REGULATORY T CELLS

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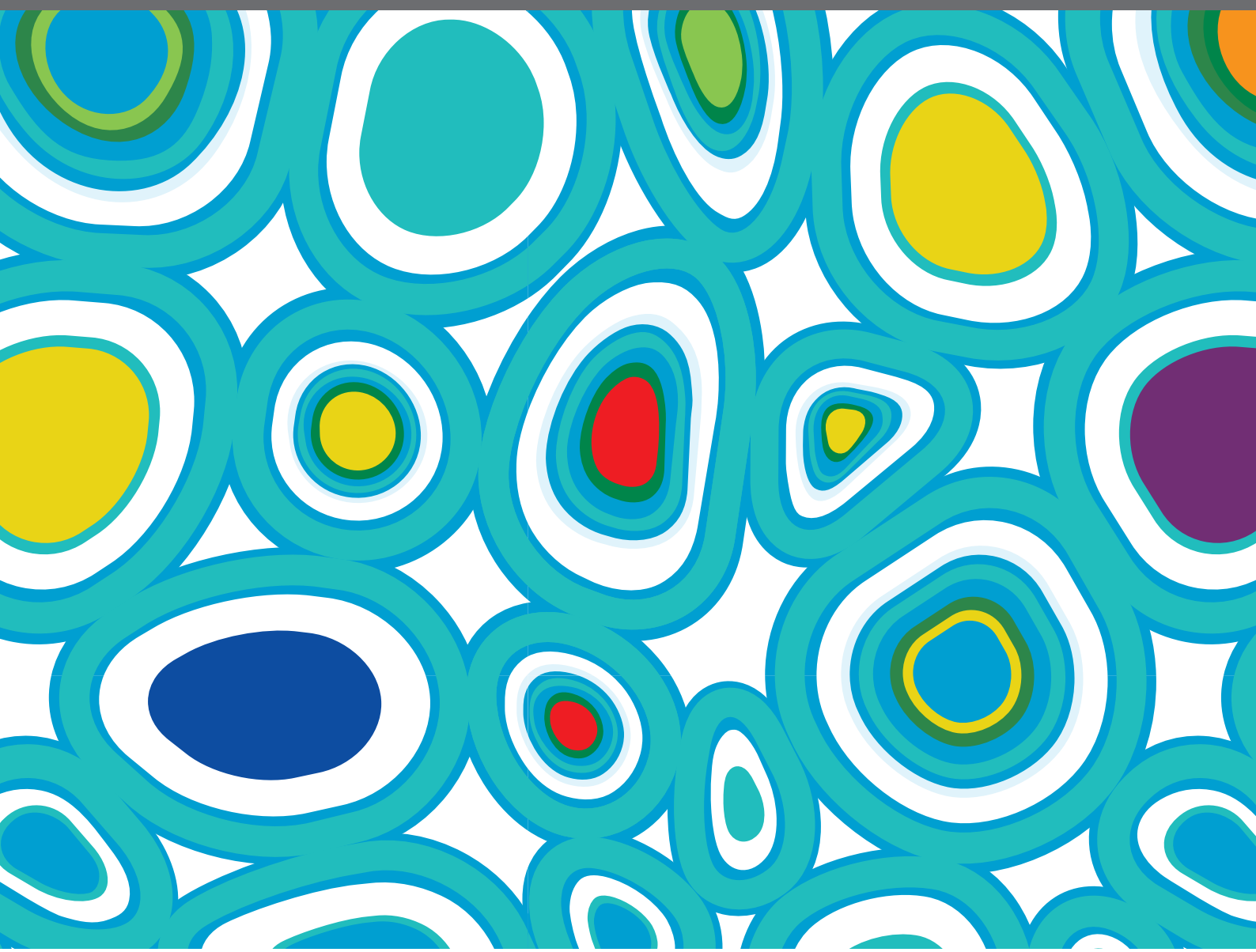

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REGULATORY T CELLS

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EDITORIAL
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Editorial: Regulatory T Cells

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Keywords: regulatory T cells, transplantation, immunotherapy, cell biology, immune balance

