

Re-educating immunity in respiratory allergies: the potential for hematopoietic stem cell-mediated gene therapy

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Abstract Respiratory allergies represent a significant disease burden worldwide affecting up to 300 million people globally. Medication and avoidance of known triggers do not address the underlying pathology. Traditional immunotherapies for allergy aim to reinstate immune homeostasis but require years of treatment and have poor long-term efficacy. Novel approaches, such as gene-engineered hematopoietic stem cell transplantation, induce profound antigen-specific tolerance in autoimmunity. Recent evidence shows this approach may also have therapeutic utility for allergy. Here, we review the mechanisms of antigen-specific tolerance and the potential of stem cell-mediated gene therapy to induce tolerance in allergic respiratory diseases.

Keywords Allergy · Autoimmunity · Tolerance · Gene therapy

Introduction

Allergic diseases result from dysregulated T helper type 2 (Th2) immune responses to innocuous environmental antigens. In the respiratory system, this manifests as allergic

rhinitis (AR) in the upper respiratory tract and as atopic asthma (AA) in the lower respiratory tract. Symptoms of respiratory allergies are typically managed by avoidance of known triggers or chronic use of medication, but these do not address the underlying pathogenesis. A key clinical challenge is to restore immune homeostasis in order to control entrenched allergen-specific memory T and B cell responses. Here, we discuss the mechanisms of antigen-specific tolerance that contribute to immune homeostasis and explore a novel approach to restore T and B cell tolerance.

Immune dysregulation in respiratory allergies

Allergic sensitization and Th2-skewed immune responses underlie most forms of AR and AA. Alarmins such as interleukin (IL)-33 and thymic stromal lymphopoietin elicited from respiratory epithelium by allergens or other stimuli promote a Th2-skewed local environment [1] in which IL-4, -5, and -13 from T cells and type 2 innate lymphoid cells promote both AR and AA [2–6]. IL-4 and -13 perpetuate Th2-skewed respiratory inflammation by recruitment of inflammatory monocytes and macrophages to sites of allergen exposure. IL-5 augments eosinophil development in bone marrow and airway eosinophilia leading to epithelial damage [3]. In addition, Th2-activated basophils and mast cells release mediators that induce airway mucus hypersecretion and smooth muscle hyperplasia [7]. Immunoglobulin (Ig)E is an important contributor to respiratory allergic diseases and IL-4 contribute to IgE-mediated effects by promoting activation of allergen-specific B cells and class-switching for IgG/IgE production [8, 9]. Although circulating IgE⁺ B cells are rare, cognate allergen encounter induces expansion and differentiation into IgE-secreting plasma cells [10]. Allergen crosslinking of surface IgE bound to FcεRI on basophils and mast cells leads to

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degranulation and release of histamines and other preformed mediators that can precipitate acute bronchospasm and AR symptoms [8]. Anti-IgE (omalizumab) therapy reduces occurrences of acute AR and asthma exacerbations by reducing circulating IgE [11]. Additionally, because FcεRI expression is regulated by circulating IgE levels, omalizumab indirectly downregulates surface expression of FcεRI on basophils and dendritic cells (DC) [12, 13].

Standard therapies such as corticosteroids are limited by poor patient compliance, leading to reduced disease control [14]. Newer therapies that include “biologics” such as omalizumab or those that neutralize Th2-derived IL-4, 5, or 13 [4, 5] provide some therapeutic benefit for AR and AA. However, global blockade of effector molecules or chronic use of immune modulators is not ideal due to toxicity and compromised immunity to pathogens as well as cost. Rather, immunotherapies that effectively ablate the established and self-perpetuating pathogenic T and B cell responses underlying allergies, either individually or in combination, to restore immune regulation are the ultimate goal for treatment.

