Cell Death and Differentiation (2010) 17, 567–576

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Review

Granzymes and perforin in solid organ transplant rejection

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Rejection of solid organ allografts by the recipient immune system is mediated, to a major extent, by T cell effector mechanisms. Granzymes and perforin are protein regulators of cytotoxic T lymphocyte-mediated target cell death. In this review, I discuss clinical data implicating granzymes and perforin in acute and chronic solid organ transplant rejection, as well as data from cell and animal experiments that support a main role for these effector molecules in allograft rejection.
Cell Death and Differentiation (2010) **17,** 567–576; doi:10.1038/cdd.2009.161; published online 30 October 2009

Overview of Solid Organ Transplantation

Solid organ transplantation is the most effective treatment for end-stage organ failure, but the effectiveness of this procedure continues to be hindered by the rejection of transplanted organs by the recipient immune system. Although immunosuppressive drugs are provided to patients to manage rejection, there remains much room for improvement because the overall half-lives of kidney and heart transplants (two of the main solid organ transplants performed) are only 12 and 10 years, respectively.[1,2] In addition, the most widely used immunosuppressive drugs, calcineurin inhibitors (which include cyclosporine and tacrolimus) and rapamycin, are non-specific and are associated with detrimental side effects that include kidney damage, increased risk of cardiovascular disease, increased susceptibility to certain malignancies, and increased rate of certain infections.[3] Advancements in organ transplant management have focused on the induction of tolerance, a process in which immune modulation of the recipient can specifically prevent detection and/or targeting of the foreign organ. Progress is being made on the induction of tolerance in humans, although its widespread clinical use remains to be determined.[4–6]

To optimize the success of organ transplantation, a better understanding of the mechanisms by which the immune system targets allografts is needed. In general, rejection of allografts involves antibody and T cell responses. In this review, I discuss specifically the role of granzymes and perforin, which are the main effector molecules of cytotoxic T lymphocytes (CTLs), in causing acute and chronic solid organ transplant rejection.

Granzyme/Perforin Effector Pathways

Cytotoxic T lymphocytes (which include CD8 and some CD4 T cells) and NK cells are involved in the elimination of infected and foreign cells by the immune system. CD8 T cells are the best understood CTL population. Resting CD8 T cells differentiate into CTLs after an encounter with foreign peptide–MHC class I in the context of other activating signals.[7] These CTLs then migrate to sites of immune activation and induce the death of target cells. In addition to T cells, recognition of activating receptors and a lack of inhibitory signals triggers the NK cell-mediated killing of infected or foreign cells.[8]

One of the most potent death-inducing mechanisms used by CTLs and NK cells is granule exocytosis. Cytotoxic granules contain the pore-forming protein, perforin, and serine proteases named granzymes. The human genome encodes five granzymes: granzyme (Gr)A and GrB, and the orphan granzymes GrH, GrK, and GrM. The mouse genome encodes for granzymes GrA and GrB, and the orphan granzymes GrC, GrD, GrE, GrF, GrG, GrK, GrM, and GrN, but not for GrH.[9] On lymphocyte recognition of target cells, granule contents are rapidly released into the lymphocyte–target cell synapse. Perforin facilitates the entry of granzymes into the target cell cytoplasm where these serine proteases induce cell death through a number of pathways.[10]

GrB is the most extensively studied granzyme that induces cell death through the activation of caspase-dependent and -independent pathways (Figure 1a). Specifically, human GrB can directly cleave the proapoptotic protein Bid, which generates an active form of Bid that translocates to the mitochondria and induces permeability of this organelle.[11,12] This Bcl-2-regulated pathway has rapid kinetics and causes the release of cytochrome c, which leads to caspase activation, as well as the release of SMAC/Diablo, which augments caspase activation by inactivating the endogenous inhibitors of caspases.[13,14] GrB also acts on the mitochondria by inducing caspase-independent cell death through the generation of reactive oxygen species (ROS).[15] In addition to acting through a mitochondrial pathway, GrB induces apoptosis by directly

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Keywords: transplantation; granzyme; perforin
Abbreviations: AV, allograft vasculopathy; CTL, cytotoxic T lymphocyte
Received 20.8.09; accepted 24.9.09; Edited by D Granville; published online 30.10.09

