



Immunobiologic consequences of assist devices

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The development of novel materials used for implant surgery and the increasing use of implanted devices has made it evident that no material is biologically inert and that optimal use of such biomaterials requires improved knowledge of events occurring at the host–implant interface. Biocompatibility may be considered in terms of four separate but interrelated components: the adsorption of proteins and other macromolecules on the material surface, changes in the material induced by the host, local effects of the material on host tissues, and systemic or remote effects of the material on the host. Commonly utilized biomaterials, including so-called “inert compounds” such as titanium, polytetrafluoroethylene (PTFE), and acrylics, may trigger an array of iatrogenic effects including inflammation, fibrosis, coagulation, and infection. A localized host inflammatory response is a common occurrence regardless of the material used, and such responses may affect the implant adversely, for example osteolytic changes around joint implants, stress cracking of pacemaker leads, and fibrosis surrounding mammary prostheses. In situations where the biomaterial is in direct contact with the blood circulation, such as with hemodialysis, significant changes in systemic immunologic and thrombostatic functions have been described.

The discrepancy between the limited availability of donor organs [1,2] and the ever increasing

number of patients with heart failure has led to the development of left ventricular assist devices (LVADs). LVADs are being increasingly used as bridges to cardiac transplantation, with satisfactory survival rates [3–5]. The encouraging medium-term results with implantable LVAD support have stimulated the initiation of prospective, randomized, multi-center trials evaluating permanent LVAD implantation as a therapeutic modality for patients with end-stage heart failure. Because the biomaterials on the LVAD surface are exposed to the entire host circulation, it becomes mandatory to define the biology of the host–LVAD relationship beyond life-sustaining pump and to determine the effects of LVAD implantation on systemic host immunity. In this article, the authors present a brief overview of general concepts regarding biomaterial-related immunologic host responses and then delineate their findings in LVAD recipients.

Human immunologic responses to implanted biomaterials

Responses to biomaterials not exposed to elements in the systemic circulation: orthopedic implants

Aseptic loosening of prosthetic joints occurs in 25% of prosthetic implants and is characterized histologically by granuloma formation and bone resorption [6,7]. Although the precise cause remains unclear, it is thought to reflect an immunologic host response to small particles of acrylic cement or shards of polyethylene generated by

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micromotion at the bone–prosthesis interface. Detailed histologic examination of tissues from the areas of osteolysis demonstrates a synovium-like pseudomembranous layer at the cement surface together with the presence of macrophages and multinucleated giant cells invading the cortices. The pseudomembranous layer contains macrophages with ingested acrylic cement or polyethylene particles, plasma cells and lymphocytes, and fibroblastic reactive zones. These features closely resemble the histologic characteristics of both rheumatoid arthritis and foreign body reactions [8,9]. Explant cultures of pseudomembrane and synovial tissue derived from patients undergoing prosthesis revision demonstrate high levels of macrophage-derived proinflammatory products such as interleukin (IL)-1, tumor necrosis factor (TNF)- α , and prostaglandin E2 (PGE2). Moreover, regional lymph nodes appear to contain sinuses filled with macrophages containing fragments of polymer, suggesting that localized macrophage activation may lead to systemic immune responses against the biomaterials in the prosthesis. Such responses may account for the observed cutaneous and other systemic features of sensitization accompanying implantation of metallic orthopedic devices [10–12].

Responses to biomaterials exposed to elements in the systemic circulation: hemodialysis

The consequences of exposure of the host circulation to dialysis membrane are a result of two principal series of interactions: adsorption of serum proteins onto the membrane and activation of immune pathways by protein or cellular components. Immediately after the start of hemodialysis, serum proteins such as C3b, coagulation factor XII, IgG, albumin, and fibrinogen are adsorbed onto the dialyzer membrane [13–16]. Activation of factor XII results in the release of bradykinin and kininogen, and such changes may be accompanied by hypotension and cardiovascular collapse, particularly when accompanied by simultaneous use of angiotensin-converting enzyme (ACE) inhibitors. Binding of C3b results in the generation of the anaphylatoxins C3a and C5a and is accompanied by an early transient drop in the number of circulating polymorphonuclear leucocytes (PMN) [13–16]. The transient neutropenia appears to be related to complement-induced changes in the expression of the β_2 -integrin molecule CD11b/CD18 and the selectin receptor CD62L (L-selectin) on the surface of

PMN [17], leading to increased PMN adhesiveness and trapping of these cells in the pulmonary vasculature. Moreover, the dialysis membrane induces PMN activation and degranulation, as indicated by release of myeloperoxidase, lactoferrin, and elastase [18,19].

In addition to effects on PMN number and function, hemodialysis directly affects circulating mononuclear cells. Circulating monocytes become activated, as demonstrated by increased surface expression of various cell markers, such as CD11b/CD18, human leukocyte antigen (HLA)–DR and CD14, and heightened production of monocyte-derived pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α [20–22]. However, despite this aberrant state of activation, the circulating monocytes in hemodialysis patients demonstrate a paradoxically decreased functional state as indicated by reduced phagocytosis, impaired antigen presentation, and reduced cytokine secretion after appropriate stimulation. A similar situation exists with circulating lymphocytes. Despite evidence of T-cell and B-cell activation accompanying hemodialysis, patients undergoing this procedure demonstrate reductions in CD4 and CD8 T-cell levels [23,24], and functional T-cell and B-cell defects [24,25]. These immunologic defects appear to have important consequences, principally by increasing susceptibility to infections [26]. Significantly, infectious diseases represent one of the most important causes of mortality in hemodialysis patients, accounting for 36% of all deaths [27].