Mechanisms of immunological tolerance

Central tolerance is a key process through which self-reactive T or B cells are removed from the repertoire during their thymic or bone marrow development, respectively. However, central tolerance is incomplete and other mechanisms, collectively termed peripheral tolerance, are required to control potentially self-destructive immune responses. Indeed, autoreactive T and B cells are present in healthy individuals indicating the effectiveness of peripheral tolerance to control reactivity of such lymphocyte clones under most circumstances [15, 16]. For allergens, because they are not expressed in the thymus or bone marrow, immune responsiveness must be controlled entirely by peripheral tolerance mechanisms. Cell-intrinsic peripheral T cell tolerance is a consequence of interactions between T cells and antigen presenting cells (APC). In the steady state, DC and other APC exist in a non-activated state expressing low levels of costimulatory molecules [17]. Under these conditions, cognate antigen presentation to T cells leads to abortive activation, limited proliferation, deletion of a large proportion of the antigen-specific T cell pool, and induction of unresponsiveness in residual undeleted T cells [18–20]. Deletion is mediated by Bim-dependent apoptosis [21] and unresponsiveness is maintained, at least in part, by cytotoxic T lymphocyte-associated molecule 4 and programmed cell death protein-1 [19]. Although DC are key inducers of steady-state T cell tolerance, other APC such as B cells may also contribute [22]. It is possible, in normal healthy individuals, that everyday environmental allergen exposure under non-inflammatory steady-state conditions limits immune sensitivity by inducing allergen-

specific T cell deletion and/or anergy through the mechanisms described above.

In addition to cell-intrinsic T cell tolerance, APC under some circumstances induce differentiation or expansion of several subtypes of suppressive regulatory T cells (Treg), including FoxP3⁺ induced Treg [23]. IL-10- and transforming growth factor-β-producing Treg [24], and more recently defined regulatory B cells (Breg) [25], dampen T cell activation and effector function. Interestingly, Treg that might normally suppress allergen-driven responses are either underrepresented or dysfunctional in patients with established AR/AA [26, 27]. These observations form the basis of ongoing clinical trials aiming to combine specific antigens with APC *ex vivo* to induce long-term antigen-specific regulation in allergy (NCT01711593).

The mechanisms of peripheral T cell tolerance are quite well characterized but it is less clear whether analogous peripheral tolerance checkpoints exist for B cells. Our understanding is based almost exclusively on murine models of B cells specific for self-antigen(s), but insights have emerged. As defined for T cells, peripheral tolerance in B cells appears to similarly rely on clonal deletion and anergy induction with chronic antigen stimulation as a key contributor. Chronic antigen stimulation leads to reversible B cell receptor (BCR) desensitization and anergy induction [28]. Upon antigen removal, B cell responsiveness is rapidly restored [28, 29] and inhibitory signaling through phosphatidylinositol-3, 4, 5-trisphosphate 5 phosphatase 1 (SHIP-1) and Docking Protein-1 (Dok-1) is important for maintaining anergy [30, 31]. Chronic antigen stimulation renders B cells exquisitely sensitive to apoptosis, and ligation of CD40L and/or Fas promotes B cell deletion [32]. Anergic B cells have a curtailed lifespan compared to non-anergic B cells [33]. Anergic B cells are unable to solicit T cell help [34], are excluded from entry into follicles [33, 35], and are instead deleted from the recirculating repertoire. T cell help, however, overcomes the negative effects of follicular exclusion. Besides chronic antigen stimulation, cellular localization of antigen also appears to determine the “quality” of tolerance induction in B cells. Membrane-bound self-antigen, which is presumably accessible to BCR, but not soluble or secreted antigens, induces deletion of cognate cells in the periphery [36, 37]. Cellular localization may not be critical for naïve T cell inactivation, as mAb targeting of DC, where antigens are endocytosed, is highly effective [18] and this likely modulates B cell responsiveness by limiting T cell help, much as soluble antigen does [36, 38].

