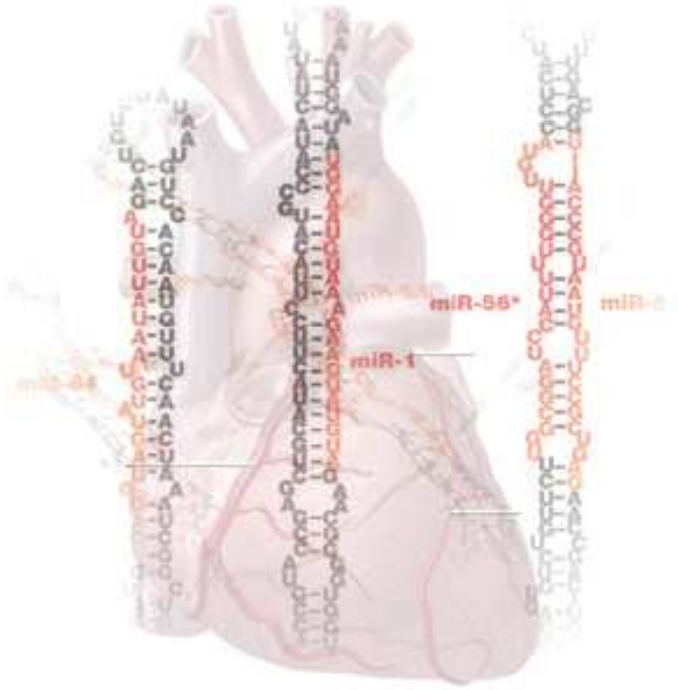
Manipulation of miRNAs can be a possible cure for CAV in heart transplant patients



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Abstract

Organ transplantation needs to be considered as an acute life saver in patients suffering from organ failure. After organ transplantation there is a chance that the transplanted organ might be rejected. After transplantation patients are required to use large amounts of immunosuppressive drugs, to suppress the organ rejection. Unfortunately even these high amounts of drugs are sometimes not able to fully repress organ rejection. In patients with heart transplantations, a disease, related to organ rejection, called cardiac allograft vasculopathy might arise. Cardiac allograft vasculopathy is characterized by vascular remodelling. The remodelling starts with intima thickening and at a later stage the disease progresses with luminal stenosis of the epicardial vessels.

The cellular immune response is considered to be the most important factor in cardiac allograft vasculopathy. The T-helper 1 response presumably plays an important role. T-helper 1 release interferon-γ, resulting in the activation of the immune response. Macrophages are activated thru the T-helper 1 response and start to infiltrate the transplanted heart and start releasing transforming growth factor-β. The release of transforming growth factor-β results in the infiltration and activation of fibroblasts. These fibroblasts deposit collagen, which is the cause of the luminal stenosis as seen in cardiac allograft vasculopathy.

So far no cure is available, but a topic of interest to which scientists have become much more aware of and which might be a possible cure to this disease, are microRNAs. MicroRNAs are small untranslated genomic clusters, which have the ability to silence their mRNA targets. This review focuses on miRNAs that play a role in the processes of the cellular immune response to give an insight into the possibility of using miRNAs to cure cardiac allograft vasculopathy.

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List of abbreviations

- CAV = Cardiac allograft vasculopathy
- ECM = Extra cellular matrix
- HLA = Human leukocyte antigen
- Htx = Heart transplantation
- IFN-γ = interferon- γ
- LPS = Lipopolysacharide
- miRNA = microRNA
- MMP = matrix – metalloproteinase
- NK cell = Natural killer cell
- NMES1 = Normal mucosa esophagus 1
- NP2 = Neuropilin-2
- RCT = Reverse cholesterol transport
- RISC = RNA-induced silencing complex
- SMC = Smooth muscle cell
- SOCS = suppressor of cytokine signaling
- TGF-β = Transforming growth factor β
- TH-1 = T-Helper cell 1
- TH-2 = T-Helper cell 2
- TLR = Toll like receptor
- VEGF = Vascular endothelial growth factor

Introduction

Organ transplantation needs to be considered as an acute life saver to patients who are suffering from organ failure; examples are liver-, kidney- and heart transplantation. The problem that may arise is (acute) transplant rejection which occurs after transplantation of the organ. To counter transplantation rejection, patients are required to take (high amounts of) immunosuppressive drugs (e.g. cyclosporine). Unfortunately even though major improvements have been made on this part, organ rejection still occurs, it just occurs at a later time period after transplantation (1).

As mentioned patients with heart transplantations (HTx) may suffer from transplantation rejection. Although over the years the chance of survival has greatly increased, 20 years ago the average 5 year survival rate was around 41 percent. Nowadays the 1 year survival rate is 88 percent for men and 77 percent for women. After five years the survival rate is 73 percent for men and 67 percent for women. This big increase is mostly due to the improvement of immunosuppressive drugs (2).

Even though the one year survival rate is high, there are still patients that die within the first 30 days after transplantation. Annual reports from the International Society for Heart and Lung Transplantation show that the most common causes of death in the first 30 days after transplantation are technical defects, graft failure and infections not related to cytomegalovirus (CMV). Patients dying after one or more years after transplantation still present infections not related to CMV, but also present cardiac allograft vasculopathy (CAV) and rare forms of lung related diseases. Of all the deaths in patients that survive the first 5 years post transplantation about 33% can be related to CAV(2)(3). The survival of patients is diminished greatly once CAV is discovered, due to the fact that currently nothing can be done to cure CAV, because it is usually discovered in the late stages of the disease. Unfortunately early discovery of CAV can be a major difficulty for clinicians. This is due to the nature of coronary artery remodeling that takes place in the early but also late stages of CAV (4).

