follows the trend shown for the RIG-I helicase pathway. The c-FLIP gene expression increases in females at 3 days post

infection, then declines with time to match c-FLIP expression in male littermates. The evaluation of expression changes of different splice variants of FLIP to fully understand how these variants regulate both autophagy and innate immune response during CVB3 infection will be of interest. FLIP expression is regulated on transcriptional, translational, and post-translational levels, as well as through protein–protein interactions. Under normal physiological conditions, FLIP is expressed at low levels and has a mostly pro-apoptotic function, and current studies point to a correlation of caspase-8 inhibition and disproportionate autophagy [94,95]. It still has to be addressed if c-FLIP can regulate autophagy directly, or if caspase-8 regulates autophagy, and FLIP contributes through the regulation of caspase-8 activity during CVB3 or other viral infections.

MICROARRAY

Mouse microarray data used in this review were previously published [64]. In brief, three representative male or female hearts were chosen day 3 and 6 after infection. The choice was based on histology score (to confirm infection), then on the quality and amount of the recovered RNA. The Affymetrix Mouse Gene 1.0st Array Chip was used for individual sample runs. Individual results were averaged by group and analyzed by the University of Vermont Bioinformatics group. Microarray data has been submitted to the Gene Expression Omnibus, and we are currently awaiting their reply.

For analysis of sex differences in human gene expression in hearts we used a publicly available gene array data set (Cardiogenomics Consortium [<http://www.cardiogenomics.med.harvard.edu>]) that was obtained from the explanted hearts of 6 patients with viral cardiomyopathy and 6 female and male patients with non-failing hearts (age between 40–70, respectively). The Affymetrix HG-U133 plus 2.0 oligonucleotide microarray platform was used for gene array. The data were also analyzed as previously published [64].

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References

1. Deter RL, De Duve C. Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. *The Journal of cell biology*. 1967; 33 (2):437–449. [PubMed: 4292315]
2. Fedorko M. Effect of chloroquine on morphology of cytoplasmic granules in maturing human leukocytes--an ultrastructural study. *The Journal of clinical investigation*. 1967; 46 (12):1932–1942.10.1172/JCI105683 [PubMed: 6073998]
3. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental cell*. 2004; 6 (4):463–477. [PubMed: 15068787]
4. Pattingre S, Espert L, Biard-Piechaczyk M, Codogno P. Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie*. 2008; 90 (2):313–323.10.1016/j.biochi.2007.08.014 [PubMed: 17928127]

5. Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. *Cell structure and function*. 2002; 27 (6):421–429. [PubMed: 12576635]
6. Mehrpour M, Esclatine A, Beau I, Codogno P. Autophagy in health and disease. 1. Regulation and significance of autophagy: an overview. *American journal of physiology Cell physiology*. 2010; 298 (4):C776–785.10.1152/ajpcell.00507.2009 [PubMed: 20089931]
7. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. *Cellular and molecular life sciences : CMLS*. 2012; 69 (7):1125–1136.10.1007/s00018-011-0865-5 [PubMed: 22080117]
8. Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. *Autophagy*. 2011; 7 (7):673–682. [PubMed: 21646866]
9. Bandyopadhyay U, Cuervo AM. Entering the lysosome through a transient gate by chaperone-mediated autophagy. *Autophagy*. 2008; 4 (8):1101–1103. [PubMed: 18927485]
10. De Meyer GR, Martinet W. Autophagy in the cardiovascular system. *Biochimica et biophysica acta*. 2009; 1793 (9):1485–1495.10.1016/j.bbamcr.2008.12.011 [PubMed: 19152812]
11. Gottlieb RA, Finley KD, Mentzer RM Jr. Cardioprotection requires taking out the trash. *Basic research in cardiology*. 2009; 104 (2):169–180.10.1007/s00395-009-0011-9 [PubMed: 19242643]
12. Hein S, Kostin S, Heling A, Maeno Y, Schaper J. The role of the cytoskeleton in heart failure. *Cardiovascular research*. 2000; 45 (2):273–278. [PubMed: 10728347]
13. Piquereau J, Caffin F, Novotova M, Lemaire C, Veksler V, Garnier A, Ventura-Clapier R, Joubert F. Mitochondrial dynamics in the adult cardiomyocytes: which roles for a highly specialized cell? *Frontiers in physiology*. 2013; 4:102.10.3389/fphys.2013.00102 [PubMed: 23675354]
14. An L, Zhao X, Wu J, Jia J, Zou Y, Guo X, He L, Zhu H. Involvement of autophagy in cardiac remodeling in transgenic mice with cardiac specific over-expression of human programmed cell death 5. *PloS one*. 2012; 7 (1):e30097.10.1371/journal.pone.0030097 [PubMed: 22253891]
15. Yan L, Vatner DE, Kim SJ, Ge H, Masarekar M, Massover WH, Yang G, Matsui Y, Sadoshima J, Vatner SF. Autophagy in chronically ischemic myocardium. *Proc Natl Acad Sci U S A*. 2005; 102 (39):13807–13812.10.1073/pnas.0506843102 [PubMed: 16174725]
16. Shimomura H, Terasaki F, Hayashi T, Kitauro Y, Isomura T, Suma H. Autophagic degeneration as a possible mechanism of myocardial cell death in dilated cardiomyopathy. *Japanese circulation journal*. 2001; 65 (11):965–968. [PubMed: 11716248]
17. Miyata S, Takemura G, Kawase Y, Li Y, Okada H, Maruyama R, Ushikoshi H, Esaki M, Kanamori H, Li L, Misao Y, Tezuka A, Toyo-Oka T, Minatoguchi S, Fujiwara T, Fujiwara H. Autophagic cardiomyocyte death in cardiomyopathic hamsters and its prevention by granulocyte colony-stimulating factor. *The American journal of pathology*. 2006; 168 (2):386–397.10.2353/ajpath.2006.050137 [PubMed: 16436654]
18. Dammrich J, Pfeifer U. Cardiac hypertrophy in rats after supraaortic constriction. II. Inhibition of cellular autophagy in hypertrophying cardiomyocytes. *Virchows Archiv B, Cell pathology including molecular pathology*. 1983; 43 (3):287–307.
19. Pfeifer U, Fohr J, Wilhelm W, Dammrich J. Short-term inhibition of cardiac cellular autophagy by isoproterenol. *Journal of molecular and cellular cardiology*. 1987; 19 (12):1179–1184. [PubMed: 3443985]
20. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, Nishida K, Hori M, Mizushima N, Otsu K. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nature medicine*. 2007; 13 (5):619–624.10.1038/nm1574
21. Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell*. 2005; 120 (2):159–162.10.1016/j.cell.2005.01.005 [PubMed: 15680321]
22. Richetta C, Faure M. Autophagy in antiviral innate immunity. *Cellular microbiology*. 2013; 15 (3):368–376.10.1111/cmi.12043 [PubMed: 23051682]
23. Kallman F, Williams RC, Dulbecco R, Vogt M. Fine structure of changes produced in cultured cells sampled at specified intervals during a single growth cycle of polio virus. *The Journal of biophysical and biochemical cytology*. 1958; 4 (3):301–308. [PubMed: 13549502]
24. Chaumorcet M, Lussignol M, Mouna L, Cavignac Y, Fahie K, Cotte-Laffitte J, Geballe A, Brune W, Beau I, Codogno P, Esclatine A. The human cytomegalovirus protein TRS1 inhibits autophagy

