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Lineage origin and transcriptional control of autoantigen-specific T-regulatory type 1 cells

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T Regulatory type-1 (TR1) cells represent an immunosuppressive T cell subset, discovered over 25 years ago, that produces high levels of interleukin-10 (IL-10) but, unlike its FoxP3+ T regulatory (Treg) cell counterpart, does not express FoxP3 or CD25. Experimental evidence generated over the last few years has exposed a promising role for TR1 cells as targets of therapeutic intervention in immune-mediated diseases. The discovery of cell surface markers capable of distinguishing these cells from related T cell types and the application of next generation sequencing techniques to defining their transcriptional make-up have enabled a more accurate description of this T cell population. However, the developmental biology of TR1 cells has long remained elusive, in particular the identity of the cell type(s) giving rise to *bona fide* TR1 cells *in vivo*. Here, we review the fundamental phenotypic, transcriptional and functional properties of this T cell subset, and summarize recent lines of evidence shedding light into its ontogeny.

KEYWORDS

autoimmunity, T-regulatory (Treg) cells, T-regulatory type 1 (TR1) cells, peptide-MHC, nanomedicine, T-follicular helper cells (Tfh)

Introduction

The TR1 cell subset has been implicated in the maintenance of peripheral tolerance against immune-mediated pathologies. TR1-like cells were first documented in severe combined immunodeficiency (SCID) patients that did not develop graft-versus-host disease (GvHD) after receiving HLA-mismatched fetal liver hematopoietic stem cell transplants (1). Subsequent work by Groux et al. using antigen-activated $CD4^+$ T cells cultured in the presence of IL-10 led to the identification of a distinct T cell subset, thereafter named TR1, that could prevent the development of experimental colitis in an IL-10- and transforming growth factor beta (TGFβ)-dependent manner (2).

Unfortunately, the paucity of information on TR1 cell-specific surface markers or transcription factors have hampered the execution of detailed studies on the role and function of this T cell subset in the maintenance or breakdown of self tolerance. The use of relatively non-specific markers of TR1 cell identity, leading to the implication of this subset in various immunological processes (i.e., sometimes relying exclusively on IL-10 expression), has muddied progress in this area. Fortunately, the last decade has witnessed the discovery of phenotypic and molecular features of 'TR1-ness' that have allowed a better definition of TR1-like cells in various experimental settings. These developments, coupled to recent methodological developments in *in vitro* TR1 cell generation (3–7), and the discovery of pharmacological approaches capable of eliciting the formation and expansion of antigen-specific TR1 cells *in vivo* (8), have exposed TR1 cells as attractive targets for therapeutic intervention in immune-mediated diseases.

Despite this progress, significant knowledge gaps remain, including a detailed understanding of the developmental biology processes responsible for the genesis of this T cell subset *in vivo*. The use of novel technologies, including mass cytometry and next-generation-sequencing to address these various gaps are beginning to shed light into these areas of scientific inquiry. In this review, we summarize current knowledge on the phenotypic and molecular hallmarks of TR1 cells and key developmental processes underlying TR1 cell genesis, including recent evidence pointing towards T follicular helper (Tfh) cells as TR1 cell precursors (9, 10).

Phenotype

TR1 cells were initially described as $CD4^+$ T cells producing high levels of IL-10 and IL-5, intermediate levels of TGFβ and INFγ and low levels of IL-4 and IL-2, and were capable of suppressing specific immune responses *in vitro* and *in vivo*, in an IL-10-dependent manner (2). With rapid IL-10 production kinetics, detectable even 4 hours post-activation, and a peak of production at 24h (11), IL-10 became the hallmark cytokine for the TR1 population and, together with the absence of FoxP3 expression, used to identify TR1 cells in early studies. We now know that these criteria are insufficient, given that other $CD4^+$ T cell types such as Th1 (12, 13) or Th2 (14) can produce IL-10 and acquire immunoregulatory properties; such cells do not belong to the TR1 subset. For example, Lönnberg et al. claimed a Th1 origin for TR1 cells in a chronic *Plasmodium* infection model, solely on the basis of presence of IL-10-expressing cells within the infection-induced Th1 pool, and on the assumption that TR1 cells are simply IL-10/IFNγ-co-expressing cells (15). In fact, further transcriptomic analyses of the $IL\text{-}10^+$ and $IL\text{-}10^-$ Th1 cells of these mice revealed the presence of only two differentially expressed genes between these subsets (*Trib2* and *BC017643*). In another study, also in a chronic *Plasmodium* infection model, Soon et al. reported a similar outcome; 34% of Th1 lineage cells co-expressed *Ifng* and *Il10* (16). It is thus likely that, based on the evidence provided, the $IL\text{-}10^+$ cells that arose in these mice were IL-10-expressing Th1 cells, rather than true TR1 cells. This indicates that the assignment of TR1ness cannot merely rely on IL-10 expression.