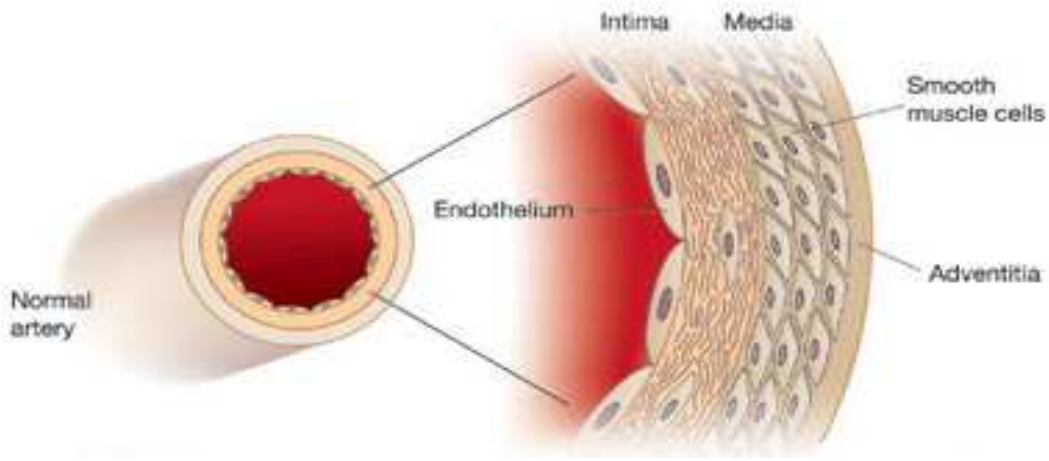

Figure 1. Different layers of an artery (30)

A healthy coronary artery consists of three layers. From the inside to the outside these layers are the tunica intima, tunica media and tunica adventitia (fig. 1). The intima in normal arteries is built from a monolayer of endothelial cells, which faces the lumen of the artery, in a coronary artery this layer is thicker than in regular arteries. This layer resides on a basement membrane overlying an extra cellular matrix (ECM) substrate(5). Bound to this layer lies the media, this layer is demarcated by an internal elastic lamina. The media is composed of relative quiescent smooth muscle cells (SMC) that are admixed with ECM. The function of the media is to maintain the vasomotor tone. To accomplish this task the media relaxes or contracts based on hormonal and metabolic stimuli(6). Above this layer lies the adventitia; it is composed of mostly myofibroblasts, some inflammatory cells, autonomic nerve fibers and associated ECM. It is believed that the primary role of the tunica adventitia is a structural supportive role, although there is research that suggests the tunica adventitia plays a role in the regulation of the vasomotor tone of arteries in general (7).

As mentioned previously the coronary arteries of Htx patients suffering from CAV undergo vascular remodeling. This means that in the beginning intima thickening occurs and at a later stage it is characterized by the luminal stenosis of epicardial vessels (8).

The presumed reason for the pathogenesis of CAV is multifactorial. The cellular immune response is currently seen as the most important factor against the allograft. Of the cellular immune response, T-helper 1 (TH-1) cells seem to play the most important role in the case of CAV (9). It is also presumed that macrophages play an important role, but for now their role has not been studied thoroughly (10). Another component of the cellular immune response involves cytokines. Although the cytokine production is severely reduced in HTx patients due to the uptake of large amounts of immunosuppressive drugs, detectable amounts of interferon-γ (IFN-γ) are still present. IFN-γ is known to play a key role in TH-1 proliferation and thus it is believed that this cytokine plays a mediating role in CAV (11)(12).

Eventually the lining of the intima of the coronary artery in CAV can become completely fibrotic. A study done by Huibers *et al.* showed a small increase in mRNA expression of fibrotic factors (e.g. TGF-β) in the tunica intima when compared to the tunica media. Anti-fibrotic factors did not show any changes between CAV and control arteries. The biggest discovery was the localization of TGF-β. The expression of TGF-β seems to be coming from infiltrating T-cells present in the intima. They suggest that the increased amount of TGF-β might result in a pro-fibrotic response, which would explain why at some point the intima becomes completely fibrotic (13).

Unfortunately the pathogenesis of CAV is usually detected at the end stage of the disease (14). As mentioned clinicians have a hard time detecting the disease due to the current technique limitations (15). The current technique uses an angiogram to measure the diameter of the lumen of the coronary artery. The result of the measurement is then compared to a normal reference diameter (16). The narrowing is mostly a result of hyperplasia of the tunica intima, in combination with scarring of either the tunica adventitia or tunica intima. This approach works perfect in early stage atherosclerosis, but unfortunately this approach does not work with early-stage CAV (17). The reason for this is that due to the specific nature of vascular remodeling in CAV angiograms cannot detect early stage CAV. The remodeling causes a vascular compensatory enlargement, where the intima undergoes morphological changes.

Yet this enlargement causes the artery to appear as a regular artery on an angiogram, due to the media and adventitia remaining relatively unaffected. Mostly once severe intima thickening has occurred you are able to detect CAV. You are then able to easily detect this with regular angiograms, due to narrowing of the lumen of the artery.

