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Mediators and mechanisms of immune checkpoint inhibitor associated myocarditis: insights from mouse and human

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Summary

The broad application of immune checkpoint inhibitors (ICIs) has led to significant gains in cancer outcomes. By abrogating inhibitory signals, ICIs promote T cell targeting of cancer cells but can frequently trigger autoimmune manifestations, termed immune related adverse events (irAEs), affecting essentially any organ system. Among cardiovascular irAEs, immune related myocarditis (irMyocarditis) is the most common and carries the highest morbidity. The currently recommended treatment for irMyocarditis is potent immunosuppression with corticosteroids and other agents, but this has limited evidence basis. The cellular pathophysiology of irMyocarditis remains poorly understood, though mouse models and human data have both implicated effector $CD8^+$ T cells, some of which are specific for the cardiomyocyte protein α-myosin. While the driving molecular signals and transcriptional programs are not well defined, the involvement of chemokine receptors such as CCR5 and CXCR3 has been proposed. Fundamental questions regarding why only approximately 1% of ICI recipients develop irMyocarditis and why irMyocarditis carries a much worse prognosis than other forms of lymphocytic myocarditis remain unanswered. Further work in both murine systems and with human samples are needed to identify better tools for diagnosis, risk-stratification, and treatment.

Keywords

Immune checkpoint inhibitors; myocarditis; cardio-oncology; immune related adverse events

Introduction

Immune checkpoint inhibitors (ICIs) are a class of cancer drugs which represent one of the most substantial advances in oncologic therapy in decades. By unleashing the remarkable power of the immune system, clinical outcomes across multiple tumor types have improved, sometimes vastly. Yet the activation of otherwise-restrained immune effectors can lead to

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a multitude of off-target consequences across the body, some of which occur in the heart. The most common of the cardiac toxicities from ICI therapy is immune-related myocarditis (irMyocarditis), which can be fatal in up to 50% of patients. This review is intended to achieve two goals: first, to summarize the current understanding of the mechanisms at play in irMyocarditis; and second, to posit hypotheses about why it carries greater morbidity than other types of cardiac inflammation and why it occurs in only a very small subset of ICI recipients. This discussion is intended to stimulate further investigations into the underlying biology which, in turn, will aid the development of new therapeutic approaches to help patients suffering from this highly morbid condition.

Background and history of immunotherapy

Immune checkpoints are signaling molecules (receptors and ligands) that transmit inhibitory signals towards immune cell activation or function. The mechanisms used by cytotoxic T lymphocyte–associated protein 4 (CTLA-4) to inhibit T cell responses to tumor cells was elucidated in late 1990s[1, 2], and subsequent work on the programmed cell death 1 (PD-1) receptor demonstrated a similar role in tumor immunosurveillance[3]. ICIs act to inhibit these negative regulators of T cell activation and function, thus allowing those cells to effectively target and destroy tumor cells[4]. The clinical impact of this class of drugs has revolutionized the landscape of oncological treatment. The first FDA approved treatment for an immune checkpoint inhibitor was in 2011 for the treatment of metastatic melanoma with the CTLA-4 targeting antibody ipilimumab[5], and many approvals have followed in the subsequent 12 years[6]. There are currently eight FDA–approved ICIs, and they are routinely used in the treatment of more than 20 cancer types, with indications in metastatic disease and also as adjuvant and/or neoadjuvant therapy[6]. With many ongoing trials, the number of eligible cancers and specific indications is likely to grow[7].

ICI toxicities

Through their mechanism of immune activation, ICIs can offer effective cancer treatment, but they can also trigger a wide range of autoimmune side effects collectively known as immune-related adverse events (irAEs)[8]. These irAEs represent clinical challenges unique to ICI therapy, with the most commonly reported toxicities including thyroiditis, pneumonitis, colitis, hepatitis, and dermatitis (rash)[9]. IrAEs are estimated to occur in up to 60% of ICI recipients[10], though the majority are not clinically severe. The rate of severe irAEs requiring hospitalization is 2.6% per patient treatment year[11]. Toxicities can occur any time during treatment but they develop most often during the first three months after ICI initiation. Risk factors for irAEs requiring hospitalization appear to include younger age and treatment with combination immune therapy[12]. The most common irAE that can be fatal is colitis, with a mortality rate of 2–5%[13].

Notably, tumor response to ICI therapy has been shown to correlate with risk of irAEs. In a post-hoc analysis of patients in seven combined trials who received ICIs for treatment of urothelial cancer, those who developed irAEs had an overall increase in survival rate[13]. Additionally, a multicenter cohort study of patients receiving ICIs for non-small cell lung cancer demonstrated that patients who developed multi-system irAEs had improved

progression-free survival compared to those who developed single irAE[14]. Such findings suggest that the immune activation responsible for anti-tumor effect of the treatment may also confer risk of significant toxicity.

Management of irAEs is dependent on severity and organ system involved[15]. For irAEs that require treatment, glucocorticoids remain the first-line therapy, with higher dosing recommended for those with more clinically severe irAEs. The treatment response can vary widely, from complete resolution to chronic illness requiring long-term immunosuppression[9].

