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T helper-9 cells and Interleukin-9 in transplantation: The open question

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ABSTRACT

The role of main TCD4+ lymphocyte subsets including T helper 1 (Th1), Th2, Th17, and T regulatory cells in transplantation has already been described; however, the implication of newly defined lineages such as Th22, Th9, and T follicular helper cells in alloimmune responses remain to be elucidated. In addition to the low number of studies, most evidence about the role of these cells in transplantation has been obtained from experimental studies, which might be insufficient or irrelevant for clinical interpretations. In the present article, we have reviewed the studies that have investigated the role of Th9 and its principal cytokine interleukin-9 (IL-9) in allograft rejection and tolerance induction. However, the findings tend to be controversial since some investigations demonstrate positive effects of Th9 on transplantation outcomes whereas others are suggestive of its detrimental influences. A similar challenge is presented by IL-9 as both advantages and disadvantages of IL-9 expression in allografts have been reported. Moreover, different organs appear to be affected in different ways by Th9 cells and IL-9. Therefore, more research particularly in human patients is required to provide sufficient data for drawing a concrete conclusion about the implication of Th9 and IL-9 in transplantation.

Contents

Abbreviations: Th9, T helper 9; IL-9, Interleukin 9; IRF-4, Interferon Regulatory Factor-4; BATF, Basic leucine zipper ATF-like Transcription Factor; TGF-β, Transforming growth factor-β.

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1. Introduction

In recent years, organ transplantation and stem cell transfusion have been accepted as therapeutic options for treating patients with end-stage organ failure and hematopoietic malignancies, respectively; however, the graft and recipients survival have not yet reached satisfactory levels [1]. Therefore, it is still required to define more details about the alloreactivity, in particular the principal components involved in graft tolerance or rejection, and those plausible for manipulation. Thus far, T lymphocytes as the key directors of immune responses have gained the most interest in developing immunosuppressive agents and immunotherapy methods. The implication of T helper 1 (Th1), Th2, Th17, and T regulatory (Treg) cells in transplant outcome have already been studied, but the role of newly defined T cell subsets, for instance, Th9, Th22, and T follicular helper (Tfh) cells remain to be clarified [2]. One of the least studied T lymphocyte lineages is Th9 whose involvement in allograft's destiny has barely been understood, and the few findings are surrounded by discrepancies and uncertainties. Therefore, we aimed to review the studies that have focused on the direct or indirect role of Th9 cell and its main cytokine interleukin-9 (IL-9) in solid organ and stem cell transplantations.

2. T helper 9 cells

Th9 cells, first defined in 2008 by Veldhoen et al. were initially supposed to be derived from Th2 lineage but later it was found that they lack the key features of Th2 cells such as IL-4 and 5 production, and expression of nuclear transcription factor GATA binding protein 3 (GATA3) [3]. On the other hand, since Th9 lymphocytes require transforming growth factor-beta (TGF-β) for differentiation and anti-inflammatory function, they were suggested to be associated with the Treg cell subset [4]. Indeed, as Th9 cells are differentiated in the presence of IL-4 and TGF-β cytokines, they might be considered a lineage between these two subsets. T cell plasticity that has been described in many contexts may explain shared characteristics of Th2, Th9, and Treg cells [5]. Additionally, Th9 cells have been shown to display both pro-and anti-inflammatory properties, the finding which is further supportive of a probable swing between subsets. Accordingly, one research has introduced two distinct $CD96^{high}$ and $CD96^{low}$ Th9 cell phenotypes with opposing inflammatory properties, since the former cells did not cause colitis in the Rag1−/− mice whereas transfer of the latter resulted in a substantial colonic inflammation. In addition, $CD96^{low}$ Th9 cells transfusion led to skin allograft rejection in the same animal model. Of note, lower CD96 expression has been observed in human IL-9+ compared with IFN-γ+ T cells [6].

The other lymphocyte lineage associated with Th9 cells are type 2 innate lymphoid cells (ILC2) which can produce IL-9 in addition to IL-5 and 13 cytokines; these cells might participate in the pathogenesis of allergic lung diseases similar to Th9 lymphocytes. Moreover, ILC2 cells need IL-9 for appropriate function as IL-9 neutralization resulted in reduced cytokine secretion by these cells [7].

Apart from IL to 4 and TGF-β, other cytokines such as IL-1β, IL-6, IL-10, IL-21, and IL-25 have been found to promote Th9 cell generation [4,8]. The co-stimulatory molecules expressed by immune cells might also be involved in Th9 development; for example, the interaction of OX40 (TNF receptor superfamily 4) with the OX40 ligand could enhance Th9 differentiation and IL-9 expression by TCD4+ cells [9]. The other molecules proposed to have a role in Th9 cells generation include glucocorticoid-induced TNFR-related protein (GITR) [10], TNF-like ligand 1A (TL1A) [11], toll-like receptor-2 (TLR2) [12], and Activin A [13] (Fig. 1).

In contrast to Th1, Th2, Th17, and Treg cells, which express specific transcription factors T-box expressed in T cells (T-bet), GATA3, Retinoic acid-related orphan receptor gamma t (RORγt), and Forkhead box P3 (Foxp3) respectively, no specific transcription factor has been identified in Th9 cells. Nonetheless, PU.1, interferon regulatory factor-4 (IRF-4), IRF-8 and basic leucine zipper ATF-like transcription factor (BATF) have been found to be expressed by this subset [14–17] (Fig. 1). Although these transcription factors are useful in distinguishing Th9 cells, it is worth noting that they could be expressed by a variety of immune cells, particularly other T helper lymphocytes [18,19].