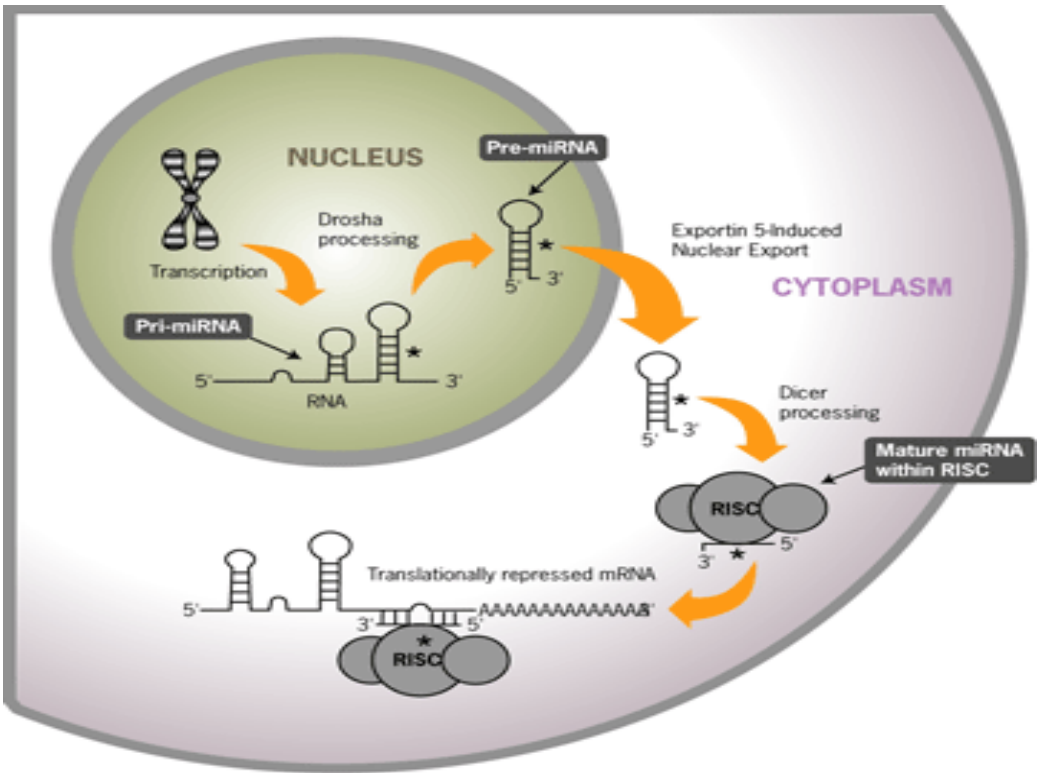

Figure 2. miRNA processing and activity (Reference: website Ambion Life technologies)

If CAV can be diagnosed in its early stages it might be possible to slow-down or stop the process of fibrotic infiltration into the lining of the wall of the coronary arteries. A topic of interest to which scientist have become much more aware of are microRNAs (miRNAs). miRNAs are small un-translated pieces of RNA and are highly conserved among eukaryotic species. miRNAs are located in genomic clusters and an extensive expression and processing mechanism results in the creation of these RNA species (fig. 2).

Once the primary transcript is expressed by RNA polymerase II and III it is then processed into pre-miRNA by an enzyme called Drosha (18). This pre-miRNA is then capable of being shuttled into the cytoplasm. Once in the cytoplasm it will be further processed by Dicer (a RNAse III enzyme), causing the pre-miRNA to turn into a 19- to 24-base-pair product (19). This product is ready to be incorporated into the RNA-induced silencing complex (RISC). RISC has the ability to recognize complementary mRNA transcripts by using the so called seed sequence of miRNA. These sequences make it possible for miRNA to silence the mRNA or target it for degradation of the mRNA (20).

So far it has already been shown that miRNAs are key regulators in T-cell lineages and they seem to play a role in the induction and function of T-helper cell lineages. This review will examine miRNAs related to the immunological process leading to the deposition of fibrosis into the tunica intima related to CAV.

Immunological response in CAV

One of the most important pathological factors of CAV is the cellular immune response. This response consists especially of TH-1 cells. Activated TH-1 cells release multiple cytokines, but most of these cytokines are repressed due to immunosuppressive drugs. One of these cytokines that does not seem to be repressed is IFN-γ. IFN-γ is known to activate macrophages, a cell type which is also involved in the allograft response seen in CAV. Macrophages can be activated in multiple ways, but in this review the focus will be on Toll like receptor (TLR) activation and activation through IFN-γ. Both pathways induce an inflammatory response resulting in the deposition of fibrosis through activated fibroblasts. Fibroblasts can be activated by transforming growth factor β (TGF- β). Once activated fibroblasts start producing collagen, which results in the fibrosis seen in CAV (21). A rather unconventional cell which is usually not considered to be part of an allograft rejection process are natural killer (NK) cells. NK cells are known to scan the surface of cells and look for missing human leukocyte antigen (HLA) receptors, if a cell without a HLA receptor is found, the NK cell will kill this cell. Previously it was thought that NK cells lack any memory function, but recently investigators discovered that NK cells in fact do have a memory function and related this memory function to CAV.

Function of miRNA-155 during the T-helper 1 response

As mentioned previously miRNAs play a key role in all kinds of immune processes, which could mean that they also play an important role in the pathogenesis of CAV. One important factor in the pathogenesis of CAV is T cell infiltration, which results in endothelial dysfunction. The occurrence of endothelial dysfunction is critically determined by the release of IFN-γ by TH-1 cells. IFN- γ is known as a pro-inflammatory cytokine and the release of this cytokine results in further proliferation of T-helper cells (22). This response is possibly one of the causes for the fibrotic infiltration seen in CAV.