Defining the role of different B cell-extrinsic control mechanisms is experimentally challenging because of the distinct, but difficult to dissect, roles of helper T cells and Treg in regulation of B cell responses. In the development of effective B cell responses, including activation, class-switch recombination (CSR), and somatic hypermutation (SHM), decision

points for becoming memory B cells or plasma cells leading to generation of large quantities of antibodies are all reliant on the provision of T cell help. Treg can indirectly regulate plasma cell differentiation by limiting follicular T helper cell (Tfh) development, thus inhibiting germinal center formation [39, 40]. While this potentially occurs at the T cell priming stage, it has emerged that a specialized subset of Treg that appears to act principally within the GC, follicular regulatory T cells (Tfr [41]) are important in control of help provided by Tfh during the GC reaction [42]. While less well understood, Tfr and/or other Treg also directly suppress peripheral B cell responses in a contact-dependent manner independently of their control of Tfh [42–44].

Current approaches to allergen-specific immunotherapy

Allergen-specific T cells and antibodies are often present in healthy individuals and a loss of “regulation” underpins conversion to clinical atopy [26, 27]. Therefore, many immunotherapies focus on restoring regulation. Allergen-specific immunotherapy (SIT) is the only clinically approved approach to restoring tolerance in patients with allergic disease. During SIT, patients are exposed to increasing doses of allergen over extended periods of time [45]. The procedure is time-consuming leading to poor compliance and, while some genuine therapeutic benefit is achieved, outcomes are not overly robust or long-lasting [45]. This does suggest that more effective immunotherapies would lead to real patient benefit. Newer and more rapid modalities, such as intralymphatic immunotherapy, are being developed [46]. In humans, SIT is associated with induction of IL-10 and TGF- β -producing Treg, switching of allergen-specific Ig isotypes to IgG₄, and, possibly, induction of unresponsiveness in allergen-specific T cells [45]. For T cell responses, low level but continuous encounter with allergen induces differentiation of IL-10-producing T regulatory 1 (Tr1) cells that suppress allergen-specific T cell responses [47]. Tr1-derived IL-10 has also been shown to abrogate enhanced allergen-specific IgE in vitro [47]. SIT also reduces IL-4 production [48], either through regulation or inactivation of T cells. IL-10-producing regulatory B cells that may appear later in course of SIT, possibly as a result of early Tr1 induction [47], might also inhibit T cells. Some T cell hyporesponsiveness to allergens may also be induced by cell-intrinsic inhibition of T cell receptor (TCR) signaling [49]. For B cells, while allergen-specific antibody production increases with SIT, this reflects increased production of inhibitory IgG₄ [47, 50]. Decreases in circulating IgE requires long-term SIT treatment, and induction of deletion or anergy in allergen-specific B cell populations has not been shown experimentally.

The challenge of antigen-specific memory

Effective immunotherapy of allergic disease requires successful control of established memory and effector T and B cell populations; however, this is not a challenge unique to allergy. Memory T cells (Tmem) are difficult to control in autoimmune disease where they persist for extended periods and their pathogenicity can be rapidly reawakened [51, 52]. Tmem are more sensitive to antigenic stimulation [53], less costimulation-dependent [54], and, perhaps, more resistant to apoptosis [55] than naïve T cells rendering them more difficult to control. For example, Tmem are resistant to the effects of cytotoxic drugs and immune-depleting regimes [56] and are less effectively controlled by conventional immunosuppression [52, 57]. Tmem are a barrier to transplant tolerance induction and resist costimulation blockade [58, 59] and are also resistant to the suppressive effects of Treg in both mice and humans [60, 61], potentially limiting the effectiveness of immunotherapies that attempt to reintroduce or expand Treg.

Similarly, memory B cells (Bmem) and plasma cells also present a challenge for immunotherapy. Bmem are more sensitive to antigenic stimulation through higher expression of Toll-like receptors, transmembrane activator and calcium-modulator and cytophilin ligand interactor (TACI) and CD80/86 than naïve B cells [62, 63]. Bmem proliferate more extensively with faster kinetics [64], are more resistant to apoptosis [65], and more readily differentiate into plasma cells [66] in response to antigen than naïve B cells. Under some circumstances, Bmem can receive help “in trans” from non-cognate T cells or can be T cell help-independent [63, 67]. Indeed, costimulation blockade, suppression of T cells by cyclosporine, or even loss of CD4⁺ T cells after HIV infection fails to prevent secondary antibody responses [68–70]. Plasma cells persist in bone marrow niches and produce affinity-matured antibody for extended periods [71, 72]. Like Tmem, plasma cells are resistant to the effects of cytotoxic agents and irradiation [73, 74] suggesting they may also be resistant to other approaches. Loss of BCR from the surface of plasma cells [75] means that BCR-dependent antigen-specific approaches (e.g., deletion/anergy) might not be effective and downregulation of surface proteins including, for example CD20, limit targeting by anti-B-cell therapeutic antibodies (e.g., rituximab) [76].