Th9 cells of mice have been shown to express both IL-9 and IL-10 cytokines; however, the most prominent feature of human Th9 cells is IL-9 production [20]. IL-9 was first described as a Th2 cytokine, but later it was revealed to be mainly produced by Th9 and to a lesser extent from Treg, natural killer, and mast cells [4,21]. IL-9 belonging to the IL-2 cytokines family, binds to the common γ chain plus cytokine-specific α chain (IL-9Rα). IL-9/IL-9R engagement leads to the phosphorylation of Janus kinase (JAK)1/JAK3, and subsequent activation of signal transducer and activator of transcription (STAT)1 and STAT5 homodimers as well as STAT1/STAT3 heterodimers. These molecules then transfer to the nucleus and induce transcriptional modulations [22]. Although many non-immune cells such as airway smooth muscle and epithelial cells are affected by IL-9 [23], Th17, Treg, mast cells, and monocytes are the main immune cells targeted by this cytokine. IL-9 has been shown to induce Th17 cells proliferation, improve Treg cells' suppressive function [24], promote growth and cytokine production in mast cells [25], and enhance TGF-β expression/IL-12 downregulation in monocytes [26].

The implication of Th9 cells and IL-9 in immune-related diseases was first demonstrated in the pathogenesis of asthma. Gene expression of *il9* was found to be enhanced in the asthma-like model of mice [27] leading to an IL-33-mediated inflammation and excessive mucus secretion [28] while neutralization of IL-9 mitigated the hyper-reactive airway symptoms [29] and prevented chronic airway remodeling [30]. Moreover, the involvement of IL-9 in developing food allergy and anaphylactic reaction has been reported [31].

The footprint of Th9 cells has been observed in the pathogenesis of certain autoimmune models such as EAE [32] and ulcerative colitis [33]. In addition, an increased frequency of peripheral blood memory CD4 cells with the capacity of transition into IL-9+ IL-17 + cells was shown in diabetic patients [34].

The significance of Th9 cells in anti-tumor defense has not yet been fully understood. The available data suggest certain advantages of the presence of Th9 cells in the tumor microenvironment whereas others reveal their detrimental role in cancer development. For instance, the transfer of Th9 cells into the mice model of melanoma resulted in reduced tumor growth [35]; moreover, decreased number of Th9 cells was reported in the blood