Effects of the LVAD–host interface on localized elements of the host immune system

Deposition of monocytes/macrophages on LVAD surface

The clinical success of LVAD implantation has nevertheless been accompanied by significant complications, including thromboembolic events in as many as 30% of cases [28,29]. This complication is reduced to < 4% [30] in LVAD types, which incorporate a textured surface lining that supports the growth of neointima-type cells [31,32]. To begin to understand the nature of this cellular lining, the authors investigated the cellular phenotypes present on the LVAD surface. The majority of the cells detected after cell detachment and resuspension were of monocyte/macrophage lineage. Resting monocytes or activated macrophages were identified, respectively, as either round, quiescent CD14-positive cells or elongated,

often multinucleated, CD68-positive cells with prominent cytoplasmic processes. The macrophage lineage cells were functionally activated as defined by NF κ B expression [33] and augmented production of cytokines and coagulation factors [34].

Monocyte–T-cell interactions on the LVAD surface result in aberrant T-cell activation

Interspersed among the cells of monocyte/macrophage lineage were T lymphocytes, which expressed strong immunoreactivity for CD3, CD4, and CD25 (IL2 receptors), consistent with activated helper T cells [35]. Moreover, incubation of T cells from the LVAD surface with LVAD material and exogenous IL-2 caused a sevenfold increase in T-cell proliferation compared with culture in medium alone, consistent with expansion of *in vivo* activated T-cells. Similarly, T-cell aggregates from the LVAD surface could be sustained in culture for up to 3 weeks in the presence of IL-2 and LVAD material but not in the absence of either. Finally, using the RT-PCR technique to determine the cytokine profile expressed by the neointimal cells on the LVAD surface, all samples studied demonstrated mRNA for both the Th1 cytokine IL-2 and the Th2 cytokine IL-10, as well as for the monocyte-derived cytokine IL-1 [35]. Together, these observations emphasize the monocyte–T-cell interactions that occur on the LVAD surface and indicate that the consequences of these interactions are to induce prominent T-cell activation via IL2 receptor–dependent pathways.

Systemic consequences of aberrant immune activation accompanying LVAD implantation: induction of T-cell defects and B-cell hyperreactivity

Circulating T cells from LVAD recipients demonstrate heightened levels of CD95 (Fas) expression, spontaneous proliferation, and spontaneous apoptosis in vivo

To determine whether this aberrant T-cell activation on the LVAD surface influenced systemic T-cell immunity in LVAD recipients, the authors next examined the phenotype and function of circulating T cells in LVAD recipients. Circulating T cells from LVAD recipients demonstrated a heightened state of *in vivo* activation,

as defined by surface expression of the activation marker CD95 (Fas), associated with a pathway of cellular apoptosis. CD95 expression was increased to a similar extent on both CD4 and CD8 T cells from LVAD recipients in comparison to controls, ($70\% \pm 6\%$ versus $22\% \pm 4\%$, $P < 0.001$, and $69\% \pm 7\%$ versus $7\% \pm 2\%$, $P < 0.001$, respectively). Reflecting this heightened state of *in vivo* activation, T cells from LVAD recipients had significantly higher levels of spontaneous proliferative activity in comparison with T cells from NYHA class IV controls, and this activity increased in parallel with duration of LVAD implantation.

The authors next investigated whether this heightened state of T-cell activation in LVAD recipients was associated with increased levels of T-cell apoptosis *in vivo*. Spontaneous T-cell apoptosis, defined by binding of annexin V to phosphatidylserine present on T-cell membranes of cells undergoing early phases of apoptosis, was significantly higher in both CD4 and CD8 T cells from LVAD recipients than in those from NYHA class IV heart failure controls ($39\% \pm 5\%$ versus $4\% \pm 1\%$, $P < 0.001$, and $45\% \pm 4\%$ versus $2\% \pm 1\%$, $P < 0.001$, respectively). Similar abnormalities were observed on T cells from recipients of either TCI or Novocor devices. This enhanced state of T-cell apoptosis *in vivo* was confirmed by analysis of DNA isolated from freshly obtained T cells. A characteristic fragmentation pattern of apoptosis was observed in DNA from circulating T cells of all LVAD recipients, but not in DNA from T cells of any control individuals.

Circulating T cells from LVAD recipients demonstrate defective proliferation after triggering via the T-cell receptor complex

Despite the high levels of spontaneous proliferation, T cells from LVAD recipients showed defective proliferative responses after activation, specifically, via the T-cell receptor (TCR) complex. After TCR engagement by allogeneic mixed lymphocyte culture, the mean stimulation index (SI) of T cells from LVAD recipients was 74% lower than that of T cells from NYHA class IV controls ($P < 0.001$). Similarly, after TCR ligation with anti-CD3 Mab, the mean SI of T cells from LVAD recipients was 83% lower than that of T cells from NYHA class IV controls ($P < 0.001$). In contrast, T-cell activation by pathways other than TCR triggering caused similar increases in

T-cell proliferation in both LVAD recipients and controls.

