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Temporal expression of cytokines and B-cell phenotypes during mechanical circulatory support

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Abstract

Background: Allosensitization during mechanical circulatory support (MCS) is a well-described phenomenon, although its mechanism remains unknown. Although immune-mediated interactions from devices or blood transfusions have been proposed, the role of inflammation in this development is less clear. This study was undertaken to further investigate the temporal association of cytokines and B-cell phenotypes in the MCS population.

Methods: Adult patients who received the Heartmate II (Thoratec, Pleasanton, Calif) at our center between September 2012 and March 2015 were prospectively followed after device implantation. Blood draws for anti-human leukocyte antigen (HLA) antibody, cytokine expression, and B-cell immunophenotyping were performed before implantation and for 3 weeks postoperatively. Time courses for cytokines and B-cell subsets were expressed using visual representations of median levels as heat maps, and mixed modeling analysis was used to model changes with time and patient factors.

Results: Twenty patients who received the Heartmate II (Thoratec) were analyzed during the study period. Four patients showed measureable levels of anti-HLA antibody during the follow-up period, although 3 of these had evidence of antibodies preoperatively. Analysis of cytokine trends revealed early (interleukin [IL]-6, IL-8, and IL-10) and late peaking (IL-3, IL-4, fibroblast growth factor 2, and CD40L) patterns. Upregulation of switched memory, transitional, and plasma blast B cells occurred over time. Right ventricular assist device use and low Interagency Registry for Mechanically Assisted Circulatory Support score were associated with decreased mature naive and increased antibody-secreting cells.

Conclusions: MCS device implantation was associated with increased inflammatory cytokines and maturation of B-cell phenotypes. No patients developed de novo HLA antibodies, whereas

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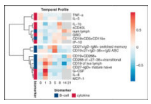
several showed increases in anti-HLA antibody levels detected before implantation. This suggests that inflammation and maturation of existing sensitized B cells might play an important role in the pathogenesis of allosensitization in MCS.

Graphcial Abstract



Significant changes in antibody-producing B cells and inflammatory cytokines during MCS.

Keywords

mechanical circulatory support; ventricular assist device; device mediated sensitization; device-mediated inflammation; B-cell maturation

Allosensitization in patients bridged to heart transplantation with mechanical circulatory support (MCS) devices (MCSDs) is a well-known phenomenon.[1–3] This development of circulating antibodies directed against human leukocyte antigens (HLAs) leads to increased time on the transplant waiting list and the potential to develop donor-specific antibodies after transplantation.[4,5] Along with multiparity, previous cardiac surgery using homografts, and a history of blood transfusions, MCSDs are now considered a significant and independent risk factor for allosensitization.[6,7]

The exact mechanism of allosensitization during MCS, however, is poorly understood. Originally noted during the era of the first-generation ventricular assist devices Abbreviations and Acronyms (VADs), allosensitization had previously been attributed to host interactions with the neointimal surfaces that formed within the relatively large chambers of these early pumps.[8–10] However, we continue to see sensitization, albeit at a lower rate, persist in the current era of smaller, axial flow and centrifugal designs.[1] Regardless of cause, management of allo-antibodies is a vexing issue. Current recommendations for desensitization therapy in this patient population are class IIb on the basis of level of evidence C.[10] Despite this lack of clarity afforded by randomized clinical trials, most programs treat their patients regardless of cause when panel reactive antibodies are>50%.[11] This is despite the known risks, medication toxicities, and infections associated with intravenous Ig infusions, plasmapheresis, and/or B cell-directed therapies such as rituximab.

Understanding the mechanism by which MCSD-mediated sensitization occurs might shed light not only on the clinical significance of these antibodies, but also on the best method of treatment if warranted. The role of the effects of inflammation on central and extramedullary B-cell lymphopoiesis has received heightened scrutiny in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. Toll-like receptors, pattern recognition receptors that are integral to the function of the innate immune system, are increasingly recognized as a key link between environmentally based inflammation and autoimmunity.[8,12,13] Anti-HLA antibody formation in the MCSD patient, however, seems to be assumed to be triggered by an immune-mediated process, either affected by the contact

of blood to neointimalized surfaces of devices or because of blood product use.[8,9] The inflammatory nature of MCSDs is undeniable but poorly studied. The goals of the current study, thus, were to document the inflammatory nature of MCSDs through the temporal expression of various cytokines during device support. Simultaneously, B-cell maturation phases were identified taking into account the various phenotypes present during device support. By correlating these findings, we sought to further investigate the possible link between MCSD-mediated inflammation and antibody-secreting B-cell development.

METHODS

This study was a prospective observational study of adult patients (age >18 years) who underwent Heartmate II (HMII, Thoratec, Pleasanton, Calif) device implantation between September 2012 and March 2015 at our center. Consenting patients received HMII implantation and postoperative management as a part of standard clinical practice. Additional peripheral blood draws for cytokine and B-cell expression assays were performed at baseline, on postoperative days 1, 3, 5, and 8, and then weekly thereafter. This study was reviewed and approved by the institutional review board at the University of California, Los Angeles.