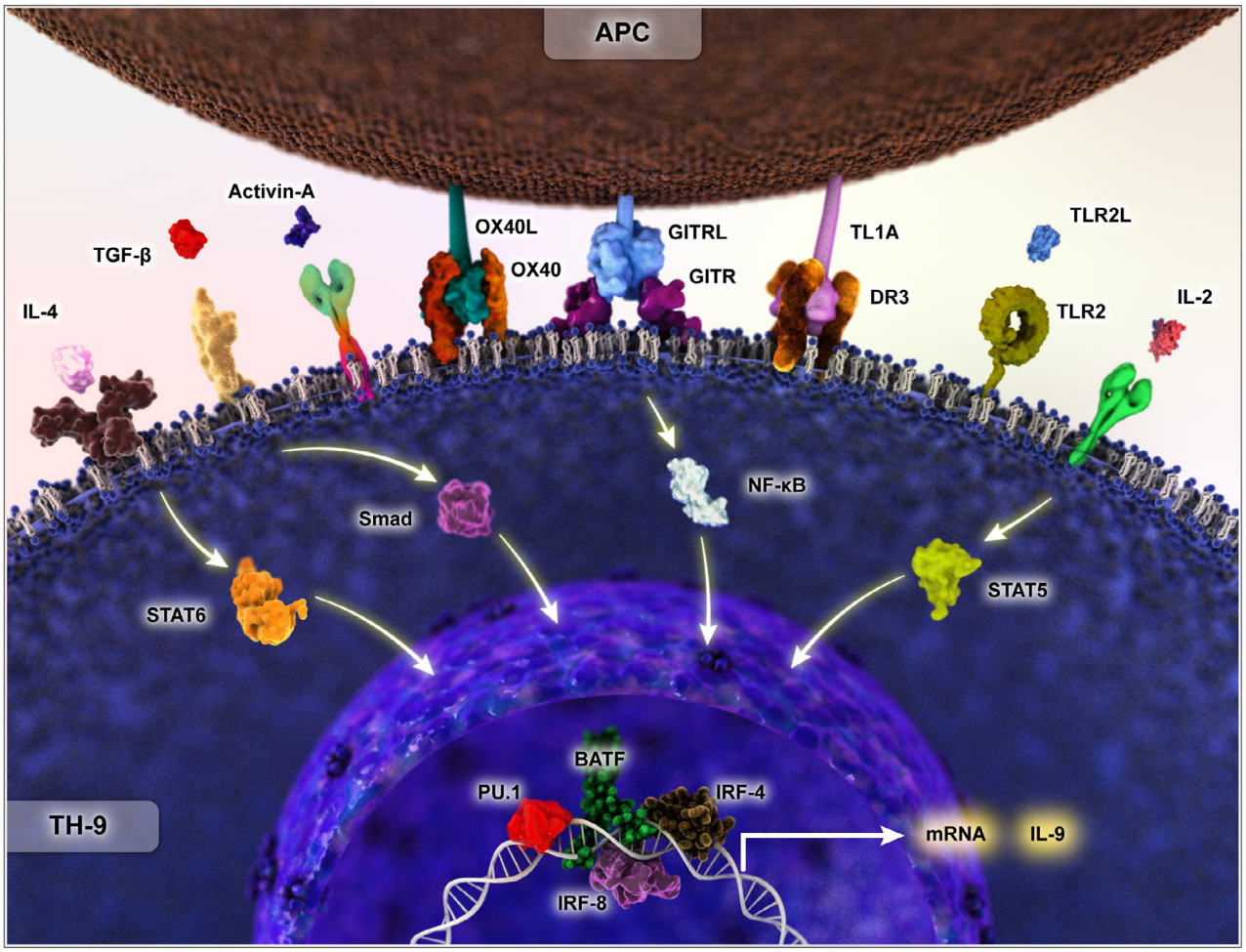

Fig. 1. Cytokines and receptors involved in Th9 differentiation and IL-9 expression. Transforming growth factor-β (TGF-β) interleukin-4 (IL-4); Interferon regulatory factor-4 (IRF-4); basic leucine zipper ATF-like transcription factor (BATF); Janus kinase (JAK); Signal transducer and activator of transcription (STAT); Glucocorticoid-induced TNFR-related protein (GITR); TNF-like ligand 1A (TL1A); Toll-like receptor-2 (TLR2); Death receptor 3 (DR3).

circulation of patients with melanoma [36]. On the other hand, there is evidence of tumor growth promotion in the presence of Th9 cells as IL-9 have been found to enhance tumor cell survival and drug resistance in experimental models of large B cell lymphoma [37], hepatocellular carcinoma [38], and certain solid tumors such as breast and colon cancers [39]. It was therefore hypothesized that the beneficial effects of Th9 in tumor defense might be mediated by other immune cells recruited by Th9 cells to the tumor site whereas detrimental observations are probably induced by Treg enhancement or direct anti-apoptotic effects of Th9 on tumor cells.

Th9 cells also contribute to the anti-parasitic defense given that impaired Th9 development or IL-9 deficiency resulted in uncontrolled helminth infections whereas adoptive transfer of Th9 cells to the Rag1-deficient mice significantly improved anti-parasitic immune defense. Indeed, Th9 cells and IL-9 have been shown to be required for IL-5 and IL-13 induction, mucosal mast cells activation, stimulation of mucus production and granuloma formation, as well as eosinophil and basophil recruitment in gastrointestinal worm infections [40–42].

Nonetheless, one of the least studied areas about the Th9 cells' function is organ transplantation. In fact, the encouraging results obtained from initial studies proposing a tolerogenic role for Th9 and IL-9 were scrutinized by the findings indicating their presence in rejected allografts and considerable association with Th17 cells. Therefore, although the overall advantages of Th9 cells seem to outweigh their disadvantages, further investigations are required to elucidate the exact role of this subset in transplantation outcomes. Herein, we review the results of investigations about Th9 cells and IL-9 implication in allograft rejection and tolerance but at first, briefly discuss the evidence of immunoregulatory effects of IL-9.

2.1. Immunomodulatory role of IL-9

Interleukine-9 is secreted from a wide range of immune cells and affects a variety of cell types in different ways [43]. One of the most affected cells by IL-9 are mast cells which have been shown to be capable of maintaining immune homeostasis in tissues by expressing co-inhibitory molecules and secretion of regulatory soluble mediators [44]. According to the role of IL-9 in recruitment and proliferation of mast cells, IL-9-blocking is assumed to aggravate pro-inflammatory responses. For example, administration of IL-9-deficient Treg cells or anti-IL-9 monoclonal antibodies to the nephrotoxic serum nephritis-prone mice prevented mast cell recruitment to the renal draining lymph nodes and neutralized the protective effect of Treg cells against nephritis. These findings suggest that IL-9 promote Treg cells' suppressive function probably through mast cell recruitment; nonetheless, it should be noted that IL-9 deficiency does not affect the general immunosuppressive activity of Treg cells [45]. Similar results were found in an experimental model of skin transplantation as

tolerance induction by Treg cells failed to develop in mast-cell-deficient mice as well as by administration of anti-IL-9 antibodies in wild type [46].

These findings were supported by another study in which purified mice CD4+Foxp3+ cells inhibited the function of CD3+ effector T cells in vitro efficiently; however, adding IL-9-neutralizing antibodies to the co-culture led to the proliferation of effector T cells and increased cytokine production (e.g. IL-2, IL-6, IL-17, IFN-γ, TNF, macrophage inflammatory protein 1 [MIP1]). On the other hand, administration of the recombinant IL-9 improved Treg cells' suppressive function and reduced cytokine secretion from effector T cells. Furthermore, the in vivo experiment using autoimmune encephalomyelitis (EAE) model showed that IL-9R−/− mice immunized with suboptimal doses of myelin oligodendrocyte glycoprotein (MOG) developed an earlier and more severe form of disease compared to the wild type. Moreover, peripheral blood T cells of IL-9R−/− mice displayed a remarkable proliferative response to MOG stimulation. IL-9R-deficient mice had also higher proportions of Th1 cells in blood circulation and brain tissue, while their Treg cells showed diminished suppressive activity in co-culture with T effector cells [24].