Emerging genetic evidence which indicates similar mechanisms of immune dysregulation might underlie autoimmunity and allergy [77, 78] suggesting shared immunotherapeutic challenges that are borne out in clinical practice [79]. It is possible the limitations of many immunotherapies result from a reliance on efforts to boost or reinstate immune “regulation” where this has failed through genetic or environmental influences. Alternative procedures that include strong elements of cell-intrinsic tolerance may therefore be more fruitful.

The potential of “engineered” antigen expression to control immune memory

When antigen is constitutively expressed throughout life, both central and peripheral tolerance contribute to a profound antigen unresponsiveness that “prophylactically” prevents induction of immunity [36, 80, 81]. However, induction of “therapeutic” tolerance in established populations of naïve and/or memory and effector T and B cells must be mediated through peripheral tolerance mechanisms alone. Studies from our laboratory show memory T cells are highly susceptible to inactivation by antigen-expressing APC in the steady state. Similar to naïve T cells, transfer of memory and effector CD8⁺ and CD4⁺ T cells to mice where cognate antigen is constitutively expressed by DC results in abortive expansion, deletion, and persistence of a residual unresponsive T cell population [82, 83]. Expression of antigen in MHC class II-positive APC leads to an outcome where model antigen- or autoantigen (e.g., insulin)-specific CD8⁺ Tmem are more rapidly inactivated through what appears to be different mechanisms as TCR is persistently downregulated and effector function is lost within 3 days of transfer [84, 85]. Whether Bmem might be similarly controlled remains unclear. However, B cells specific for T-independent type II antigens, and possibly self-antigens with highly repetitive epitopes such as collagen that is implicated as a self-antigen in autoimmune arthritis, are inactivated by direct BCR engagement with cognate antigen [86].

Using “engineered” hematopoietic stem cells for tolerance induction

Autologous hematopoietic stem cell transplantation is applied as an “immunotherapy” for severe autoimmune diseases [87]. In this context, pathogenic immune cells are depleted by highly toxic, immunoablative conditioning and hematopoietic stem/progenitor cells (HSPC) are provided for hematopoietic support and reconstitution of the immune system. This provides a “reset” of the immune system. Despite efficacy, immune ablation and associated toxicities rule this procedure out for anything but severe disease. Additionally, relapse is common due to incomplete depletion of pathogenic immune cells or a failure of the “immune reset” to counter the effects of underlying genetic disease susceptibilities or environmental effects [88].

Transfer of bone marrow (BM) or HSPC gene-engineered to encode antigen provides the opportunity to exploit enforced antigen expression for tolerance induction which is effective for model antigens, autoantigens, or allergens [88] as this generates a de novo tolerogenic environment in recipients [89]. When myeloablative and/or immunoablative conditions are used, the immune system is reconstituted with a repertoire where both central and peripheral mechanisms establish T

and B cell tolerance toward the encoded antigen. This is associated with failure to develop cognate T cell responses, disease-associated antibodies and with protection from disease development in autoimmune and other disease models [89–92].

Studies of the effect of engineered antigen expression using HSPC transfer in the specific context of allergy are limited. However, both T and B cell responses, including induction of IgE production, are prevented when BM that encodes ubiquitous expression of an allergen is transferred [93]. Here, T and B cell tolerance are likely mediated by both central and peripheral mechanisms [88, 94]. Interestingly, in a different setting, development of anti-factor VIII and -factor IX antibodies that cause hypersensitivity and/or inhibit replacement therapy for hemophilia A and B, respectively, is partially controlled when B cells transgenically expressing factor VIII or IX fused to an IgG backbone are transferred [95]. As this is potentially mediated through peripheral tolerance mechanisms, similar effects might be possible through peripheral tolerance induced by antigen-encoding HSPC transfer under immune-preserving conditions.