Editorial on the Research Topic

Regulatory T Cells

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that maintain tolerance to self-antigens, prevent autoimmune disease, and limit anti-tumor immunity. Over recent years, Tregs have demonstrated great potential as either a target or immunotherapy in a variety of diseases, such as autoimmunity, cancer, transplantation and graft-versus-host disease (GVHD). In clinical trials, Tregs have been infused as an adoptive cellular therapy in diabetes, GVHD and organ transplantation. For example, in Nanjing Medical University in China, Dr. Ling Lu and his team have infused exvivo expanded nTregs to liver transplant recipients in combination with a reduction in immunosuppression to promote immune tolerance. As of writing, 5 patients out of 17 have ceased immunosuppression with the majority of the remaining patients receiving a reduced dose. Some other trials are also ongoing, both in GVHD and transplantation, including the Phase IIb TWO Study in Oxford. However, some challenges must be overcome before the implementation and widespread use of Treg therapy. This Research Topic describes the latest advances in the basic science and clinical application of Tregs, including insights into the use of Tregs for the treatment of tumors and autoimmune diseases, and new strategies for Treg therapy in organ transplantation.

The phenotypic and functional diversity of Tregs has important implications for their biology and function. Tregs can mirror conventional T cell subsets, and include naïve, effector, and memory subpopulations. Here, Sjaastad et al. from the University of Minnesota focus on the ontogeny, phenotype and function of different effector Treg subsets, which is important for the future development of tools for specific subgroups and their functions.

Tissue Tregs, also known as tissue-resident Tregs, do not recirculate in the blood or lymphatics and are adapted to the specific tissue environment. In this Research Topic Shao et al. review the phenotype, function, and cytokine expression of these Tissue Tregs. In local tissues, Tregs can restrain immune responses, maintain tissue homeostasis, and promote tissue recovery. With the increasing number of chronic tissue inflammatory diseases and immune deficiency diseases, understanding the role that Tregs play in either the control or perpetuation of these pathologies is crucial.

Current studies suggest that Tregs may be a potential treatment for ischemia-reperfusion injury (IRI). Butyric acid (BA) is a product formed by intestinal microorganisms after decomposing indigestible food and is a potential negative immunomodulator. Chen et al. show that the heme oxygenase 1(HO-1)/STAT3 signaling pathway is related to the inhibitory effect of BA on the differentiation of Th17 cells. BA regulates the differentiation of Th17 cells into Tregs and reduces renal IRI. This provides a route for BA to inhibit inflammation by regulating the balance of Tregs to Th17 cells.

Human amniotic mesenchymal stem cells (hAMSCs) have the potential for multi-directional differentiation and the ability to promote immune regulation and tissue repair in a number of diseases. Heren, Deng et al. provide convincing evidence for the hAMSC and Treg combined activity. The authors find that Tregs improve the function of hAMSCs by regulating the TGF-β/IDO signaling

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pathway, increasing cytokine expression of hAMSCs, and enhancing the effect of liver cirrhosis treatment. This suggests that co-infusion of hAMSCs and Tregs may provide a promising method for the treatment of liver cirrhosis.

Tregs can impair anti-tumor responses and reduce the efficacy of immunotherapies. In the past few years, there has been interest in targeting Tregs to enhance anti-tumor responses. As TNFα plays a key role in phenotypic stabilization and inhibition of human and mouse Tregs, tumor necrosis factor receptor 2 (TNFR2) has recently been identified as a target for anti-cancer immune checkpoint therapy Moatti and Cohen have reviewed and analyzed the results of recent studies on TNFR2 function in Tregs. This signaling pathway may provide a new route for anti-tumor immunotherapy.

Altering the balance of effector and regulatory cells is likely to be the key to the control of alloimmune responses in transplantation. While the most focus has been on T cell control, B cell activity must also be controlled to ensure that late alloresponses and chronic allograft dysfunction are also targeted. Attention is therefore also turning to regulatory B cell subpopulations or cells that control B cells in the lymphatics. In the accompanying article, Chong et al. summarize new discoveries in T follicular regulatory cells, regulatory B cells, and alloreactive tolerogenic B cells and their roles in the control of allo-immunity and transplant rejection.

Non-human primates (NHP) are valuable for the translation of Tregs from basic research to clinical application. Thomson and Thomson et al. have contributed crucial data over the years to assist in the translation of Treg therapeutics to the clinic, with a particular focus on alloreactive Tregs. In this Research Topic, the authors explore the approaches used to produce NHP Tregs as well as the differences between polyclonal and alloantigen reactive Tregs. Important insights are provided in the methodology for Treg production and assessment in NHPs, as well as future perspectives on their therapeutic potential.

Adoptive transfer of *ex vivo* expanded Tregs is a key method under investigation for the treatment of immune-mediated diseases. Ou et al. describe the epigenetic changes in Tregs after repeated cell stimulation which may affect the survival and function of cells. The authors call for a careful examination of the molecular changes in the production of T-cell products, and their research also highlights the importance of epigenetic analysis in evaluating the quality of Treg cell products in the future.