In light of these challenges, hampering progress in defining the significance of the TR1 cell subset in both physiology and pathology, extensive efforts were made to better describe the molecular hallmarks of the TR1 subset [Reviewed in (17)]. Notwithstanding the fact that markers strictly unique to TR1 cells remain elusive, recent advances have made it possible to more accurately identify such cells in biological samples.

Gagliani and colleagues identified co-expression of CD49b and Lymphocyte-activation gene 3 (LAG-3) as surface markers for both human and murine IL-10-producing TR1-like cell populations (18). Subsequent studies indicated that a significant fraction of these IL-10-producing $CD49b^+LAG\text{-}3^+$ T cells are co-inhibitory receptor-rich, expressing Programmed cell death-1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T-cell immunoglobulin and mucin-domain containing protein-3 (TIM-3), and Cytotoxic T-Lymphocyte antigen 4 (CTLA-4), and co-express the co-stimulatory molecule ICOS (Inducible Costimulator) and the chemokine receptor CCR5, among other molecules (7). In agreement with these observations, intestinal TR1-like cells expressing PD-1 and CCR5 were found to co-express CD49b and LAG-3 by others (19), thus supporting the use of such markers for TR1-like cell identification. Thus, as proposed elsewhere (20), TR1 cell annotation should meet the following four criteria: 1) high IL-10 production competency (co-expression of other cytokines in variable amounts depending of environmental cues is possible); 2) immunoregulatory activity; 3) absence of constitutive FoxP3 expression (expression of FoxP3 upon activation, particularly in human TR1 cells, is not an exclusion criterium); and 4) co-expression of CD49b and LAG-3 in the presence of other co-inhibitory receptors such as PD-1, TIGIT, TIM-3 or CTLA-4 among others.

Indeed, the profoundly immunoregulatory antigen-specific TR1-like cells that arise *in vivo* in response to systemic delivery of nanoparticles (NPs) coated with mono-specific disease-relevant peptide-Major Histocompatibility Complex class II (pMHCII) molecules (8, 21–23) lack FoxP3 expression and upregulate many of the markers mentioned above, including CD49b and the co-inhibitory receptors LAG-3, PD-1, TIGIT and CTLA-4, the co-stimulator ICOS, the cytokines IL-10, IL-21 and IFNγ and the chemokine receptors CCR5 and CXCR3 (9). We have shown that administration of these compounds can lead to the resolution of inflammation in various organ-specific autoimmune disease models in a disease-specific manner without impairing normal immune responses (8, 21, 22). Adoptive transfer experiments demonstrated that the cognate ($pMHCII\ tetramer^+$) T cells arising in these mice in response to therapy were largely, albeit not exclusively, responsible for the therapeutic properties of these compounds (TR1 cell-induced B-regulatory cells also contributed to disease suppression) (8, 22).

Mechanisms of action

TR1 cells need to be activated to immunoregulate. Upon recognition of their cognate pMHCII complexes on co-stimulation-competent APCs, TR1 cells become productively

activated. By actively inhibiting the antigen-presentation and pro-inflammatory properties of these APCs (in addition to direct effects on other target cell types, see below), TR1 cells can suppress both cognate and non-cognate effector T cell activation (a process referred to as 'bystander immunoregulation'). This biological activity involves the deployment of several mechanisms (Figure 1).

Production of immunoregulatory cytokines

As noted above, productive activation of TR1 cells leads to rapid and robust production of IL-10, which can suppress the function of different immune cell subsets, such as T cells, APCs and B cells. IL-10 can inhibit the proliferation of, and downregulate the production of effector cytokines by, effector T cells (24), and can induce an anergic state in T cells in a STAT3-dependent manner (25). Likewise, IL-10 can inhibit the production of pro-inflammatory mediators by professional APCs, and downregulate the expression of MHC class II molecules and co-stimulatory molecules on their surface (26). It can also promote the upregulation of the immunoglobulin-like transcripts 3 and 4 (ITL3 and 4) and HLA-G, which have been implicated in the generation of tolerogenic dendritic cells (DCs) (27). On B cells, IL-10 promotes proliferation, expression of MHC class II molecules and isotype switching to IgG4 (28).