- via its interaction with Beclin 1. *Journal of virology*. 2012; 86 (5):2571–2584.10.1128/JVI.05746-11 [PubMed: 22205736]
25. Gannage M, Dormann D, Albrecht R, Dengjel J, Torossi T, Ramer PC, Lee M, Strowig T, Arrey F, Conenello G, Pypaert M, Andersen J, Garcia-Sastre A, Munz C. Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. *Cell host & microbe*. 2009; 6 (4):367–380.10.1016/j.chom.2009.09.005 [PubMed: 19837376]
 26. Kyei GB, Dinkins C, Davis AS, Roberts E, Singh SB, Dong C, Wu L, Kominami E, Ueno T, Yamamoto A, Federico M, Panganiban A, Vergne I, Deretic V. Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. *The Journal of cell biology*. 2009; 186 (2):255–268.10.1083/jcb.200903070 [PubMed: 19635843]
 27. Orvedahl A, Alexander D, Talloczy Z, Sun Q, Wei Y, Zhang W, Burns D, Leib DA, Levine B. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell host & microbe*. 2007; 1 (1):23–35.10.1016/j.chom.2006.12.001 [PubMed: 18005679]
 28. Wong J, Zhang J, Si X, Gao G, Mao I, McManus BM, Luo H. Autophagosome supports coxsackievirus B3 replication in host cells. *J Virol*. 2008; 82 (18):9143–9153.10.1128/JVI.00641-08 [PubMed: 18596087]
 29. Yoon SY, Ha YE, Choi JE, Ahn J, Lee H, Kweon HS, Lee JY, Kim DH. Coxsackievirus B4 uses autophagy for replication after calpain activation in rat primary neurons. *Journal of virology*. 2008; 82 (23):11976–11978.10.1128/JVI.01028-08 [PubMed: 18799585]
 30. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, Janssen PM, Blanz J, von Figura K, Saftig P. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature*. 2000; 406 (6798):902–906.10.1038/35022595 [PubMed: 10972293]
 31. Kemball CC, Alirezaei M, Flynn CT, Wood MR, Harkins S, Kiosses WB, Whitton JL. Coxsackievirus infection induces autophagy-like vesicles and megaphagosomes in pancreatic acinar cells in vivo. *J Virol*. 2010; 84 (23):12110–12124.10.1128/JVI.01417-10 [PubMed: 20861268]
 32. Gorbea C, Makar KA, Pauschinger M, Pratt G, Bersola JL, Varela J, David RM, Banks L, Huang CH, Li H, Schultheiss HP, Towbin JA, Vallejo JG, Bowles NE. A role for Toll-like receptor 3 variants in host susceptibility to enteroviral myocarditis and dilated cardiomyopathy. *J Biol Chem*. 2010; 285 (30):23208–23223.10.1074/jbc.M109.047464 [PubMed: 20472559]
 33. Negishi H, Osawa T, Ogami K, Ouyang X, Sakaguchi S, Koshiba R, Yanai H, Seko Y, Shitara H, Bishop K, Yonekawa H, Tamura T, Kaisho T, Taya C, Taniguchi T, Honda K. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. *Proc Natl Acad Sci U S A*. 2008; 105 (51):20446–20451.10.1073/pnas.0810372105 [PubMed: 19074283]
 34. Gangaplara A, Massilamany C, Brown DM, Delhon G, Pattnaik AK, Chapman N, Rose N, Steffen D, Reddy J. Coxsackievirus B3 infection leads to the generation of cardiac myosin heavy chain-alpha-reactive CD4 T cells in A/J mice. *Clinical immunology*. 2012; 144 (3):237–249.10.1016/j.clim.2012.07.003 [PubMed: 22854287]
 35. Ratcliffe NR, Hutchins J, Barry B, Hickey WF. Chronic myocarditis induced by T cells reactive to a single cardiac myosin peptide: persistent inflammation, cardiac dilatation, myocardial scarring and continuous myocyte apoptosis. *Journal of autoimmunity*. 2000; 15 (3):359–367.10.1006/jaut.2000.0432 [PubMed: 11040076]
 36. Steinman RM, Inaba K. The binding of antigen presenting cells to T lymphocytes. *Advances in experimental medicine and biology*. 1988; 237:31–41. [PubMed: 3075854]
 37. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity*. 2007; 26 (1):79–92.10.1016/j.immuni.2006.10.018 [PubMed: 17182262]
 38. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *The Journal of clinical investigation*. 2005; 115 (10):2656–2664.10.1172/JCI26373 [PubMed: 16200199]
 39. Kostin S, Pool L, Elsasser A, Hein S, Drexler HC, Arnon E, Hayakawa Y, Zimmermann R, Bauer E, Klovekorn WP, Schaper J. Myocytes die by multiple mechanisms in failing human hearts. *Circulation research*. 2003; 92 (7):715–724.10.1161/01.RES.0000067471.95890.5C [PubMed: 12649263]