Data on patients including demographic characteristics and etiology of heart failure were collected for all patients. The University of California, Los Angeles Immune Assessment Core was used for all cytokine and B-cell expression assays. Cytokine and chemokine concentrations from plasma samples were assayed using the 38-multiplex MILLIPLEX Human Cytokine Chemokine Panel I (EMD Millipore, Merck KGaA, Darmstadt, Germany). In addition, HLA class I and II single-antigen Luminex antibody profiles were performed via flow cytometry on available samples as previously described.[14] Allosensitization was defined as HLA antibody production (mean fluorescence intensity>5000) during the MCSD course. B-cell multiparameter immunophenotypes were performed by staining peripheral blood mononuclear cells for surface markers using fluorochrome-tagged antibodies against CD3, CD5, CD11b, CD19, CD24, CD27, CD38, CD268, IgD, IgM, and IgG (One Lambda, Inc, Canoga Park, Calif). Data were acquired using the BD LSR Fortessa (BD Biosciences, San Jose, Calif) and analyzed using the FCS Express software (De Novo Software, Los Angeles, Calif).

Demographic information for all patients in the study was reported as mean ± standard deviation or frequency (percent of population). Linear mixed modeling was then performed in a sequential fashion to determine the effects of demographic factors such as sex, age, Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) class, necessity for right VAD (RVAD) support, and pre-VAD sensitization on cytokine and B-cell phenotypes. The INTERMACS scale identifies patients with advanced heart failure according to 7 levels depending on hemodynamic profile and level of end organ damage.[15] A detailed explanation and full report of our statistical methods are available in an online supplement, and can be viewed at https://nwisn.github.io/B-cell/supplement.html. In brief, we were interested in examining whether group effects were more salient at particular time points. Because our sample size did not permit testing a full multifactorial model, we restricted our investigation to linear mixed effect models consisting of a group factor,

time, and the interaction of that factor with time. For all mixed effect models, a random intercept accounted for within-subject variability. We also performed post hoc testing using the estimated marginal means at each time point, again assessing each factor separately. Statistical significance was adjusted for multiple testing, and we controlled all results at a false discovery rate of 10%. All significant associations were graphed separately over time, and are available for complete review in our online supplement (https://nwisn.github.io/B-cell/supplement.html).

Next, to determine temporal trends of cytokine and B-cell phenotypes and identify patterns, hierarchical clustering was performed over time, using standard clustering packages in R (R Foundation for Statistical Computing, Vienna, Austria). After generating clustering groups according to biomarker and over time and sorting all phenotypes appropriately, time courses were visualized by displaying individual levels at each time point as a heatmap. The heatmaps were made with R using the "pheatmap" package[16,17]; the individual levels displayed were computed by first double-standardizing the data set (a preferred method in bioinformatics), which converts each of the biomarker levels to a z-score, red denoting greater than average and blue denoting less than average values, respectively. For example, a red box at the first time point indicates that the mean value of the cytokine at that time point is greater than the mean value of all other values for all-inclusive time points. In this way, trends over time can be appreciated according to changes in color, while still maintaining the relative dynamic range of each biomarker. R (R Foundation for Statistical Computing) was used for mixed effect analyses and figure creation for immunophenotype time course and multiplex cytokine analysis.

RESULTS

A total of 20 patients received HMII support and consented to participate in this study during the study period. The average age of these recipients was 58 ± 15 years, and 75.0% were male. The average INTERMACS score was 2.45 ± 0.69 and biventricular support was required in 25% of cases. Baseline demographic information for our patient population is noted in Table 1.

Allosensitization

Of the 20 patients included in the study, 10 patients had HLA antibody profiles available. Among these 10, 4 (40%) showed allosensitization post VAD implantation, as evidenced by the presence of circulating HLA antibodies (≥5000 mean fluorescence intensity). Of these, 3 had evidence of antibodies pre-VAD insertion. The remaining patient had his first anti-HLA antibody profile tested 7 months after device insertion. Figure 1 shows, for each patient, the (1) average mean fluorescence intensity for all detected antibodies, and (2) number of unique detected antibodies before VAD implantation and after implantation just before transplantation (both $P>.50$). Although no patient who expressed 0 antibodies at baseline became sensitized de novo in our series, those with at least 1 preexisting antibody did go on to express new anti-HLA antibodies during their follow-up as indicated in the Figure 1.

Temporal Expression During MCSD

In clustering analysis by time, the optimum number of clusters that best fit our data with maximized silhouette width was 4. Thus, 4 distinct temporal patterns of biomarkers were noted: (1) those with early peak on postoperative day 1 followed by a late decrease, (2) those with early decrease followed by a peak approximately 2 weeks postimplantation, (3) those with an intermediate peak at approximately 5 days postimplantation, and (4) those with a steady rise throughout the entire 3-week follow-up period. These clusters are shown in Figure 7 in our online statistical supplement (https://nwisn.github.io/B-cell/supplement.html).

The temporal expression of cytokine expression in the 20 study patients pre- and post-MCSD insertion is shown in Figure 2. Early peaking cytokines with peaks on postoperative day 1 included interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, granulocyte-colony stimulating factor, and IL-10, whereas IL-1b, IL-3, IL-4, growth-related oncogene, and CD40L peaked later during the MCSD course. B-cell phenotype over the interval of MCSD therapy is similarly expressed in heat map form shown in Figure 3. When examining B-cell phenotypes in our population overall, several phenotypes were noted to rise and peak in the immediate postoperative setting with a subsequent downtrend, including several mature naive populations ($CD19^+24dim38dim$, $CD27\text{-}IgD^+$) and $CD19^+268^+$ cells. Those with peaks at the intermediate time points included antigen-secreting $CD19^+CD27^+$ cells, and select switched memory populations ($CD19^+24^+hi$, $CD27^+IgD\text{-}IgM^+$). Finally, several populations were noted to have late peaks included several transitional populations ($CD19^+24^+38^+$, $CD5^+$, $CD19^+CD5^+CD11b^+$) and plasma blasts ($CD27^+38^+$).