Circulating T cells from LVAD recipients demonstrate increased susceptibility to activation-induced cell death after T-cell receptor engagement

Because pre-activated T cells expressing CD95 (Fas) are susceptible to activation-induced cell death (AICD) after triggering via the TCR complex, the authors next investigated whether the observed defects in T-cell proliferative responses in LVAD patients after TCR engagement might be related to AICD. A flow cytometric assay was used to detect the proportion of apoptotic T cells in a given individual (LVAD recipient or NYHA class IV control), defined by annexin V binding to surface phosphatidylserine, which underwent cell death, defined by propidium iodide staining, after 24 hours of culture with either medium or anti-CD3 Mab. The increase in T-cell death after anti-CD3 activation was then compared in both experimental groups, LVAD patients and NYHA class IV controls. After activation of resting T cells with anti-CD3 Mab, the proportion of annexin V positive CD4 T cells undergoing cell death (propidium iodide positive) increased by a mean of 3.2-fold among LVAD patients compared with only 1.2-fold in heart failure controls ($P < 0.05$). These results clearly demonstrate that circulating CD4 T cells from LVAD recipients have increased susceptibility to AICD in comparison with those from heart failure controls.

LVAD implantation as a model for diseases of immune deficiency

The development of defects in T-cell immunity in LVAD recipients is reminiscent of another disorder of acquired immune dysfunction, infection with the human immunodeficiency virus type 1 (HIV-1) [36]. Progressive depletion of CD4 T cells and immune dysfunction in HIV-1 infection accompanies progressive increases in viral burden within cells of various lineages, including T cells, macrophages, and dendritic cells [37–39]. One proposed mechanism is inappropriate induction of apoptotic T-cell death resulting from HIV-mediated interactions between CD95 (Fas) and CD95L (FasL) [40–43]. HIV-infected dendritic cells, a lineage highly specialized for cellular activation through the TCR complex, appear to

be particularly effective at inducing T-cell expression of CD95 and delivering apoptosis-inducing signals to uninfected T cells [44,45]. Because activation via the TCR complex increases CD95L expression [46], T cells that express high surface levels of CD95 are particularly susceptible to AICD after TCR engagement by antigen-presenting cells (APCs).

Some aspects of the pathogenesis of AICD in LVAD recipients may resemble those in HIV-infected individuals. Cells of monocyte or dendritic lineage are present on the LVAD surface at the time of explantation [35] and are functionally activated as defined by NF κ B expression [33] and augmented production of cytokines and coagulation factors [34]. In addition, pre-activated T cells expressing IL-2 receptors are interspersed among the macrophages and dendritic cells isolated at the time of LVAD explantation [35]. These results suggest that APCs that are aberrantly activated by the implanted LVAD deliver excessive co-stimulatory signals to T cells, inducing surface expression of CD95 and other markers of cellular activation. Rather than proliferate appropriately in response to subsequent antigenic stimulation, these cells presumably undergo AICD as a result of interactions between CD95 and newly expressed CD95L.

Alterations in T-cell cytokine profiles in LVAD recipients: loss of Th1, but not Th2, cytokine mRNA expression

Because T cells producing Th1-type cytokines (IL-2 and IFN- γ) have been reported to be selectively susceptible to CD95 (Fas)-mediated apoptosis [47,48–51], the authors next used the RT-PCR technique to compare the pattern of Th cytokine gene expression in LVAD recipients and heart failure controls. Freshly obtained circulating PBMC from each of 12 NYHA class IV control patients expressed mRNA for both Th1-type cytokines (IL-2 and IFN- γ) and Th2 type cytokines (IL-10 and TGF- β). In contrast, freshly obtained PBMC from each of 12 LVAD recipients expressed mRNA for Th2-type cytokines (IL-10 and TGF- β), but not for the Th1-type cytokines IL-2 and IFN- γ . Expression of IL-4 or IL-5 mRNA was not detected in any LVAD or control patient. These results suggest that the heightened levels of T-cell apoptosis in LVAD recipients leads to a selective loss of T cells producing Th1-type cytokines and unopposed T-cell production of Th2-type cytokines.

B-cell hyperreactivity in LVAD recipients: high frequency of antiphospholipid and anti-HLA antibodies

Because induction of autoimmunity, polyclonal B cell activation, and production of auto-antibodies have been postulated to result from both excessive circulating apoptotic waste [52–54] and from a predominance of circulating Th2-type cytokines [55,56], the authors investigated whether LVAD recipients demonstrate prominent B-cell hyperreactivity. LVAD recipients had significantly higher frequencies of circulating anti-phospholipid and anti-HLA antibodies in comparison with NYHA class IV controls awaiting cardiac transplantation. Circulating anti-phospholipid antibodies were detected in nine of 20 LVAD recipients (45%), but in none of 20 heart failure controls ($P < 0.0001$). Similarly, the frequencies of IgG antibodies against major histocompatibility complex (MHC) class I and class II antigens were significantly higher in LVAD recipients than in heart failure controls awaiting transplantation (43% versus 3% and 33% versus 3%, respectively, both $P < 0.0001$).