40. Porrello ER, Delbridge LM. Cardiomyocyte autophagy is regulated by angiotensin II type 1 and type 2 receptors. *Autophagy*. 2009; 5 (8):1215–1216. [PubMed: 19955853]
41. Oberst A, Green DR. It cuts both ways: reconciling the dual roles of caspase 8 in cell death and survival. *Nature reviews Molecular cell biology*. 2011; 12 (11):757–763.10.1038/nrm3214
42. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature*. 2011; 471 (7338):363–367. nature09852 [pii]. 10.1038/nature09852 [PubMed: 21368763]
43. Lee JS, Li Q, Lee JY, Lee SH, Jeong JH, Lee HR, Chang H, Zhou FC, Gao SJ, Liang C, Jung JU. FLIP-mediated autophagy regulation in cell death control. *Nat Cell Biol*. 2009; 11 (11):1355–1362. ncb1980 [pii]. 10.1038/ncb1980 [PubMed: 19838173]
44. He MX, He YW. A role for c-FLIP(L) in the regulation of apoptosis, autophagy, and necroptosis in T lymphocytes. *Cell Death Differ*. 2013; 20 (2):188–197.10.1038/cdd.2012.148 [PubMed: 23175183]
45. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, MacFarlane M, Hacker G, Leverkus M. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell*. 2011; 43 (3): 449–463. S1097-2765(11)00452-7 [pii]. 10.1016/j.molcel.2011.06.011 [PubMed: 21737330]
46. Xiao J, Moon M, Yan L, Nian M, Zhang Y, Liu C, Lu J, Guan H, Chen M, Jiang D, Jiang H, Liu PP, Li H. Cellular FLICE-inhibitory protein protects against cardiac remodelling after myocardial infarction. *Basic research in cardiology*. 2012; 107 (1):239.10.1007/s00395-011-0239-z [PubMed: 22202974]
47. Kataoka T. The caspase-8 modulator c-FLIP. *Critical reviews in immunology*. 2005; 25 (1):31–58. [PubMed: 15833082]
48. Irmeler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. *Nature*. 1997; 388 (6638):190–195.10.1038/40657 [PubMed: 9217161]
49. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinel E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature*. 1997; 386 (6624):517–521.10.1038/386517a0 [PubMed: 9087414]
50. Dohrman A, Russell JQ, Cuenin S, Fortner K, Tschopp J, Budd RC. Cellular FLIP long form augments caspase activity and death of T cells through heterodimerization with and activation of caspase-8. *Journal of immunology*. 2005; 175 (1):311–318.
51. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grutter MG. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem*. 2002; 277(47):45162–45171. M206882200 [pii]. 10.1074/jbc.M206882200 [PubMed: 12215447]
52. Ritthipichai K, Nan Y, Bossis I, Zhang Y. Viral FLICE inhibitory protein of rhesus monkey rhadinovirus inhibits apoptosis by enhancing autophagosome formation. *PloS one*. 2012; 7 (6):e39438.10.1371/journal.pone.0039438 [PubMed: 22745754]
53. Iyer AK, Azad N, Talbot S, Stehlik C, Lu B, Wang L, Rojanasakul Y. Antioxidant c-FLIP inhibits Fas ligand-induced NF-kappaB activation in a phosphatidylinositol 3-kinase/Akt-dependent manner. *J Immunol*. 2011; 187 (6):3256–3266.10.4049/jimmunol.1002915 [PubMed: 21856935]
54. Halder SM, Stamler JS. S-Nitrosylation at the interface of autophagy and disease. *Mol Cell*. 2011; 43 (1):1–3.10.1016/j.molcel.2011.06.014 [PubMed: 21726803]
55. Buskiewicz IA, Huber SA. MDA5: The Almighty for Myocardium. *Circulation Heart failure*. 2013; 6 (2):153–155.10.1161/CIRCHEARTFAILURE.113.000137 [PubMed: 23513048]
56. Jounai N, Takeshita F, Kobiyama K, Sawano A, Miyawaki A, Xin KQ, Ishii KJ, Kawai T, Akira S, Suzuki K, Okuda K. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc Natl Acad Sci U S A*. 2007; 104 (35):14050–14055.10.1073/pnas.0704014104 [PubMed: 17709747]

