

Functional regulatory T cells and allergen immunotherapy

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Purpose of review

Burgeoning literature ascribing roles for regulatory T cells (Treg) in controlling clinical allergy frequently relies on phenotypic markers rather than functional suppression assays. This review examines evidence for functional Treg in controlling immune responses to allergen with particular focus on allergen-specific immunotherapy.

Recent findings

Over the last year, use of recently identified Treg markers has facilitated more precise delineation and isolation of Treg subtypes. Advanced flow cytometry allows better discrimination and tracking of effector T cells (Teff) and Treg in co-culture assays for Treg function. These approaches enable critical appraisal of the evidence for Treg mediation of the observed changes in immune reactivity to allergen accompanying immunotherapy. Recent studies suggest that different mechanisms of immune regulation may occur at different times of immunotherapy, with Treg playing a more important role early in treatment. Additionally, advanced imaging techniques reveal increased Treg numbers in allergen-exposed mucosal tissue after therapy consistent with an enhanced local functional role.

Summary

These findings support a mechanistic role for Treg early in allergen immunotherapy. Research is required to clarify the precise Treg subsets involved, their specificity and how their activity can be enhanced during immunotherapy by appropriate allergen form, optimal site of administration and use of adjuvants and adjunct therapies.

Keywords

allergen immunotherapy, allergy, regulatory T cells

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Introduction

Regulatory T cells (Treg) are clearly pivotal for maintenance of immune self-tolerance, and evidence also suggests that they are important for promoting immune homeostasis following response to exogenous antigens, including allergens. As reviewed herein, there are reports of Treg deficiency in allergic individuals and of enhanced Treg activity following successful allergen-specific immunotherapy. However, interpretation and comparison of many studies are difficult due to the use of different Treg nomenclature, markers for identification and assays for evaluation. Importantly, clear evidence of relevant Treg function is often lacking. This review will focus on recent studies providing insight into the mechanisms by which human Treg may down-regulate the allergen-specific immune response following allergen-specific immunotherapy (SIT). Elucidation of these mechanisms will suggest strategies for targeting and enhancing Treg function for more pronounced and sustained efficacy of allergen-specific immunotherapy.

Delineation and modes of action of Treg

CD4⁺ T cells with suppressive function are broadly classified as either naturally occurring, thymic-derived regulatory T cells (nTreg) or induced or adaptive Treg that acquire suppressive function following antigen stimulation in an appropriate cytokine milieu in the periphery (iTreg) [1]. nTreg were first described in 1995 when a thymic-derived CD4⁺ T-cell subset constitutively expressing high levels of the IL-2 growth factor receptor α chain, CD25, was shown to control development of autoimmunity in mice [2]. The presence of an equivalent nTreg subset in humans was supported by the demonstration of similar in-vitro cell contact-dependent suppressor function by a subset of T cell receptor (TCR)-stimulated peripheral blood CD4⁺CD25⁺ T cells [3]. Reversal of the hypoproliferative state and regulatory function of these cells was achieved by increasing the strength of signal by CD28 co-stimulation or addition of IL-2. Plasticity of Treg is an important consideration when targeting Treg for immunotherapy. A critical role for the transcription factor Foxp3

in nTreg differentiation, function and maintenance through adult life is shown in mice with mutated or deleted *Foxp3* which develop fatal autoimmune-like disease with hyper-responsive CD4⁺ T cells [4–7]. Similarly, humans with a mutation in the *Foxp3* gene develop fatal immunopathology including autoimmune disease and allergy (IPEX syndrome) [8,9].

Treg control immune responses by inhibiting the activation, proliferation and effector functions of a wide range of target cells including innate cells, antigen-presenting cells and effector T cells (Teff; see review [10]). Modes of action include engagement of inhibitory cell surface molecules, production of inhibitory cytokines, induction of cytolysis, and metabolic disruption. Whereas nTreg represent the major regulatory cell population for control of immune self-tolerance, peripheral induction of iTreg may be more important for regulation of immune response to exogenous antigens. In-vitro assays of Treg function demonstrate predominant cell contact-dependent mechanisms for nTreg function, and for iTreg, suppression predominantly via immunoregulatory cytokines including IL-10 (Tr1 cells [11]) and TGF- β (Th3 cells [12]). However, interpretation of many studies of Treg function, especially using clinical samples, is difficult due to overlap of surface marker expression between Treg and Teff, especially when activated.