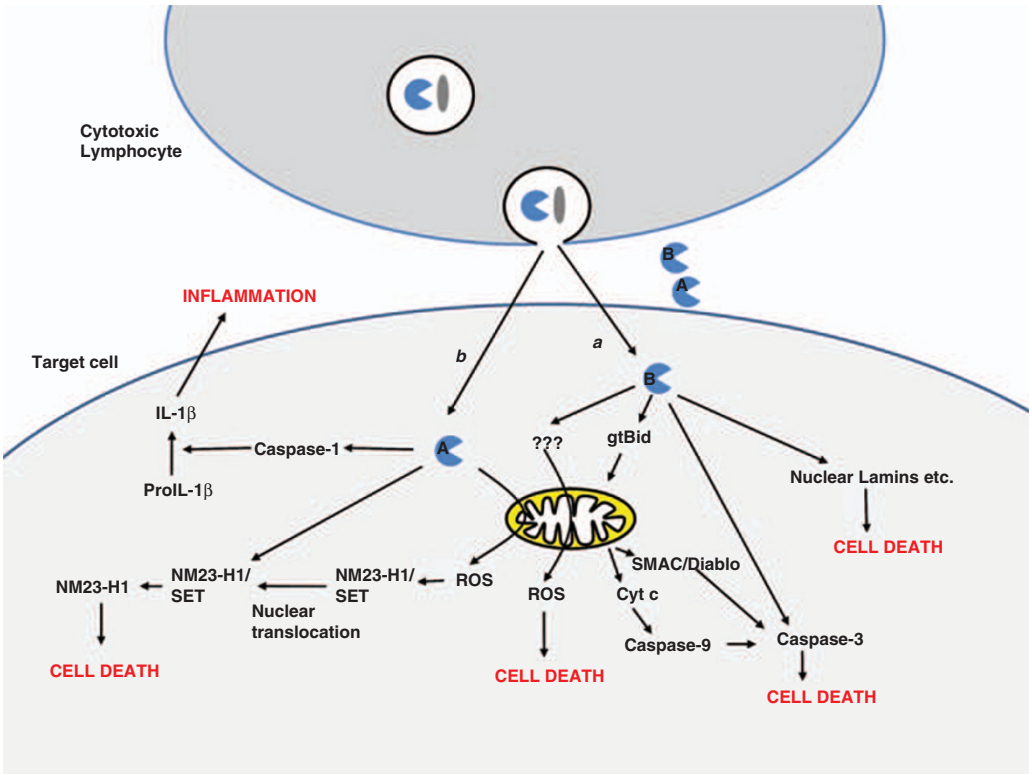

Figure 1 Granzyme A and B (GrA and GrB)-mediated cell death pathways. On recognition of foreign cells, activated CTLs release granzymes and perforin into the lymphocyte–target cell synapse. Perforin facilitates the entry of granzymes into the target cell cytoplasm. (a) Once inside the target cell, GrB induces cell death through a number of mechanisms. GrB-mediated cleavage of Bid results in the generation of an active form of Bid, which causes mitochondrial permeability. Downstream release of cytochrome c initiates caspase activation. SMAC/Diablo is also released by mitochondrial permeabilization and this molecule augments caspase activity by preventing caspase inhibition by endogenous inhibitors. GrB also directly cleaves and activates caspase-3. In the absence of caspase activation, GrB can induce cell death through the induction of ROS from mitochondria as well as through the direct cleavage of structural proteins, such as nuclear lamins. (b) GrA acts directly on mitochondria to induce the production of ROS. Generation of ROS induces the translocation of the SET complex, which contains the SET protein that binds to and inhibits the endonuclease NM23-H1 in the nucleus. GrA cleaves and inactivates SET in the nucleus, which allows NM23-H1 to cleave DNA into large fragments. In addition to these death-inducing properties of GrA, this serine protease induces inflammation through the production of IL-1β through a caspase-1-dependent mechanism

cleaving and activating caspase-3.[16] In situations in which caspase activity is blocked, GrB can directly cleave and inactivate proteins involved in cellular structure and function, such as nuclear lamins, and in this way contribute to the dismantling of target cells through a caspase-independent mechanism.[17] In all, GrB induces cell death through several pathways that may maximize its effectiveness in clearing pathogens that evolve strategies to evade specific pathways.

GrA is also expressed by CTLs and NK cells. Cell death induced by this protease is morphologically distinct from apoptosis and is characterized by cell rounding and cleavage of DNA into relatively large fragments.[18] This trypsin-like protease induces caspase-independent death *in vitro* by triggering the generation of ROS and releasing the endonuclease, NM23-H1, from inhibition (Figure 1b).[19–21] These processes lead to the cleavage of DNA into large fragments in the target cell nucleus. Interestingly, GrA induces the generation of ROS through direct cleavage of the mitochondrial matrix complex I protein, NDUSF3.[22] Other granzymes can also induce cell death *in vitro* and the specific pathways are just beginning to be defined.[23–25]