57. Du XJ, Fang L, Kiriazis H. Sex dimorphism in cardiac pathophysiology: experimental findings, hormonal mechanisms, and molecular mechanisms. *Pharmacology & therapeutics*. 2006; 111 (2): 434–475.10.1016/j.pharmthera.2005.10.016 [PubMed: 16439025]
58. Petrea RE, Beiser AS, Seshadri S, Kelly-Hayes M, Kase CS, Wolf PA. Gender differences in stroke incidence and poststroke disability in the Framingham heart study. *Stroke; a journal of cerebral circulation*. 2009; 40 (4):1032–1037.10.1161/STROKEAHA.108.542894
59. Tegos TJ, Kalodiki E, Sabetai MM, Nicolaides AN. The genesis of atherosclerosis and risk factors: a review. *Angiology*. 2001; 52 (2):89–98. [PubMed: 11228092]
60. Huber SA. Role of estrogen in suppressing autoimmunity in coxsackievirus B3-induced myocarditis. *Future Virology*. 2010; 5 (3):273–286.10.2217/fvl.10.19 [PubMed: 20963181]
61. Fairweather D, Cooper LT Jr, Blauwet LA. Sex and gender differences in myocarditis and dilated cardiomyopathy. *Current problems in cardiology*. 2013; 38 (1):7–46.10.1016/j.cpcardiol.2012.07.003 [PubMed: 23158412]
62. Woodruff JF. Viral myocarditis. A review. *The American journal of pathology*. 1980; 101 (2):425–484. [PubMed: 6254364]
63. Reddy J, Massilamany C, Buskiewicz I, Huber SA. Autoimmunity in viral myocarditis. *Current opinion in rheumatology*. 2013; 25 (4):502–508.10.1097/BOR.0b013e3283620036 [PubMed: 23656709]
64. Roberts BJ, Dragon JA, Moussawi M, Huber SA. Sex-specific signaling through Toll-Like Receptors 2 and 4 contributes to survival outcome of Coxsackievirus B3 infection in C57Bl/6 mice. *Biology of sex differences*. 2012; 3 (1):25.10.1186/2042-6410-3-25 [PubMed: 23241283]
65. Murphy PJ, Campbell SS. Sex hormones, sleep, and core body temperature in older postmenopausal women. *Sleep*. 2007; 30 (12):1788–1794. [PubMed: 18246988]
66. Bouma W, Noma M, Kanemoto S, Matsubara M, Leshnower BG, Hinmon R, Gorman JH 3rd, Gorman RC. Sex-related resistance to myocardial ischemia-reperfusion injury is associated with high constitutive ARC expression. *American journal of physiology Heart and circulatory physiology*. 2010; 298 (5):H1510–1517.10.1152/ajpheart.01021.2009 [PubMed: 20173041]
67. Straface E, Vona R, Gambardella L, Ascione B, Marino M, Bulzomi P, Canu S, Coinu R, Rosano G, Malorni W, Franconi F. Cell sex determines anoikis resistance in vascular smooth muscle cells. *FEBS letters*. 2009; 583 (21):3448–3454.10.1016/j.febslet.2009.09.052 [PubMed: 19804776]
68. Sobolewska A, Gajewska M, Zarzynska J, Gajkowska B, Motyl T. IGF-I, EGF, and sex steroids regulate autophagy in bovine mammary epithelial cells via the mTOR pathway. *European journal of cell biology*. 2009; 88 (2):117–130.10.1016/j.ejcb.2008.09.004 [PubMed: 19013662]
69. Coto-Montes A, Tomas-Zapico C, Martinez-Fraga J, Vega-Naredo I, Sierra V, Caballero B, Huidobro-Fernandez C, Soria-Valles C, Tolivia D, Rodriguez-Colunga MJ. Sexual autophagic differences in the androgen-dependent flank organ of Syrian hamsters. *Journal of andrology*. 2009; 30 (2):113–121.10.2164/jandrol.108.005355 [PubMed: 18930906]
70. Lista P, Straface E, Brunelleschi S, Franconi F, Malorni W. On the role of autophagy in human diseases: a gender perspective. *J Cell Mol Med*. 2011; 15 (7):1443–1457.10.1111/j.1582-4934.2011.01293.x [PubMed: 21362130]
71. Gottlieb RA, Mentzer RM Jr. Cardioprotection through autophagy: ready for clinical trial? *Autophagy*. 2011; 7 (4):434–435. [PubMed: 21187717]
72. Kanamori H, Takemura G, Goto K, Maruyama R, Ono K, Nagao K, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Kawasaki M, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion. *American journal of physiology Heart and circulatory physiology*. 2011; 300 (6):H2261–2271.10.1152/ajpheart.01056.2010 [PubMed: 21421825]
73. Przyklenk K, Undyala VV, Wider J, Sala-Mercado JA, Gottlieb RA, Mentzer RM Jr. Acute induction of autophagy as a novel strategy for cardioprotection: getting to the heart of the matter. *Autophagy*. 2011; 7 (4):432–433. [PubMed: 21187719]
74. Wang M, Crisostomo PR, Markel TA, Wang Y, Meldrum DR. Mechanisms of sex differences in TNFR2-mediated cardioprotection. *Circulation*. 2008; 118 (14 Suppl):S38–45. 118/14_suppl_1/S38 [pii]. 10.1161/CIRCULATIONAHA.107.756890 [PubMed: 18824767]