TGFβ has also been implicated in TR1-mediated immunoregulation. This cytokine suppresses T cell proliferation via various mechanisms, such as by inducing the downregulation of cyclins and IL-2 and the upregulation of cyclin-dependent-kinases (CDKs) (29–31). TGF-β can also suppress the formation of effector CD4+ or CD8+ T cells by inhibiting the expression of the master Th1 and Th2 cell transcriptional regulators (T-bet and GATA-3, respectively) (32, 33) or the IL-12Rβ2 chain (34).

The contribution and importance of both cytokines, IL-10 and TGFβ, to the immunosuppressive activity of TR1 cells is exemplified by the fact that, blockade of these cytokines inhibits TR1 cell-mediated immunoregulation in various experimental settings, including pMHCII-NP-treated animals (2, 8).

An additional cytokine that has been implicated in TR1-mediated immunoregulation is IL-21. Whereas IL-10 is directly responsible for most of the regulatory properties of pMHCII-NP-induced TR1 cells, IL-21 contributes to sustaining IL-10 expression in TR1 cells and is directly responsible for TR1-induced Breg cell formation (8).

Engagement of co-inhibitory and co-stimulatory molecules

Engagement of the TR1 cells' co-inhibitory (i.e., LAG-3, CTLA-4, TIGIT or PD-1) and co-stimulatory receptors (i.e., ICOS) by the corresponding ligands on target cells, such as APCs, is also thought to play a role in their immunoregulatory activity. Indeed, all these molecules are upregulated on the TR1-like cells induced by

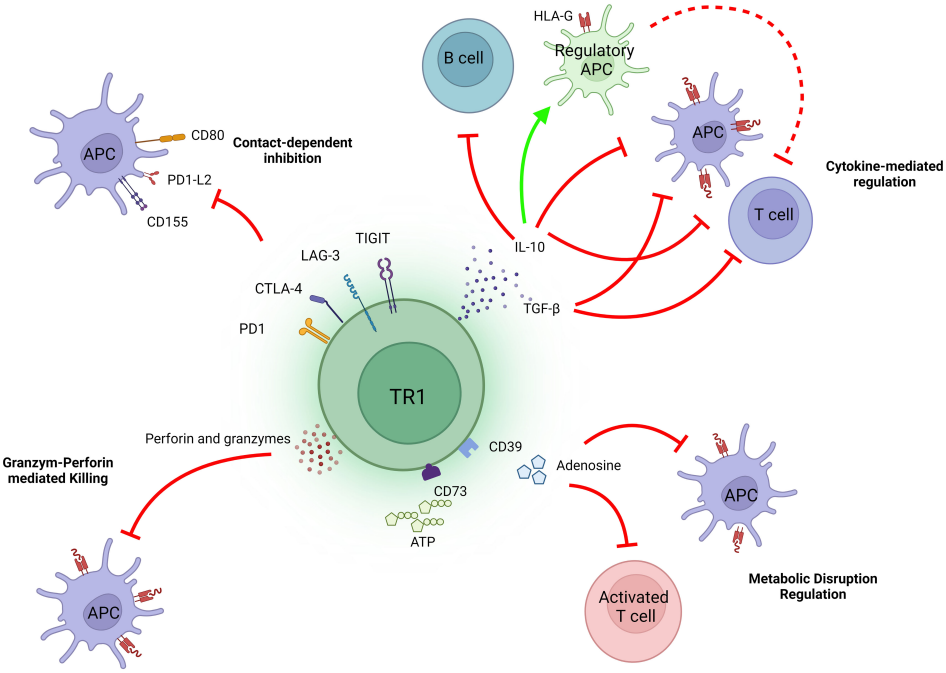

FIGURE 1
Mechanisms of action of TR1 cells. The immunosuppressive activity of TR1 cells is largely, albeit not exclusively, mediated through the release of IL-10 and TGFβ. Both cytokines can directly inhibit effector T cells and APCs, the latter also having a large indirect impact on effector T cell function. Additionally, IL-10 can imprint regulatory properties in APCs, such as by promoting the upregulation of tolerogenic molecules like HLA-G. The co-inibitory molecules expressed on TR1 cells, including CTLA-4, PD-1, TIGIT and LAG-3, can also result in contact-dependent inhibition of APCs and consequently, inhibit APC-induced T cell activation. Upon recognition of cognate pMHCII complexes on APCs, TR1 cells can also kill these cells via perforin and granzymes. In addition, these TR1 cells can inhibit T cell function by producing adenosine.