Presence of HLA-DR3 predisposes LVAD recipients to B-cell hyperreactivity

The authors next sought to determine whether production of anti-MHC antibodies in LVAD recipients was influenced by either perioperative transfusion of blood products or by host genetic factors. Of the patients who received more than 6 platelet units, 63% were found to develop IgG antibodies against MHC class I antigens by 4 months of LVAD implantation compared with 8% of those receiving less than 6 units ($P < 0.01$). Perioperative red blood cell transfusions did not influence the production of these antibodies, presumably because donor red blood cells contain less contaminating MHC class I-expressing T cells than donor platelets. In contrast to anti-MHC class I antibodies, development of IgG antibodies against MHC class II antigens was not influenced by either the number of perioperative platelet or red blood cell transfusions, presumably because contaminating T cells in the absence of activation do not express MHC class II antigens.

The authors next investigated whether development of anti-MHC class II antibodies in LVAD recipients was influenced by inheritance of particular HLA-DR types. The median time to developing anti-MHC class II IgG antibodies was

found to be significantly shorter for LVAD recipients with HLA-DR3, 33 days, than for those without this HLA-DR type, 103 days ($P = 0.03$). By 50 days post-LVAD implantation, 80% of HLA-DR3 individuals had developed anti-MHC class II IgG antibodies compared with only 30% of DR3-negative persons. HLA-DR3 type was also associated with shorter time to developing anti-MHC class I IgG antibodies, although this did not reach statistical significance. No other HLA-DR type significantly influenced onset of anti-MHC antibody production. In additional studies, circulating anti-phospholipid antibodies were detected in seven of nine (78%) HLA-DR3 LVAD recipients compared with only five of 15 (33%) LVAD recipients who were not HLA-DR3 ($P < 0.05$). Together, these results indicate that inheritance of HLA-DR3 increases susceptibility of LVAD recipients to development of B-cell hyperreactivity.

LVAD implantation as a model of autoimmunity

A similar discordance between defects in T-cell immunity and B-cell hyperreactivity characterizes two other immunologic disorders, systemic lupus erythematosus (SLE) [57–59] and infection with the human immunodeficiency virus type-1 (HIV-1) [60,61]. A proposed mechanism to account for the coexistence of T-cell defects and autoimmunity in these disorders is inappropriate induction of apoptotic T-cell death [62–64] due to heightened interactions between CD95 (Fas) and CD95L (FasL) [65–68]. Because these interactions appear to result in a selective loss of Th1 cytokine-producing CD4 T cells in both SLE [69,70] and HIV-1 infection [71,72], the residual populations of CD4 T cells may induce B-cell hyperreactivity and dysregulated immunoglobulin synthesis by unopposed production of Th2 cytokines. The results support the concept that excessive T-cell apoptosis is a general mechanism underlying B-cell hyperreactivity and suggest that this process is amplified in individuals with inheritance of immunogenetic haplotypes encoding high-level production of additional proapoptotic factors.

Maintenance of T-cell homeostasis and peripheral tolerance to self-antigens after repeated lymphocyte stimulation is regulated by AICD. Activated CD4 T cells undergo AICD as a result of interactions between CD95 (Fas) and CD95L (FasL) molecules co-expressed after TCR engagement [73–75]. After crosslinkage of CD95, the

cytoplasmic domain of this receptor binds the adaptor molecule FADD [76,77], enabling interactions with the protein-binding domain of another protein termed FLICE (caspase 8). Activation of FLICE leads to catalytic activation of a cascade of caspases, with the ultimate result of cellular apoptosis [78,79]. The binding of FLICE to FADD can be competitively inhibited by another recently identified protein, FLIP, which negatively regulates apoptosis [80,81]. Cellular levels of FLIP are high in naive T cells, which are resistant to CD95-mediated apoptosis, but are inhibited in activated T cells by IL-2 [82]. Because IL-2 also enhances transcription of CD95L (FasL) [82], these observations provide an explanation for the selective sensitivity of IL-2 producing Th1 cells, and resistance of Th2 cells, to AICD after CD95 engagement [73–75]. The absence of Th1-type cytokine mRNA expression in circulating mononuclear cells from LVAD recipients could, therefore, be consistent with a selective loss of IL-2 producing Th1 cells in these people consequent to induction of CD95-mediated apoptosis. Selective loss of Th1 cells through apoptosis would explain the high prevalence of disseminated fungal infections *in vivo* [83–85] as well as the observed defects in cell-mediated immunity *in vitro* in LVAD recipients. Because SLE patients also demonstrate a predominantly Th2-type cytokine profile with loss of IL-2 producing capability, a similar process may underlie the defects in cellular immunity that exist in this disorder.