Cardiovascular irAEs

The broadening use of ICI therapy has led to a growing appreciation of the effects of checkpoint inhibitor therapy on various components of the cardiovascular system including the myocardium, conduction system, pericardium, and vascular network. IrMyocarditis is the most studied cardiovascular toxicity due to the significant mortality risk[13, 16, 17]. The first description of cardiac toxicity in humans was in early clinical trial reporting of checkpoint inhibitors. A single case of irMyocarditis was noted among 207 recipients of anti-PD-L1 antibody during a phase 1 trial[18], followed by a death from myocarditis after the use of ipilimumab in the adjuvant setting for melanoma[19]. Broad recognition of cardiac toxicity from ICI use occurred following the reporting of two cases of fulminant myocarditis following treatment with ipilimumab and nivolumab therapy[20]. IrMyocarditis is an uncommon irAE, estimated to occur in 0.3–1.7% of ICI recipients[21–24], but is associated with a striking mortality rate of up to 50%, which is far higher than non-ICI-associated myocarditis or other non-cardiac irAEs[13, 16, 17, 25] (Table 1). The median onset of irMyocarditis after ICI initiation ranges from 27–65 days, with up to 80% of the cases occurring during the first twelve weeks of treatment[21, 26, 27]. Risk factors for the development of irMyocarditis remain poorly defined, though combination ICI therapy has been proposed[16]. Concurrent non-cardiac irAEs occur in 42% of patients, with myositis the most common. Nearly all myocarditis cases present with troponin elevation and abnormal ECG, though only half have reduced left ventricular systolic function[25]. Major adverse cardiovascular events (MACE, defined as a composite of CV death, cardiogenic shock, cardiac arrest, hemodynamically significant complete heart block) may occur in up to 46% of patients[26]. Fatality rates are estimated to be between 20–50%[27]. Additionally, fatality rates have been reported to be higher in patients who develop myocarditis after combination ICI therapy (67%) versus monotherapy (36%)[16, 28]. In summary, irMyocarditis is uncommon but highly morbid, and tools for risk-stratification prior to and during the use of ICI therapy remain elusive.

ICIs have been associated with other cardiac complications as well. Pericardial disease is estimated to occur in 0.3–1.4% of patients receiving ICIs[28]. Compared to patients who received traditional chemotherapy for metastatic disease, those on immunotherapy are four times as likely to develop pericardial disease (defined as pericardial effusion or pericarditis)[28]. Conduction disturbances and arrhythmias, both atrial and ventricular, occur with increased frequency after ICI therapy[29–31]. Furthermore, ICI use is associated with a greater than 3-fold increase in the subsequent incidence of atherosclerotic cardiovascular

events and accelerated progression of thoracic atherosclerotic plaque burden[32]. Takotsubo (stress) cardiomyopathy has also been described among ICI recipients, though a causal relationship is not clear[33]. Hence, while myocarditis appears to be the most common and most morbid cardiovascular toxicity, ICI therapy appears capable of affecting nearly every part of the cardiovascular system. A large knowledge gap exists regarding all of these toxicities, including identification of the risk factors, optimal diagnostic approaches, and effective therapies.

Treatment of ICI myocarditis

Treatment of irMyocarditis relies on the use of immunosuppressive therapy, but there are currently no prospective data to guide such management. Multiple professional societies have published treatment guidelines, with each recommending early initiation of corticosteroids upon diagnosis of irMyocarditis[12, 15, 34]. Despite this consensus across guideline documents, the data supporting the efficacy of corticosteroids are limited. In a multi-center registry of 35 irMyocarditis patients, the administration of lower doses of corticosteroids was associated with higher residual troponin and higher rate of MACE[34]. In a retrospective analysis, patient outcomes were optimized by the receipt of early (≤24 hours from diagnosis) and high dose (>500 mg of methylprednisolone-equivalent) cortiocosteroids[35]. In addition to corticosteroids, various other immunosuppressive treatments have been described, including the anti-proliferative agent mycophenolate mofetil, monoclonal antibodies such as infliximab (targeting TNF-α) and alemtuzumab (targeting CD52), polyclonal antibodies against T cells (anti-thymocyte globulin), intravenous immunoglobulin, the Janus kinase (JAK) inhibitor ruxolitinib, and the CTLA-4-Ig fusion protein abatacept, which inhibits T cell co-stimulation[12, 15, 34, 36–40]. Most often these additional agents are used as secondary therapy in patients deemed non-responsive to corticosteroid therapy. Of note, the use of infliximab has been associated with higher cardiovascular mortality in this population[41]. A recent report summarizing 40 irMyocarditis patients has provided support for non-corticosteroid therapies[41]. An early cohort of 10 patients treated with high-dose corticosteroids and a variety of other immunosuppressive agents (including abatacept, mycophenolate mofetil, tacrolimus, and plasmapheresis) experienced a mortality rate of 60%. However, upon applying a protocol in which most patients promptly received abatacept (with dosing guided by receptor occupancy monitoring), ruxolitinib, lower-dose corticosteroids, and the aggressive use of mechanical ventilation, the mortality was reduced to 3%. These results are hypothesis-generating and randomized trial data are awaited.

The current reliance on corticosteroids as the first-line treatment of irMyocarditis carries significant risks and long-term side effects including hyperglycemia, decreased bone density, psychiatric disturbances, skin changes, and suppression of the intrinsic hypothalamic-pituitary-adrenal axis, among others[42]. Additionally, corticosteroids act broadly upon many cellular mediators of the immune system and therefore it is likely that they suppress cells and signals that are not directly involved in irMyocarditis pathogenesis. As such, their use risks overly immunosuppressing an already vulnerable population of cancer patients. Finally, the use of corticosteroids for the treatment of irAEs may lead to worsened overall survival in some groups[43]. These collective harms highlight the need to identify more targeted and less

toxic therapies for irMyocarditis. The recent evidence in support of abatacept has stimulated the initiation of two clinical trials to prospectively test its safety and efficacy (clinical trials NCT05195645 and NCT05335928). However, identification of the most appropriate therapy for irMyocarditis will be best founded upon an understanding of the underlying cellular and molecular biology of the condition, thereby allowing for selection of agents which selectively target the involved mediators.