Antigen-encoding HSPC transfer inactivates established immune memory

Most preclinical studies using autologous engineered BM/HSPC transfer for antigen expression have used highly toxic myeloablative “conditioning,” or agents that deplete mature T cells or other leukocytes, in order to achieve high levels of “engraftment” and to facilitate reconstitution with a tolerant repertoire [93, 94]. A major requirement, however, is reduced toxicity as well as preservation of host immunity so that only the unwanted, detrimental immune repertoire is targeted. Therefore, immune-preserving conditioning is required to both achieve the therapeutic goals and to permit memory responses to be studied. As a consequence, immune rejection of engineered HSPC that occurs if they express the encoded antigen must be overcome. This can be achieved by stringently restricting antigen expression to differentiated APC (e.g., CD11c⁺ DC) [89], or by including minimal, short-term (3 weeks) treatment with conventional immunosuppression [96] which both facilitate long-term engraftment of transferred HSPC.

Based on previous proof-of-principle studies demonstrating memory and effector T cells are susceptible to tolerance induction [82–84], we proposed the tolerogenicity of antigen-expressing APC could be exploited to address the challenges of Tmem [88]. Taking advantage of the protection afforded by antigen targeting [89], it was shown that pre-existing memory CD8⁺ T cell responses were ablated after transfer of antigen-encoding HSPC under immune-preserving conditions [89, 97]. Both deletion and inactivation contributed to loss of

memory CD8⁺ T cell responses [85, 89, 97] and this was as demonstrated in adoptive transfer studies [82]. Importantly for treatment of established allergic disease, we have now demonstrated that Th2-skewed allergen-specific memory T cell responses are effectively “turned off” by engineered HSPC transfer [98]. In this setting, ablation of Th2 immunity reversed established airways inflammation and alleviated airways hyperresponsiveness [98] (Fig. 1). This has mastered some of the challenges associated with terminating Tmem responses, but given the importance of IgE in allergy, similar studies need to be performed to study effects on Bmem and allergen-specific IgE-producing plasma cells. It is likely that the level and distribution of antigen and the relative contributions of T vs B cell tolerance will be important determinants of outcome. Additionally, Treg are induced or expanded in many gene therapy-induced tolerance settings [99] including HSPC-mediated tolerance [100] and this could be an important component to be explored for optimal therapeutic outcomes. For control of B cell or Bmem responses, induction of Tfr may be important, but an understanding of the conditions that induce Tfr is only beginning to emerge [101].

Moving forward with HSPC-mediated therapies, targeting antigen to specific APC types, cellular locations or achieving specific levels of expression or engraftment may all be important determinants of the effectiveness of HSPC-mediated

tolerance induction. For engineered HSPC transfer, some studies have explored cell-specific promoters and these appear to be of value for restorative therapy of hemoglobinopathies, for example, where very high lineage-restricted therapeutic protein expression levels are desirable [102]. For sensitized hosts, which have an established memory repertoire against the encoded antigen, targeting antigen expression away from HSPC may be crucial [89] as discussed above. Although targeting APC per se is not essential for T cell tolerance, it is effective when engineered HSPC are transferred [89, 103], but there may be other benefits in targeting different APC subsets. For example, the microanatomical localization of different APC subtypes might deliver antigen specifically to either T or B cells. Antigen expression by B cells or follicular DC might most effectively deliver tolerogenic antigen to B cells due to their co-localization, whereas DC resident in T cell areas such as CD8⁺ DC in mouse are possibly most effective for targeting T cells. Likewise, antigen delivery to different DC subsets or other APC [84, 85] indicates intrinsic differences exist in function that extend into tolerogenic potential [104–106]. Whether this affects HSPC-mediated immunotherapies remains unclear but studies examining the effects of engineered HSPC on B cell responses or antibody production [90, 91] have to date typically employed “ubiquitously targeted” antigen expression. For lower respiratory tract