The other T cell subset correlated with IL-9 is Th17 lymphocytes. IL-9 appears to be involved in the differentiation of Th17 cells since replacement of IL-6 by IL-9 induced successful Th17 generation in presence of TGF-β. In addition, neutralization of IL-9 with anti-IL-9 antibodies reduced IL-17 secretion from IL to 6/TGF-β-induced Th17 cells, suggesting a contribution of IL-9 to the development and function of Th17 lymphocytes [24]. In line with this, one experiment demonstrated that IL-9 neutralization or IL-9 receptor deficiency was associated with a reduced number of Th17 cells in CNS tissue and consequently, attenuated symptoms of EAE in mice [32]. IL-9 not only enhances the differentiation of Th17 cells but also seems to be produced by these cells. One study introducing Th17 cells as a source of IL-9 secretion revealed an autocrine effect of IL-9 in the expansion, function, and survival of these cells. It also demonstrated the anti-inflammatory properties of Th17-secreted IL-9 since the transfer of IL-9-deficient Th17 cells to the nude mice caused a more severe form of autoimmune gastritis accompanied by a higher lymphocyte count compared to the Th17 transfusion from wild type. This effect was attributed to the reduced recruitment of mast cells to gastrointestinal mucosa [47]. Finally, Wu et al. observed that PBMCs from patients with tuberculosis stimulated with the Mycobacterium Tuberculosis (TB)-specific antigen, early secretory antigenic target-6 (ESAT-6), expressed lower mRNA levels of IFN-γ, IL-12 and IL-23 cytokines, but higher amounts of IL-9 mRNA. Furthermore, IL-9 reduced IFN-γ expression in PBMCs from patients with latent TB infection while neutralization of IL-9 restored the IFN-γ production. Therefore, it was concluded that IL-9 may contribute to the inefficiency of Th1 cells and developing TB infection [48]. According to these findings, IL-9 appears to promote Treg cells immunosuppressive activity while modulating the proinflammatory function of Th17 and Th1 subsets, which propose an immunoregulatory role for this cytokine.

3. Cell and organ transplantation

In recent years, solid organ transplantation has been considered a standard treatment for patients with end-stage organ failure, mainly renal, hepatic, or cardiac dysfunction. Cell therapy and stem cell transplantation have also brought fresh hopes for treating certain hematologic malignancies, congenital immune deficiencies, various genetic disorders and bone marrow failure [49]. Thanks to the considerable advances in surgical methods and pharmaceutical progression, the survival of organs and recipients have been improved significantly; however, graft loss occurs frequently, and long-term suppression of the immune system to induce tolerance towards the allograft could eventually lead to substantial adverse effects in particular metabolic disorders and susceptibility to infectious diseases and various malignancies [50]. Therefore, it is necessary to come up with new ideas to induce sustainable tolerance with the least side effects to improve the survival and quality of life of the recipients.

One of the most plausible methods of tolerance induction is immune cell therapy and the most studied subset showing some promising results is the Treg subset. Treg cells infusion has improved graft tolerance, particularly in liver transplant recipients but it has not yet been accepted as a routine treatment mainly due to the technical difficulties, risk of unspecific immunosuppression, and T cells plasticity between Treg and Th17 subsets [51]. For this reason, there is an interest to explore and exploit other immune cells with comparable immunomodulatory properties and less risk of immunodeficiency. Th9 cells might be considered as a candidate for tolerogenic T cell therapy; however, at first, their precise role in various transplants should be clarified.

4. Skin transplant

Skin grafting is generally performed in case of deep wounds due to cancer, severe burning, and certain infections. Due to the abundance of immunologic cells particularly specified dendritic cells and T lymphocytes in the skin, skin allograft is assumed to provoke substantial alloimmune responses [52]. Based on an extensive serial analysis of gene expression (SAGE) in tolerant tissue which showed a considerable overexpression of mast cell genes [53], Lu et al. conducted an experiment on skin allograft focusing on mast cells collaboration with Treg cells and the main mediator of this correlation, IL-9. They showed a crucial function of mast cells in Treg-induced peripheral tolerance since mast-cell-deficient mice rejected the allograft despite an intravenous infusion of allogeneic cells (donor-specific transfusion (DST)) and administration of anti-CD154 (CD40 ligand) antibodies. Interestingly, intradermal injection of bone-marrow-derived mast cells to mast-cell-deficient mice restored the tolerance towards skin allograft in the presence of DST and anti-154 antibodies. Moreover, it was shown that a high expression of the *il9* gene in Treg cells was responsible for mast cell recruitment and tolerance induction because these effects were reversible when anti-IL-9 antibodies were used [46]. Similarly, Gorczynski et al. found that interaction of CD200 with CD200 receptor1 (R1) suppressed alloreactivity in skin graft through Treg induction and infiltration of mast cells; however, anti-IL-9-treated mice showed a decreased frequency of mast cells in skin allograft and reduced tolerance induction by CD200/CD200R1 engagement. This study provided further evidence for the critical role of Treg-secreted IL-9 in mast cell recruitment and tolerance induction [54].

Contrarily, Th9 cells injection to Rag1-deficient mice showed unfavorable results since transfer of CD44+IL-9-producing Th9 cells led to skin allograft rejection in a rate comparable with Th1-mediated rejection. Likewise, $CD96^{low}$ Th9 cells transfusion to the same model resulted in pathologic features of inflammation in skin allograft [6].

5. Kidney transplant

Kidney transplant is the most studied type of organ transplantation, which has helped describe the most fundamental principles of alloreactivity; however, the implication of Th9 cells in developing rejection or tolerance towards renal allograft has rarely been investigated. Our initial understanding about the role of IL-9 as a T cell growth factor in renal allograft rejection dates back to

1998 when Chang Li et al. examined the gene expression of IL-9 in 26 human renal transplant biopsies taken with suspicion of rejection. Of note, in contrast to the other common γ chain receptor cytokines including IL-2, IL-4, IL-7, and IL-15, there was no detectable IL-9 gene expression in allograft tissue. This finding refuted the direct involvement of IL-9 in renal allograft rejection [55]. Similarly, one experiment evaluated gene expression of γ chain signaling cytokines and their receptors in rat allograft during the first week of renal transplantation. Interestingly, IL-9 was expressed in normal kidneys but it was almost undetectable during rejection. IL-9Rα was barely expressed in normal kidney but showed a transient, mild increase on day 3 post-transplant [56].