In addition to the classic DNA fragmentation that accompanies cellular apoptosis, a consequence of FLICE activation after CD95 ligation is the export of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane by a process that involves inhibition of mitochondrial phosphatidylserine decarboxylation [86]. Outer leaflet phosphatidylserine serves as a signal for engulfment of apoptotic cells by macrophages [87] and may be shed from the cell surface within plasma membrane vesicles [86]. Because high levels of T-cell CD95 expression and apoptosis occur in patients with SLE [65,66], it has been postulated that B-cell hyperreactivity and auto-antibody production in these patients is a consequence of heightened macrophage presentation of autoantigens, which have been altered by the apoptotic process, to Th2-type CD4 T cells. In direct support of this hypothesis is the recent observation that intravenous injection of syngeneic apoptotic thymocytes in mice induces the

production of anti-nuclear and anti-phospholipid antibodies as well as glomerular IgG deposition [88]. In this context, LVAD implantation serves as an iatrogenic example of another human disorder of B-cell hyperreactivity accompanying excessive T-cell apoptosis.

The association between HLA-DR3 and antibody production in LVAD recipients provides a striking genetic parallel to patients with SLE, in whom elevated frequencies of HLA-DR3 [89,90] or of HLA-DR3-associated extended haplotypes [91,92] have been well documented. In particular, cumulative observations have shown that inheritance of HLA-DR3 is associated with an extended haplotype containing both C4 null alleles [89–92] and a particular TNF- α promoter polymorphism [93–95] associated with high levels of TNF- α gene transcription [96] and cytokine production [97], particularly after monocyte stimulation with lipopolysaccharide (LPS) [98]. The LPS receptor, CD14, is used by monocytes to recognize and phagocytose apoptotic cells [99], suggesting that HLA-DR3 individuals may produce higher levels of TNF- α after CD95-mediated T-cell apoptosis than HLA-DR3-negative individuals. Because binding of TNF- α to TNF receptor-1 molecules is accompanied by further augmentation of the FADD/FLICE-dependent cell death pathway [100], particularly in chronically activated T cells where IL-2 expression inhibits FLIP transcription [101], this process is likely to lead to a vicious cycle of progressively increasing apoptosis of CD4 Th1 cells, unopposed Th2 cytokine production, and B-cell hyperreactivity in HLA-DR3 individuals.

Clinical consequences of T-cell defects and B-cell hyperreactivity in LVAD recipients

LVAD recipients demonstrate in vivo evidence of defects in cell-mediated immunity: increased prevalence of disseminated Candidal infections

We next investigated whether the *in vitro* defects in cellular immunity identified in LVAD recipients were related to infectious complications *in vivo*. In a Candidal infection prevalence study, among 78 NYHA class IV heart failure patients listed as UNOS status I and awaiting cardiac transplantation, the presence of LVAD implantation was associated with a significantly increased risk of developing disseminated Candidal infection, defined as positive blood cultures either alone or in association with positive cultures at extravascular sites. By 3 months post-LVAD

implantation, 28% of patients with an LVAD had developed disseminated Candidal infection compared with only 3% of those without device implantation ($P = 0.0029$). Since the risk of developing disseminated Candidal infection persisted throughout the duration of LVAD implantation, with 34% of patients developing an infection by 6 months and 45% of patients by 9 months, this strongly argued that the risk for Candidal infection was increased by the presence of the implanted device rather than any possible effects of surgery. Moreover, no cases of disseminated fungal infection were observed in an additional 425 consecutive patients undergoing cardiac bypass surgery at the College of Physicians and Surgeons of Columbia University over the past 12 months by the surgical team principally involved in LVAD implantation.

The clinical consequences of LVAD-related immune dysfunction, particularly disseminated fungal infections, are very serious complications of device implantation. To prevent induction of defects in host immunity and limit such infectious complications, novel strategies need to be developed. In contrast to HIV-1 disease, where the cause of the severe immune dysfunction is multifactorial, the immune defects accompanying LVAD implantation appear to be far more limited and may, therefore, be more amenable to reversal by therapeutic intervention. One potential approach to prevent AICD is the use of cyclosporine A or FK506, two drugs that inhibit mRNA transcription of FasL after T-cell activation via the TCR complex [102]. The authors are currently evaluating these and other approaches to reduce the abnormal immune activation present in LVAD recipients.

Presence of IgG anti-MHC Class I antibodies increases waiting time to cardiac transplantation

Antibodies in the serum of a cardiac allograft recipient that are directed against donor HLA class I MHC antigens constitutively expressed by allograft endothelium portend a significant risk for early graft failure (ie, within the first 24 to 48 hours) and poorer patient survival as a result of complement-mediated humoral rejection [102–104]. Because T lymphocytes constitutively express MHC class I antigens, the presence of preformed lymphocytotoxic antibodies, particularly of IgG isotype, detected in a routine T-cell crossmatch is considered a contraindication to solid organ transplantation [102]. To identify

patients at high risk of having a positive donor-specific crossmatch, cardiac transplantation candidates are screened for anti-MHC class I antibodies reactive with lymphocytes from a panel of volunteers representative of the major HLA allotypes, collectively referred to as measurements of panel-reactive antibodies (PRA). Because patients with high PRA levels are considered to be “sensitized” to various alloantigens and require donor-specific crossmatches before transplantation at the College of Physicians and Surgeons of Columbia University, the authors investigated the effects of IgG anti-MHC class I antibodies on waiting time to cardiac transplantation. As expected, LVAD patients with IgG anti-MHC class I antibodies had a significantly longer waiting time than those without these antibodies (175 versus 90 days, $P = 0.009$) [105]. In contrast, the presence of IgG anti-MHC class II antibodies did not affect the waiting time to transplantation (139 versus 114 days, $P = 0.50$).