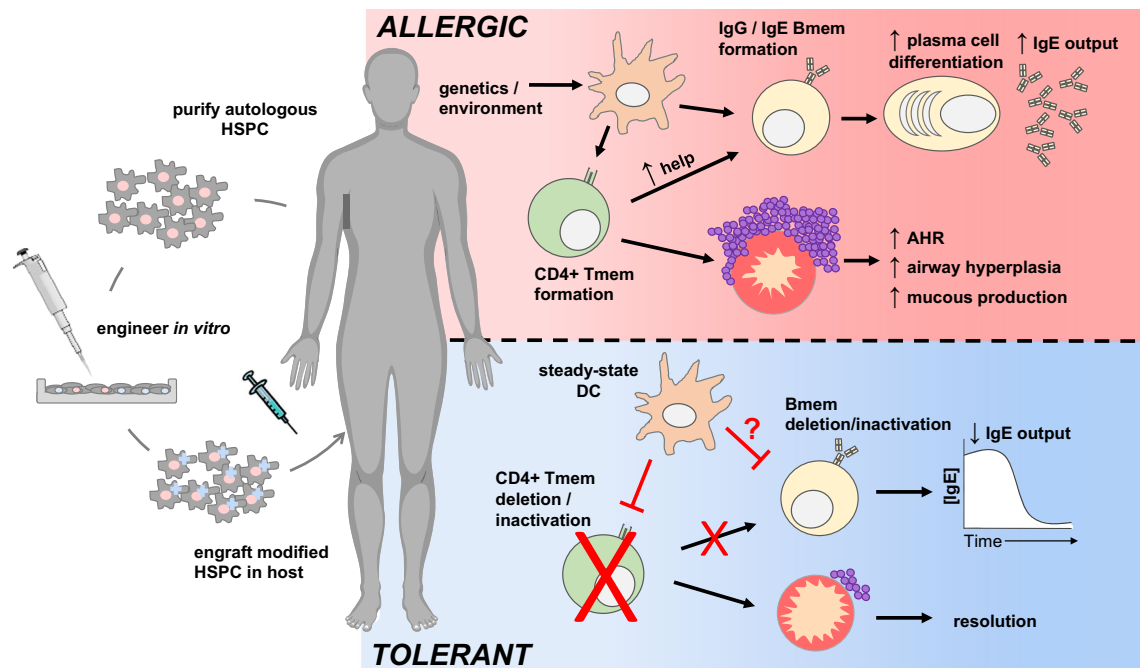


Fig. 1 Allergen-encoding HSPC transfer has the potential to resolve respiratory allergic disease. Genetic and environmental determinants promote antigen-presenting cell induced activation of allergen-specific CD4⁺ T cells, Th2 differentiation and B cell activation, and class switching to IgE. Continued allergen exposure expands memory CD4⁺ T cell (Tmem) and B cell (Bmem) populations, plasma cell differentiation, and allergen-specific IgE accumulation. Allergen-specific T cells induce airway remodeling, hyperresponsiveness, and chronic

inflammation and allergen-specific IgE promotes mast cell and basophil mediated localized inflammation. Genetic engineering and transfer of autologous hematopoietic stem/progenitor cells (HSPC) lead to generation of steady-state dendritic cells that express allergen. Steady-state antigen presentation leads to deletion and inactivation of cognate allergen-specific Tmem and, potentially, Bmem, ultimately resolving allergic airways inflammation

disease, ablation of pathogenic T cell responses is likely to be of major benefit. For upper respiratory tract disease (e.g., allergic rhinitis), reversal of IgE production and clearance of circulating IgE may be necessary. T cell tolerance is achieved at modest levels of chimerism [84, 89, 97, 98] which could be achieved with minimally toxic conditioning regimes. Whether deletion/inactivation of B cells is effectively achieved at such low levels of chimerism is yet to be determined; it appears this may be the case [107]. Advantageously, it is likely circulating IgE can be readily depleted with a single dose or short course of anti-IgE antibody (omalizumab). Effective ablation of T and B cell responsiveness would prevent re-emergence of IgE production. Thus, high levels of engraftment/chimerism are requiring myeloablation, which are able “mop-up” pathogenic antibodies, to be necessary.