Approximately 10% of human peripheral blood CD4⁺ T cells co-express CD25, but only those with highest CD25 expression (approximately 1–2% of CD4⁺ T cells) are functionally suppressive [3]. Unlike their murine counterparts, human peripheral blood CD4⁺CD25⁺ T cells demonstrate a broad spread in level of CD25 expression, with no clear separation of a highly positive population. Human Treg cannot be reliably distinguished from activated CD4⁺ Teff based on expression of high levels of CD25 alone. Similarly the transcription factor Foxp3, being intranuclear, cannot be used to identify and isolate viable nTreg for functional studies. Whereas the majority of human CD4⁺CD25^{hi} peripheral blood cells express Foxp3 [13], these could comprise iTreg as well as nTreg as both Foxp3⁺ and Foxp3⁻ iTreg populations are described [14]. Moreover, the demonstration of transient expression of Foxp3 by activated human Teff without gain of suppressor function or change in cytokine profile [15,16] precludes use of this marker alone for reliable Treg delineation. In the future, detection of epigenetic markers may provide more reliable identification of a permanent Treg lineage since epigenetic modification of the *Foxp3* gene such as by demethylation is required for stable Foxp3 expression [17].

Other Treg surface markers include cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) [18], glucocorticoid-

induced tumour necrosis factor receptor family-related gene (GITR) [19] and lymphocyte activation gene-3 (LAG-3) [20], but again, expression of these markers is also induced on activated effector CD4⁺ T cells and none is Treg-specific. nTreg themselves show altered expression of several markers, including those shared with naïve and activated conventional T cells, as they differentiate from naïve, recent thymic emigrants to effector and terminal effector Treg in the periphery [1].

To date, human Treg have been isolated from peripheral blood for functional studies using a limited set of surface markers. With the demonstration that CD127 (IL-7 receptor α chain) expression correlates inversely with Foxp3 expression and suppressor function of human CD4⁺ T cells, viable human nTreg (CD4⁺CD25^{hi}CD127^{lo}) can be distinguished from in-vivo activated T cells (CD4⁺CD25^{hi}CD127^{hi}) and purified from fresh peripheral blood [21,22]. Double staining confirms that the CD127^{lo} subset of CD4⁺CD25^{hi} cells is Foxp3⁺ [23[•]]. However, when utilizing in-vitro stimulated cell culture assays, additional markers such as CD62L are required to differentiate nTreg from activated conventional CD4⁺ T cells since CD127 is down-regulated on naïve T cells after activation [24]. Co-staining for CD39 has been successfully used to enrich functional CD4⁺CD25⁺Foxp3^{hi} cells from allergen-stimulated human peripheral blood mononuclear cells (PBMC) cultures [23[•]]. CD39 is an ectonucleotidase, which together with CD73, hydrolyses ATP to generate the immunosuppressive molecule, adenosine [25,26,27[•]].

Evidence for a role of Treg in controlling the allergic phenotype

Despite difficulties in clearly identifying Treg populations as discussed above, numerous studies provide evidence for a functional role for Treg in actively maintaining peripheral tolerance to allergens in nonatopic healthy individuals and suggest that immunotherapy could restore this tolerant state in allergic individuals. Whereas the peripheral T-cell repertoire of healthy individuals and those with allergic diseases includes recognition of the same dominant T-cell epitopes of allergens, early seminal studies concluded that the immune responses of these two groups are distinguished by differences in the balance between allergen-specific Treg and Th2 cells [28,29]. In a natural model of immune regulation, multiple bee stings in healthy nonallergic beekeepers induced clonal switching from dominant venom antigen-specific Th1 and Th2 cells towards dominant IL-10-secreting 'Tr1' cells, with persistence of this switch until return to baseline by 3 months after cessation of allergen exposure [30]. In studies of seasonal allergy, peripheral blood CD4⁺CD25⁺ T-cell inhibition of in-vitro allergen-induced proliferation and cytokine release