Association of Biomarkers With Factors

In mixed-model analyses, several factors were shown to affect the expression of cytokines and B-cell phenotypes during MCSD. Patients with low INTERMACS score (INTERMACS 1 or 2) were found to have a generalized increase in levels of IL-1b and $CD19^+27^+38^+$ antigensecreting cells (Figure 4). Patients requiring biventricular support had increased early levels of granulocyte-colony stimulating factor and IL-8, along with again increasing levels of $CD19^+27^+38^+$ antigen-secreting cells (Figure 5). However, use of an RVAD was associated with decreasing $CD19^+CD268^+$ cells overall. As might be expected, younger patients had a generally increased number of overall lymphocytes throughout their course. Interestingly, older patients appeared to have later peaking $CD27^+38\text{-}CD268^+$ transitional cells, with a bimodal peak at approximately 2 weeks compared with approximately 1 week in younger patients. A similar trend was seen among $CD27\text{-}IgD^+$ mature naive cells. A complete list of all significant interactions identified is shown section 2.5 of the online supplement (https://nwisn.github.io/B-cell/supplement.html).

DISCUSSION

The increasing use of MCSD as a bridge to transplantation has led to close scrutiny of HLA antibody status in the patients being bridged with devices because of the risk of antibody-mediated rejection from donor-specific antibodies. As such, many data have been accumulated in these patients with the observation made that a number of these

patients seem to become sensitized.[1–4] Although varying hypotheses as to why this occurs have been generated, none have convincingly shown the mechanism behind an adaptive, antigen-specific immune response. However, although inert, these devices undoubtedly exert an innate, inflammatory effect. In the current study, no de novo allosensitization was noted among any patients studied; however, increases in anti-HLA antibodies in patients with preexisting sensitization was noted, in concert with a pattern of increases in proinflammatory cytokines and maturation of antibody-producing B cells. This suggests a potential role for inflammatory-mediated immune responses in allosensitization noted among MCSD patients.

The production of antibody by antibody-secreting plasma blasts and plasma cells requires the complex coordination of B-cell development. T cell-dependent and T cell-independent antigens might be recognized by the naive B-cell receptor of a mature B cell, and trigger activation and differentiation into active plasma blasts or translocation to germinal centers for further development and affinity maturation.[18] This development might be influenced by a variety of extrinsic factors including cytokines/chemokines, direct T-cell activation or immunogenic signals, and stimulants of toll-like receptors. Furthermore, recognition of previously encountered antigens by memory cells and subsequent transformation into antibody-secreting cells are also subject to similar external influences.[18] Thus, it follows that allosensitization might result from: (1) neoantigen presentation during MCSD and subsequent antibody synthesis, or (2) overcoming anergic signaling or otherwise promoting quiescent B-cell activation via extrinsic factors induced by MCSD, such as increases in levels of proinflammatory cytokines/chemokines.

Previous studies have done much investigation into the immunologic properties of MCSD, and a variety of immunologic mechanisms have been proposed to explain allosensitization. Shear stress exerted by flow patterns through the device have been implicated in endothelial and platelet activation, both of which have links to HLA antibody formation.[19,20] The biomaterials and grafts used, although designed to be inert, have been implicated in the potential for neoantigen exposure and subsequent presentation to B cells.[21,22] Furthermore, it has been highlighted that left VAD surfaces become colonized with T cells, macrophages, and monocytes throughout the course of MCSD.[8,9] The nature of this “pseudo-intima” formed involves aberrant T-cell activation with defective proliferative mechanisms, and subsequently increased B-cell activity, in part mediated through CD40-CD40 ligand interactions.[23]

The introduction of newer generation devices with continuous flow mechanisms, smaller priming volumes, and textures with improved biocompatibility have led to reductions in the incidence of allosensitization in most contemporary reports.[1,3] However, MCSD persists as a significant risk factor for allosensitization despite controlling for known confounders such as blood product transfusions. In fact, Drakos and colleagues paradoxically correlated increasing rates of transfusion of leukocyte-filtered blood products with decreased rates of allosensitization, which might speak to a potential immunomodulatory effect of blood transfusions.[24] Of note, the finding that no patients were de novo sensitized in our report of modern-era MCS is noteworthy. We had previously reported this finding in an earlier report of VAD use at our center, and in a larger contemporary report, Coppage and

colleagues reported a<10% de novo sensitization rate with the use of a leukoreduced transfusion protocol during VAD support.[25,26] Although early studies of VAD-mediated sensitization report higher rates of de novo sensitization, improvements in antibody-detecting technologies such as the Luminex platform used in the current study might be more sensitive in detecting subclinical levels of antibody before VAD implantation.[27] This pattern of increasing HLA antibodies among patients with previous antigen exposure and the persistence of allosensitization despite improvements in adaptive immune triggers with modern MCSDs suggests an additional mechanism for allosensitization.