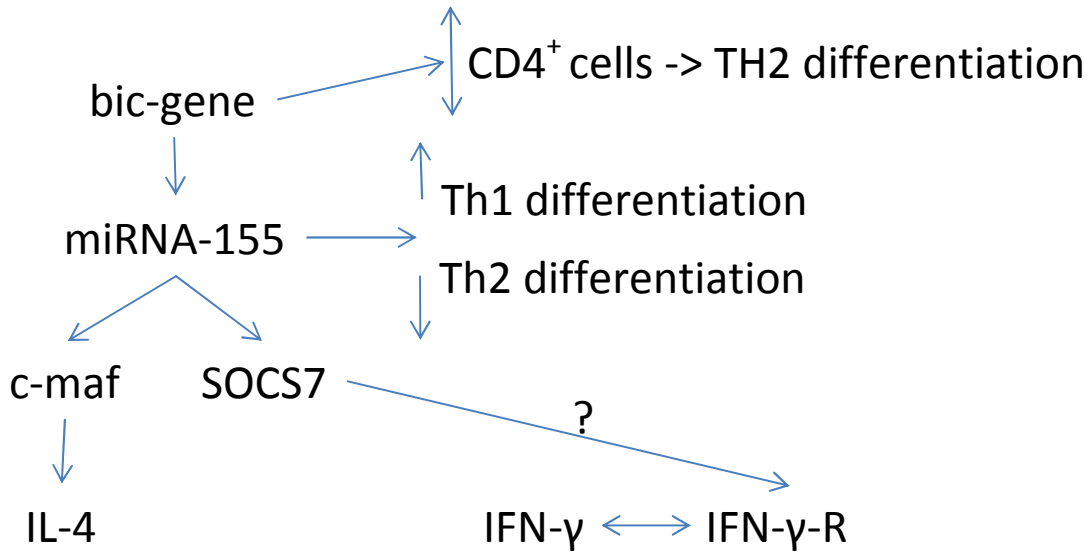

Figure 3. miRNA-155 pathway

A miRNA that is related to this inflammatory response is miRNA-155. Figure 3 shows how miRNA-155 is related to this inflammatory response. MiRNA-155 encoded by the bic gene, is so far the only known miRNA related to influence CD4+ T cell differentiation. miRNA-155 is known to target c-Maf, which is a transcription factor that promotes interleukine-4 (IL-4). Another target is the suppressor of cytokine signaling 1 (SOCS1) in CD4+ T-cells. A study done by Banerjee *et al.* shows how miRNA-155 is related to Th1 differentiation in CD4+ cells both in vivo as in vitro. Their study shows that when miRNA-155 is overexpressed in CD4+ T cells it results in an increased differentiation of TH-1 cells. A different study showed that the lack if miRNA-155 in CD4+ T cells results in an increased differentiation of TH-2 cells. The difference in T-helper cell differentiation was not limited to an in-vitro model. Experiments done in bic deficient mice showed that CD4+ cells from these mice were more biased towards TH-2 than towards TH-1 differentiation (23).

Banerjee *et al.* also did some studies on IFN-γ to see if there is evidence supporting that miRNA-155 is influencing IFN-γ expression, especially since IFN-γ is a key element of CD4+ T-cell subset differentiation. They did some computational studies to see if there is actually any evidence that miRNA-155 is related to IFN-γ. They found that the receptor of IFN-γ (IFN-γR) is a putative target of miRNA-155. It is believed that IFN-γR plays an important role in the differentiation of TH-1 cells through regulating IFN-γ. Evidence shows that during TH-1 maturation the β-chain of the IFN-γR is down-regulated. Blocking this down-regulation results in impaired TH-1 cell differentiation. Their results show that an overexpression of miRNA-155 results in a diminished expression of IFN-γR, which is an effect that they saw in TH-1 cells, but this effect was not seen in TH2 inducible conditions. They also tested if miRNA-155 significantly disrupts the IFN-γ signaling. They noticed that there is no significant difference in TH-1 differentiation when they use anti- IFN-γ antibodies or just a miRNA-155 antagomir. The only difference that they noted is that the repression of TH-1 differentiation is greater with the antagomir than with the anti-IFN-γ antibody, suggesting that IFN-γR is one of miRNA-155's targets (24).

This study would suggest that if it is somehow possible to repress the expression of miRNA-155 in CAV, you would be able to get more CD4+ T-cells to differentiate into TH-2 cells, resulting in a response that is more tolerate towards organ transplantations (25).

miRNAs related to the memory capability of natural killer cells

Another component of the innate immune system is the NK cell. The role of NK cells is believed to be scanning the surface of other cells for 'self' MHC class receptor molecules. When these 'self' MHC class receptor molecules are not found on the target cell, the NK cell will be activated. Activated NK cells release perforin to lysate the cell and activate an inflammatory cytokine release, mostly IFN-γ. As mentioned previously, IFN-γ plays a critical role in the process of endothelial dysfunction, an important process in the occurrence of CAV. It was always assumed that the NK cells were not able to acquire any memory and thus not participating in the rejection of transplanted organs; recent studies have shown that NK cells in fact can acquire memory, a feature also found in T-cells that is linked to graft rejection (26).

Cooper *et al.* studied the characteristics of the NK cells and if it was possible to 'teach' these NK cells. In their study they expanded NK cells from mice *ex vivo*. To expand the NK cells they used IL-12 and IL-18 as stimulatory interleukins and used IL-15 as a survival factor, to make sure these cells did not die. After expansion these cells were returned into Rag -/- mice. Rag -/- mice lack T- and B-cells and are thus a perfect model to study NK cell transfers. The NK cells that were returned were phenotypically similar to the NK population that was already present in these mice. The biggest notable difference was that after re-stimulation there was a significant difference in the expression level of IFN-γ in these retransferred NK cells. This suggests that the NK cells have the ability to acquire memory and possibly are a factor in CAV due to the increase in IFN-γ levels (27).

Bezman *et al.* did a study to discover if miRNAs are responsible for the regulation of NK cells. First they determined which miRNAs are both expressed in mouse NK cells as in human NK cells. They did this to validate their study. After determining which miRNAs are relevant, they created a mouse model in which they used a cre-lox-cre system to knock out either Dicer or Dgcr8. The use of a cre-lox-cre system was needed, because a conditional germ line knockout results in a lethal phenotype. The researchers discovered that in the Dicer and DGcr8 knockout mice the amount of selected miRNA was reduced by 50-75%. After confirming that their cre-lox-cre system works, they wanted to find out what miRNAs actually regulate in NK cells.

To uncover the relationship between miRNA and NK they treated isolated NK cells from the knockout mice and treated them with an apoptotic marker to assess apoptosis. They discovered a significant increase in apoptotic cells when compared to control cells. In addition they also examined the turnover rate of NK cells lacking Dicer and/or Dgcr8. They discovered that the turnover rate in the mutated NK cells was lower when compared to wild type.

The investigators also wanted to observe if miRNAs play a critical role in the maturation of NK cells. They analyzed the expression of maturation markers CD11b and CD27. In mature NK cells the expression level of CD11b is high and the level of CD27 is low. The cells that express the markers in those ratios are considered to be the most mature. It seemed that indeed the amount of mature NK cells was significantly lower in the mutated mice than of the control mice.

Finally to find out if miRNAs play a role in the activation of NK cells they tried to activate NK cells, by introducing ITAM-containing adapter proteins. These adapter proteins associate with the Natural Killer cell Receptor and activate the NK cell. Once activated the NK cell starts to

degranulate and produce cytokines (e.g. IFN-γ), which results in the killing of the target cell. Several studies have suggested that miRNAs regulate targets downstream of ITAM. Thus they wanted to find out what the effect on ITAM is when Dicer- or Dgcr8 deficient NK cells were activated. Their results show a decreased amount of produced IFN-γ from activated NK cells through ITAM-containing receptors in these Dicer or Dgcr8 deficient NK cells. They also discovered when the deficient NK cells were stimulated with IL-12 and IL-18 that normal amounts of IFN-γ were produced. Thus unlike the ITAM-containing receptors, the cytokine receptors still work perfectly and are able to produce a normal IFN-γ response, despite the lack of Dicer and Dgcr8.