75. Yoon S, Woo SU, Kang JH, Kim K, Kwon MH, Park S, Shin HJ, Gwak HS, Chwae YJ. STAT3 transcriptional factor activated by reactive oxygen species induces IL6 in starvation-induced autophagy of cancer cells. *Autophagy*. 2010; 6 (8):1125–1138. [PubMed: 20930550]
76. Du L, Hickey RW, Bayir H, Watkins SC, Tyurin VA, Guo F, Kochanek PM, Jenkins LW, Ren J, Gibson G, Chu CT, Kagan VE, Clark RS. Starving neurons show sex difference in autophagy. *The Journal of biological chemistry*. 2009; 284 (4):2383–2396.10.1074/jbc.M804396200 [PubMed: 19036730]
77. Cosper PF, Leinwand LA. Cancer causes cardiac atrophy and autophagy in a sexually dimorphic manner. *Cancer Res*. 2011; 71 (5):1710–1720.10.1158/0008-5472.CAN-10-3145 [PubMed: 21163868]
78. Isensee J, Witt H, Pregla R, Hetzer R, Regitz-Zagrosek V, Noppinger PR. Sexually dimorphic gene expression in the heart of mice and men. *J Mol Med (Berl)*. 2008; 86 (1):61–74.10.1007/s00109-007-0240-z [PubMed: 17646949]
79. Onyimba JA, Coronado MJ, Garton AE, Kim JB, Bucek A, Bedja D, Gabrielson KL, Guilarte TR, Fairweather D. The innate immune response to coxsackievirus B3 predicts progression to cardiovascular disease and heart failure in male mice. *Biology of sex differences*. 2011; 2:2.10.1186/2042-6410-2-2 [PubMed: 21338512]
80. Rabouille C, Strous GJ, Crapo JD, Geuze HJ, Slot JW. The differential degradation of two cytosolic proteins as a tool to monitor autophagy in hepatocytes by immunocytochemistry. *The Journal of cell biology*. 1993; 120 (4):897–908. [PubMed: 8432730]
81. Alvarez BV, Johnson DE, Sowah D, Soliman D, Light PE, Xia Y, Karmazyn M, Casey JR. Carbonic anhydrase inhibition prevents and reverts cardiomyocyte hypertrophy. *The Journal of physiology*. 2007; 579 (Pt 1):127–145.10.1113/jphysiol.2006.123638 [PubMed: 17124262]
82. Zhaorigetu S, Yang Z, Toma I, McCaffrey TA, Hu CA. Apolipoprotein L6, induced in atherosclerotic lesions, promotes apoptosis and blocks Beclin 1-dependent autophagy in atherosclerotic cells. *J Biol Chem*. 2011; 286 (31):27389–27398.10.1074/jbc.M110.210245 [PubMed: 21646352]
83. McLaughlin L, Zhu G, Mistry M, Ley-Ebert C, Stuart WD, Florio CJ, Groen PA, Witt SA, Kimball TR, Witte DP, Harmony JA, Aronow BJ. Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. *The Journal of clinical investigation*. 2000; 106 (9):1105–1113.10.1172/JCI9037 [PubMed: 11067863]
84. Li YY, Ishihara S, Aziz MM, Oka A, Kusunoki R, Tada Y, Yuki T, Amano Y, Ansary MU, Kinoshita Y. Autophagy is required for toll-like receptor-mediated interleukin-8 production in intestinal epithelial cells. *International journal of molecular medicine*. 2011; 27 (3):337–344.10.3892/ijmm.2011.596 [PubMed: 21225224]
85. Johnson TP, Tyagi R, Patel K, Schiess N, Calabresi PA, Nath A. Impaired toll-like receptor 8 signaling in multiple sclerosis. *Journal of neuroinflammation*. 2013; 10:74.10.1186/1742-2094-10-74 [PubMed: 23787171]
86. Campbell GR, Spector SA. Toll-like receptor 8 ligands activate a vitamin D mediated autophagic response that inhibits human immunodeficiency virus type 1. *PLoS Pathog*. 2012; 8 (11):e1003017.10.1371/journal.ppat.1003017 [PubMed: 23166493]
87. Wang JP, Cerny A, Asher DR, Kurt-Jones EA, Bronson RT, Finberg RW. MDA5 and MAVS mediate type I interferon responses to coxsackie B virus. *J Virol*. 2010; 84 (1):254–260. JVI.00631-09 [pii]. 10.1128/JVI.00631-09 [PubMed: 19846534]
88. Barral PM, Sarkar D, Fisher PB, Racaniello VR. RIG-I is cleaved during picornavirus infection. *Virology*. 2009; 391 (2):171–176.10.1016/j.virol.2009.06.045 [PubMed: 19628239]
89. Fredericksen BL, Keller BC, Fornek J, Katze MG, Gale M Jr. Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *J Virol*. 2008; 82 (2):609–616.10.1128/JVI.01305-07 [PubMed: 17977974]
90. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M Jr. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol*. 2008; 82 (1):335–345.10.1128/JVI.01080-07 [PubMed: 17942531]