pMHCII-NP therapy (9). Whereas engagement of co-inhibitory receptor ligands on APCs by the TR1 cells' co-inhibitory receptors may contribute to the suppression of the APC's function, engagement of co-stimulatory receptor ligands (along with cognate pMHCII) elicits the productive activation of the TR1 cells, leading to secretion of the TR1 cells' immunoregulatory cytokines. In turn, these molecules have immunoregulatory effects on APCs and other downstream cellular targets.

LAG-3 negatively regulates T cell activity. Structurally similar to the CD4 co-receptor, LAG-3 recognizes MHCII molecules with higher affinity than CD4 (35). Recent evidence has shown that engagement of LAG-3 by stable pMHCII complexes transduces intracellular inhibitory signals to the T cell, without interfering with the recognition of these complexes by the T cells' TCR or CD4 molecules (36). Although such a mechanism helps understand how LAG-3 upregulation by an effector T cell might suppress its activation, a T cell-intrinsic inhibitory role for LAG-3 on regulatory T cell activity/function (37) seems counter-intuitive, as it would suppress the Treg cell, suggesting the existence of alternative mechanisms. One possibility is that the interaction between LAG-3 on Treg cells and pMHCII on APCs exclusively results in suppression of the latter, perhaps by failing to transduce intracellular inhibitory signaling into the former. The finding that such interaction results in the inhibition of dendritic cell (DC) activation (38), supports this possibility.

CTLA-4, a member of the CD28 family, binds to the co-stimulatory ligands CD80/86 with higher affinity than CD28, inhibiting the activation of the latter. In addition, the CTLA-4–CD80/86 interaction promotes the dephosphorylation of CD3 and CD28 signalling intermediates through the Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2), promoting T cell inhibition (39). Although CTLA-4 is dispensable for peripheral Treg cell expansion, it is necessary for immunoregulatory activity (40). There is also evidence indicating that CTLA-4 (along with PD-1) plays an active role in the regulatory activity of TR1 cells (41). It therefore seems likely that the role of CTLA-4 expression on TR1/Treg cells is different than that of CTLA-4 upregulation by effector T cells, although this remains to be determined (42).

The PD-1 receptor binds PD-L1 or PD-L2, expressed predominantly on APCs. Upon interaction with PD-L2, PD-1 on effector T cells recruits SHP-1 and SHP-2 phosphatases, which in turn reduce T cell activation and induce Treg cell differentiation (43). On DCs, the PD1–PD-L2 interaction inhibits the expression of molecules associated with DC maturation such as CD80, CD86 or CD40 and induces IL-10 expression, thus promoting the induction of an immunosuppressive DC phenotype (44).

Although the intracellular domain of TIGIT contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) capable of recruiting SHIP-1 and thus suppress T-cell (and NK cell) activation (45), binding of TIGIT to CD155 or CD112 on APCs (with high and low affinity, respectively), inhibits the engagement of the CD226 co-stimulator on T cells (46). In addition, this interaction induces a tolerogenic phenotype in DCs, by promoting IL-10 and suppressing IL-12 production (47).

Binding of the co-stimulator ICOS on TR1 cells to its ligand, ICOS-L, on B cells, DCs or macrophages (in the context of a cognate TCR-pMHCII interaction) promotes TR1 cell activation, leading to secretion of regulatory cytokines such IL-10 (48–50).

Extracellular generation of adenosine

TR1 cells, including those arising in response to pMHCII-NP therapy (9) express ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73), which hydrolyze extracellular adenosine triphosphate (ATP) released during T cell activation (51). This leads to the generation of adenosine (52, 53), which binds to the G protein-coupled adenosine receptor A2 ($A_{2A}R$). This interaction elicits a signalling cascade that suppresses effector T cell proliferation and cytokine production (54). On APCs, binding of adenosine to $A_{2A}R$ promotes IL-10 expression and inhibits both their maturation and their ability to secrete pro-inflammatory cytokines (55).