Presence of IgG anti-MHC class II antibodies is a risk factor for high-grade cellular rejections post-transplantation

As shown in Table 1, the presence of IgG anti-MHC class II antibodies detected at the time of transplantation was highly predictive of early high-grade cellular rejection in the post-transplant period [106]. The median time for a high-grade rejection was 70 days for patients positive for IgG anti-MHC class II antibodies. In contrast, the actuarial freedom from rejection never fell below 50% in more than 1700 days of follow-up for patients without IgG anti-MHC class II antibodies (odds ratio > 24.3 , $P = 0.006$). The presence of IgG anti-MHC class I antibodies was also a moderate risk factor for a high-grade rejection, however this was not statistically significant ($P = 0.08$). Additionally, neither the presence of IgM anti-MHC class I nor IgM anti-MHC class II antibodies at the time of transplant influenced the time to a high-grade cellular rejection ($P = 0.94$ and $P = 0.79$, respectively). By Cox Proportional Hazard modeling for multivariable analysis, the only risk factors identified to predict an early high-grade cellular rejection were the presence of pretransplant IgG anti-MHC class II antibodies ($P = 0.018$) and, to a lesser extent, IgG anti-MHC class I antibodies ($P = 0.086$). None of the other variables tested in this analysis were predictive of rejection in LVAD recipients, including T-cell PRA, matching at the HLA-DR, -B, or -A

Table 1

Influence of preformed anti-HLA antibodies in cardiac allograft recipients at risk for sensitization (n = 88) on cumulative annual rejection frequency post-transplantation

Pre-formed antibody type	Cumulative annual rejection frequency (number of 3A or 3B rejections/year)		
	Positive	Negative	P value
IgG anti-HLA class II	1.29	0.48	0.02
IgG anti-HLA class I	0.611	0.291	0.09
IgM anti-HLA class II	0.468	0.328	0.88
IgM anti-HLA class I			

Cumulative high-grade (3A/3B) rejections were modeled by the method of Wei, Lin, and Weissfeld [126], computing robust variance estimates allowing for the dependence among multiple event times.

loci, ischemic time, or donor age. Additionally, those patients with IgG anti-MHC class II antibodies at the time of transplantation had higher cumulative annual rejection frequencies than those without these antibodies (0.846 versus 0.169 high-grade rejections per patient year of follow-up). Among the demographic and immunologic variables examined, including the other antibody types, only the presence of pretransplant IgG anti-MHC class II antibodies was predictive of a higher cumulative annual rejection frequency ($P = 0.002$).

The mechanism by which the presence of pretransplant IgG anti-MHC class II antibodies relates to the post-transplant development of earlier and more frequent high-grade cellular rejections remains conjectural at present. Recent cumulative evidence has emerged that the indirect pathway of CD4 T-cell activation plays a major role in acute and chronic cardiac allograft rejection, due to continuous shedding of donor alloantigenic HLA peptides and their processing by host APCs, such as macrophages and B cells. Acute cardiac cellular rejection is accompanied by the appearance both in the circulation and in the allograft of recipient T cells reactive with donor HLA-DR peptides presented by self-APCs [107]. Primary rejections appear to be invariably accompanied by indirect recognition of a dominant HLA-DR allopeptide [107,108], whereas recurrent rejections appear to be accompanied by intermolecular spreading and T-cell recognition of multiple donor HLA-DR alloantigenic determinants [108]. Similar patterns of progressive intramolecular and intermolecular HLA-DR epitope spreading can be detected in cardiac transplant recipients developing accelerated transplant-related coronary artery disease [109]. This diversification of the immune response has been postulated to be a result of activation of antigen-specific B cells by soluble HLA-DR molecules, and the subsequent

efficient presentation of multiple HLA-DR allopeptides by self B cells to CD4 T cells [110–112]. Therefore, the relationship between recurrent high-grade cellular rejections and pre-existing IgG anti-MHC class II antibodies documented in this study may in fact indirectly reflect the presence in sensitized cardiac transplantation candidates of circulating memory B cells with reactivity to allogeneic HLA-DR molecules.

Therapeutic interventions for B-cell hyperreactivity

Regimen of intravenous immunoglobulin together with intravenous cyclophosphamide is superior to plasmapheresis for reduction of allosensitization

Recent studies have suggested that pooled human intravenous immunoglobulin (IVIg) is an effective modality to reduce allosensitization [113–117]. Postulated mechanisms include the presence in IVIg of anti-idiotypic antibodies [118–120], antibodies against membrane-associated immunologic molecules such as CD4 or CD5 [121,122], or soluble forms of HLA molecules [123,124]. We investigated the effects of IVIg on serum reactivity to HLA class I molecules in LVAD recipients, and compared these effects with plasmapheresis, an alternative modality for reduction of alloreactive antibodies [105,125].