by autologous CD4⁺CD25⁻ T cells was significantly lower for grass pollen-allergic patients compared with nonatopic healthy controls; this decrease was accentuated during the pollen season [29] and shown to be allergen-specific [31[•]]. Similar observations were reported in birch pollen-allergic patients [32]. Likewise, atopic asthmatic patients showed an insufficient CD4⁺CD25⁺ Treg response and an excessive IL-4 response compared with sensitized asymptomatic individuals when PBMC were stimulated with the perennial house dust mite (HDM) aeroallergen *in vitro* [33]. The asymptomatic group had higher percentages of CD4⁺CD25⁺ T cells and CD4⁺IL-10⁺ T cells and enhanced IFN- γ and IL-10 cytokine production in allergen-stimulated PBMC cultures. As discussed in more detail elsewhere in this issue, several studies investigating pathogenesis of food allergy point to a functional role for CD4⁺CD25⁺ Treg in the development of normal tolerance to food allergens and the spontaneous resolution of milk allergy [34–36]. A higher frequency of milk allergen-specific CD4⁺CD25⁺CD27⁺T cells in peripheral blood correlated positively with a clinical phenotype of mild disease and increased likelihood of acquisition of clinical tolerance with ageing [37].

Consistent with the growing appreciation of the importance of environmental factors on expression of the allergic phenotype, recent studies raise the tantalizing possibility that maternal farm exposure, including stables, barns and consumption of farm milk possibly enhancing particular microbial exposure, may provide a form of natural immunotherapy redirecting the immune response to aeroallergens from a Th2-dominant to Treg-dominant response [38]. Treg function has been shown to be more efficient in those with farming exposure. A potential immunoregulatory component of the farming environment was identified recently when it was shown that grass arabinogalactan from cowshed dust given intranasally protects mice from developing allergic airway inflammation [39]. This group previously reported decreased Treg numbers (CD4⁺CD25^{hi}), mRNA expression of markers (GITR, LAG-3) and Treg function in infants at high risk of allergic diseases based on maternal atopy compared with low risk infants [40].

Analysis of Treg function in allergen-specific immunotherapy (SIT)

Clinically effective SIT is associated with decreases in allergen-specific T-cell proliferation, as well as in Th2-type cytokine production and inflammatory cell activity (see reviews [41,42]). In parallel, levels of allergen-specific IgG₄ increase and, with time, allergen-specific IgE levels decrease. With these observations and evidence of defective Treg response to allergen in allergic patients as described above, many have sought to deter-

mine whether clinically successful SIT is associated with enhanced Treg activity. Early studies of Treg in SIT were limited to analyses based on a restricted number of cell surface molecules and examination of T-cell regulation in bulk cultures of allergen-stimulated PBMC or short-term T-cell lines. CD4⁺CD25⁺ T cells were shown to be the major producers of the increased IL-10 seen in allergen-stimulated PBMC following SIT for HDM and grass pollen [43,44], but it is unclear whether these were a population of recently activated conventional T cells or Treg. Functional Treg activity was suggested by the observation that peripheral blood CD4⁺ cells taken post-SIT inhibited proliferation of allergen-stimulated PBMC collected from individuals before therapy [45], and a role for IL-10 in mediating this suppression was consistent with its ablation by anti-IL-10 blocking antibodies [46]. In a seminal study of sublingual immunotherapy (SLIT) for birch pollen allergy, further evidence that a Treg population mediated SIT-induced suppressor function was the loss of suppressor activity at 4 weeks' treatment when CD25⁺ cells were first depleted from PBMC, and increased PBMC expression of Foxp3 mRNA at this time [47]. This field has advanced over the last year as additional Treg markers have been described (see above) enabling more precise identification of Treg subtypes. Improved methods for isolating Treg for subsequent functional analysis have also been established and commercial kits developed. Another advance has been the utilization of 5,6-carboxyfluorescein diacetate succinimidylester (CFSE) labelling to track dividing Treg during in-vitro co-culture assays of functional Treg. This is important since human-naïve CD45 RA⁺ Treg can potentially proliferate under suitable conditions [48].