Mechanisms of ICI myocarditis

The recognition that ICI therapy can lead to a highly morbid form of myocarditis has stimulated investigations into the underlying pathophysiologic mechanisms. Challenges to such work include the infrequency of the condition and the limited availability of human myocardial tissue from affected patients. Multiple mouse models have been developed, though their mechanistic fidelity to human irMyocarditis remains unclear[44]. Broadly, the insights derived from studies from both mouse and human samples can be grouped into one of three categories: the cellular mediators involved, the participating molecular signals and soluble factors (such as cytokines and chemokines), and T cell receptor (TCR) clonality and specificity (Figure 1). Clarity about the specific roles played by each towards the incitement, progression, and resolution of irMyocarditis will provide key guidance towards improved diagnostic, risk-stratification, and treatment approaches.

Cellular mediators

Mouse models: Several mouse models have supported a role for an array of immune cell types in irMyocarditis pathogenesis. Nearly three decades ago, analysis of mice lacking the gene for CTLA-4 (*Ctla-4*$^{-/-}$) showed cardiac infiltration of both CD4$^+$ and CD8$^+$ T cells, macrophages, and rare B cells[45]. A more recently developed genetic model using mice heterozygous for *Ctla4* and deficient for *Pdcd1* (which encodes PD-1) found increased cardiac infiltration of CD4$^+$ and CD8$^+$ T cells and CD68$^+$ macrophages; notably, NK and B cells were not enriched[46]. The heart tissue of these *Ctla4*$^{+/-}$*Pdcd1*$^{-/-}$ mice has been more deeply phenotyped using single-cell RNA-sequencing (scRNA-seq)[47]. Among sorted CD45$^+$ immune cells, activated T cells comprised 34%, compared to only 2% in control (wild-type mice). Within the T compartment, activated effector CD8$^+$ T cells and proliferating CD8$^+$ T cells were enriched in the myocarditis sample relative to control[47]. Among non-T lineages, myeloid cells constituted 36% of all CD45$^+$ cells in myocarditis, up from a control baseline of 22%. Interestingly, B cells accounted for 52% of the immune cells in control tissue, but only 4% in the setting of myocarditis, suggesting a limited initial role, if any, for these cells[47]. This report also demonstrated a critical role for CD8$^+$ T cells in disease pathophysiology through two complementary approaches. First, the administration of CD8$^+$ T cell-depleting antibodies, but not CD4$^+$ T cell-depleting antibodies, led to a significant survival benefit. Second, the adoptive transfer of whole splenocytes, but not CD8 T cell-depleted splenocytes, conferred fatal myocarditis to the recipients[47]. Consistent findings were also seen in an inducible model of irMyocarditis in which cardiac pathology is triggered the injection of anti-PD-1 antibodies into immunocompetent A/J mice; higher numbers of both CD4$^+$ and CD8$^+$ T cells, monocytes, macrophages, and natural killer cells were seen in cardiac tissue of affected animals[48]. Analysis of the infiltrated T cells showed increased numbers of effector cells (with surface CD62L$^-$CD44$^+$ surface phenotype), and

reduced numbers of both naïve ($CD62L^+CD44^-$) and memory ($CD62L^+CD44^+$) cells; this was true for both $CD8^+$ and $CD4^+$ T cells[48]. Additional evidence supporting a role for effector $CD8^+$ T cells comes from studies of *Pdcd1*$^{-/-}$ mice (deficient for PD-1) on the MRL (Murphy Roths large) background, which develop spontaneous myocarditis[49]. Hearts of these mice contain increased frequencies of $CD4^+$ and $CD8^+$ T cells compared to controls, and effector memory cells comprise nearly 30% of the cardiac $CD8^+$ T cell compartment in the MRL/*Pdcd1*$^{-/-}$ mice versus less than 10% in controls[50]. Collectively, work using murine models supports a scenario in which effector $CD8^+$ T cells are the primary driver of pathology in the cardiac tissue, but other immune cell types, including $CD4^+$ T cells, macrophages, and potentially NK cells, may have contributory roles that remain poorly defined. As differences in the cardiac cellular landscape have been noted across different murine irMyocarditis models, additional investigations to clarify which shares the greatest biological similarity to human irMyocarditis would be of significant value.

Human: Histopathological analyses of human irMyocarditis heart tissue have also yielded insights into the cellular drivers. In their landmark report, Johnson and colleagues described a dense lymphocytic infiltration into the myocardium, including large numbers of $CD68^+$ macrophages and $CD3^+$ T cells; both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells were observed[20]. The absence of multinucleated giant cells confirmed that this condition was not giant cell myocarditis, which carries a similar fulminant course. Other reports have confirmed the presence of a lymphohistiocytic infiltrate, and some have noted a predominance of $CD8^+$ over $CD4^+$ T cells[51–53]. Champion and Stone reviewed and classified ten cases as either histologically low or high grade based on the density of $CD3^+$ cells[54]. Increased numbers of macrophages were also associated with high grade cases, suggesting a possible pathogenic role. Furthermore, the ratio of myocardial $CD68^+$ macrophages to $CD3^+$ T cells was higher in irMyocarditis than in moderate-severity cardiac allograft transplant rejection, a potential indication of distinct pathophysiologic mechanisms[54]. Rare B cell infiltration has only infrequently been observed, suggesting that the pathology is primarily T cell-mediated[20, 52, 53, 55]. Together these reports strongly suggest that $CD8^+$ T cells and $CD68^+$ macrophages are major cellular participants in the pathophysiology of ICI myocarditis, with $CD4^+$ T cells potentially contributing to a lesser degree. B cells are likely minimally locally involved in mediating cardiac damage, though this does not exclude a remote role in secondary lymphoid organs. Comparing pathologic findings across different studies is challenged by the current lack of a validated and broadly utilized ICI myocarditis pathology grading scheme; efforts should be made to address this gap.