The physiological roles of perforin and granzymes have been studied using gene knockout mice. Perforin-deficient mice have increased susceptibility to certain viruses and develop autoimmunity secondary to viral infection.[26–30] In comparison, GrB and GrA knockout mice are susceptible to a limited number of pathogens, although GrA/GrB double knockout mice may closely resemble perforin knockout mice in their defective clearance of viral infections.[31–33] The immunological defects observed in GrB and perforin knockout mice are likely a result of impaired induction of target cell death because perforin-deficient T cells are unable to kill most cell types in *ex vivo* killing assays and GrB-deficient T cells have a significant delay in their ability to induce target cell death in similar assays.[34] The mechanisms by which GrA contributes to immunity are still being investigated, but may involve the induction of target cell death and/or of inflammatory cytokine production. Although GrA induces cell death *in vitro*, a recent report questioned the ability of murine GrA to induce target cell death *in vivo* and showed instead that it induces inflammation by stimulating the production of IL-1β from monocytes.[35]

CD8 T Cells and NK Cells in Acute Allograft Rejection

CD8 and CD4 T cells are present in acutely rejecting allografts along with a small number of NK cells.[36,37] Interestingly,

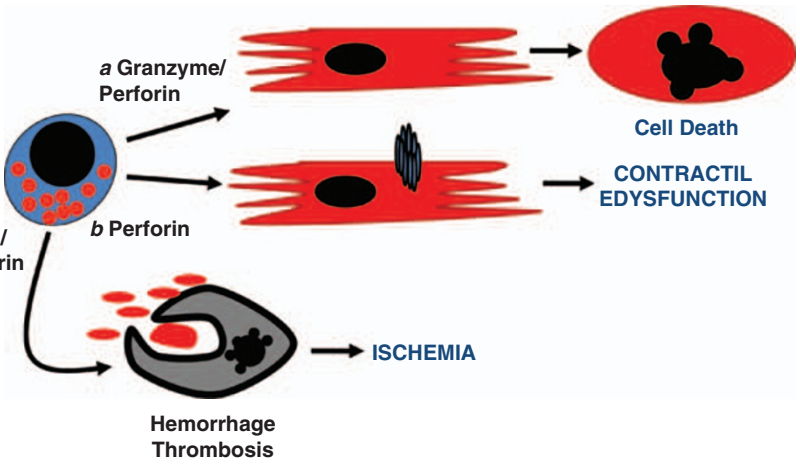

Figure 2 Role of granzymes and perforin in acute rejection. (a) Granzymes/perforin act together to directly induce cell death of allograft parenchymal cells, thereby leading to organ failure. (b) In heart allografts, the formation of plasma membrane pores by perforin alone may cause cardiac myocyte contractile dysfunction through the dysregulation of cytoplasmic ion concentrations. (c) Granzyme B (GrB) and perforin act together to induce endothelial cell death within solid organ allografts. The destruction of the graft microvasculature in this manner leads to hemorrhage and thrombosis, thereby contributing to acute rejection by causing ischemic organ failure

memory CD8 T cells may be one of the earliest leukocytes to enter allografts and trigger the rejection process.[38] In animal models of transplantation using complete MHC-mismatched strains of mice in the absence of immunosuppression, antibody-mediated depletion of CD8 T cells does not alter the kinetics of acute cardiac allograft rejection, but does change the rejection process from one that involves graft infiltration by mononuclear cells to one characterized by excessive infiltration by neutrophils and eosinophils.[39] As acute rejection is most often associated with mononuclear cell infiltration in clinical settings, these data support the involvement of CD8 T cells in acute organ transplant rejection. In addition, CD8 T cell deficiency prevents the acute rejection of heart allografts in a minor histocompatibility antigen-mismatched mouse model.[40] In addition to T cells, NK cells may also contribute to graft rejection because of their ability to respond to 'missing-self', a process by which the absence of inhibitory signals derived from binding to self peptide–MHC molecules triggers NK cell activation.[41–43] The above studies establish a role for CTLs and NK cells in causing acute allograft rejection.

Granzymes and Perforin in Acute Allograft Rejection

Histological studies have shown the abundance of GrA, GrB, and perforin in many types of acutely rejecting allografts.[44–49] In addition, increased expression of granzymes and perforin is predictive of the development of acute rejection episodes.[45,50] Further evidence for a role of GrB in acute transplant rejection comes from the observation that there are higher levels of the endogenous GrB inhibitor, PI-9, within the tubular epithelial cells of kidney allografts with grade IA rejection than in those with more severe acute rejection as defined by grade IB or greater.[51]