Despite staining for Foxp3 or additional markers such as CD127^{lo} to more clearly delineate Treg, it is clear that analysis of total Treg in peripheral blood fails to demonstrate significant changes associated with SIT as highlighted in recent studies. Mobs *et al.* [49^{••}] performed a longitudinal analysis of allergic patients receiving 12 months' birch pollen SIT and observed no significant change in the percentage of CD4⁺CD25⁺CD127^{lo} cells in total peripheral blood CD4⁺ cells and no seasonal effect on levels during this time. Yamanaka *et al.* [50^{••}] found no difference in the percentage of CD4⁺CD25^{hi}Foxp3⁺ cells/CD4⁺ T cells between patients receiving 6 months' peptide SLIT for Japanese cedar pollen allergy compared with untreated allergic patients and Grindebacke *et al.* [51^{••}] observed no difference in the percentage of CD25⁺ or CD25^{hi} cells within the peripheral blood CD4⁺ cell fraction between birch pollen-allergic patients with or without 6 months of injection SIT. In all of these three studies there was evidence of clinical efficacy of SIT. These findings point to the difficulty in distinguishing an

allergen-specific Treg response by analysing total Treg numbers in peripheral blood.

In contrast, SIT-induced quantitative changes in Treg populations can be revealed by ex-vivo allergen stimulation. In the study by Mobs *et al.* [49^{••}], although proportions of peripheral blood CD4⁺CD25⁺CD127^{lo} cells were unchanged, in-vitro Bet v 1-stimulated PBMC showed changes in the frequency of IL-10-secreting cells detected by ELISPOT assay, termed Bet v 1-specific Tr1 cells. The Tr1 frequency increased progressively from 3–6 months then decreased at 12 months, although levels were still higher than at pretreatment. Allergen-induced Treg may play a more prominent role in the initial phases of SIT. Tr1 cells were identified in Japanese cedar pollen allergen-stimulated PBMC cultures by double staining for CD4 and IL-10 secretion, with an increased percentage after 6 months' SIT compared with untreated pollen-allergic controls [50^{••}]. Using CFSE staining to distinguish nondividing CD4⁺CD25⁺Foxp3⁺ cells in 7-day HDM-stimulated PBMC cultures, consistent with an allergen nonresponsive or anergic Treg population, our group showed an increase in levels of these cells at 6 and 12 months' HDM SLIT compared with pretreatment [52^{••}]. Importantly, back-gating showed that the CD4⁺CD25⁺Foxp3⁺ cells were CD127^{lo}, allowing sorting by flow cytometry and confirmation of suppressor function. The percentage of these cells decreased to pretreatment levels at 2 years despite persistent clinical efficacy. Similar kinetics of allergen-induced Treg were evident in a study of oral peanut SIT [53[•]] when levels of CD4⁺CD25⁺Foxp3⁺ cells in 6-day allergen-stimulated PBMC cultures increased approximately 1.5-fold at 6 and 12 months of treatment then returned to baseline by 20 months. Together these findings suggest that different mechanisms of regulation may occur at different times of SIT, with Treg playing a more important role early in treatment. Anergy or deletion of allergen-specific Teff could explain persistence of decreased Th2-type response later in treatment.

Further insight into the mechanisms of Treg function during allergen-specific immunotherapy comes from recent advances in Treg isolation techniques. To more carefully exclude CD25⁺ activated Teff when analysing CD4⁺CD25⁺ Treg function, Grindebacke and colleagues [51^{••}] used suboptimal concentrations of commercial anti-CD25-labelled magnetic beads, and O'Hehir *et al.* [52^{••}] sorted CD4⁺CD25⁺CD127^{lo} cells by flow cytometry, confirming Foxp3 expression in a small sample of purified cells. In both these studies, suppressor function of the separated Treg was demonstrated by decreased allergen-stimulated proliferation and cytokine production in CD4⁺CD25⁺ T-cell co-cultures. Interestingly in the study by O'Hehir's group, greater efficiency of Treg suppression of IFN- γ and IL-2

production compared with IL-5 production was shown in Teff co-cultures, perhaps explaining why suppression of IFN- γ but not IL-5 production was observed by Grindebacke's group [51^{••}].