Temporal analysis of the cytokines assayed during MCS in the current study revealed early- and late-peaking phases of cytokine expression. Early after implantation, large spikes in MCP, IL-6, IL-8, and IL-10 concentrations are seen. IL-8 is known to be a chief driver of the innate immune response related to the inflammatory response of MCSD insertion.[28] IL-6 expression has been heavily associated with B-cell activity and differentiation, and has been shown to be mediated by CD40-CD40 ligand interactions, previously shown to be upregulated by MCSD.[29,30] MCP-1 is known recruit and direct monocyte/macrophage activity, but also has evidence in directing T-helper cells responses and might directly interact with IL-4 to promote helper T-cell activity and subsequent antibody production from quiescent B cells.[31]

Still later-peaking cytokine responses included growth factors such as vascular endothelial growth factor, fibroblast growth factor (FGF), and epidermal growth factor, along with IL-4, −5, −3, and 1b. FGF is a multifunctional cytokine with multiple downstream targets and effects including angiogenesis, epithelial cell, and fibroblast proliferation. Interestingly, FGF production has been shown to correlate with B-cell lymphomas and decrease with treatment.[32] CD40 ligand was also found to be increased in later time periods, again likely representing the effects of the “neointima” formation previously described, and a possible immunologic link leading to increased B-cell activity.[9,23]

Previous studies have shown a correlation between polytetrafluoroethylene and dacron grafts with inflammation around the conduits within 4 weeks of implantation as seen by the abundance of inflammatory cells, in particular multinucleated giant foreign body cells.[33] Patients receiving hemodialysis have also been shown to have higher concentrations of chemokines MCP-1, macrophage inflammatory protein 1a, and IL-8 in the periphery as well as in the circulation along with increased CD11b expression on leukocytes compared with healthy controls.[34] MCSDs have components of dacron grafts as well as artificial blood-carrying circuits that are many fold larger than that present in standard hemodialysis units. In fact, if one considers the leukocyte dysfunction with predisposition to infection with subsequent upregulation of various inflammation meditators in hemodialysis patients there are many corollaries to our experience with MCSD patients.[35]

During the study period, naive B-cell phenotypes largely decreased whereas transitional, plasma blast, and switched memory cells increased, consistent with an overall picture of B-cell maturation. The association of B-cell phenotypes with various comorbidities is also noteworthy, with the general theme of increasing plasma blast and transitional cell populations with worse illness or concomitant RVAD support, being possibly consistent with

a heightened state of inflammation. An additional potential immunologic link between the inflammatory response and B-cell phenotype changes might be found in the B-cell activating factor (BAFF) pathway, which directly stimulates the proliferation of B cells and might provide signaling to overcome quiescence.[36] Although not directly assayed, members of the BAFF cytokine family (other tumor necrosis factors) were noted to be upregulated during late MCS. A decrease in BAFF receptor carrying B cells ($CD268^{+}$) were simultaneously noted throughout support. An inverse relationship between BAFF and BAFF receptor cells has been previously described, thus indicating increases in soluble BAFF levels during MCSD support as a possible mechanism explaining allosensitization.[36]

This study has several weaknesses that might limit the strength of conclusions drawn. First, only 3 weeks of postoperative cytokine and antibody levels were routinely examined in all patients, and because of variable treatment courses, values were not available for all patients studied. This does limit the association of biomarkers with clinical status over longer periods and the progression of heart failure. Because sensitization is likely a dynamic process, it is unclear if additional patients might have become sensitized if followed for longer periods of MCS. In addition, the population analyzed represents a critically ill heart failure cohort who have greatly varying clinical courses; thus, controlling for postoperative treatment courses and transfusion requirements was not possible and might have influenced results.

CONCLUSIONS

In the current study, MCSDs were associated with a pattern of increased inflammation and inflammatory cytokine release during the early support period, mirrored by a change in B-cell phenotypes that favors mature, antibody-producing B-cell populations. However, de novo HLA allosensitization was not observed in our cohort; instead, patients with a predisposition to sensitization began secreting more antibodies during MCSD support. The link between inflammation and B-cell maturation has been extensively reported in the literature. Thus, a reasonable hypothesis follows that MCSD-mediated sensitization might not be a direct result of adaptive immunity and neoantigens produced during MCSD support, but rather the result of inflammatory upregulation in susceptible individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Acronyms

BAFF	B-cell activating factor
FGF	fibroblast growth factor
HLA	human leukocyte antigen

HMII	Heartmate II (Thoratec, Pleasanton, Calif)
Ig	immunoglobulin
IL	interleukin
INTERMACS	Interagency Registry for Mechanically Assisted Circulatory Support
MCP	monocyte chemoattractant protein
MCS	mechanical circulatory support
MCSD	mechanical circulatory support device
RVAD	right ventricular assist device
VAD	ventricular assist device

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Central Message

Inflammatory changes and maturation of existing sensitized B cells, rather than exposure to neoantigens, might play an important role in the pathogenesis of allosensitization in MCS.

Perspective

Previous studies have proposed an adaptive immune response to neointimal device surfaces as the mechanism for allosensitization in MCS. Demonstration of inflammatory markers with concomitant maturation of B cells suggests an inflammatory contribution to allosensitization. Further studies will be necessary to understand the significance of these antibodies and the appropriate method of treatment.