Unfortunately no specific miRNA has yet been discovered that is specifically related to the regulation of NK cells, however Bezman *et al.* discovered that the following miRNAs are highly expressed in naïve NK cells, as well as in CD8+ cells from both humans and mice: miRNA142-3p, miRNA142-5p, miRNA150, miRNA16, miRNA23a, miRNA15b, miRNA29a, miRNA30b and miRNA26a. There are also three miRNAs that are shared between effector CD8+ cells and NK cells, which are: miRNA21, miRNA221 and miRNA222. Interestingly they also discovered that miRNA146a is expressed in NK cells, which is also known to play a key role in the regulation of TLR4 (28).

miRNAs related to multiple inflammatory pathways in macrophages

Even though not much is yet known about the relationship between macrophages and CAV, it is known that they are involved (29). It is believed that macrophages are the so called 'end-effector' candidate. They are called like this, because it is believed that they are potently activated by IFN-γ, once activated they can produce reactive oxygen species, which can cause injury of allograft vascular endothelium (30). Lastly they can produce growth factors (e.g. TGF-β), which are believed to be a possible explanation to the proliferation of the neointimal cells (31).

TLRs are located on the surface of macrophages. These receptors are part of a family of trans-membrane receptors which reside on epithelial cells, dendritic cells, macrophages and T- and B-lymphocytes. Due to the fact that TLR's are one of the oldest components of the innate immune system they are able to recognize conserved ligands from microbial pathogens like bacteria (32). The activation of TLR's is thought to occur through both exogenous as endogenous ligands. Endogenous ligands are believed to be released as a result of ischemic/reperfusion (I/R) injury. The release of these endogenous ligands results is now known to create an inflammatory environment favoring graft rejection (33).

miRNA-146 is a negative regulator of TLR-4 signaling

TLR-4 is of special interest, since once activated on a macrophage it results in an inflammatory response. This response results in the expression of TNF-α and IL-6, through activation of transcriptional factor NF-κB (34). The activation of TLR-4 also results in the activation of the transcription factor IRF3. The activation of IRF3 results in an amplification of IFN-associated responses (35).

A study done by Methe *et al.* shows that out of a group of 38 patients which received an HTx, 13 developed allograft endothelial dysfunction. These patients also showed a significantly increased expression of TLR-4. They did a similar experiment in mice, which resulted in similar effects. They suggest that the upregulation of TLR-4 is a first step in the process that leads to the rejection of the heart transplant (36).

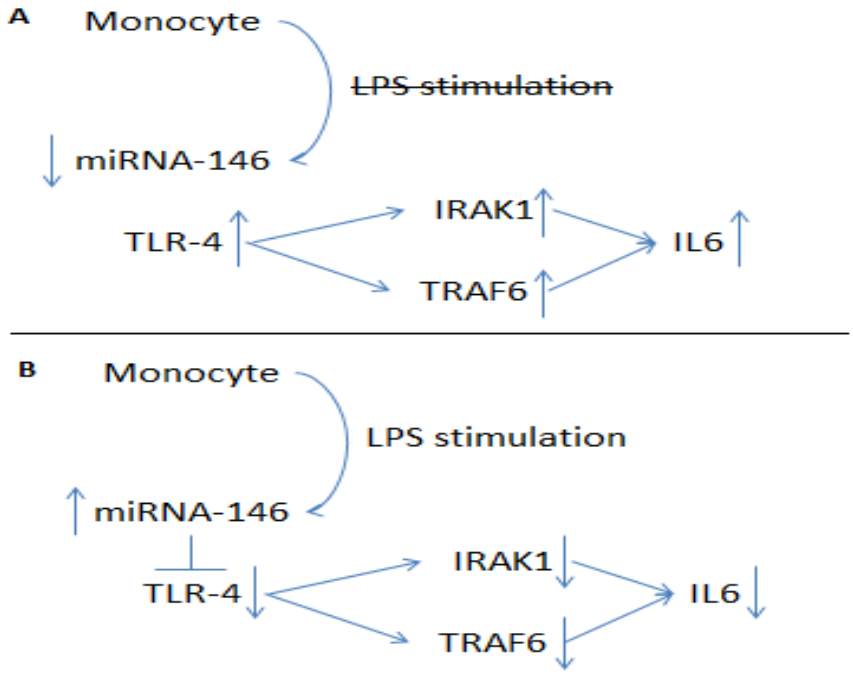

Figure 4. miRNA-146 pathway without (a) and with (b) stimulation of LPS

To prevent an overwhelming proinflammatory response TLRs need to be tightly regulated. Recent studies have shown that miRNAs seem to play a crucial role in the regulation of TLRs. The miRNA-146 family regulates TLR-4. A study done by Lederhuber *et al.* shows that miRNA-146 negatively regulates TLR-4. In their study they investigate if miRNA-146 plays a regulatory role for TLR-4 expression in both monocytes derived from cord blood, as well as monocytes derived from adult blood. To stimulate the monocytes they used lipopolysaccharide (LPS), which is a component found on gram-negative bacteria and recognized by TLR-4. Their results show that after stimulation of LPS in these monocytes, miRNA-146 was up regulated. Obviously TLR-4 was also up regulated after stimulation with LPS. They also checked to see if there was any difference in up regulation between neonatal monocytes and adult monocytes, but there were no significant differences.

Different data is showing conclusive evidence that miRNA-146 targets IRAK1 and TRAF6. These two molecules are found in the signal cascade activated by the TLR. Once activated this cascade results in a pro-inflammatory response, by releasing IL-6. The study of Lederhuber *et al.* is showing that after 24 hours of LPS stimulation, the amount of detectable IL-6 is significantly reduced, which supports the claim that miRNA-146 is a negative regulator of TLR-4 signaling (fig. 4) (37).

miRNA-147 involvement in the TLR-4 regulatory response

Liu *et al.* created a study to discover if a specific miRNA plays a role in the regulatory response in monocytes by the activation of TLR-4. Their target was miRNA-147. They did this study in mice and discovered that in the mouse pre-miRNA-147 is located in exon 4 of the normal mucosa esophagus specific 1 gene (NMES1). They also tested if mature miRNA-147 is derived from the same NMES1 transcript, which it did.