Analyses of the peripheral blood of patients with irMyocarditis may also be informative towards the underlying pathobiology. Patients with myocarditis have a reduced blood absolute lymphocyte count and a higher neutrophil-to-lymphocyte ratio at the time of presentation than do ICI-treated control patients[56]. Furthermore, the magnitude of the decrement in absolute lymphocyte count and the rise in neutrophil-to-lymphocyte ratio are each associated with the occurrence of major adverse cardiac events[56]. Zhu and colleagues have recently published the first comprehensive analysis of peripheral blood mononuclear cells (PBMCs) in irMyocarditis patients, utilizing time-of-flight mass cytometry (CyTOF)

and scRNA-seq to provide deep phenotyping[50]. An important strength of this study was the use of two different control populations: one comprised of patients treated with ICIs and without any consequent irAEs, and a second comprised of patients treated with ICIs who developed non-myocarditis irAEs. While no differences were seen in the irMyocarditis group in the blood frequency of the major circulating lineages (including T cells, macrophages/monocytes, NK cells, B cells, or neutrophils), a particular circulating $CD8^+$ T cell subset – effector memory cells re-expressing CD45RA (Temra cells) – was increased in irMyocarditis blood relative to each control group[50]. These Temra cells expressed higher levels of genes associated with activation and cytotoxicity, such as granzyme B and IL-32, and demonstrated increased clonal expansion, suggesting that this population had been activated in response to specific antigen stimulation[50]. While this study highlighted a clonal, cytotoxic $CD8^+$ T cell population expanded in the blood of irMyocarditis patients, the relevance of these cells towards the disease pathobiology remains uncertain, especially as this population was found at only modestly lower frequencies in both control groups[46]. There are currently no data regarding Temra cells in human irMyocarditis heart tissue, thus precluding more definitive conclusions about the direct pathogenic role of these cells. However, peripheral Temra cells may serve as a valuable biomarker to aid diagnosis and risk-stratification of irMyocarditis patients, though further work is needed to validate such uses.

Signaling molecules and soluble mediators

Mouse models: The repertoire of molecular signals driving irMyocarditis is not known. Yet as T cells achieve full activation through synergistic signaling by the TCR and co-stimulatory proteins[57], the determination of key co-stimulatory signals in irMyocarditis has been pursued. Treatment of *Ctla4*$^{+/-}$*Pdcd1*$^{-/-}$ irMyocarditis mice with the CTLA-4-Ig fusion protein abatacept, which disrupts the binding of CD80/CD86 on antigen presenting cells to CD28 on T cells, led to reduced intracardiac immune cell infiltration and a striking improvement in overall animal mortality[46]. These findings implicate co-stimulation by CD28 as a key driver of irMyocarditis in this model system. The identification of further activating signals, ideally those with less ubiquity than CD28 so as to limit broad T cell inhibition when targeted, should be a key goal of future work.

Limited data exist regarding the soluble factors involved in the pathogenesis of murine irMyocarditis. The *Ctla4*$^{+/-}$*Pdcd1*$^{-/-}$ genetic model, which has very limited non-cardiac pathology, showed no change in the serum levels of various cytokines and chemokines compared to *Ctla4*$^{+/+}$*Pdcd1*$^{-/-}$ controls, including the inflammatory cytokines interferon-γ and TNF-α[48]. In contrast, the inducible model utilizing administration of anti-PD-1 antibodies into immunocompetent A/J mice demonstrated higher transcriptional expression of *Ifng* and *Tnf* (encoding cytokines interferon-γ and TNF-α, respectively) within intracardiac $CD8^+$ T cells[56–58]. One possibility to explain the discrepancy is that there are local intracardiac alterations of cytokine levels, but such differences are not detectable in the systemic circulation. Furthermore, the inflammatory cytokine IL-1β has been found at increased levels in two other irMyocarditis models[58, 59]. Finally, $CD8^+$ effector cells from the heart and blood of MRL/*Pdcd1*$^{-/-}$ mice show increased transcriptional expression of *Ccl5* (encoding CCL5, the ligand for the chemokine receptor CCR5, known to support

recruitment of T cells to the heart[60, 61]), and cardiac $CD8^+$ cells had increased levels of *Ccr5* (encoding CCR5). However, the identification of increased levels of cytokines and chemokines does not necessarily imply mechanistic involvement; additional investigation is needed to further define their roles. The identification of soluble factors with pathogenic contributions to irMyocarditis is a particularly attractive goal because inhibitors of many of these mediators are already FDA-approved for other clinical uses, which would allow for more streamlined investigations into their utility as irMyocarditis treatments.

Human: In agreement with data from mice[46], several studies of human irMyocarditis patients have suggested clinical benefit from abatacept treatment[36, 37, 40, 62]. These findings point to co-stimulation of T cells by CD28 in disease pathogenesis, though it remains unclear if it is either necessary or sufficient. Two reports have also described the use of the JAK inhibitor ruxolitinib[36, 40], though it was used in combination with other therapies which complicates the interpretation of its efficacy. As ruxolitinib acts to block the intracellular signaling cascade triggered by multiple cytokines, the specific soluble factors relevant to irMyocarditis pathophysiology remain undefined. One substantial advantage of utilizing abatacept and ruxolitinib is their existing FDA approval for other indications, thus accelerating their use in irMyocarditis clinical research.