Granzyme/perforin pathways are the main mechanisms by which CTLs induce cell death in allografts, as exemplified by cell culture studies using cardiac myocytes, renal tubular epithelial cells, and vascular endothelial cells. Perforin alone induces cardiac myocyte shortening and destruction *in vitro*, and GrA acts in concert with perforin to accelerate myocyte destruction (Figure 2a).[52] Interestingly, the pore-forming properties of perforin alone may also contribute to myocyte contractile dysfunction by causing extracellular calcium influx (Figure 2b). Consistent with these *in vitro* findings, GrA expression is correlated with decreased diastolic function in heart transplant patients.[53] With regard to renal allografts, Miltenburg *et al*.[54] isolated and cultured graft-infiltrating lymphocytes from kidney biopsy material and showed that they were cytolytic toward donor tubular epithelial cells from the same biopsy material but not toward third-party tubular epithelial cells. The determination that cytolytic activity was inhibited by blocking MHC class I suggested the involvement of CD8 T cells. Subsequent studies determined that these graft-infiltrating CTLs induce allogeneic tubular epithelial cell death through a perforin-dependent pathway, as cell death was prevented with concanamycin A but not by inhibition of the Fas pathway.[55] Finally, in the mouse, GrC is involved in the delayed T cell-mediated killing of allogeneic targets in the absence of active GrA and GrB,[56] and this orphan granzyme is partly involved in the T cell-mediated cell death of tubular epithelial cells in an autoimmune model.[57] Although the human genome does not encode GrC, it will be interesting to determine the role of the related granzyme in humans, GrH, in allogeneic T cell responses.

Vascular endothelial cells activate allogeneic T cell responses and are also an important target of CTLs in all solid organ allografts.[58] Endothelial cell-reactive CTLs can be cultured from endomyocardial biopsies, and immune-mediated microvascular damage leads to ischemic organ failure by causing hemorrhage and thrombosis.[59] GrB acts in concert with perforin to induce rapid cell death of human endothelial cells *in vitro*, and GrB alone is capable of inducing endothelial cell death at later time points by proteolyzing extracellular proteins required for adhesion-mediated cell survival.[60,61] Zheng *et al*.[62] and Kreisel *et al*.[63] investigated the mechanisms by which CD8 T cells kill human and mouse vascular endothelial cells, respectively. In both instances, CD8 T cells induced cell death of allogeneic endothelial cells

predominantly through a GrB/perforin pathway and there was minimal contribution of the FasL pathway. This may be due to the expression of an endogenous inhibitor of the Fas pathway, c-FLIP, in endothelial cells.[64,65] Using a humanized mouse model of T cell-mediated endothelial cell injury in which human endothelial cell engraftment in SCID/beige mice is followed by the adoptive transfer of allogeneic human T cells, Zheng *et al*.[66] also determined that human T cell induction of allogeneic endothelial cell death is inhibited by Bcl-2 *in vivo*. Finally, the susceptibility of endothelial cells to GrB/perforin-mediated injury is likely to be tightly regulated by cytokines because IFNγ signaling in graft cells reduces GrB/perforin-induced microvascular damage in a mouse model of kidney transplantation.[67]

Animal models have been used to determine the role of granzymes and perforin in acute organ transplant rejection (Table 1). Although it is clear that CTLs predominantly use granzyme/perforin pathways to induce allogeneic target cell death, experiments using complete MHC-mismatched animal models of organ transplant rejection in the absence of immunosuppression have failed to identify a requirement for perforin/granzyme pathways in allograft rejection in this setting.[68,69] Specifically, in these models there is no difference in cardiac allograft survival (as determined by palpation) or kidney allograft rejection (as determined by histology) in perforin or granzyme knockout recipients compared with wild-type counterparts. This may be explained by a predominance of antibody-mediated rejection in these models. Indeed, acute rejection of heterotopic heart transplants that occurs in complete MHC-mismatched recipients in the absence of immunosuppression is due in large part to antibody-mediated graft injury.[70,71] Clinically, antibody-mediated rejection is implicated in 5.6–23% of rejecting ABO-matched kidney allografts, 30–60% of ABO-mismatched kidney allografts, and 3–28% of heart allografts (although there was an incidence as high as 52% in one report).[72,73] These data indicate that there is a substantial contribution of T cell-mediated rejection (as reflected by the large proportion of clinical cases in which antibody-mediated rejection is not implicated) that may not be accurately reflected in complete MHC-mismatched mouse models in the absence of immunosuppression. CTL cytotoxic effector pathways are likely to be the main mechanism of rejection in patients undergoing this type of cellular rejection. In fact, immunosuppression with cyclosporine might preferentially inhibit humoral immune responses in some cases,[74] potentially resulting in an increased contribution of CD8 T cell responses to allograft rejection in the presence of this immunosuppressive drug. This has been observed in a murine aortic allograft model of vascular rejection.[75] Therefore, in allografts in which antibody-mediated rejection is implicated, the immune effector pathways involved probably include both antibody and T cell-mediated graft injury, whereas the effector pathways involved in allografts that are undergoing rejection in the absence of antibody-mediated graft destruction include mainly T cell pathways such as CTL and cytokine-mediated graft cell death and dysfunction. Individual patient differences in the quality of immune responses and susceptibility to immunosuppressive drugs may underlie the respective contribution of each effector pathway in specific patients. Understanding the