On the other hand, de Vries et al. evaluating three groups of living (n = 8), cardiac-dead (n = 7), and brain-dead (n = 9) donors demonstrated that upon reperfusion, brain-dead renal allografts released significantly more cytokines including granulocyte-colony stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP-1), IL-6, IL-16 and IL-9 compared to the other groups. Besides, pretransplant pathological examination revealed greater CD3+ and CD68+ cells infiltration (representing T lymphocytes and macrophages population, respectively) in the brain-dead kidneys [57]. However, despite increased secretion of IL-9 from ischemic tissue, it is not clear whether it promotes inflammation or protects the tissue by preserving the balance between pro-and anti-inflammatory cytokines. Similar observations have been reported in liver transplantation as levels of anti-inflammatory cytokine IL-10 elevated during surgery [58]. Accordingly, Kortekaas et al. evaluated the tissue expression of IL-9 in human brain-dead and living-donor kidneys pre-transplant and post-reperfusion. Interestingly, neither deceased nor living donors displayed considerable expression alterations. Besides, anti-IL-9 antibodies were administrated to the ischemia–reperfusion model of mice by clamping renal and vein arteries. Noteworthy, structural damage including tubular, cortical, and medullar necrosis as well as protein cast aggregations were observed in kidneys of anti-IL-9-treated mice compared to the control group. This finding was consistent with a possible protective function of IL-9 against ischemia–reperfusion injury [59].

6. Liver transplant

Liver transplantation is considered a life-saving treatment for patients with end-stage hepatic diseases such as hepatocellular carcinoma, cirrhosis, and incurable hepatitis [60]. We have already discussed the role of cytokines in liver transplantation [58]; the most prominent studies about the implication of IL-9 in liver transplantation outcomes have been conducted by Fábrega and his colleagues. In one study, they compared the serum levels of IL-9 between healthy subjects (n = 34) and stable recipients with more than 7 years of rejection-free survival (n = 30), which showed significantly higher amounts of IL-9 in stable patients. Moreover, the IL-9 levels were associated with lower concentrations of cyclosporine (<80 ng/mL) or tacrolimus (<5 ng/mL) in blood circulation, suggesting a tolerogenic role for IL-9 in liver transplant [61]. In the other study, serum amounts of IL-9 in healthy controls (n = 34), liver transplant recipients with acute rejection episodes (n = 15), and stable recipients (n = 35) were evaluated on days 1, 7, and between days 12 and 18 post-transplant. Similar to the previous study, both stable and rejecting recipients had significantly higher IL-9 levels compared to the healthy individual. However, there was no considerable difference between rejecting and stable patients, which declines the participation of IL-9 in allograft rejection [62]. Likewise, allogeneic liver transplantation between rats showed a very low expression of IL-9 in graft tissue without any difference between pre- and post-transplant values. IL-9R expression was hardly detectable in normal liver but slightly increased on day 7 [56].

7. Islet cell transplant

Islet cell transplantation holds a potential cure for type 1 diabetes; however, many islet recipients do not show long-lasting insulin independence, mainly due to alloreactive injuries [63]. To find out the implication of IL-7, IL-9, and IL-15 cytokines in islet transplant outcome, Chang Li et al. compared IL-2−/−/IL-4−/− double knockout (DKO) model with wild-type mice. Following transplantation, DKO mice exhibited allograft rejection along with upregulated tissue expression of IL-7 and IL-15 genes but there was no trace of IL-9 expression in the allograft. Similarly, islet cell transplant into the wild-type mice resulted in allograft rejection without any detectable IL-9 gene expression [55]. Furthermore, one human study evaluating the serum levels of 94 different cytokines and chemokines before and after islet cell transplantation in three groups of long-term graft function (n = 6), temporary graft function (n = 3), and insufficient engraftment (n = 4) showed no association between IL and 9 concentrations and transplant outcomes. Of note, IFN-α and Leukemia inhibitory factor (LIF) were shown as the most valuable biomarkers predicting insulin dependence post-transplant [64].

8. Heart transplant

Despite all improvements in heart transplant survival during the past 50 years, many aspects of alloimmune responses including newly described T cell subsets effects on cardiac graft remain undefined. In one experiment conducted by Poulin et al. wild type (WT) and IL-9-knock-out mice were compared for their ability to reject cardiac allograft after CD8+ T cell depletion in recipients. As a result, both groups rejected the allografts within 9 days; however, histological analysis showed eosinophilic infiltration into the myocardium solely in the wild-type animals. It was therefore concluded that IL-9 in spite of being involved in eosinophil recruitment might not be required for allograft rejection. In addition, heart transplantation from an IL-9-overexpressing model to the WT resulted in <30 days of survival while more than 50 days of functional allograft survival was recorded when transplantation was performed from WT mice. Remarkably, administration of IL-9-neutralizing antibodies to the recipients from IL to 9-overexpressing mice restored the situation. This study also demonstrated that blocking IL-4 and IL-5 cytokines by monoclonal antibodies in the IL-9-overexpressing model prevented allograft rejection, inhibited leukocyte infiltration, and improved the histological features. These findings, although suggestive of a negative role for IL-9 in heart transplantation, indicated a more prominent influence of IL-4 and IL-5 cytokines on the allograft compared to IL-9 [65].