Alterations in the circulating cytokine profile of human ICI myocarditis have been reported. Broad markers of inflammation such as C reactive protein (CRP) are often increased[63–65], though the diagnostic value of such nonspecific markers is limited. Elevated serum levels of the pro-inflammatory cytokine IL-6 have been reported by multiple groups, though the source of the cytokine and the cardiac response have not been clarified[63, 64, 66, 67]. Measurement of IL-6 levels may contribute to irMyocarditis diagnosis, as its elevation in serum conferred a sensitivity of 100% and specificity of 71%[66]. These values, however, were calculated when comparing irMyocarditis patients to healthy controls (i.e. without cancer and without exposure to ICIs, both of which can alter circulating cytokine levels[68, 69]); as such, this analysis should best be considered hypothesis-generating. Indeed, whether IL-6 functions as a pathophysiologic mediator in irMyocarditis remains unclear, though the use of tocilizumab, a monoclonal antibody specific for the IL-6 receptor, as part of a successful irMyocarditis treatment regimen has been reported[70–72]. Multiple other cytokines including IL-8, IL-10, CXCL9, CXCL10, CXCL12, CXCL13, and VEGF-A may also increase during irMyocarditis, but a systematic analysis including controls with cancer and exposed to ICIs has not yet been reported[63, 64, 66, 67].

Owing mainly to the difficulty of obtaining fresh human heart tissue, only four studies have directly studied such tissue from patients with irMyocarditis[20, 47, 73, 74]; two have employed bulk RNA sequencing to identify genes and pathways that are differentially expressed in irMyocarditis[73, 74]. The first report of gene expression changes in irMyocarditis used a panel of 579 genes involved in immune responses to compare transcript expression from irMyocarditis heart samples relative to liver and lung metastasis samples from the same patient[74]. Heart tissue had higher expression of 55 genes relative to liver, and 16 relative to lung. Among the most upregulated genes in the heart were multiple genes involved in chemotaxis (*CCL13, CCL19, CXCL11,* and *CXCR3*), a co-stimulation molecule (*TNFRSF4*), genes related to interferon signaling (*IRF1* and *IRF4*), and cytokine receptor

subunits (*IL2RB* and *IL12RB1).* Notably, the chemokines *CXCL9* and *CXCL11* and their receptor *CXCR3* were each expressed in the heart at higher levels than liver and lung. Interpretation of these findings is limited by the fact that the gene expression comparisons were made between different tissue types (from the same patient), rather than between irMyocarditis heart tissue and normal non-inflamed heart tissue. Unbiased bulk RNA-seq analysis has also been used to characterize the genetic programs activated in irMyocarditis. Comparing biopsy samples from irMyocarditis to those from patients with viral myocarditis and others with dilated cardiomyopathy yielded 3784 genes that were upregulated in irMyocarditis compared to at least one of the other conditions, 1125 of which were upregulated in both comparisons[73]. Among the genes with the highest magnitude increase in transcript level were the chemokines *CXCL9* and *CXCL11*[74]. Two members of the guanylate binding protein family, *GBP5* and *GBP6*, were also highly upregulated in irMyocarditis samples; these genes are induced by interferon-**γ** signaling[75]. Genetic pathways involved in cell division, RNA splicing, interferon-**γ** responses were upregulated in irMyocarditis versus viral myocarditis. Together, both studies of transcriptional changes in irMyocarditis highlighted genes involved in interferon signaling and chemotaxis. Notably, despite their distinct experimental approaches, both reports nominated the CXCR3 signaling axis, which has been suggested to support intracardiac recruitment of immune cells in the setting of cardiac allograft rejection[76, 77], though this is controversial and other reports have not demonstrated such a role[78–80]. Further work is needed to identify which cardiac cells express this protein in the setting of irMyocarditis and to determine if this is a key chemotactic signal utilized to orchestrate the immune response.

TCR clonality and specificity

Mouse models: While infiltrating effector T cells are a central histologic feature of irMyocarditis, the immunologic drivers of their response has been a central focus of research in the field. Numerous unanswered fundamental questions exist: What antigens are driving the T cell responses? Are these self-antigens, neoantigens from the tumor, or non-self antigens (e.g. virally-derived)? Are cardiac T cells responding to the same antigens as T cells in the tumor (or other sites)? This has been pursued by independent groups using two different mouse models, and both have yielded the same auto-antigen: the cardiac structural protein α-myosin[47, 48]. T cells (both $CD4^+$ and $CD8^+$) carrying TCRs specific for α-myosin were detected in the hearts of mice with myocarditis provoked by the repeated administration anti-PD-1 antibodies into immunocompetent A/J mice[68]. Moreover, such α-myosin-specific $CD4^+$ and $CD8^+$ T cells were detected in the blood, spleens, mediastinal lymph nodes, and the hearts of naïve mice without myocarditis. These α-myosin-specific T cells expressed higher levels of PD-1 on their surface compared to the remainder of the T cells. Additionally, using the *Ctla4*$^{+/-}$*Pdcd1*$^{-/-}$ genetic model of irMyocarditis, three distinct α-myosin-specific TCRs were identified; one recovered from a highly abundant T cell clone in *Ctla4*$^{+/-}$*Pdcd1*$^{-/-}$ hearts, and two recovered from expanded T cell clones in the hearts of recipient immunodeficient *Rag1*$^{-/-}$ mice following the adoptive transfer of whole splenocytes from *Ctla4*$^{+/-}$*Pdcd1*$^{-/-}$ myocarditis mice[47]. Collectively, these findings support a mechanistic model in which rare α-myosin-specific T cells exist in mice prior to the development of myocarditis, and are capable of efficient activation through the binding

of anti-PD-1 antibody. Additional work is needed to identify other auto-antigens in murine models and clarify which TCR clonotypes are necessary or sufficient to drive irMyocarditis.