Table 1 Role of cytotoxic T cells and granule effector molecules in acute heart and kidney transplant rejection in mouse models

Effector molecule or cell	Experimental method of effector elimination	Organ transplanted	Type of antigen mismatch in model	Immunosuppression	Effect on allograft (reference number)
CD8	Antibody-mediated elimination	Heart	Complete MHC	No	No effect on allograft survival. Change from mononuclear cell infiltration to polymorphonuclear cell infiltration[39]
CD8	Gene knockout	Heart	Minor histocompatibility antigen	No	Inhibition of acute rejection[40]
Perforin	Gene knockout	Heart	Complete MHC	No	No effect on allograft survival or cellular infiltrate[68]
Perforin	Gene knockout	Kidney	Complete MHC	No	No effect on histopathology or arteritis[69]
Perforin	Gene knockout	Heart	MHC class I	No	Prolongation of allograft survival[68]
Perforin	Gene knockout	Heart	Minor histocompatibility antigen	No	Prolongation of allograft survival[78]
Granzyme A/B	Gene knockout	Kidney	Complete MHC	No	No effect on histopathology or arteritis[69]
Granzyme B	Gene knockout	Heart	Minor histocompatibility antigen	No	No effect on allograft survival[60]

regulation of both effector pathways is required to optimize future therapeutic approaches, such as co-stimulatory blockade and tolerance induction, that are effective in preventing both immunological responses.[76]

Given the contribution of CTL cell effector pathways in allograft rejection as outlined above, it is important to understand the role of specific effector molecules in this process. In a murine heterotopic heart transplant model across minor histocompatibility antigen-mismatched strains, which is a situation in which there is minimal antibody-mediated targeting of allografts, acute heart transplant rejection is significantly delayed in perforin-deficient recipients.[77,78] Perforin is also required for the acute rejection of mouse heterotopic heart allografts performed across MHC class I disparate strains.[68] Although these experiments show that perforin is required for the cellular rejection of solid organ allografts, there is no difference in the rate of acute cardiac allograft rejection in GrB cluster-deficient recipients (which lack GrB and have reduced expression of GrC, GrF, and GrD) compared with wild-type counterparts.[60] This could be due to redundancy in the granzyme pathways and it will be important to determine the rejection of cardiac allografts in GrA/GrB double knockout mice in this model.

In addition to inducing cell death directly, GrA may be pro-inflammatory in allografts through the induction of IL-1β production. Neutralization of IL-1 with a soluble receptor antagonist slightly prolongs heterotopic heart transplant survival, and blocking IL-1 signaling acts in synergy with cyclosporine to prolong heart transplant survival.[79,80] The recent identification that murine GrA induces IL-1β production from monocytes suggests that there may be additional non-cytotoxic properties of granzymes that may contribute to organ transplant rejection by increasing leukocyte recruitment and activation, as well as vascular cell dysfunction.[35]

Granzymes and Perforin in Chronic Allograft Rejection

To date, advances in immunosuppressive regimens have decreased graft failure because of acute rejection, but have had limited impact on chronic rejection.[1] This difference in efficacy could reflect distinctions in the pathological mechanisms driving acute and chronic rejection, although there are clearly similar immunological processes involved. Chronic rejection is defined as late graft loss because of host anti-graft immune responses,[81] and it is represented mainly by allograft vasculopathy (AV) and parenchymal fibrosis. AV is a vascular condition characterized by immune-mediated arterial intimal thickening and dysfunction. These vascular changes result in vessel occlusion and resultant ischemic graft failure.[82] Fibrosis of the graft parenchyma is observed as a reflection of chronic rejection in most solid organ allografts and is one of the structural changes that defines chronic allograft nephropathy (CAN) in kidney allografts. The exact mechanisms underlying the development of parenchymal fibrosis remain to be fully defined, but may include early graft damage due to acute rejection episodes, ischemia resulting from AV, or production of fibrosis-inducing cytokines by the immune responses.[81]

Chronic rejection involves T cell responses. Analysis of clinical specimens of AV has shown that both CD8 and CD4 T cells accumulate in allograft arteries.[83] There is also a small number of NK cells that infiltrates the arteries of heart allografts.[84] Intimal thickening of complete MHC-mismatched aortic allografts does not develop in Rag− and CD4 T cell-deficient recipient mice.[85] Although CD8 T cells do not seem to be required for the development of intimal thickening in mouse models of AV that use complete MHC-mismatched strains of mice in the absence of immunosuppression,[85] CD8 T cells are required for the development of AV in a complete MHC-mismatched model that includes the treatment of mice with a clinically relevant cyclosporine-based immunosuppressive regimen.[75] The mechanisms by which T cells cause AV involve both the direct cytokine-mediated dysfunction of graft cells as well as CTL-mediated vascular cell damage.[86,87] The role of CTL responses in AV and CAN is discussed below, and specific studies are summarized in Table 2.