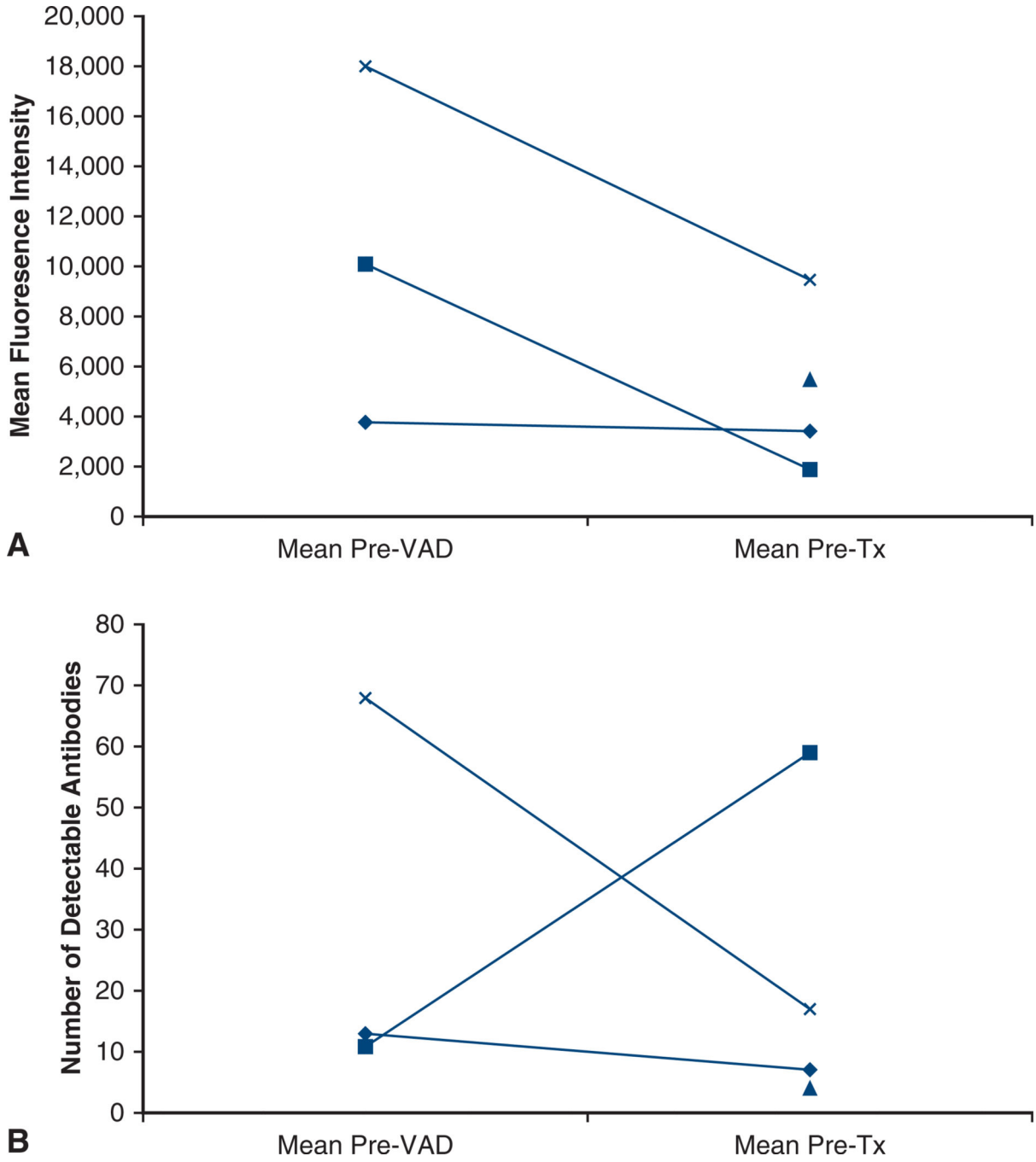

FIGURE 1.

MFI patterns for HLA antigens before and after LVAD implantation. A, Mean MFI values for all antibodies present and (B) total number of detectable, unique antibodies present before and after VAD implantation for available patients who became sensitized (≥5000 MFI) during VAD therapy. No significant trends were observed in the number and average intensity of antibodies during VAD support. *VAD*, Ventricular assist device; *Tx*, treatment.