Next they wanted to delineate the kinetics of miRNA-147 in activated macrophages, to see if and when miRNA-147 gets transcribed. They used LPS to activate peritoneal macrophages. miRNA-147 started to get up-regulated after two hours of stimulation with LPS. The up-regulation continued to increase for 24 hours. Interestingly they also tested the up-regulation of pri-miRNA-147 and discovered that pri-miRNA-147 was only up-regulated for 6 hours and started to degrade thereafter.

To find out if miRNA-147 is specific to just TLR-4 they stimulated TLR2 and 3 to see if there are any differences compared to TLR-4. They discovered that when only TLR2 or TLR3 is activated, the amount of miRNA-147 is lower than when TRL4 is activated. The activation of TLR2 or TRL3 results in the activation of either NF-κB (in the case of TLR2) or IRF3 (in the case of TLR3). Whereas the activation of TLR4 results in the activation of both transcription factors, suggesting that both transcription factors need to be activated to get the highest expression levels of miRNA-147.

Liu *et al.* also asked themselves the question if miRNA-147 actually plays a regulatory role in the inflammatory response, given the fact that it is up-regulated during the activation of both inflammatory related transcription factors (NF-κB and IRF3). To give answer to this question they transfected the macrophages with miRNA-147, which results in an overexpression of miRNA-147 and then activated them using LPS. The results show that the addition of miRNA-147 constructs causes a decrease in the expression of TNF-α and IL-6, when compared to controls. Next they knocked down miRNA-147 to see what the response was when no miRNA-147 is present. The amount of TNF-α and IL-6 was markedly increased in the miRNA-147 knocked down macrophages. This data suggests that miRNA-147 plays part in a negative feedback loop that prevents excessive inflammatory responses (38). If this response in fact can be reduced in the transplanted heart it might be able to repress CAV and thus result in increased graft survival.

miRNA-155 plays an important role in macrophage TGF-β release

A different study done by Louafi *et al* focuses on the release of growth factors by macrophages. One of the growth factors that is known to be released by macrophages is TGF-β (39). As mentioned previously the release of TGF-β is believed to give a possible explanation for the proliferation of neointimal cells. This proliferation results in the narrowing of the lumen, which is one of the phenotypes seen in CAV (5).

Using bioinformatics the investigators discovered that miRNA-155 is a possible mediator of TGF-β by targeting SMAD2. The receptor of TGF-β phosphorylates both SMAD2 and SMAD3, after the receptor is activated. After phosphorylation SMAD2 and 3 bind to SMAD4 after which the whole complex moves into the nucleus. Other studies have shown that in human fibroblasts activated TGF-β down regulates miRNA-155(40). Interestingly in murine endothelial cells TGF-β seems to up-regulate miRNA-155, a process that seems to be the key for epithelial-mesenchymal transition and tight junction dissolution (41).

To discover the relationship of miRNA-155 and TGF-β in macrophages Louafi *et al.* first wanted to find out what the effects of miRNA-155 on the macrophage is. To do so they transfected a monocytic cell line with lentiviral transduced miRNA-155 gene that once activated will result in an overexpression of miRNA-155. Results show that the expression of the SMAD2 protein is significantly lower than the control, while mRNA levels remained similar. They also looked at the other binding partners of SMAD2 to see if miRNA-155 also affect them. Their results show that the protein expression of both SMAD 3 and SMAD 4 remained similar to the controls.

They also wanted to check if the reduction of the SMAD2 protein also had a similar effect of the phosphorylated form of SMAD2. The reason behind this was that if P-SMAD2 was not significantly reduced the effect of the activation of the TGF-β would still be similar even if regular SMAD2 protein was reduced. Their results did show that miRNA-155 reduction of SMAD2 also significantly reduced the amounts of P-SMAD2 proteins found inside the cell.

Next they wanted to find out what the effects of miRNA-155 were on the TGF-β dependent transcriptional activation. This would give an answer if overexpression of miRNA-155 is sufficient enough to disrupt the transcriptional activities related to the TGF-β pathway. They studied the gene SERPINE1, which is a gene related to fibrosis and is known to be activated through the TGF-β pathway by SMAD proteins (42). Their results show that the overexpression leads to a reduction of SERPINE1. To confirm that infact the repression of SMAD2 through miRNA155 results in the reduction of SERPINE1, they also blocked SMAD2 by using RNAi. The results from that experiment were similar to the miRNA experiment, suggesting that miRNA-155 has the ability to alter gene transcription through the regulation of SMAD2.

Lastly they wanted to broaden their study to see which genes are affected by stimulating their monocytic cell line with the cytokine TGF-β and see what affect overexpression of miRNA-155 has on these particular genes. The genes that they studies were all TGF-β dependent. They studied genes which were involved in fibrosis, angiogenesis and genes related to immunity. Their results showed that when stimulated with TGF-β significant increase in expression to genes involving fibrosis were seen. Whereas angiogenesis was hardly affected and genes related to immunity were

just slightly affected in these monocytic cell lines. Interestingly in the cells that overexpressed miRNA-155 the effects of the TGF-β induced expression was inhibited (39). Which again suggests that miRNA-155 is a regulator of TGF-β induced expression and thus might be a target for curing CAV, which can possibly result in graft survival.

The role of miRNA-29 in fibroblast activation

Coronary arteries in patients with CAV can become completely fibrotic. This fibrosis is usually considered to be the end stage of CAV and is the result of proliferating fibroblasts. These fibroblasts start to produce excessive amounts collagen, resulting in the observed fibrosis in these patients. TGF-β seems to play an important role in mediating the fibrotic pathway. In CAV it is believed that T-cells release TGF-β, which results in the activation of fibroblasts. Activated macrophages also play an important role in CAV, by producing Matrix-metalloproteinases (MMP), these MMPs result in more TGF-β activation, which in turn results in more fibroblast activation (13).

Unfortunately no studies so far have been done about the relationships of miRNAs involved in the fibrotic process of CAV. However one particular miRNA seems to play a key role in the fibrotic process in two different organs. In both renal fibrosis as in pulmonary fibrosis miRNA-29 seems to have a relationship with TGF-β and play a key role in the fibrotic process.