Granzyme and perforin-mediated killing

Another mechanism that TR1 cells may use to regulate T cell activity involves the killing of cognate APCs (i.e., expressing the TR1 cells' target pMHCII) via granzyme A and B and perforin (56, 57). By killing APCs, TR1 cells can thus suppres the activation of other T cell specificities and promote bystander immunoregulation. However, our work in mice treated with autoimmune disease-relevant pMHCII-NPs suggest that this mechanism is not always at play. For example, the antigen-specific TR1 cells emerging in these mice upon pMHCII-NP therapy did not significantly upregulate perforin and did not kill antigen-expressing or peptide-pulsed APCs (B cells or DCs) *in vivo* (8), unlike the case for the regulatory CD8+ T cells arising in pMHCI-NP-treated animals (58).

Molecular and transcriptional regulation of TR1 cell specification

Extensive efforts over the last two decades have sought to define the molecular and transcriptional mechanisms orchestrating TR1 formation. Unlike the case for the FoxP3+ Treg cell subset, where expression of FoxP3 is central to the acquisition of its immunoregulatory properties, there is no known unique master transcriptional regulator of TR1 cell development. Notwithstanding this limitation, experimental evidence has implicated a number of cytokines, kinases and transcription factors in the generation of TR1 cells *in vitro*. Although TCR engagement in the presence of IL-10 appears to play a major role, other signals are also required.

There is evidence suggesting that superantigens (59, 60) and pMHCII multimers can induce the expression of IL-10 in CD4+ T cells (61–63). It has also been shown that high-avidity TCR–pMHCII interactions favour the production of IL-10 by T cells (64), affecting both the number of cells expressing IL-10 and the immunoregulatory properties of such cells (65). Molecularly, TCR activation leads to the engagement of intracellular signalling

pathways that eventually activate the interferon regulatory factor 4 (IRF4) transcription factor via Ras or the inducible tyrosine kinase (ITK) kinases. IRF4 has been shown to promote *Il10* gene expression in different CD4+ T cell types, including Th2, Th1 (66), Tfh cells (67) and Tregs (68). Indeed, it has been reported that IRF4 contributes to the development of an IL-10-producing CD4+ T cell that co-expresses LAG-3 and CD49b (69). As noted below, IRF4 is absolutely required for pMHCII-NP-induced TR1 cell formation, albeit through a different mechanism (i.e., it is dissociated from its *Il10* transactivating function) (9). The transcription factor Eomes, which can promote *Il10* expression in T-bet-expressing cells (70), and the Th17 transcription factor Rorα, which can transactivate the *Il10* gene (71), might also be implicated in TR1 formation. However, pMHCII-NP-induced TR1 cells do not upregulate Eomes or Rorα, suggesting that neither of these transcription factors are required for TR1 cell specification.

Although productive TCR ligation is required for TR1 activation, TR1 cell genesis requires additional cues. Early studies by Groux and colleagues using both human and murine CD4+ T cells cultured in presence of IL-10 indicated that these culture conditions promoted the development of an anergic T cell population that included TR1-like cells (2), highlighting a prominent role for this cytokine in TR1 cell generation, at least *in vitro*. It was subsequently proposed that the IL-10 that contributes to TR1 cell generation *in vivo* derives from a tolerogenic DC population (72, 73). Indeed, a human DC population expressing high amounts of IL-10 has been identified (27). This DC population, named DC-10, can induce TR1 cells *ex vivo* with increased efficacy, as compared to other experimental approaches, and such cells have been used to generate and expand TR1 cells for use in clinical trials [Reviewed in (5)].

IL-27 has also been implicated in the generation of murine TR1 cells. IL-27 is an IL-12 family cytokine that is produced by activated APCs (74) and can induce IL-10 expression in murine T cells (75–77), especially in the presence of TGFβ, with which it synergizes (78). Binding of IL-27 to the IL-27 receptor (IL-27R) activates the STAT1 and STAT3 signalling pathways and promotes the expression of the transcription factors c-Maf and AhR, which cooperatively promote *Il10* and *Il21* expression (78, 79). STAT3-induced *Il10* expression also involves the upregulation of *Egr2* (encoding the Early Growth Response 2 transcription factor (EGR-2)) and EGR-2's downstream target *Prdm1* (encoding the zinc finger-containing transcription factor Blimp-1) (80). Although Blimp-1 has been primarily implicated in plasma cell differentiation (81), it has also been shown to regulate *Il10* gene expression in T cells (82, 83). However, and notwithstanding the fact that the generation of terminally differentiated TR1 cells in response to pMHCII-NPs requires Blimp-1, this role is dissociated from Blimp-1's *Il10* transactivating function (9) (see below). In fact, there is evidence suggesting that IL-27-induced TR1 cell formation does not require IL-27-induced IL-10 (84) and that IL-27 contributes to TR1 cell formation by inducing changes in chromatin accessibility via IRF1 and BAFT (85). Although IL-27 can induce the formation of IL-10-expressing T cells from naïve human CD4+ precursors (86), it remains to be determined whether these cells are *bona fide* TR1 cells.