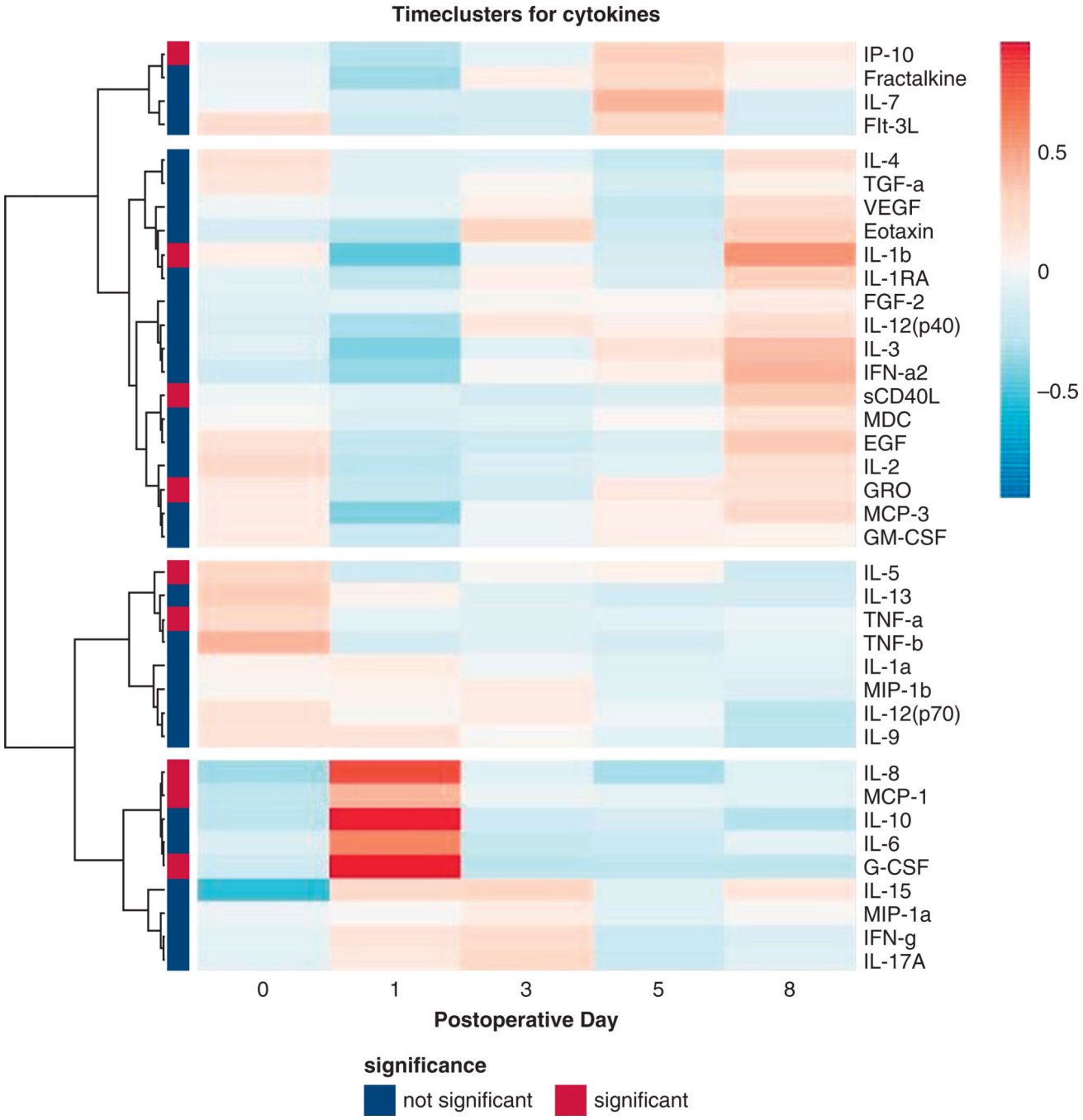

FIGURE 2.

Temporal expression of cytokines during MCS. Heat map box colors depict a comparison between mean levels at each time point, scaled to z-score, with reference to the mean of the entire cohort (*red* = high, *blue* = low). The corresponding color is derived from the –log (*P* value) computed using the Wilcoxon test. Cytokines with similar heat map profiles were clustered together per the "pheatmap" algorithm in R. Clusters of early-peaking cytokines can be visualized including IL-8, MCP-1, and G-CSF, whereas clusters of later-peaking cytokines include IL-1b and CD40L. *MCS*, Mechanical circulatory

support; *IL*, interleukin; *MCP*, monocyte chemoattractant protein; *IP-10*, interferon gamma-induced protein 10; *Flt-3L*, FMS-like tyrosine kinase 3 ligand; *TGF*, tumor growth factor; *VEGF*, vascular endothelial growth factor; *FGF*, fibroblast growth factor; *IFN*, interferon; *CD*, cluster of differentiation; *MDC*, macrophage-derived chemokine; *EGF*, epidermal growth factor; *GRO*, growth-related oncogene; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *TNF*, tumor necrosis factor; *MIP*, macrophage inflammatory protein; *G-CSF*, granulocyte-colony stimulating factor.