Human: In humans, questions about TCR specificity in irMyocarditis were first addressed through an assessment of TCR-β sequences in two patients[20]. TCR-β sequences were shared between T cells in the heart and those in the tumor, as well as those in skeletal muscle. In one patient, the two most abundant T cell clones in the heart were also found in tumor, but only after cancer therapy; these clones were also among the most abundant in skeletal muscle. In the second patient, a the single most abundant T cell clone from the heart was found in both pre- and post-therapy tumor and in skeletal muscle[20]. Collectively, these data are consistent with a scenario in which some cardiac, intratumor, and skeletal muscle T cells (derived from a common clonal origin) share specificity for a shared common antigen found in all three tissues. T cell clonality was further explored through an analysis of TCR-β sequences from the heart, liver, and a lung metastasis of an irMyocarditis patient[74]. Of the 3,147 unique TCR-β sequences recovered from the heart, 33% were shared with those from the lung metastasis and 4% were shared with those from the liver; 3% were shared across all three tissues. The T cell repertoire from the lung metastasis had a greater number of clones with high frequency (i.e. was more “clonal”) than the heart. These findings provide support for the suggestion that at least a subset of intracardiac T cells share antigen specificity with T cells in the tumor.

The most detailed investigation of T cell clonality in irMyocarditis evaluated immune responses in both a murine genetic model of irMyocarditis and in human patients[47]. Two key observations were made from human samples. First, T cells specific for the cardiac structural protein α-myosin were found in the blood of both healthy controls and irMyocarditis patients (as determined by T cell clonal expansion in response to α-myosin peptides *in vitro*). Second, α-myosin-specific T cells were identified in the heart tissue of all three irMyocarditis patients analyzed, and also in the skeletal muscle of the two patients for whom this was assessed. This study was the first to identify an antigen specificity for intracardiac T cells in human irMyocarditis[47]. The α-myosin-specific T cells, however, were not among the most abundant clonotypes in the heart (or skeletal muscle) among any of the patients, thereby suggesting that α-myosin is very unlikely to be the sole antigen driving T cell responses in irMyocarditis. As α-myosin is expressed by a substantial fraction of tumors (for example, in 37 of 91 melanoma tumors[47]), these findings remain compatible with the hypothesis that a shared antigen (or at least shared epitope) drives T cell responses in the tumor and the heart in irMyocarditis. However, the TCRs of tumor-infiltrated T cells were not sequenced to determine if they had shared clonotypes with T cells found in blood, heart, and skeletal muscle.

The identification of α-myosin-specific T cells in the hearts of irMyocarditis patients begets the question of how such auto-reactive T cells could escape mechanisms of central tolerance. Curiously, α-myosin transcripts are essentially absent from human thymic epithelial cells which normally function to express the repertoire of self-antigens and guide the elimination of auto-reactive developing T cells[81]. This observation would predict that α-myosin-specific T cells would be present in humans, which has indeed been observed in both healthy controls and myocarditis patients[47, 81]. The presence of such cells even in healthy controls

strongly suggests the utilization of peripheral tolerance mechanisms to prevent cardiac autoimmunity under normal physiologic circumstances. One such mechanism is likely to be inhibitory signaling by immune checkpoint molecules such as PD-1 and CTLA-4, the abrogation of which by ICI agents then leads to irMyocarditis. However, while the prevalence of α-myosin-specific T cells in the human populations is not known, the occurrence of irMyocarditis in only approximately 1% of ICI recipients suggests that the disinhibition of α-myosin-specific T cells is very unlikely to be the sole pathophysiologic mechanism. Further work towards the identification of other TCR antigen specificities in irMyocarditis is fundamental towards answering this question. Additionally, it is possible that while self-antigen-specific T cells may first initiate the intracardiac immune response, other "bystander" T cells which are not specific to cardiac antigens may be recruited into the heart and mediate damage[47, 48]. Finally, intracardiac T cells in irMyocarditis may be specific to self but not necessarily cardiac antigens; rather, they may have specificity for tumor antigens and be recruited to the heart due to the presence of cardiac metastases[82].

ICI myocarditis compared to other types of myocardial inflammation

The pathophysiologic relationships between irMyocarditis and other forms of myocardial inflammation, such as viral (lymphocytic) myocarditis, giant cell myocarditis, and cardiac allograft rejection, have not been explored. To assess if viral infection may trigger or in some way contribute to irMyocarditis, Johnson and colleagues investigated if sequences from the genomes of 472 viruses known to infect humans could be detected from cardiac tissue; elements from herpes simplex virus 1 were detected in one irMyocarditis patient, and elements from Epstein-Barr virus were detected in another[20]. Yet the presence of viral genetic features does not necessarily implicate these viruses in the pathogenesis of irMyocarditis, as they may have been dormant bystanders within the heart or present only in the small volume of blood contaminating the heart sample and within not the cardiac tissue itself. Other cases of irMyocarditis showed no evidence of many of the most common viruses known to infect the heart[51, 74]. To date, there are no data to support a direct role for viral infection in the pathogenesis of irMyocarditis, yet a comprehensive and protocolized investigation inclusive of a large number of patients has not been performed.