Using mechanical injury models of arterial intimal thickening, Ross *et al.* initially showed that endothelial injury leads to an aberrant reparative response in arteries characterized by rapid smooth muscle cell migration from the media into the intima, whereupon these cells proliferate rapidly to form occlusive lesions.[88] The signals mediating this type of intimal thickening involve the production of growth factors by vascular smooth muscle cells, infiltrating leukocytes, and platelets. As AV is characterized by similar rapid intimal thickening and lesions containing large numbers of smooth muscle cells and leukocytes, immune-mediated endothelial injury could be an initiating event in the development of this vascular condition.[89] In support of this notion, there is extensive endothelial cell apoptosis in human specimens of AV.[90] Interestingly, the number of apoptotic luminal endothelial cells is highest in arteries with mild AV (which perhaps resembles an early stage of AV) and lower at later stages. Extensive endothelial cell death is also observed in allograft arteries early after heterotopic heart transplantation in a rat model.[91] Separate studies also showed that infiltrating T cells express perforin in allograft arteries.[92] Many of the observed perforin-expressing T cells localized to endothelial cells that displayed morphological characteristics of damage, and perforin-containing granules were seen to be polarized toward the endothelial surface.[93] Finally, GrB is also abundant in human specimens of AV and is spatially associated with apoptotic cells. Its increased abundance in allograft arteries is associated with increased intimal thickening.[94]

The above data suggest that CTLs may initiate the development of AV by inducing endothelial cell death through a GrB/perforin pathway. To study this experimentally, we examined the effects of perforin or GrB deficiency on the development of AV in a mouse heterotopic heart transplant model in which minor histocompatibility antigen-mismatched strains of mice were used to prevent early antibody-mediated graft destruction. There was a significant reduction in AV in hearts transplanted into perforin knockout recipients compared with wild-type counterparts, and this was related to a significant reduction in early endothelial cell apoptosis in coronary arteries of hearts transplanted into perforin knockout recipients compared with wild-type counterparts.[78] Similarly, in hearts transplanted into GrB cluster knockout recipients, there was a significant reduction in the extent of intimal

Table 2 Role of cytotoxic T cells and granule effector molecules on AV in mouse models

Effector cell or molecule	Experimental method of effector elimination	Transplanted tissue/organ	Type of antigen mismatch in model	Immunosuppression	Effect on AV (reference number)
CD8	Gene knockout	Aortic allograft	Complete MHC	No	None[85]
CD8	Antibody-mediated elimination	Aortic allograft	Complete MHC	Yes—cyclosporine	Reduced intimal thickening[75]
Perforin	Gene knockout	Kidney	Complete MHC	No	No effect on intimal thickening[69]
Perforin	Gene knockout	Heterotopic heart	Minor histocompatibility antigen	No	Reduced intimal thickening[78]
Granzyme A/B	Gene knockout	Kidney	Complete MHC	No	No effect on intimal thickening[69]
Granzyme B	Gene knockout	Heterotopic heart	Minor histocompatibility antigen	No	Reduced intimal thickening[60]