We first evaluated the efficacy of monthly IVIg courses, at 2 g/kg, together with monthly infusions of IV cyclophosphamide (0.5 to 1.0 g/m²), on reduction of reactivity of circulating IgG antibodies for allogeneic HLA class I molecules. Data were obtained from 16 patients who received one to three monthly courses of IVIg (total 28 courses). Each course of IVIg was evaluated as an independent event, and the effects of each IVIg course on IgG anti-HLA class I antibodies during

the ensuing 4 weeks were analyzed. Within 1 week after infusion of IVIg in four divided daily doses, the reactivity of circulating IgG antibodies for allogeneic HLA class I molecules was reduced by a mean of 33% (range 14% to 52%, $P < 0.01$). This was the maximal level of reduction in alloreactivity during the 4 weeks post-IVIg infusion, with the efficacy of IVIg progressively decreasing by the end of week 4 to a mean reduction in alloreactivity of $8\% \pm 7\%$. Sequential courses of IVIg did not cause an additive effect on reduction of reactivity of circulating IgG antibodies with allogeneic HLA class I molecules. Each course resulted in a similar level of reduction in alloreactivity compared to baseline, with mean decreases of 38%, 36%, and 35% accompanying first, second, and third courses of IVIg, respectively. Six of the 16 highly sensitized patients were found to be resistant to treatment with IVIg at 2 g/kg, with a mean reduction of only 4% in reactivity of circulating IgG antibodies with allogeneic HLA class II molecules per treatment course in this group. These patients were subsequently treated with one to two courses of high-dose IVIg therapy, 3 g/kg in four divided daily doses. In each patient treated, high-dose IVIg therapy reduced reactivity of circulating IgG antibodies with allogeneic HLA class I molecules. Alloreactivity in this group was reduced by a mean of 20% (range 16% to 24%) per treatment course ($P < 0.05$).

We next compared the effects of IVIg (2 g/kg) with plasmapheresis on reduction of reactivity of circulating IgG antibodies with allogeneic HLA class I molecules in LVAD recipients. Four sensitized patients received one to two monthly courses of plasmapheresis, administered two to three times per week (total six courses). Reactivity of circulating IgG antibodies with allogeneic HLA class I molecules was not significantly reduced within the first 2 weeks after initiation of plasmapheresis. Maximal reduction in alloreactivity, $38\% \pm 11\%$, occurred by the fourth week of plasmapheresis. These results show that IVIg has earlier onset of action, and greater efficacy, in reducing IgG anti-HLA alloreactivity compared with plasmapheresis.

Therapy with IVIg together with IV cyclophosphamide shortens the waiting time to cardiac transplantation in sensitized patients

We next investigated whether treatment with IVIg (2 g/kg) together with IV cyclophosphamide

(0.5 to 1.0 g/m²) to reduce alloreactivity in sensitized recipients impacted on waiting time to transplantation. The first three highly sensitized LVAD recipients to receive desensitization therapy had unsuccessfully been waiting for cardiac transplantation for a mean of 303 ± 25 days before the onset of therapy as a result of repeated positive donor-specific crossmatches (mean 33, range 24 to 43). After initiation of IVIg/cyclophosphamide therapy, with or without additional immunodepletion using plasmapheresis, all patients obtained negative donor-specific crossmatches and were successfully transplanted in a mean duration of 99 ± 8 days. On the basis of these results, a formal protocol was established to initiate monthly courses of IVIg therapy (2 g/kg) with IV cyclophosphamide after initial detection of allosensitization. The duration from listing to cardiac transplantation was then compared among 28 sensitized patients who did not receive IVIg treatment and 16 sensitized patients who received one to two courses of IVIg (2 g/kg) together with IV cyclophosphamide after detection of anti-HLA class I IgG antibodies. None of these patients received additional plasmapheresis. Whereas the mean duration to cardiac transplantation was 7.1 months (range 0.2 to 17.9 months) in patients with IgG antibodies against HLA class I molecules, this was significantly reduced to 3.3 months (range 0.3 to 6.2 months) in sensitized recipients receiving one to two courses of IVIg (2 g/kg) ($P < 0.05$). No patient in either group was transplanted across a positive donor-specific IgG T cell crossmatch. This duration was similar to the waiting time to transplantation in 27 unsensitized patients, 3.1 months (range 0.3 to 10.7 months).

Post-transplant intravenous cyclophosphamide pulse therapy in sensitized cardiac transplant recipients reduces immunologic markers of alloreactivity

The post-transplant induction of immunologic markers of allograft rejection were next compared in sensitized cardiac allograft recipients who were treated with cyclosporine/steroid-based triple immunosuppressive regimens incorporating either intravenous cyclophosphamide pulses or oral mycophenolate mofetil. For this analysis, each treatment group contained 16 selected patients matched for comparable pretransplant values of anti-HLA class I and class II antibodies. In comparison with mycophenolate mofetil, treatment for

Table 2

Intravenous pulse therapy with cyclophosphamide is superior to mycophenolate mofetil for reduction of cumulative annual rejection frequency in sensitized cardiac allograft recipients

Preformed antibody type	Cumulative annual rejection frequency (number of 3A or 3B rejections/year)		
	Intravenous cyclophosphamide	Mycophenolate Mofetil	P value
IgG anti-HLA (total)	1.29	0.48	0.02
IgG anti-HLA class II	0.611	0.291	0.09
IgG anti-HLA class I	0.468	0.328	0.88

Cumulative high-grade (3A/3B) rejections were modeled by the method of Wei, Lin, and Weissfeld [13], computing robust variance estimates allowing for the dependence among multiple event times.