A study done by Qin *et al.* investigates the relationship of miRNA-29 and TGF-β in renal fibrosis. They hypothesize that miRNA-29 is negatively regulated by the TGF-β/SMAD3 signaling pathway. To get a clear answer to this hypothesis they first look at SMAD3 to see if it regulates miRNA-29. To do so they used a well characterized renal fibrosis mouse model of UUO nephropathy in which SMAD3 was knocked out and compared it to a wild type mouse. Their results showed that in the WT mouse the amounts of miRNA-29 were significantly reduced when compared to the SMAD3 KO. Interestingly the SMAD3 WT mouse suffered from severe renal fibrosis, whereas the KO mouse was lacking severe renal fibrosis. This would suggest that miRNA-29 is regulated by TGF-β via the SMAD3 pathway.

As mentioned previously SMAD3 once activated co-localizes together with SMAD2. The researchers thus wanted to be sure that in fact SMAD3 is the negative regulator of miRNA-29 and not SMAD2. They used mouse embryonic fibroblasts and knocked out either SMAD2 or SMAD3 and stimulated the cells by adding TGF-β. The results show that in fact the amount of miRNA29 is only significantly increased in the SMAD3 KO fibroblasts.

Next they wanted to find out the effect of overexpressing miRNA-29 would be. They overexpressed miRNA-29 *in vivo* and looked at both the mRNA and protein expression of collagen I and collagen III. Overexpression of miRNA-29 resulted in almost no expression of both collagens I and III. They also looked at the expression levels of both collagens when miRNA-29 was knocked down. Their results show that when miRNA-29 is knocked down, the amount of collagen expression is largely enhanced.

They also wanted to see if the *in vivo* results of miRNA-29 overexpression were reproducible *in vitro*. For this they again used the UUO nephropathy mouse model. In this mouse model they did a

gene transfer of miRNA-29, they transferred the gene using a Dox-inducable plasmid system. They transferred the plasmid into the normal left kidney using an ultrasound-microbubble-mediated gene transfer technique. After the gene transfer was completed they ligated the left ureter to induce the necropathy resulting in kidney fibrosis. Interestingly the control mice had the standard fibrosis, while the mouse with the gene transfer into the kidney showed a prevention of renal fibrosis.

Lastly they also wanted to see if it is also possible to halt the progressive fibrosis, by inserting the plasmid after 4 days of the disease onset. Their results show that after inserting the plasmid with the miRNA-29 gene into the left kidney inhibited the progressive fibrosis. This shows that it is fact possible to halt the disease even after it started, by using gene therapy (43).

As mentioned previously miRNA-29 is not just confined to the kidneys. A study done by Cushing *et al.* shows that miRNA-29 also plays a role in pulmonary fibrosis. TGF-β also plays an important role in pulmonary fibrosis, just like in renal fibrosis. In this study the investigators try to unravel which miRNAs play a role in pulmonary fibrosis.

First they treated C57Bl/6 mice with bleomycin. Bleomycin is known to induce pulmonary fibrosis in both humans and mice. The investigators used miRNA array analysis to unravel which miRNA's were involved in ECM deposition/remodeling in pulmonary fibrosis. Their results show that several miRNAs, such as miRNA-184 and 192, were altered in the bleomycin treated mice. Interestingly they discovered that miRNA-29 was significantly reduced in these treated mice.

Next the investigators questioned how miRNA-29 was expressed and if the expression is consistent with a role in the regulation of profibrotic genes during the development of fibrosis. Their results show that miRNA-29 is down regulated while the expression of profibrotic genes is increased. These genes include ECM collagen (Collagen III) and basement membrane collagen (Collagen IV). Inversely the moment the expression of profibrotic genes is decreased, miRNA-29 expression in increased.

The investigators also wanted to find out if miRNA-29 regulates other fibrotic related genes. They used IMR-90 cells, a human fetal lung fibroblast cell line, in which they silenced miRNA-29 expression. After 48 hours the contents of these cells were placed on an array and a genome wide analysis was performed. The results show that 830 genes were altered. As expected among these genes all the direct targets of miRNA-29 were up-regulated. They also looked at the other genes that were significantly altered and among these genes were genes coding for components of BM, integrins and genes that are involved in proteolysis and ECM remodeling.

Lastly they also wanted to see if miRNA-29 is a mediator of the TGF-β effect on the expression of pro-fibrotic genes. Their results show that on TGF-β activation, miRNA-29 is down regulated. Interestingly miRNA-29b, which is a subfamily of miRNA-29, showed more than a 2 fold down reduction, when compared to the other miRNA-29 subfamilies. This suggests that just like with renal fibrosis, miRNA-29 plays a negative regulatory role in the TGF-β pathway in pulmonary fibrosis (44). Possibly since miRNA-29 plays a role in different organs, it might also play a role in the heart. If it in fact does play a role in the heart it might also play a similar negative regulatory role and thus be target against CAV to reduce the amount of fibrosis seen in CAV related Htx patients.

miRNAs able to reverse graft rejection

Being able to stop the process of CAV or being able to reverse the process, would be a life saver for people who had a Htx and are diagnosed with CAV. miRNAs can play a pivotal role in halting this disease. Unfortunately it is not as simple as finding one miRNA to cure this disease, as CAV is a multifactorial disease.

That the manipulation of miRNAs can in fact be beneficial towards graft survival has been shown in a study by Tang *et al.* In this study they investigated if miRNA's can be beneficial towards graft survival in corneal transplants. They believe that after transplantation a process known as lymph angiogenesis causes the immune system to respond against the transplanted corneal graft. This process results in newly formed lymph vessels to appear near the transplant, these newly formed lymph nodes are then believed to transport donor-derived antigen-presenting cells. The donor-derived antigen-presenting cells are transported to the nearest lymph node resulting in the immune response against the graft.