9. Stem cell transplant

Hematopoietic stem cell transplantation (HSCT) is the treatment of choice for life-threatening hematological disorders. Graft versus host diseases (GVHD) is the most concerning complication post-HSCT which in acute or chronic forms affects several organs particularly the gastrointestinal system, liver, lung, skin, and eyes. Many immune cells and mediators have already been introduced to be involved in developing GVHD [66], but the impact of Th9 and IL-9 is still under study. For instance, idiopathic pneumonia syndrome (IPS), a fatal feature of GVHD, is supposed to be induced by IFN-γ+ Th1 cells. However, it has been shown that intranasal administration of IL-33 to the murine model of IPS resulted in an

increased number of lung ILC2 expressing *il9* and PU.1 transcripts which prevented IPS probably through IL-9 secretion [67]. On the other hand, Cocho et al. comparing the gene expression of 84 cytokines, chemokines, and their receptors in the conjunctival epithelium of 20 GVHD-dry eye patients and 14 healthy individuals found significantly upregulated expression of IL-6, IL-9, IL-10 and, IFN-γ in patients group. As a result, the expression level of IL-9 together with IL-6, epidermal growth factor receptor (EGFR), and nicotinamide phosphoribosyltransferase (NAMPT) were suggested as a diagnostic biomarker with high sensitivity and specificity for evaluation of ocular status post-HSCT [68].

Likewise, measuring plasma cytokine concentrations in nine acute myeloid leukemia (AML) patients who underwent HSCT demonstrated higher levels of IL-9 on day 0 in those who later developed acute GVHD (n = 3) compared to those without (n = 6) (157 vs.36 pg/ml). In addition, the mean IL-9 levels 4 days before transplantation were greater in patients with acute GVHD compared to the others (117 vs. 21 pg/ml). There was no statistically significant difference between other cytokines in two groups of patients [69]. In contrast, Pang et al. comparing 11 HSCT recipients with acute GVHD and 20 without during the first months of transplantation found higher percentages of Th9 cells and increased levels of IL-9 in stable recipients compared to the acute GVHD patients. Moreover, serum IFN-γ/IL-9 ratios positively correlated with the severity of acute GVHD. This study also demonstrated a slower reconstitution for Th9 lineage and IL-9 post-HSCT (started on day 60 and reached a normal level on day 90) compared to the Th1 and Th2 subsets and their cytokines [70]. In line with this, Mangus et al. found that rapamycin-resistant murine Th9 cells significantly reduced both CD4+ and CD8+ T cells engraftment and inhibited allospecific IFN-γ production from these cells. Notably, rapamycin-resistant Th9 cells persisted in vivo and maintained high IL-9 expression, preserving their capability to inhibit IFN-γ-driven alloreactivity [71].

To study the significance of miR-155 as a regulator of T helper cells differentiation in GVHD, Xie et al. examined serum samples of 19 stable HSCT recipients, 25 patients with acute and 20 with chronic GVHD. They found significantly upregulated expression of miR-155 in both acute and chronic GVHD; moreover, the recipients with high miR-155 expression showed increased serum levels of IFN-γ, IL-17, and IL-9 than those with lower expression. There was also an association between the proportions of Th17 and Th9 cells, and miR-155 levels. The patients with acute GVHD had higher Th17 and Th9 percentages at disease onset and Th9 percentage was elevated in chronic GVHD patients with higher miR-155 levels [72]. Finally, an experiment showed that transfusion of the in vitro-differentiated Th9 lymphocytes could exert a considerable graft versus leukemia effect in mice models of B cell malignancies, which were lethal if treated with bone marrow transplantation only. This result suggested Th9 cells as a probable candidate for post-HSCT cell therapy in malignant hematopoietic disorders [73].

10. Lung and intestine transplants

Unfortunately, due to a lack of research, it is not possible to define the contribution of Th9 cells and IL-9 in lung transplantation; however, the results of allergy studies are suggestive of a proinflammatory role for these cells in the lung tissue. For instance, it has been demonstrated that decreased expression of PU.1 by a conditional deletion in mice impaired IL-9 production in the lungs, which resulted in attenuated allergic pulmonary inflammation [15]. Th9 cells have also been shown to be implicated in airways remodeling. An experimental model of IL-9-overexpressing mice that were exposed to intranasal Alternaria alternata extract showed a higher accumulation of collagen and fibronectin in lung tissue compared to the wild type. Moreover, the concentration of the eosinophil chemoattractant RANTES and the profibrotic mediator connective tissue growth factor (CTGF) was higher in the bronchoalveolar lavage of challenged IL-9-overexpressing mice. These results suggest profibrotic effects of IL-9 in airway remodeling, possibly via eosinophil recruitment and CTGF induction [74]. There have also been attempts to reduce lung inflammation using microbiota-derived short-chain fatty acids (SCFAs) such as butyrate to enhance Foxp3 expression. Foxp3 has been shown to bind the *il9* gene locus and repress IL-9 production [75].

Although there is no research on the implication of Th9 cells in intestinal transplantation, evidence suggests that IL-9 deficiency might attenuate acute and chronic colitis. It has already been shown that PU.1 deficiency in mice T cells was protective against intestinal inflammation, and anti-IL-9 antibodies were able to mitigate experimental colitis. In addition, the patients with ulcerative colitis had more PU.1- and IL-9-expressing T lymphocytes [33,76]. As mentioned earlier, transcription factors BATF, BATF3 and TL1A are involved in Th9 cells differentiation and IL-9 secretion. Accordingly, it was found that Batf3−/− Th9 cells caused reduced inflammation compared to the wild-type cells. In addition, TL1A promoted IL-9-dependent, Th9 cell-induced intestinal and lung inflammation whereas, IL-9-neutralizing antibodies attenuated TL1A-driven mucosal inflammation [77]. These findings indicate a role for Th9 and IL-9 in mucosal inflammation, which might negatively influence the lung and intestine transplants.