Whereas Grindebacke *et al.* [51^{••}] found no difference in Treg-mediated inhibition of allergen-stimulated Teff proliferation and production of IFN- γ between birch pollen-allergic patients with or without 6 months' birch pollen SIT, IL-10 production in Treg co-cultures was increased in the SIT-treated group but not controls. IL-10 production was increased when CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells were cultured together but not when CD4⁺CD25⁺ or CD4⁺CD25⁺ T cells were cultured alone. These results suggest that either Treg only produce IL-10 when in contact with Teff or that Treg can induce differentiation of Teff to IL-10-producing Tr1 cells. Addition of anti-IL-10 receptor antibodies to the Treg co-cultures increased levels of IFN- γ for the SIT-treated patient group and not controls, but blocking IL-10 had no effect on Treg suppression of allergen-induced Teff proliferation. In contrast, in the study of Japanese cedar pollen SLIT by Yamanaka *et al.* [50^{••}], addition of IL-10-neutralizing antibody or anti-IL-10 receptor antibody ablated the SIT-induced decreased allergen-specific CD4⁺ T-cell proliferation in a CFSE-tracked assay. More clearly pointing to a functional role for Tr1 cells in SIT, Mobs *et al.* [49^{••}] purified viable CD4⁺IL-10⁺ cells from peripheral blood using a commercial IL-10 Secretion Assay Cell Enrichment and Detection Kit which allows the separation of IL-10-producing cells without having to fix for intracellular cytokine staining. Lack of Foxp3 expression confirmed that the purified IL-10-producing cells were not nTreg. When co-cultured with CD4⁺CD25⁺ Teff, the purified CD4⁺IL-10⁺ Tr1 cells suppressed allergen-specific proliferation in a cell number-dependent manner, and addition of blocking antibodies confirmed that suppressor function was mediated by IL-10. A role for TGF- β in Treg suppression of Teff allergen-specific proliferation at 6 months' HDM SIT was demonstrated by O'Hehir's group by addition of recombinant human soluble TGF- β receptor II/Fc chimeric protein to co-cultures [52^{••}]. Whether the TGF- β in these co-cultures was produced by the Treg themselves or by the Teff induced by Treg was not determined. However, membrane-bound TGF- β may have mediated suppression since there was no change in culture supernatant TGF- β levels.

Enhanced activity of IL-10-secreting Treg could also mediate the observed changes in allergen-specific antibody isotype from predominantly IgE to IgG₄ seen in SIT. In an elegant in-vitro study [30], both allergen-specific Tr1 cells (IL-10-secreting cells purified from allergen-stimulated PBMC) and CD4⁺CD25⁺ T cells (purified from fresh PBMC and confirmed Foxp3⁺) from

nonallergic donors induced IgG₄ and suppressed IgE production at the mRNA and protein level in purified B-cell co-cultures. The pivotal role for IL-10 in these experiments was demonstrated by blocking IL-10 or its receptor. Treg may also control allergen-specific antibody production by direct lysis of B cells [54].

Due to sampling limitations, most clinical studies of Treg examine the circulating pool in peripheral blood, but some recent studies provide evidence for local regulatory effect of Treg. Examination of oral epithelium biopsies revealed increased numbers of Foxp3⁺ cells for grass pollen SLIT-treated compared with placebo-treated patients [55[•]]. Previous studies had demonstrated increased numbers of Foxp3⁺CD4⁺ and Foxp3⁺CD25⁺ cells [56], and IL-10⁺ and TGF-β⁺ cells [57] in the nasal mucosa following injection SIT compared with untreated allergic patients, consistent with dissemination of Treg to mucosal sites of allergen exposure. Treg express multiple chemokine receptors [58] which could influence their migration to different compartments. Preferential expression of CCR4, CCR5, CCR6 and CCR8 by CD4⁺CD25⁺ Treg compared with CD4⁺CD25⁺ Teff is described [59–61] and in-vitro allergen stimulation can alter chemokine receptor expression by Treg [62]. Following HDM SIT, CD4⁺IL-10⁺ PBMC showed increased expression of the peripheral tissue trafficking receptors CCR5 and CD49d [43] and in another study of pollen SLIT, PBMC Tr1 cells co-expressed skin and gut-homing cell surface markers including β7 integrin, CLA, CCR10 and CCR4 [50^{••}]. Treg trafficking to regulate mucosal sites may be impaired in allergic patients due to defective chemokine intracellular signalling. Decreased CCL1-mediated chemotaxis of CD4⁺CD25⁺CD127^{lo} PBMC from allergic asthmatic patients compared with healthy controls was associated with decreased phosphorylation of protein kinase B (AKT) but unaltered expression of the CCL1 receptor, CCR8 [63]. Further studies are required to fully explore this important aspect of potential Treg response to SIT.