Despite all these observations, largely if not exclusively generated *in vitro*, our *in vivo* work has demonstrated that IL-27 is not required for pMHCII-NP-induction of TR1-like cells (8). We have proposed that IL-27 and pMHCII-NPs lie upstream and downstream of the TR1 precursors (Tfh cells, see further below); whereas IL-27 would elicit both Tfh and TR1 cell formation from naïve precursors, pMHCII-NPs would just be able to promote the conversion of Tfh cells into TR1-like cells (9).

IL-21 is another cytokine that has been implicated in TR1 cell genesis. This cytokine, produced by antigen-stimulated CD4+ T cells and NKT cells, signals via the IL-21R, composed of the IL-21Rα chain and the common receptor γ_c chain (87), leading to activation of the STAT3 signalling pathway. The transcription factor c-Maf, upregulated by IL-27 among other stimuli, promotes IL-21 expression in TR1 cells (79, 88). In turn, IL-21 promotes the expression of *Il10* and *cMaf* expression in an autocrine manner (79, 89).

In addition to the molecules discussed above, other cytokines and transcription factors have been reported to contribute to IL-10 production by T cells and, consequently, may play a role in TR1 cell specification. For instance, IL-6, which signals through STAT1 and STAT3, can upregulate the transcription factors c-Maf, IRF4 or AhR (90), which are known to participate in *Il21 and/or l10* expression in different T cell types (66–69, 78, 79). In fact, IL-6, together with TGFβ, can induce IL-10 production in Th17 cells (91, 92). Type-1 Interferons have also been reported to promote IL-10 expression in CD4+ T cells (93–95) or TR1 cell development in anti-CD3 mAb/IL-10-treated mice (96).

The co-stimulator ICOS may also play an important role in TR1 cell specification, homeostasis or function, perhaps by promoting *cMaf* and *Il10/Il21* expression (88, 97).

In summary, research to date has provided valuable information regarding the transcriptional control of *Il10*, encoding the hallmark TR1 cytokine, but has not yet been able to define the key transcription factors, co-stimulators and cytokines that control TR1 cell development from their precursors. As summarized below, the recent identification of Tfh cells as precursors of TR1 cells *in vivo*, coupled with definition of the transcriptional changes that underlie this transdifferentiation process, offer a unique opportunity to carefully map the molecular events responsible for TR1 cell formation.

Challenges hampering studies on the developmental biology of TR1 cells

Given the challenges associated with the lack of TR1 cell-specific markers and our inability to reliably identify this T cell subset *in vivo* until recently, it is unclear whether the TR1-like cells that have been described to arise *in vitro* and/or *in vivo* in response to various cues do so from a single or various precursors [reviewed in (98, 99)].

As noted above, both human and mouse T cells can be differentiated into TR1-like cells *in vitro*. *In vitro*-activated naïve T cells from either species can give rise to anergized IL-10-

producing CD4+ T cells when cultured in the presence of exogenous IL-10 (2), DC-10 cells (100) or IL-27 (79, 101).

Several other lines of evidence have suggested that TR1 cells arise from memory T cell precursors. Repetitive administration of anti-CD3 monoclonal antibodies (mAb) to mice can induce TR1-like cell formation *in vivo* (102). *In vitro* stimulation of memory-like $CD4^{+}CD44^{high}FoxP3^{-}$ T cells in the absence of polarizing cytokines can also elicit TR1-like cell specification (103). Likewise, extracellular matrix components have been reported to guide the formation of TR1-like cells from human memory $CD4^{+}$ T cells *in vitro* (104), and others have shown that the precursors of TR1 cells are contained within the memory $CD4^{+}$ T cell pool, in both humans and mice (105).