11. Discussion

T helper lymphocytes play a substantial role in developing alloimmune responses towards transplanted organs; therefore, they are considered as the most eligible targets for immunosuppressive manipulations [78]. Th1 cells have been shown to elicit cellular immune responses to the allograft through proinflammatory cytokines production (e.g. IFN-γ, IL-12, TNF-α) and recruitment of macrophages, natural killer cells, and other lymphocyte subsets. On the other hand, Th2 cells despite the expression of certain anti-inflammatory cytokines (e.g. IL-4, IL-10) contribute to the development and progression of antibody-mediated alloimmune responses [79]. T helper follicular cells have also been shown to participate in the differentiation and activation of alloreactive plasma cells and antibody production [80]. The other proinflammatory subset supposed to be implicated in graft rejection is Th17, which is usually studied in correlation with Treg cells population; it has been demonstrated that to have a stable functional allograft, it is essential to establish a balance between Th17 and Treg cells frequencies in recipients [81,82]. Additionally, Foxp3+ Treg cells are considered to be the most appropriate candidate for immunotherapy and tolerance induction in transplant recipients since in vivo expansion and transfer of Treg cells could improve allograft survival, particularly in liver transplants [83]. Besides, the tolerogenic potential of other regulatory subsets such as Th3, Tr1, and B regulatory cells has been investigated in various inflammatory disorders [84]. Recent findings about the immunomodulatory effects of IL-9 and Th9 cells suggest them as a probable candidate for immunotherapy post-transplant; however, further development of this hypothesis requires a precise definition of Th9 cells role in transplantation. Unfortunately, apart from the limited number of conducted studies, the results have been obtained from research in different organs, using various methods (in vitro, in vivo, experimental, inhuman), applying diverse techniques of evaluation, including inadequate numbers of subjects, and during different pre- or post-transplant periods, which makes it difficult to draw a concrete conclusion. Nonetheless, the overall findings propose a positive contribution of IL-9

in transplantation as experiments of Gorczynski, Lu and Eller demonstrated the essential role of Treg-secreted IL-9 in mast cell recruitment and subsequent immunoregulatory effects [45,46,54]. However, IL-9 has exerted various influences on different types of allografts (Fig. 2). For instance, IL-9 improved allograft tolerance in experimental models of skin transplantation but showed no association with the outcome of islet cell transfer [55,64]. Heart transplantation from the IL-9-overexpressing mice resulted in enhanced allograft rejection whereas the IL-9-deficient model rejected the cardiac allograft similar to the wild type [65]. On the other hand, subtle advantages were observed in renal transplantation as there was no detectable IL-9 gene expression in allograft with acute rejection, and anti-IL-9-treated mice showed more damage following ischemia–reperfusion than the control group [55]. Although one study showed an increased release of IL-9 from brain-dead donor kidneys following ischemia–reperfusion [58], another investigation reported no considerable change in IL-9 expression upon reperfusion [59]. Furthermore, serum levels of IL-9 were elevated in liver transplant recipients in both stable and rejecting groups without any association between rejection and IL-9 concentrations [62]. Despite upregulated expression of IL-9 in dry-eye due to the chronic GVHD, IL-9 secretion from ILC2 cells in the lung had protective effects against idiopathic pneumonia syndrome [68,69]. Besides, Ognjanovic and Xie demonstrated elevated levels of IL-9 and increased proportion of Th9 cells in acute GVHD [69,72] while Pang found higher percentages of Th9 cells and IL-9 levels in stable recipients and Mangus showed benefits of rapamycin-resistant murine Th9 cells for HSCT outcome [70,71].

In addition, there is evidence of an opposing function beteweenTh9 and Th1 cells, which further supports the tolerogenic effects of this subset [48,70,71]. Therefore, Th9 cells might be considered as a probable option for tolerance induction at least in certain organs. Yet, advantages and disadvantages of Th9 therapy should be studied thoroughly, for instance, Th9 transfer might cause fewer adverse effects than Treg cells therapy due to the lower probability of differentiation into Th17 cells in an inflammatory environment; it may also induce less unspecific immunodeficiency in recipients. However, all these have to be examined in preclinical models. Th9-depleted or IL-9-knockout mice could serve as useful experimental donors and recipient models to study the consequences of Th9 or IL-9 deficiency in different organs transplantation. Infusion of purified Th9 cells or administration of different amounts of IL-9 to the experimental models of organ