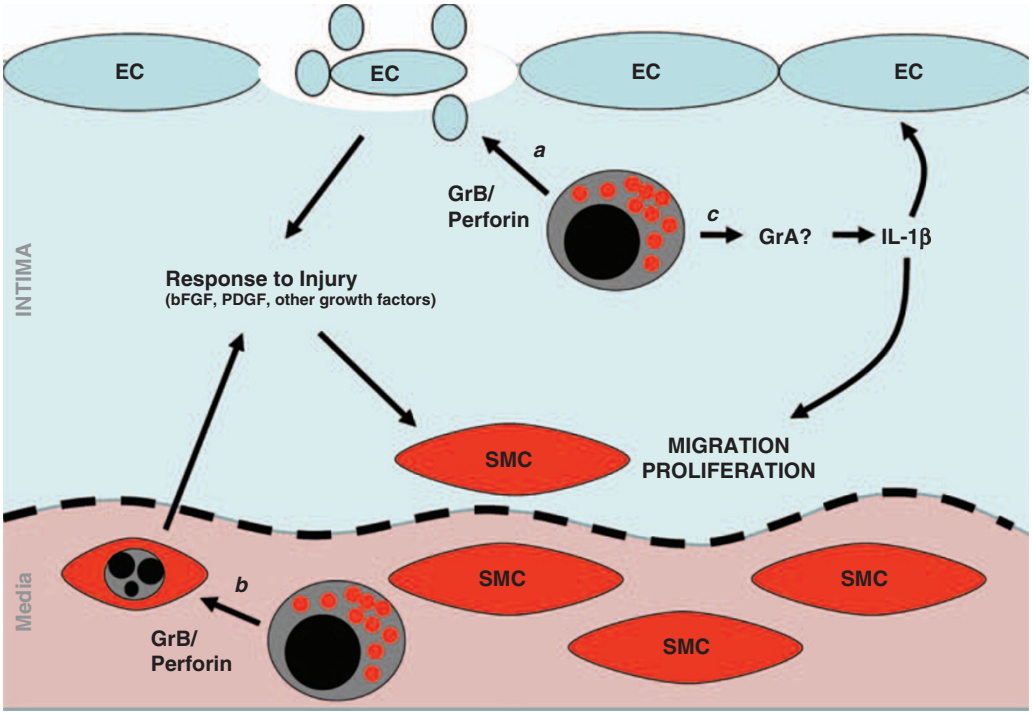

Figure 3 Role of granzymes and perforin in AV. (a) Cytotoxic T lymphocytes infiltrating allograft arteries induce cell death of luminal endothelium through a GrB/perforin pathway. This endothelial destruction induces a response to injury in the artery that involves the production of growth factors, which induce the migration and proliferation of medial smooth muscle cells in the arterial intima. (b) In addition to endothelial damage, induction of smooth muscle cell death by CD8 T cells may also be mediated by GrB and perforin. Smooth muscle cell death may also initiate a response to injury characterized by intimal migration and proliferation of this cell type. (c) GrA may also potentiate inflammation within allograft arteries by inducing the production of IL-1β. This could augment intimal hyperplasia within allograft arteries by stimulating smooth muscle cell proliferation and/or or inhibiting cell death. Il-1β may also activate endothelial cells to produce immune-stimulating cytokines, chemokines, and cell surface adhesion molecules

thickening in allograft coronary arteries compared with hearts in wild-type recipients.[60] However, there was slightly more AV in allograft arteries in GrB cluster knockout compared with perforin knockout mice. Taken together, the above studies indicate that perforin and GrB contribute to the pathogenesis of AV by causing early endothelial cell death (Figure 3a). In addition, perforin-dependent but GrB-independent pathways are involved. The identity of these pathways has not been determined, but they could involve GrA. Finally, as neither perforin nor GrA/B deficiency affects intimal thickening in a mouse kidney allograft model using complete MHC-mismatched strains of mice in the absence of immunosuppression,[69] the granzyme/perforin pathway is likely to predominate in the initiation of AV in allograft arteries that are targeted mainly by T cell effector responses, whereas antibody-mediated targeting of allograft arteries also contributes to AV in grafts in which antibody responses are present.

In addition to the induction of endothelial cell death, immune-mediated smooth muscle cell death may also contribute to the development of AV (Figure 3b). Using mechanical injury models, Reidy *et al.*[95] showed that smooth muscle cell death can also initiate a response to injury within the arteries, thereby leading to intimal hyperplasia. Recent studies using transgenic mice that express diphtheria toxin receptor on vascular smooth muscle cells failed to observe the initiation of widespread intimal thickening in response to high

levels of smooth muscle cell death under baseline conditions induced by the administration of diphtheria toxin, perhaps suggesting that the type of smooth muscle damage may be important in determining the specific effects on vascular pathology.[96] In the setting of transplantation, CD8 T cell-mediated smooth muscle cell death has been suggested to contribute to the development of AV in a clinically relevant mouse model of AV.[97] Consistent with these experimental data, GrB localizes to apoptotic smooth muscle cells in clinical specimens of AV and this cytotoxic protease is able to induce smooth muscle cell death in a perforin-independent manner by cleaving extracellular matrix proteins *in vitro*.[94,98]

Although the above data establish a role for granzymes and perforin in the pathogenesis of AV and chronic heart allograft failure, the potential involvement of these effector molecules in the development of chronic kidney allograft rejection is less clear. Mouse models have determined that chronic rejection is reduced in renal allografts that lack MHC expression.[99] CD8 T cells are observed in biopsies from chronically rejecting kidneys and this T cell subset is associated with fibrotic changes in renal allografts.[100] However, there do not appear to be detectable increases in expression of granzymes or perforin in kidney allografts undergoing chronic rejection.[101,102] Nevertheless, because allograft damage resulting from acute rejection episodes contributes to the subsequent development of CAN, early allograft damage through granzyme/perforin-mediated pathways might initiate, in part, the development of CAN in renal allografts.[103] In addition, fibrotic changes in kidney allografts may be secondary to ischemia resulting from AV.[81] Expression studies that have failed to identify altered expression of granzymes and perforin in chronic kidney allograft rejection may not have been able to properly evaluate changes specifically in allograft arteries. The potential role of these CTL effector pathways in AV in kidney allografts requires further investigation.