Engineering HSPC for clinical immunotherapeutic application

For gene therapy, HSPC are harvested from bone marrow or from the blood of “mobilized” patients (reviewed in [108]), cultured in “activating” growth factors and transduced with viral vectors prior to re-infusion to patients. Recent reviews provide detailed overviews of HSPC engineering and production [109, 110], but key points are provided here. For engineering HSPC, the requirement for stable incorporation of genetic material into the genome has focused efforts on retroviral and lentiviral vectors. Early studies employed Moloney murine leukemia virus-based gamma retroviral vectors (γ RV) [111], but HIV-based lentiviral vectors (LV) which have the advantage of transducing non-dividing cells were developed in the mid-1990s [112]. γ RV-engineered HSPC have been used in several clinical settings and in ADA-SCID have provided beneficial outcomes [113] [114]. Safety concerns arising after a series of X-SCID and Wiskott-Aldrich syndrome (WAS) patients developed leukemias following γ RV-engineered HSPC treatments provided impetus for the clinical testing of self-inactivating (SIN)-RV [115] and LV [116, 117]. Due to their lower genotoxicity profile and improved safety relative to γ RV, LV are considered the most suitable current approach to gene-engineering of HSPC. Initially, viral vectors employed endogenous viral promoters that led to ubiquitous gene expression in transduced cells although some SIN-RV and LV with lineage or cell-specific promoters have been tested clinically [115, 116]. Currently, clinical application of engineered HSPC is restricted to serious, life-threatening diseases; however, rapid advances foreshadow ways in which gene therapy can be feasible for immune modulation in less serious diseases. For example, improving transduction efficiency and survival [118], preservation of “stemness” [119], and effective expansion [120] of engineered HSPC all increase the feasibility of HSPC engineering. Importantly,

engineered HSPC restore B cell homeostasis and tolerance induction clinically in WAS [121], providing strong support for HSPC-mediated tolerogenic therapies. Technologies such as zinc finger nuclease (ZFN), TALEN, and CRISPR/Cas gene editing are emerging as powerful tools and these might be effectively applied for incorporation of tolerogenic gene constructs in HSPC [122–124]. These approaches used for engineering of induced pluripotent stem cells and their differentiation to HSPC [125, 126] might be predicated as future approaches to HSPC-mediated gene therapy. Ultimately, HSPC-mediated gene therapy might be applied without need of ex vivo transduction if this can be achieved in vivo through direct injection of antigen-encoding vectors [127, 128].

Future directions

While the results for model allergens and autoantigens are encouraging, allergy and autoimmunity are usually perpetuated by responses directed against multiple allergens or antigens. Whether there is a requirement to inactivate responses to all targeted allergens/antigens or just those that comprise the major components of responsiveness will need to be determined. One key question is whether Bmem cells are reliant on antigen-specific T cell help and whether establishing T cell tolerance impairs their responsiveness. Indeed, does induction of T cell tolerance alone sufficiently limit naïve B cell responses to prevent sensitization for IgE production? Future studies also need to examine the capacity of antigen-encoding HSPC to purge naïve or Bmem responses and whether this provides therapeutically meaningful effects. Importantly, novel approaches to safer transplantation are required to provide opportunities for engineered HSPC to be used in allergy given the great potential of this approach.

Summary

Autoimmunity and allergy affect a substantial proportion of the population and current therapies are unsuccessful in resolving the immunological drivers of disease. Understanding of peripheral tolerance induction has improved greatly providing insights for the purging of the pathogenic immune memory that perpetuates allergy. Transfer of engineered BM/HSPC-encoding antigen is emerging as an effective means to inactivate undesirable memory T cell responses, and this approach may have great potential for application to severe allergic respiratory diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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