While appearing similar histologically, irMyocarditis carries a much worse clinical prognosis than conventional lymphocytic myocarditis or cardiac allograft rejection[13, 17, 54, 83, 84]. The reasons for this high clinical morbidity are not well understood, but several possibilities can be proposed. First, compared to other forms of cardiac inflammation, the immune response in irMyocarditis may be more intense (i.e. capable of recruiting and activating more immune cells). In support of this idea, irMyocarditis was associated with a denser lymphohistiocytic infiltrate with a higher ratio of macrophages to T cells than allograft rejection[79–81]. Additionally, distinct gene expression patterns are seen when comparing cardiac tissue bulk RNA-seq results from irMyocarditis versus virally-mediated myocarditis, suggesting at least partially non-overlapping immune mechanisms[73]. The cellular basis for these changes has yet not been explored. Secondly, irMyocarditis may be less likely to diminish with time or with treatment by immunosuppression. Indeed, numerous cases of irMyocarditis refractory to potent immunosuppression have been reported[62, 85, 86]. The mechanisms by which activated T cells (and presumably other

involved immune cells) escape or withstand the inhibitory effects of immunosuppression remain unknown. One possibility is that the T cells have been durably reprogrammed by ICI exposure to become resistant to inhibitory signals. Supporting this hypothesis is the observation that therapeutic effects of ICIs can extend well beyond their physical presence predicted by pharmacokinetics, in some cases for years[87]. Another potential explanation for the more refractory clinical course of irMyocarditis is that most non-ICI-associated lymphocytic myocarditis is thought to be due to acute or subacute viral infections[88]. Upon activation of the immune response, the viral antigens are cleared and the immune response can then resolve. However, the driving antigens in irMyocarditis may be self-antigens or tumor neoantigens that cannot be cleared and thereby promote persistent immune responses. A third and final possibility for the severity of irMyocarditis is that the pathogenic immune cells in irMyocarditis may utilize distinct mechanisms of cardiac injury (i.e. that are more damaging) or target different regions of the heart than in other types of cardiac inflammation. In sum, the cellular and molecular basis for the clinical severity of irMyocarditis remains poorly defined; further studies are necessary to deeply phenotype the cardiac inflammation and identify the driving signaling networks, the focused targeting of which may yield clinical benefits.

Gaps in knowledge and conclusions

While much has been learned in recent years regarding the clinical presentation and prognosis of irMyocarditis, very few studies have examined human biological samples from affected patients, and consequently the underlying pathophysiologic mechanisms remain poorly understood. Perhaps the most fundamental unanswered question is why only approximately 1% of ICI recipients develop irMyocarditis[21, 22]. Could the incidence be based upon some feature intrinsic to patients that exists (and therefore can be measured) prior to the start of ICI therapy? Or is it instead related to an event that transpires once on ICI therapy? At least four possibilities exist that may explain this observation. First, disease development may rely on the presence of T cells with TCR specificity for cardiac antigens. Such T cells are known to exist in humans, for example with specificity to α-myosin[47, 89] or β-adrenergic receptors[90, 91], but the prevalence of such auto-reactive T cells in the population is not known. The administration of ICIs in patients carrying such cells may be sufficient to break the mechanisms of peripheral tolerance normally restraining these cells from attacking self. Alternatively, the presence of such T cells alone may be insufficient and aberrant expression of the cardiac antigens by the tumor (as has been described with melanoma, for example[47]) may also be required as an inciting step. Determining the range of antigen specificities of intracardiac T cells during irMyocarditis is key towards addressing this possible etiologic mechanism. Second, viral or other infections may be triggering events for irMyocarditis. Direct viral infection of the heart is thought to cause the majority of non-ICI-associated lymphocytic myocarditis[92], and elements from viral genomes have been detected in some irMyocarditis myocardial samples[20]. Additionally, viral proteins may share close similarity with human cardiac proteins, potentially leading to autoimmune manifestations even when the heart is not directly virally infected, as has been suggested for Coxsackie infections[93] and Covid-19[94, 95]. Third, underlying genetic susceptibility may influence the development and severity of irMyocarditis. Germline variants are well-

established risk factors for a variety of autoimmune conditions[96]. Furthermore, the first genome-wide association study of irAEs recently identified a genetic variant near the *IL7* gene locus conferring increased risk[97]. Further studies with larger cohorts are likely to highlight further loci that confer increased risk, though such investigations of irMyocarditis specifically will be challenged by the infrequency of this particular irAE. Fourth and finally, the host microbiome may play a role in the development of irMyocarditis. A growing body of work has supported the influence of microbiome features on the occurrence and severity of irAEs[98–102], though the mechanisms remain unresolved. There are currently no data on the relationship between the microbiome composition and irMyocarditis incidence or severity.

While research regarding treatment of irMyocarditis will improve the morbidity and mortality of this condition, preventing its development in the first place should be prioritized. Ongoing and future work aimed at deciphering the fundamental biology of irMyocarditis will clarify why it develops in only a very small subset of ICI recipients. Such knowledge will be critical towards risk-stratifying cancer patients not only after they develop irMyocarditis, but at earlier points such as after the initiation of ICI therapy, or, ideally, prior to even starting such therapy. Accurate determination of risk will allow patients and providers to make informed decisions about the optimal treatment strategy, which in some cases may be the avoidance of ICI therapy altogether.