4 to 6 months with intravenous pulses of cyclophosphamide protected against IL2-receptor positive T-cell outgrowth from biopsy sites during the first post-transplant year ($P < 0.01$ by regression analysis). Moreover, cyclophosphamide prevented the post-transplant induction of IgG antibodies against HLA class II, but not class I, antibodies (defined as increase by $>10\%$ above pretransplant values). Whereas nine of 16 (56%) mycophenolate mofetil-treated patients produced increased levels of anti-HLA class II IgG antibodies, only two of 16 (13%) cyclophosphamide-treated patients showed an increase in anti-HLA class II IgG antibodies ($P = 0.009$). These data indicate that post-transplant use of intravenous cyclophosphamide in sensitized patients is superior to mycophenolate mofetil at preventing recipient T-cell and B-cell responses to donor HLA class II alloantigens.

Post-transplant intravenous cyclophosphamide pulse therapy in sensitized cardiac allograft recipients prolongs rejection-free interval and decreases cumulative rejection frequency

We next compared cardiac allograft rejection in sensitized recipients ($N = 74$) treated with cyclosporine/steroid based triple immunosuppressive regimens incorporating either intravenous cyclophosphamide pulses or oral mycophenolate mofetil [125]. Immunosuppression using intravenous pulses of cyclophosphamide in sensitized recipients for 4 to 6 months post-transplantation

significantly prolonged the rejection-free interval compared with mycophenolate mofetil (Table 2). Overall, only four of 26 (15%) cyclophosphamide-treated patients developed one or more high-grade rejections within the first post-transplant year compared with 22 of 48 (46%) patients treated with mycophenolate mofetil ($P = 0.009$). Cyclophosphamide treatment had the same effect on sensitized recipients with either pre-formed IgG anti-HLA class I antibodies ($P = 0.02$) or class II antibodies ($P = 0.04$). Moreover, treatment with cyclophosphamide reduced the cumulative annual rejection frequency by 63%, from 0.94 rejections per year for sensitized patients treated with mycophenolate mofetil to 0.35 rejections per year ($P = 0.03$). The latter value is within the same range as the annual rejection frequency in non-sensitized patients at our institution. Again, similar results were found regardless of whether sensitization was due to IgG anti-HLA class I or class II antibodies.

Post-transplant intravenous cyclophosphamide pulse therapy in sensitized cardiac allograft recipients is the only protective factor against rejection by multivariable analysis

By Cox Proportional Hazard modeling for multivariable analysis, the only significant protective factor against development of high-grade cellular rejection in sensitized patients was treatment with cyclophosphamide (Table 3). In comparison with cyclophosphamide, mycophenolate

Table 3

By multivariable analysis, using the Cox Proportional Hazards model, treatment of sensitized cardiac allograft recipients with mycophenolate mofetil ($n = 48$) portends a significantly higher risk for cellular rejection than intravenous cyclophosphamide ($n = 26$)

Variable	Coefficient \pm SE	P value	Risk ratio	95% CI
Mycophenolate mofetil	1.035 \pm 0.4393	0.0184	3.7	(1.19, 6.66)
Cyclophosphamide	0.908 \pm 0.5295	0.0863	1.0	(0.88, 7.00)

mofetil treatment conferred a 3.7-fold higher risk of rejection ($P = 0.009$). None of the other variables tested were protective against or predictive of rejection in this group of sensitized individuals, including matching at the HLA-DR, -B, or -A loci, ischemic time, or donor age.

Safety profile of pretransplant and post-transplant immunosuppressive therapy in sensitized cardiac allograft recipients

Treatment with intravenous cyclophosphamide has proved to be extremely safe. The incidence of cytomegalovirus (CMV) disease (defined as clinical disease together with virologic culture confirmation) was lower in cyclophosphamide-treated patients (three of 26, 12%) than in those treated with mycophenolate mofetil (10 of 54, 19%). No other viral, bacterial or fungal infections were seen in patients treated with cyclophosphamide. Intravenous pulse therapy with cyclophosphamide was frequently (> 80%) accompanied by transient nausea and vomiting, which responded to antiemetic therapy. Mesna was co-administered with cyclophosphamide and may have contributed to the absence of any cases of hemorrhagic cystitis. No malignancies have developed after 540 patient months of follow-up (range of follow-up per patient 6 to 38 months). IVIg therapy was associated with clinical manifestations of immune complex disease in four of 27 (15%) monthly courses, as evidenced by fevers, arthralgias, and maculopapular rashes. Reversible renal insufficiency (defined as > 50% increase in serum creatinine level) occurred in four cases, all of which resolved spontaneously over the ensuing 3 weeks postinfusion.

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