Th1 and Th2 cells have also been proposed as a source of TR1-like cells. *In vitro*, Th1 cells can be induced to express IL-10 when stimulated in the presence of CXCL12 (106), but such cells might have just been IL-10-producing Th1 cells rather than full-fledged TR1 cells. As noted earlier in this review, it has been suggested that chronic infection of mice with *Plasmodium* can trigger the differentiation of Th1 cells into TR1-like cells (15, 16), but the reported TR1-like cells did not appear to be *bona fide* TR1 cells. It has also been suggested that allergen-specific Th2 cells can be re-programmed into a TR1-like phenotype *in vitro* (107).

Intestinal Th17 cells can also give rise to anti-colitogenic TR1-like cells in response to anti-CD3 mAb treatment (108). Furthermore, IL-27 and IL-23 promote the up-regulation of Blimp-1 and can elicit the expression of a TR1-like phenotype in Th17 cells (109).

Collectively, the above observations suggest that TR1-like cells might arise from various T cell precursor types, but many of these studies did not use stringent criteria for definition of the TR1 cell state, or did not involve detailed transcriptional studies of the T cell pools used for experimentation or of their progeny; and when such studies were done/reported, the resulting T cell pools were transcriptionally heterogeneous. As a result, it is not possible to unambiguously assign or exclude a specific cell type as a TR1 cell precursor on the basis of these studies.

Tfh cells as a source of TR1 cells

We have taken advantage of the large pools of antigen-specific TR1 cells that arise *in vivo* in various animal models of autoimmunity upon systemic delivery of nanoparticles (NPs) coated with disease-relevant pMHCII molecules (8, 21–23), to carefully map the transcriptional events leading to TR1 cell formation *in vivo* (9, 10) (Figure 2). Early work established that pMHCII-NPs functioned by re-programming cognate antigen-experienced (i.e. memory) $CD4^{+}$ T-cells of unknown identity (excluding a role for naïve T-cells) (8, 23).

The robust and prolongued TCR signaling events that result from sustained assembly of TCR microclusters by pMHCII-NPs on autoantigen-experienced T cells leads to the expression of known TR1-like cell markers, including IL-10, IL-21, c-Maf, LAG-3, CD49b, CTLA-4, PD-1, TIGIT, CCR5, CXCR3, ICOS and OX-40, among others, in a manner that does not require APCs or IL-27 (9).

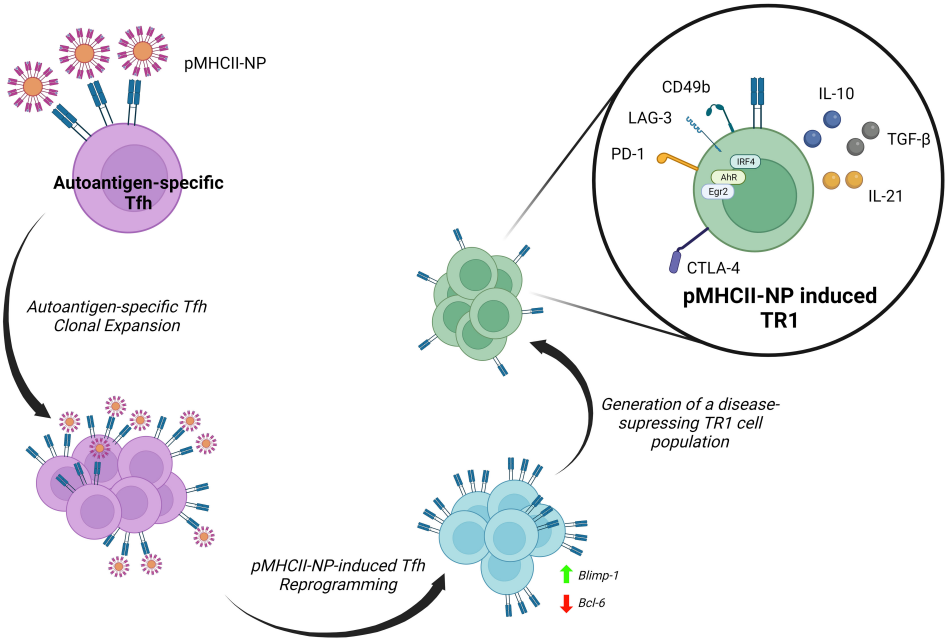

FIGURE 2
Reprogramming of Tfh cells into TR1 progeny by pMHCII-NPs. Upon binding to cognate TCRs, autoimmune disease relevant pMHCII-NPs selectively trigger the clonal expansion of Tfh cells and their immediate transdifferentiation into transitional TR1-like cells and terminally differentiated TR1 cells. pMHCII-NP encounters trigger the downregulation of master transcriptional regulators of Tfh cells, such as Bcl-6, and the upregulation of Blimp-1. Blimp-1 expression is a *sine-qua-non* requirement for the conversion of transitional TR1-like cells into the terminally differentiated, immunoregulatory TR1 cell subset (9).