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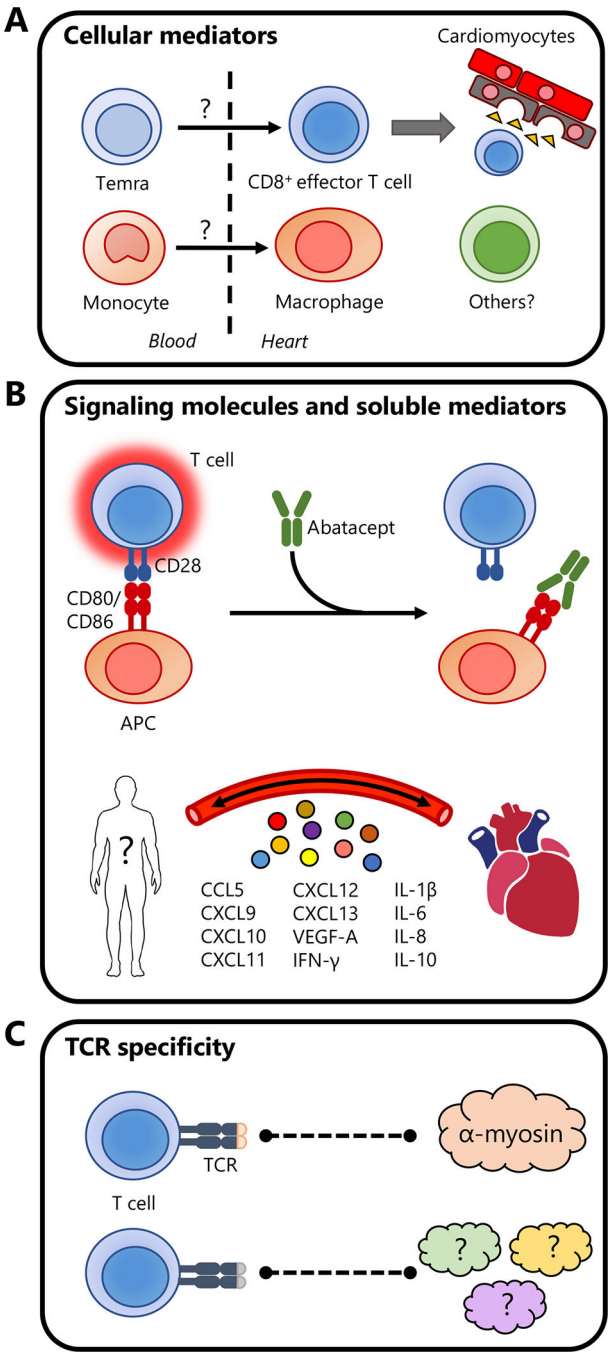

Figure 1. Potential mechanisms underlying irMyocarditis.

A, (*Top*) Increases in circulating Temra cells are found in irMyocarditis[50], though it is unknown whether these cells are the source of the intracardiac $CD8^+$ T cells that damage cardiomyocytes. (*Bottom*) Histologic analysis reveals an increased density of macrophages in irMyocarditis cardiac tissue. The role of these cells and the potential participation of other cells types in the heart remain poorly understood. **B**, (*Top*) Co-stimulation via the binding of CD80/CD86 on APCs to CD28 on T cells can be disrupted through the administration of abatacept, thereby decreasing the effector function of the T cells; whether this drug

can reduce cardiac inflammation and improve clinical outcomes in irMyocarditis is under evaluation in clinical trials. (*Bottom*) Various soluble factors, including those depicted and others, may help to orchestrate the cardiac immune response in irMyocarditis. Both the source and target of these factors are not known. **C**, T cells with specificity for the cardiac structural protein α-myosin have been identified in a mouse model and human patients with irMyocarditis but account for only a small fraction of the recovered T cells[47, 48], indicating that other specificities exist. Temra: T effector memory cells re-expressing CD45RA; APC: antigen presenting cell.

Table 1.

Consensus definition for irMyocarditis diagnosis

2021 International Cardio-Oncology Society consensus definition for the diagnosis of irMyocarditis[103]
Either pathohistological diagnosis:
Multifocal inflammatory cell infiltrates with overt cardiomyocyte loss by light microscopy of cardiac tissue samples.
Or clinical diagnosis[†,‡]:
A troponin elevation[§] (new, or significant change from baseline) with 1 major criterion or a troponin elevation (new, or significant change from baseline) with 2 minor criteria after exclusion of acute coronary syndrome or acute infectious myocarditis based on clinical suspicion.
Major Criterion:
• Cardiac magnetic resonance (CMR) imaging diagnostic for acute myocarditis (based on modified Lake Louise criteria)
Minor Criteria
• Clinical syndrome
◦ *Including fatigue, muscle weakness, myalgia, chest pain, diplopia, ptosis, shortness of breath, orthopnea, lower extremity edema, palpitations, lightheadedness/dizziness, syncope, or cardiogenic shock*
• Ventricular arrhythmia and/or new conduction system disease
• Decline in cardiac (systolic) function, with or without regional wall motion abnormalities in a non-Takotsubo pattern
• Other immune-related adverse events, particularly myositis, myopathy, or myasthenia gravis
• Suggestive CMR (meeting some but not all of modified Lake Louis criteria)

[†]Clinical diagnoses should be confirmed with CMR or endomyocardial biopsy if possible and without causing delays of treatment

[‡]In a patient that is clinically unwell, treatment with immunosuppression should be promptly initiated while awaiting further confirmatory testing.

[§]Either troponin I or troponin T can be used; however, troponin T may be falsely elevated in those with concomitant myositis.