Granzymes and Perforin as Biomarkers of Allograft Rejection

Increased GrB and perforin mRNA in peripheral blood mononuclear cells from kidney allograft recipients is associated with acute rejection episodes,[104–106] although an association was not identified in one report.[107] In addition to the examination of granzyme and perforin expression in isolation, combining their expression analysis with that of other genes in peripheral blood cells may provide additional diagnostic power. For instance, increased expression of the regulatory T cell molecule FoxP3 in peripheral blood cells has been shown to be the most precise marker of acute kidney allograft rejection.[106] Combining the expression analysis of this gene with that of granzymes and perforin may provide additional increases in the sensitivity and specificity of diagnosis. In addition to peripheral blood cells, analysis of granzyme and perforin gene expression in urine may be a useful method to monitor acute kidney transplant rejection. Detection of perforin mRNA levels above 0.9 fg per μg of total RNA in urine predicts acute rejection with a sensitivity of 83% and a specificity of 83%, and detection of GrB mRNA levels above 0.4 fg per μg of total RNA predicts acute rejection with a sensitivity of 79% and a specificity of 77%.[108] Therefore, measurement of perforin and/or GrB mRNA levels in urine may also be useful as a non-invasive biomarker of acute kidney allograft rejection.

In attempts to develop biomarkers of acute heart transplant rejection, Schoels *et al*.[109] examined mRNA expression of a number of immune-related genes in peripheral blood mononuclear cells of heart transplant recipients and determined that the expression of perforin and GrB, along with six other genes, was significantly increased in patients undergoing acute rejection episodes. Combining increased perforin expression with differential expression of four other immune genes resulted in the ability to predict acute cardiac rejection with a sensitivity of 82% and a specificity of 84%. Thus, analysis of perforin expression in peripheral blood cells may also be useful in predicting cardiac transplant rejection when combined with expression of other immune regulatory genes.[110]

GrB in Treg Cell Functions

In addition to its role in effector lymphocyte responses, GrB is also involved in immune suppression by CD4+ Treg cells. GrB in this T cell subset was found to be required for maximal suppression of CD4 effector T cell responses *in vitro*, and this was surprisingly independent of perforin.[111] Further, the suppressive effect of GrB-expressing Treg cells was attributed to contact-mediated killing of effector CD4 T cells. In support of these initial findings, recent studies have shown that GrB is required for the induction of tolerance in a skin allograft model and in Treg cell inhibition of tumor clearance.[112,113] With respect to transplantation, tolerance induced by anti-CD154 and donor-specific transfusion is reduced in GrB-deficient mice as well as in mice transgenically overexpressing SPI-6, which is the mouse homolog of the human GrB inhibitor, PI-9. Treg cells that lack functional FoxP3 and GrB fail to induce tolerance, whereas Treg cells that contain a functional gene for either molecule can prolong graft survival to varying degrees.[112]

The effect of tolerance induction on granzyme expression may depend on the type of protocol used. In a rat model of cardiac transplantation, induction of tolerance by donor-specific transfusion prevented acute rejection, but did not diminish the expression of granzymes and perforin within the graft.[114] On the other hand, analysis of T cells from patients who have undergone a tolerance-inducing protocol involving bone marrow transplantation and a non-myeloablative preparative regimen showed an increase in the number of FoxP3 T cells and a reduction in GrB-expressing T cells.[4] Therefore, it will be important to understand the expression of GrB within regulatory and effector T cell subsets in these patients as well as to reevaluate the usefulness of GrB and FoxP3 as biomarkers of rejection in individuals who have undergone tolerance-inducing protocols.

Conclusions

Granzymes and perforin are key effectors of cellular immunity. A wide array of studies has established these effector molecules as main mediators of cellular rejection of solid organ allografts. However, the determination that GrB may

participate in immunosuppressive functions of Treg cells highlights the importance of understanding, in detail, the immunological pathways in which this protease functions to maximize the success of organ transplantation. A more advanced understanding of the pathways that the immune system uses to respond to solid organ allografts may assist in the optimization of immunosuppressive or tolerizing regimens in transplantation management.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported in part by an Operating Grant from the Canadian Institutes of Health Research (CIHR), a Grant-in-Aid from the Heart and Stroke Foundation of BC and Yukon, and a New Investigator Award from the CIHR.

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