91. Shingai M, Ebihara T, Begum NA, Kato A, Honma T, Matsumoto K, Saito H, Ogura H, Matsumoto M, Seya T. Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol.* 2007; 179 (9):6123–6133. [PubMed: 17947687]
92. Buskiewicz IA, Koenig A, Huber SA, Budd RC. Caspase-8 and FLIP regulate RIG-I/MDA5-induced innate immune host responses to picornaviruses. *Future Virol.* 2012; 7 (12):1221–1236.10.2217/fvl.12.115 [PubMed: 23503762]
93. Huber S, Dohrman A, Sartini D, Budd RC. Reduced myocarditis following Coxsackievirus infection in cellular FLICE inhibitory protein–long form-transgenic mice. *Immunology.* 2006; 119 (4):541–550. [PubMed: 17010108]
94. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science.* 2004; 304 (5676):1500–1502.10.1126/science.1096645 [PubMed: 15131264]
95. Bell BD, Leverrier S, Weist BM, Newton RH, Arechiga AF, Luhrs KA, Morrisette NS, Walsh CM. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proc Natl Acad Sci U S A.* 2008; 105 (43):16677–16682.10.1073/pnas.0808597105 [PubMed: 18946037]
96. Harb JM, Burch GE. Spherical aggregates of coxsackie B4 virus particles in mouse pancreas. *Beitrage zur Pathologie.* 1975; 156 (2):122–127. [PubMed: 1212165]
97. Alirezai M, Flynn CT, Wood MR, Whitton JL. Pancreatic acinar cell-specific autophagy disruption reduces coxsackievirus replication and pathogenesis in vivo. *Cell host & microbe.* 2012; 11 (3):298–305.10.1016/j.chom.2012.01.014 [PubMed: 22423969]
98. Kembell CC, Alirezai M, Whitton JL. Type B coxsackieviruses and their interactions with the innate and adaptive immune systems. *Future microbiology.* 2010; 5 (9):1329–1347.10.2217/fmb.10.101 [PubMed: 20860480]
99. Patel KK, Miyoshi H, Beatty WL, Head RD, Malvin NP, Cadwell K, Guan JL, Saitoh T, Akira S, Seglen PO, Dinanuer MC, Virgin HW, Stappenbeck TS. Autophagy proteins control goblet cell function by potentiating reactive oxygen species production. *EMBO J.* 2013; 32 (1):201–213.10.1038/emboj.2013.233
100. Munz C. Antigen processing for MHC presentation by autophagy. *F1000 biology reports.* 2010; 2:61.10.3410/B2-61 [PubMed: 21173848]

Figure 1. Divergent role of autophagy during CVB3 infection

This schematic model of autophagy shows that early after infection with CVB3, Beclin 1, a component in the PtdIns3K complex responsible for the initiation of nucleation of the phagophore membrane, is activated enwrapping cytosolic proteins, protein aggregates, and organelles. CVB3 was shown to use the phagophore-attached membranes as scaffolds to support its own translation and replication [96,97,28]. In the next step, Atg12–Atg5–Atg16 and LC3/Atg8, which is covalently linked to the amino group of phosphatidylethanolamine (PE) are recruited to the phagophore to facilitate the phagophore expansion step. Molecular interaction studies indicated that the Atg5–Atg12 conjugate negatively regulates the type I IFN production pathway ($\perp$) by direct association with the retinoic acid-inducible gene I (RIG-I) pathway through the caspase recruitment domains (CARDs) [56]. Upon vesicle completion, most of the ATG proteins are dissociated from the autophagosome and autophagosomes subsequently fuse with endosomes to form an amphisome. It was proposed that upon fusion of endosomes with autophagosome TLR could recognize CVB3 RNA and initiate production of pro-inflammatory cytokines and type I IFN [98]. Ampisomes were recently also shown to be signaling platforms, where NADPH oxidase-driven ROS production promotes the release of the mucin granules [99]. At the next step autophagosome - endosome and/or - lysosome fuse and degrade cargo by lysosomal proteases. Fusion of autophagosome with lysosome was suggested to constitutively and efficiently deliver cytosolic proteins for MHC class II presentation [100], and therefore promote the presentation of products of lysosomal proteolysis to CD4 T cells [100]. CVB3 was suggested to suppress ($\perp$), via an unknown mechanism, the latest stages of autophagy maturation and therefore escape presentation of its RNA to TLRs [98] and/or loading of CVB3 derived antigens onto MHCII.