Lymph angiogenesis is caused by multiple factors, but the most well studied factor is vascular endothelial growth factor C (VEGF-C) and its receptor VEGFR-3 (45, 46). The activation of VEGF-C is believed to be regulated by Neuropilin-2 (NP2). NP2 is a co-receptor of VEGF-C and the investigators believe if it is possible to block NP2 by using miRNAs they might be able to reduce the risk of graft rejection. Since the investigators at the time did not know which miRNA is involved in regulating NP2 they used a silencing RNA (siRNA) construct against NP2, to mimic a negative regulating miRNA against NP2. After testing if the siRNA in fact blocked NP2 expression, they wanted to find out if blocking of NP2 expression resulted in the survival of the graft.

They found out that blocking of NP2 resulted in a reduction of lymph angiogenesis and discovered that the amount of lymphatic vessels present was reduced as well. They also wanted to see if NP2 played a role in blood vessel formation, but blocking NP2 did not seem to result in a reduction of blood vessels. Knowing that NP2 reduces lymph vessel formation they wanted to see if siRNAs are in fact able to help in graft survival. They injected the siRNA construct into the mice receiving the transplant prior to transplantation. The results show that recipient mice treated with artificial miRNA against NP2 show a significant increase in graft survival compared to non-NP2 knockdown (47). This suggests that if there is in fact a miRNA that controls the expression of NP2 it might possibly be manipulated to increase graft survival.

A study done by Rayner *et al.* shows another application where miRNAs play a positive effect on the progression of the disease. In this study the investigators researched miRNA-33 and its role in the regression of atherosclerosis. Atherosclerosis is a vascular disease in which cholesterol accumulates in the vascular wall. The deposition of cholesterol starts an immunologic reaction which results in the formation of a so called fibrotic plaque.

In this study Rayner *et al.* uses an atherosclerotic mouse model to study miRNA-33. They hypothesize that miRNA-33 plays a role in the reverse cholesterol transport (RCT). RCT is believed to be a relevant process to the removal of cholesterol out of foam cells that have accumulated into the vascular wall. Foam cells are macrophages that have taken up cholesterol. They believe that inhibition of miRNA-33 results in an increased RCT, resulting in a regression of the fibrotic plaque.

Their results show that when miRNA-33 is blocked the amount of HDL cholesterol increased. HDL cholesterol is the type of cholesterol that can be excreted by the liver and is not known to accumulate inside the vascular wall. They also wanted to see if the blocking of miRNA-33 results in the reuptake of cholesterol out of the foam cells. To see if this actually occurs they injected foam cells with labeled cholesterol in the atherosclerotic mouse model in which miRNA-33 was blocked. Their results show that after 48 hours the labeled cholesterol was located in the plasma, suggesting that blocking of miRNA-33 results in reuptake of cholesterol out of these foam cells, due to an enhancement of the RCT pathway.

Lastly the investigators wanted to find out, due to the reuptake of cholesterol out of foam cells, if the blocking of miRNA-33 might also have an effect on atherosclerosis in general. Their results show that the mice which had miRNA-33 blocked show a reduction of 35% in the atherosclerotic lesion area. Next to the reduction the fibrotic plaques themselves appear to have been subject to remodeling, making it a much more stable plaque. Whereas the mice treated with a non-targeting miRNA show no change in lesion size and still suffer from an instable plaque, which might result in a rupture of the blood vessel (48).

Closing remarks

This review has shown that multiple miRNAs are involved in multiple processes all involved in inflammatory responses seen in diseases which are similar responses as seen in CAV. This review also showed that manipulating these miRNAs can result in improving the disease state (e.g. increasing the graft survival or reduction of the atherosclerotic lesion).

As mentioned in this review there are several processes that lead to CAV. These processes are infiltration of T-cells resulting in endothelial dysfunction, as well as releasing cytokines, resulting in an inflammatory response. This inflammatory response is able to activate macrophages. These macrophages activate fibroblasts, which infiltrate the tissue and start producing collagen, resulting in fibrosis. A different response, unrelated to the inflammatory response caused by T-cells is the activation of NK ‘memory’ cells. These NK cells are also believed to play a role in the graft rejection in CAV.

The most prominent effect of the T-cell response is the infiltration of TH-1 cells that release IFN-γ. IFN-γ release results in the activation of the inflammatory response. As mentioned in this review, miRNA-155 plays an important role in regulating IFN- γ. Increased miRNA-155 levels result in a TH-1 response, while reduced amounts of miRNA-155 result in a TH-2 response. If it would be possible to block the expression of miRNA-155, it would possibly result in a lowered TH-1 response or even a TH-2 response. The TH-2 response is a more favorable towards graft survival, thus blocking miRNA-155 would possibly result in graft survival.

Unfortunately in macrophages high levels of miRNA-155 result in a decreased amount of TGF-β expression. The release of TGF-β by activated macrophages results in the activation of fibroblasts, which start producing collagen. So if miRNA-155 treatment becomes a viable treatment for patients with CAV, there needs to be looked at a mechanism where blocking miRNA-155 is based on cell type and not just a global miRNA-155 blocker.

One of the mechanisms to activate macrophages is thru TLRs. A TLR of specific interest has been shown to be TLR-4. The activation of this TLR results in an activation of the inflammatory response. This review has shown two miRNAs that play a crucial role in regulating this TLR. These miRNAs are miRNA-146 and miRNA-147. Both miRNAs have been shown to play a negative regulatory role on TLR-4, thus if it is possible to over express these miRNAs or, at least make sure to keep the expression levels of these miRNAs at a certain level, it would be possible to repress the activation of TLR-4. The repression of TLR-4 would then result in a lowered immunoresponse, which can positive for graft survival in HTx patients.

As mentioned previously, activated macrophages can activate fibroblasts, thru the release of TGF-β. As shown in this review miRNA-29 plays an important role in the release of collagen in both renal fibrosis as pulmonary fibrosis. This review shows that the over expression of miRNA-29 results in a decrease in expression of both collagen I and III. Thus if it would be possible to over express miRNA-29 in the heart as well and if the results would be similar as discussed in this review, then it might also be beneficial towards graft survival in HTx patients.

Unfortunately the miRNAs discussed in this review have not yet been tested in a setting resembling CAV. Thus these miRNAs need to be tested in a setting related to CAV to see if it is possible to improve graft survival through any of the discussed miRNAs. More research also needs to be done towards the function of other miRNAs as there are thousands of known miRNAs, without a known function, giving possibly more insight on how miRNAs might become a cure towards CAV.

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