Future prospects

Persistence of clinical efficacy after SIT is consistent with induction of specific immune tolerance and there is strong evidence for Treg playing a key mechanistic role. What is not clear is the precise Treg subsets involved, their specificity and how their activity can be enhanced during SIT. Murine studies demonstrate that nTreg require stimulation via the TCR for acquisition of suppressor function, but once activated, they can suppress in a nonspecific fashion [64]. This has important implications for SIT, when suppression of multiple Teff clones is required. Clear evidence for 'linked suppression' in SIT comes from studies using a limited panel of T-cell

epitope-based Fel d 1 peptides for therapy [65^{••}]. In another study, spectratypic analysis of TCR complementarity-determining region 3 of Tr1 cells demonstrated preservation of a highly diverse TCR repertoire after pollen SLIT [50^{••}].

Whether SIT expands pre-existing Treg populations and/or induces Treg from effector or memory T cells is not clear. Whereas analysis of Vβ gene expression in patients receiving multiple bee stings demonstrated differentiation of IL-10⁺ Tr1 cells from activated Th1 or Th2 effector cells [30], in-vitro studies showed that Foxp3⁺ T cells expanded in allergen-stimulated cultures were derived from both nondividing nTreg and dividing CD4⁺CD25⁺ cells [23[•]]. However, longitudinal studies are required to determine whether more prolonged TCR stimulation as in SIT would result in induction of a permanent Treg status (e.g. by demethylation of the *Foxp3* gene). Incorporation of newly described markers of Treg may assist Treg delineation in these studies. One such marker with potentially functional significance is the glycoprotein-A repetitions predominant (GARP) receptor which binds the latency-associated peptide (LAP)/latent TGF-β complex, thus linking with the important TGF-β regulatory circuit [66,67]. Epigenetic regulation of GARP expression is proposed to underly alternative differentiation of suppressor lineage cells into nTreg and iTreg [48].

Finally, the influence of allergen form (e.g. recombinant allergen, peptide or fusion protein) and route of administration (e.g. subcutaneous, sublingual) on development of Treg in SIT and whether adjuvants can promote expansion and function of Treg during SIT are areas of intense research [41,68[•],69[•],70,71[•]]. Adjuvants may enhance efficacy via influencing the crucial cross-talk between dendritic cells and Treg [72[•],73[•]]. Agents such as LPS can induce indoleamine-2,3-dioxygenase expression by dendritic cells with subsequent tryptophan deprivation mediating their ability to induce CD4⁺CD25⁺Foxp3⁺ Treg [74[•]]. However, the demonstration that certain inflammatory stimuli can subvert suppressor function of Treg and induce their production of the pro-inflammatory cytokine IL-17 [75[•]] raises a note of concern when manipulating the immune synapse with adjuvants. Of relevance is the reported association between increased allergen-induced IL-17 mRNA level and poorer therapeutic outcome of SLIT [76[•]]. In contrast, adjunct therapies such as all-trans retinoic acid, rapamycin, glucocorticoids and vitamin D can drive effector and memory T-cell conversion to Treg and co-administration of glucocorticoids with the active form of vitamin D (1α,25-dihydroxyvitamin D3 or calcitriol) promotes induction of Foxp3⁺ suppressor T cells [77[•]] and IL-10-secreting Tr1-like cells [78[•]]. Whereas prospects for promoting SIT efficacy by administration of ex-vivo expanded Treg [79] seem remote, innovative

protocols for enhancing Treg development, function and survival are within reach.

Conclusion

Recent studies using a panel of Treg markers to more clearly identify and isolate Tregs for functional assay support a mechanistic role for Treg early in allergen immunotherapy. Further research is required to clarify the precise Treg subsets involved, their specificity and how their activity can be enhanced during immunotherapy by appropriate allergen form, optimal site of administration and use of adjuvants and adjunct therapies.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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8 Immunotherapy and new treatments

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