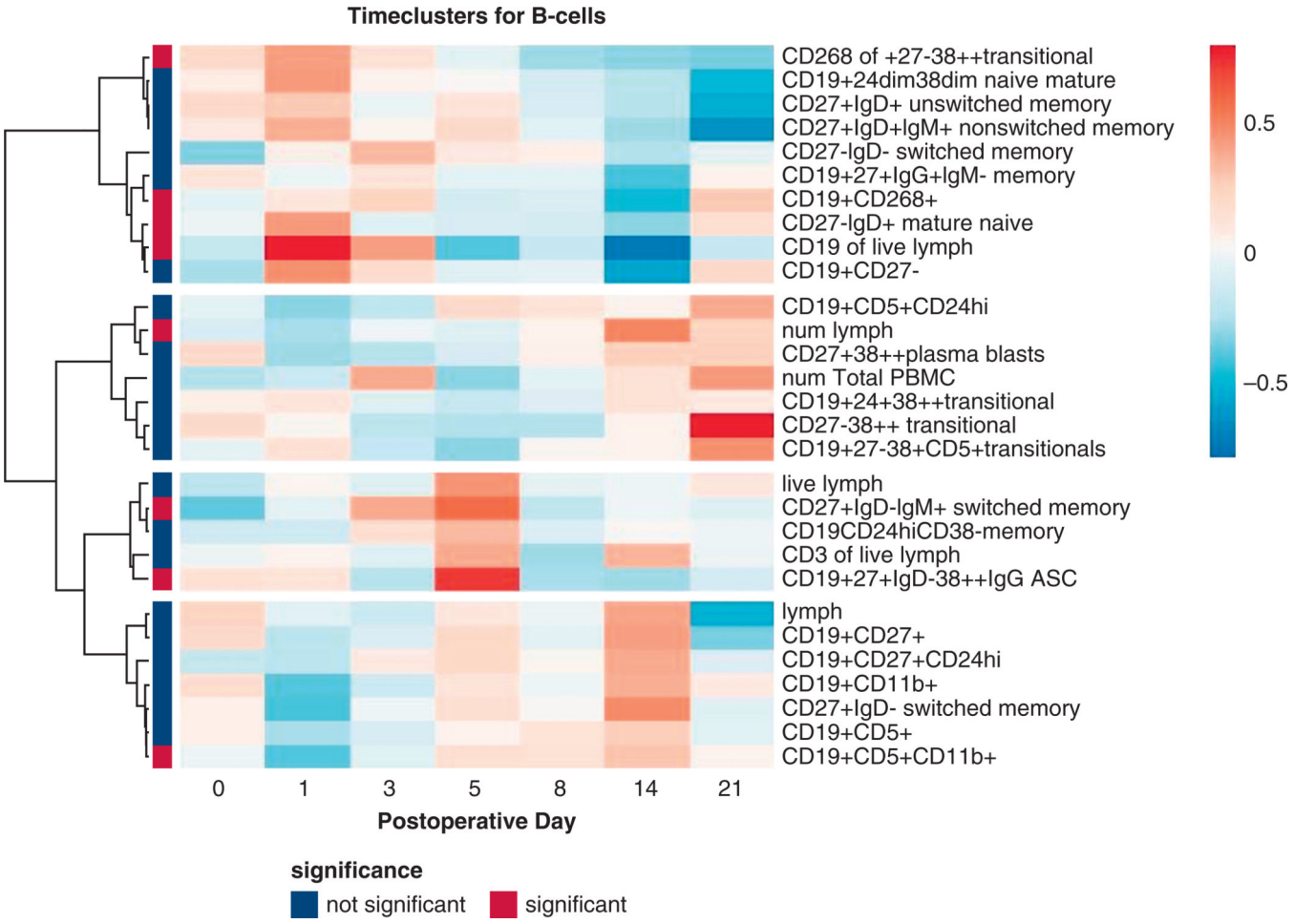

FIGURE 3.

Temporal expression of B-cell phenotypes during MCS. Heat map box colors depict a comparison between mean levels at each time point, scaled to z-score, with reference to the mean of the entire cohort (*red* = high, *blue* = low). The corresponding color is derived from the –log (*P* value) computed using the Wilcoxon test. Phenotypes with similar heat map profiles were clustered together per the “pheatmap” algorithm in R. Clusters of early-, intermediate-, and late-peaking phenotypes can be appreciated throughout MCS, in an overall pattern consistent with B-cell maturation. *MCS*, Mechanical circulatory support; *CD*, cluster of differentiation.

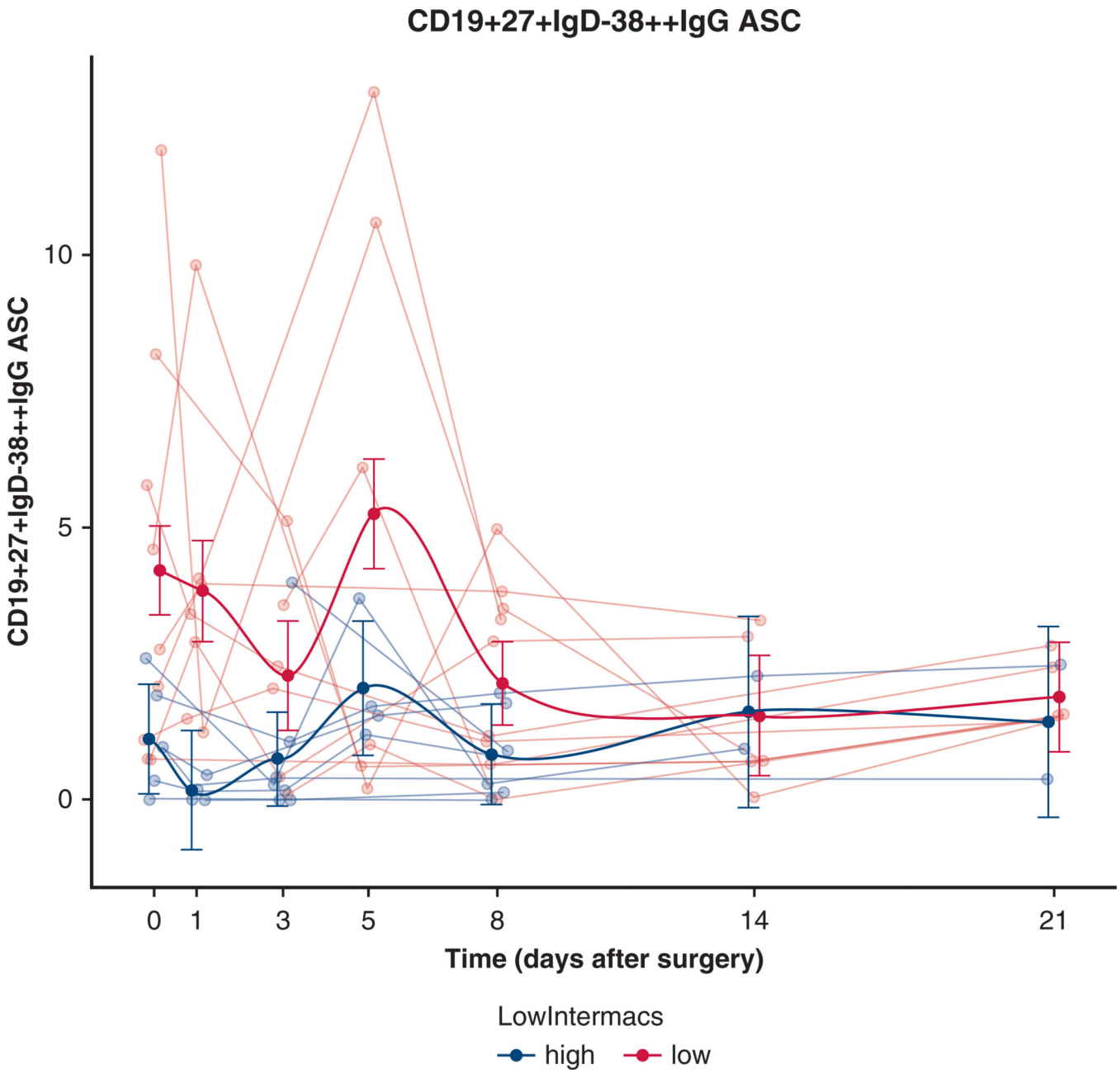

FIGURE 4.