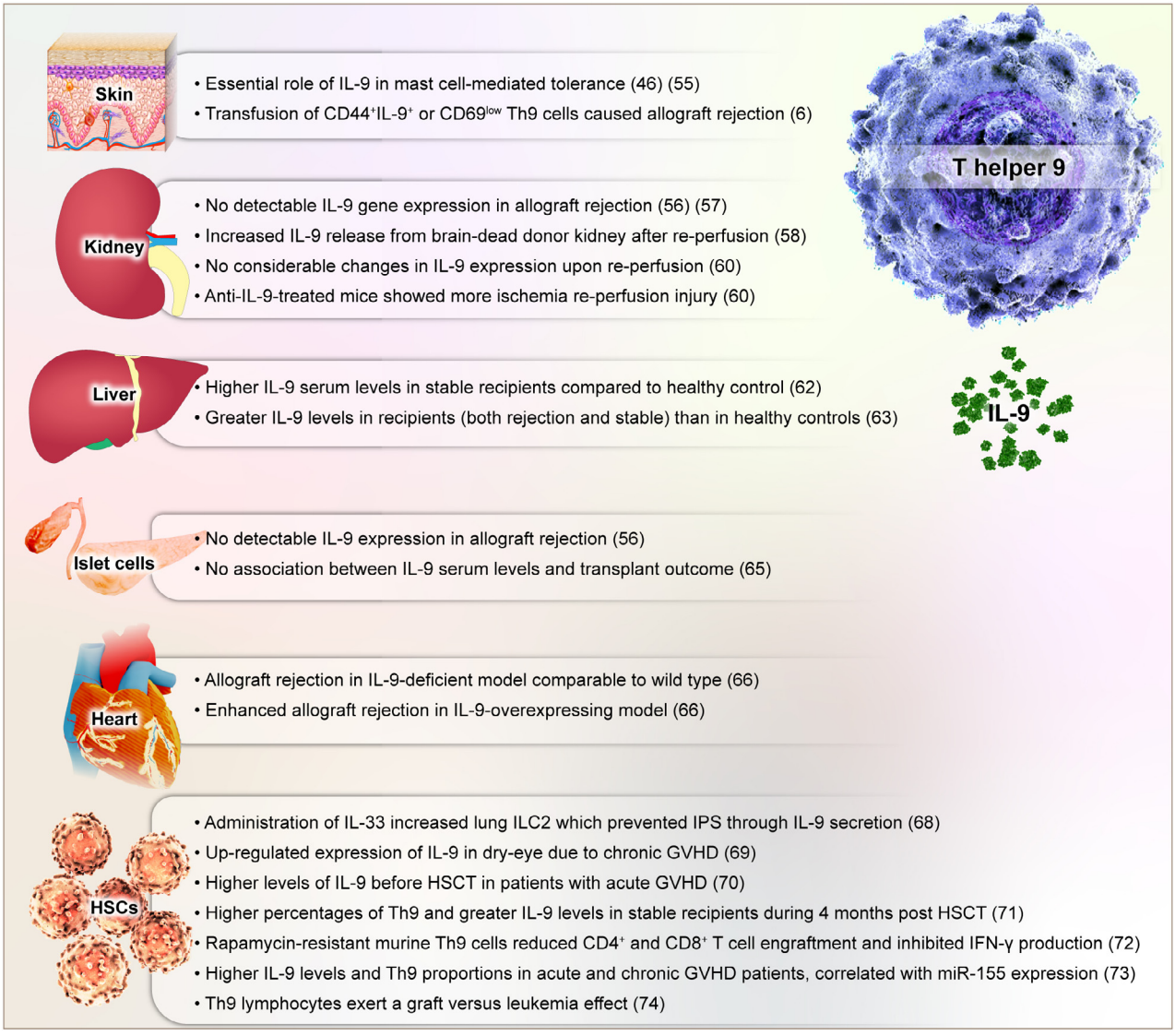

Fig. 2. Effects of Th9 and IL-9 on cell and organ transplantation

recipients might also be helpful in defining the direct implication of these cells in transplant outcome. Moreover, Th9 transfer in various stages of transplantation (i.e. before, after, early, late), in opposing conditions (e.g. stable and rejection), or to the models with immune-related disorders such as autoimmunity might help describe the potential mechanisms underlying the contradictory functions of Th9 in transplantation. In addition, simple observational studies such as determining the proportion of Th9 cells or IL-9 levels in the blood circulation of transplant recipients in different states, or investigating the expression levels of IL-9 or PU.1 in rejected allografts might provide useful information.

The other question to be answered is the effect of different immunosuppressive drugs on Th9 cells' function in vivo. For example, a rat liver transplant study demonstrated that Tacrolimus used for immunosuppression in many organ transplants inhibited IRF4 expression in grafts and splenic mononuclear cells. Since IRF-4 is a transcription factor involved in the differentiation and cytokine secretion of Th9 cells, this drug might exert specific inhibitory effects on Th9 lymphocytes [85]. On the other hand, Th9 cells do not seem to be affected by anti-tumor necrosis factor (TNF) agents since one study on rheumatoid arthritis patients showed no significant decrease in the percentage of Th9 cells after infliximab exposure [86]. Furthermore, it has recently been shown that in successful immune-check-point inhibition therapy Th9 cells count increase significantly and IL-9 secretion is promoted [87]. Th9 cells might also be affected by vitamins as one experiment demonstrated that calcitriol (1,25-dihydroxyvitamin D3) and retinoic acid antagonize each other to regulate the differentiation of Th9 cells and IL-9 production via modulating the function of transcriptional factors, in particular PU.1 [88]. Finally, deprivation of endogenous polyamines resulted in upregulated Th9-related genes, such as Il9, Irf4, and Batf3 [89]. These data certainly need to be extended by further in vitro and in vivo experiments evaluating the effect of other immunosuppressive drugs on Th9 cells' survival and function.

To summarize, Th9 cells and IL-9 have shown beneficial, neutral, or detrimental contributions to the allograft survival depending on the organ type and different stages of transplantation. Nevertheless, overall findings suggest that the immunoregulatory properties of Th9 cells and IL-9 outweigh the proinflammatory effects; therefore, they might be considered as potential candidates for tolerance induction in graft recipients. However, further investigations are required to provide sufficient data in favor of their advantages or against their application in the clinic. Until then, the question is open.

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Conflict of interest

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