In addition to *Maf*, these TR1 cells upregulate the transcription factor coding genes *Ahr, Egr2, Irf4, Nfil3, Prdm1 and Tbx21* (9), all involved in IL-10 expression (79, 110). In addition, these TR1 cells upregulate three other transcription factors that have been previously implicated in the development, maintenance or function of IL-10-expressing Treg cells (*Bhlhe40*, *Runx2* and *Vdr*) (9). Whereas the IL-10 produced by these antigen-specific TR1 cells is the direct mediator of some of their immunoregulatory properties, IL-21 contributes to the homeostatic regulation of this T-cell subset and plays a critical role in TR1-induced Breg cell formation (8). This ability of pMHCII-NPs to elicit the formation of large pools of antigen-specific TR1-like cells afforded us a unique opportunity to explore their developmental biology. This work has demonstrated that pMHCII-NP-induced TR1 cells derive from cognate Tfh cells and do so in a Blimp-1-dependent manner (9, 10) (Figure 2).

Initial work indicated that the cognate TR1-like cell pools arising in response to pMHCII-NPs expressed a transcriptional program that shared significant features with Tfh cells, raising the possibility that the latter might function as a source of the former. Subsequent single cell RNA sequencing (scRNAseq) and mass cytometry studies demonstrated that these antigen-specific TR1-like cell pools harboured a cognate Tfh-like cell subcluster, in addition to its TR1-like cell countepart. Importantly, studies of the TCR repertoires of these two cell sub-clusters indicated that they consistently harbored identical clonotypes, thus demonstrating that they were developmentally related (9, 10). This was substantiated with the use of different pMHCII-NP types in different genetic backgrounds and models of autoimmunity (9, 10).

This was further documented by demonstrating that pMHCII-NPs could elicit cognate TR1 cell formation in immunocompromised hosts transfused with purified Tfh cells, and that these compounds lacked pharmacodynamic activity in mice unable to generate Tfh cells (9). Most importantly, T cell-specific deletion of *Prdm1* (encoding Blimp-1) revealed that the Tfh-to-TR1 cell conversion evolves through a transitional (TR1-like) subset, and that expression of this transcription factor in these transitional T cells is a *sine qua non* requirement for full-fledged acquisition of the TR1 transcriptional profile and regulatory function (9). Thus, while specific deletion of *Bcl6* or *Irf4* in T-cells blunted pMHCII-NP-induced cognate CD4+ T-cell expansion and downstream TR1 generation, deletion of *Prdm1* enabled the former but completely abrogated the latter (9).

It could be argued that pMHCII-NP-induced TR1 cells are T-follicular regulatory (TFR) cells, which negatively regulate the germinal center (GC) reaction (111). However, unlike TR1 cells, TFR cells express CXCR5 (but not CCR5), Bcl-6, FoxP3 and CD25, and arise from natural FoxP3+ Treg cell precursors in a Blimp-1-independent manner (9).

Together, the data summarized above conclusively demonstrate that murine TR1 cells can arise from Tfh cells in a Blimp-1-dependent manner. Interestingly, a pool of thymus-derived self-reactive CD4+ T cells that adopt numerous hallmarks of Tfh cell identity in the periphery has been recently discovered (112). This finding raises the possibility that these cells might function as a source of a negative feedback regulatory loop (i.e., formation of autoreactive TR1 cells) to suppress autoimmunity.

Concluding statement

Since the discovery of TR1-like cells more than 25 years ago, the last decade has witnessed steady improvements in our ability to identify and phenotype this previously enigmatic CD4+ T cell subset. While knowledge gaps persist, we have gained detailed new insights into these cells' transcriptional make-up, mechanisms of action and lineage origin. Further research into the different topics reviewed in this article, as well as other aspects of the TR1 cell biology will undoubtedly help in the translational application of TR1 cells as a therapeutic approach for immune-mediated diseases.

Author contributions

PS: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review and editing. EA: Investigation, Writing – original draft.

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Conflict of interest

PS is founder, scientific officer and stock holder of Parvus Therapeutics. He is inventor on patents on pMHC-based nanomedicines and receives funding from the company.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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