ASC population during MCS according to INTERMACS profile. Patients with low INTERMACS profile (1 or 2) had significantly higher amounts of ASC populations. *ASC*, Antigen-secreting cell; *CD*, cluster of differentiation.

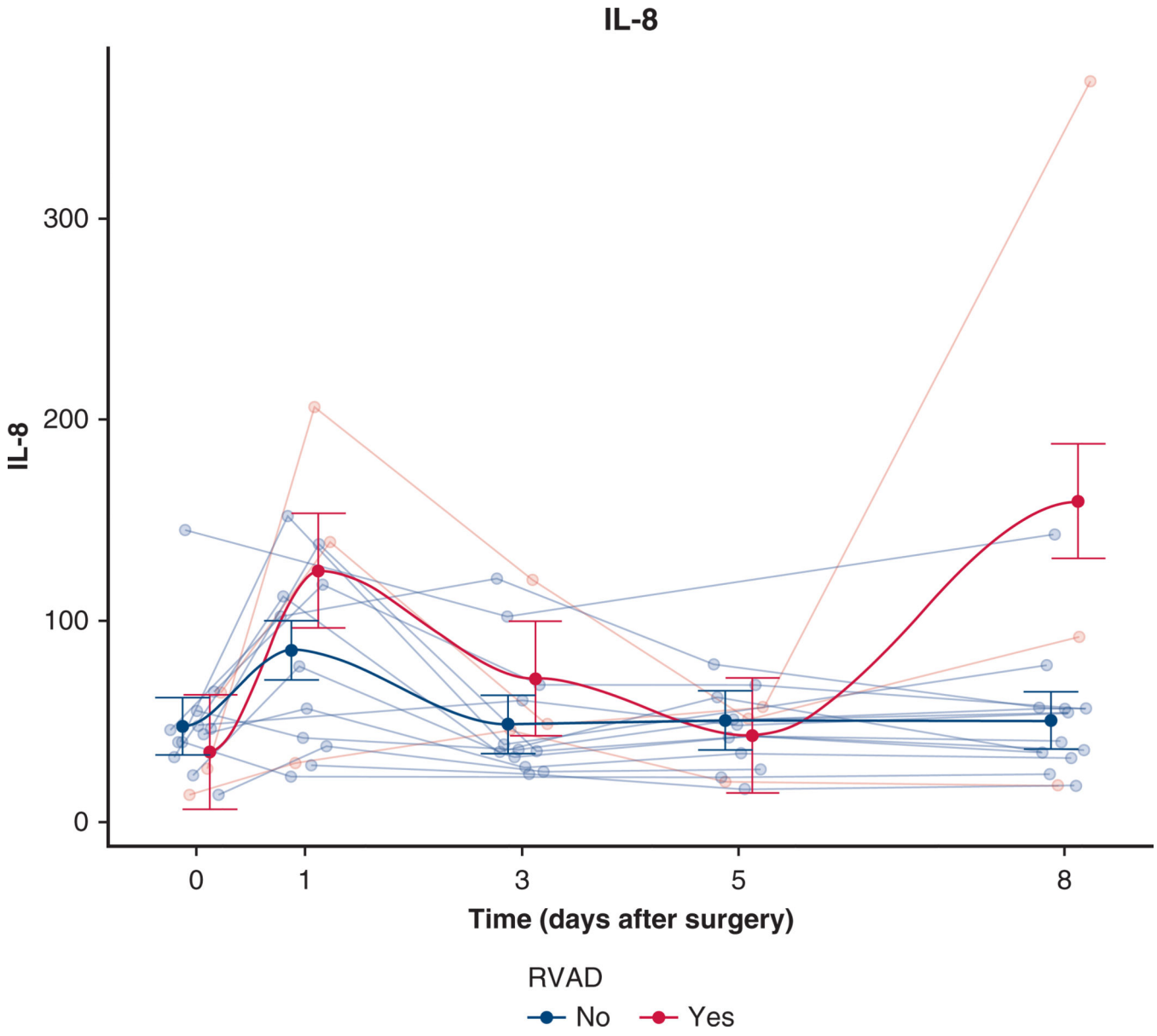

FIGURE 5.

Interleukin (*IL*)-8 expression during MCS by concomitant RVAD support. Patients with concomitant right-sided support had significantly higher amounts of IL-8 throughout the support period.

TABLE 1.

Demographic information (N = 20)

Preoperative variable	Value
Age, y	58 ± 15
Male sex	15 (75.0)
INTERMACS score	2.45 ± 0.69
Biventricular support	5 (25.0)
Diagnosis	
Ischemic cardiomyopathy	8 (40)
Nonischemic cardiomyopathy	12 (60)
HLA antibodies available	10 (50)
Evidence of sensitization	4 (20)

Data are presented as mean ± standard deviation or frequency (percent of population). *INTERMACS*, Interagency Registry for Mechanically Assisted Circulatory Support; *HLA*, human leukocyte antigen.