TABLE 1**AUTOPHAGY RELATED GENE EXPRESSION IN HUMAN HEART**

Gene name	Fold change	<i>P</i> value
<u>Gene expression in the whole heart of healthy men compared to women</u>		
Carbonic anhydrase 3	2.2	0.02
Apolipoprotein L6	−3.7	0.01
ApoJ/clusterin	−1.4	0.04
TLR 8	−1.5	0.08
RIG-I	−1.0	0.70
MDA5	−1.2	0.48
c-FLIP	1.6	0.03
<u>Gene expression in the whole heart of CVB3 infected men compared to healthy men</u>		
Carbonic anhydrase 3	1.2	0.42
Apolipoprotein L6	5.4	0.02
ApoJ/clusterin	1.2	0.01
TLR 8	7.7	0.01
RIG-I	1.4	0.02
MDA5	1.8	0.03
c-FLIP	−1.7	0.02

For analysis of sex differences in human gene expression in hearts we used a publicly available gene array data set (Cardiogenomics Consortium [<http://www.cardiogenomics.med.harvard.edu>]) that was obtained from the explanted hearts of 6 male and female patients with non-failing hearts and 6 male patients with viral cardiomyopathy (please note there are no samples available from female patients with viral cardiomyopathy). The Affymetrix HG-U133 plus 2.0 oligonucleotide microarray platform was used for gene array. The data were also analyzed as previously published [64].

TABLE 2

AUTOPHAGY RELATED GENE EXPRESSION IN MOUSE HEART

ATG5						
Male – Female		Female – Female		Male – Male		
Day (p.i.)	D ₀	D ₃	D ₆	D ₃ – D ₀	D ₆ – D ₀	D ₆ – D ₀
Fold change	-1.7	-1.3	-1.1	-1.9	-1.9	1.0
P value	0.02	0.06	0.56	0.02	0.02	0.99

RIG-I						
Male – Female		Female – Female		Male – Male		
Day (p.i.)	D ₀	D ₃	D ₆	D ₃ – D ₀	D ₆ – D ₀	D ₆ – D ₀
Fold change	1.2	-1.7	-1.0	11.1	3.1	5.6
P value	0.01	0.003	0.99	9.9 ⁻¹⁰	3.6 ⁻⁶	4.9 ⁻⁸

MDA5						
Male – Female		Female – Female		Male – Male		
Day (p.i.)	D ₀	D ₃	D ₆	D ₃ – D ₀	D ₆ – D ₀	D ₆ – D ₀
Fold change	1.1	-1.4	-1.1	11.0	3.5	7.4
P value	0.50	0.02	0.04	7.6 ⁻¹¹	1.6 ⁻¹¹	3.1 ⁻⁷

c-FLIP						
Male – Female		Female – Female		Male – Male		
Day (p.i.)	D ₀	D ₃	D ₆	D ₃ – D ₀	D ₆ – D ₀	D ₆ – D ₀
Fold change	1.2	-1.5	-1.2	2.0	1.4	1.3
P value	0.06	0.003	0.20	2.6 ⁻⁵	0.001	0.003

Mouse microarray data used in this review were previously published [64]. In brief, three representative male or female hearts were chosen. The choice was based on histology score (to confirm infection), then on the quality and amount of the recovered RNA. The Affymetrix Mouse Gene 1.0st Array Chip was used for individual sample runs. Individual results were averaged by group and analyzed by the University of Vermont Bioinformatics group. Microarray data has been submitted to the Gene Expression Omnibus, and we are currently awaiting their reply. The data represent differences in gene expression between male and female or within the same sex at day 0 (D0) - uninfected and day 3 (D3) and 6 (D6) - post infection.