Another major challenge for Treg therapy use in the clinical setting is the need for cryopreservation, which may impact Treg viability and function. Kaiser et al. examine the effects of different cryoprotectants on Tregs, finding that a medium containing 5% dimethyl sulfoxide enhances cell viability, recovery, and function after thawing. This is important data for Treg clinical translation.

Overall, the studies and overviews in this Research Topic highlight key points of progress in our understanding of Tregs and their therapeutic application. Progress has been across a broad front across all aspects of Treg biology, together with building crucial foundations for the deployment of Tregs to the clinic. Ongoing clinical trials are likely to report their results in the near future, and these results are highly anticipated.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the editorial work of this Research Topic and to this Editorial and approved it for publication.

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MINI REVIEW
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Non-human Primate Regulatory T Cells and Their Assessment as Cellular Therapeutics in Preclinical Transplantation Models

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Non-human primates (NHP) are an important resource for addressing key issues regarding the immunobiology of regulatory T cells (Treg), their *in vivo* manipulation and the translation of adoptive Treg therapy to clinical application. In addition to their phenotypic and functional characterization, particularly in cynomolgus and rhesus macaques, NHP Treg have been isolated and expanded successfully *ex vivo*. Their numbers can be enhanced *in vivo* by administration of IL-2 and other cytokines. Both polyclonal and donor antigen (Ag) alloreactive NHP Treg have been expanded *ex vivo* and their potential to improve long-term outcomes in organ transplantation assessed following their adoptive transfer in combination with various cytoreductive, immunosuppressive and "Treg permissive" agents. In addition, important insights have been gained into the *in vivo* fate/biodistribution, functional stability, replicative capacity and longevity of adoptively-transferred Treg in monkeys. We discuss current knowledge of NHP Treg immunobiology, methods for their *in vivo* expansion and functional validation, and results obtained testing their safety and efficacy in organ and pancreatic islet transplantation models. We compare and contrast results obtained in NHP and mice and also consider prospects for future, clinically relevant studies in NHP aimed at improved understanding of Treg biology, and innovative approaches to promote and evaluate their therapeutic potential.

Keywords: regulatory T cells, non-human primates, transplantation, cell therapy, immunobiology

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INTRODUCTION

NHP are a valuable resource for translational research on innovative therapies with potential for clinical application. These include strategies to promote transplant tolerance without the toxicities and morbidities associated with immunosuppressive (IS) drugs that fail to induce clinical tolerance (Knechtle et al., 2019). Mouse models of tolerance induction are inadequate predictors of tolerance in both NHP and humans (Sachs, 2003). This reflects the fact that unlike the latter species, specific pathogen-free (spf) laboratory mice do not acquire heterologous immunity, i.e., virus-induced T

Abbreviations: Ab, antibody; Ag, antigen; (a)APC, (artificial) antigen-presenting cell; ATG, anti-thymocyte globulin; Co-SB, co-stimulation blockade; Cy, cyclophosphamide; darTreg, donor antigen alloreactive regulatory T cells; IS, immunosuppression/immunosuppressive; NHP, non-human primate; PBMC, peripheral blood mononuclear cells; spf, specific pathogen free; Teff, T effector cell(s); TGFβ, transforming growth factor β; Tmem, memory T cell(s); Treg, regulatory T cell(s).

cell memory that constitutes a powerful barrier to tolerance induction (Adams et al., 2003). In addition, whereas MHC class II Ags that are key to induction of alloimmunity are absent from rodent vascular endothelium, they are expressed constitutively by these cells in large animals (Choo et al., 1997; Houser et al., 2004). Due to (i) similarities between NHP and human immune systems, (ii) cross-reactivity between drug targets in these species, (iii) the outbred nature of NHP, (iv) their natural exposure to environmental pathogens, and (v) their longevity compared with mice, NHP are important models for studying mechanisms underlying immune regulation and tolerance (Fitch et al., 2019). NHP also allow evaluation of the scalability and safety of innovative therapeutic strategies (e.g., monitoring and management of adverse events, such as potential infusion reactions and infectious complications) and associated techniques, e.g., leukapheresis in conjunction with cell therapy, that are not practiced in rodents.

NHP Treg phenotype and function resemble those of human Treg (Anderson et al., 2008; Haanstra et al., 2008). As in humans, testing of their efficacy is dependent on their isolation, massive expansion and product validation. The ability to assess the *in vivo* stability and migration of adoptively-transferred Treg is also an important translational aspect of studies of these cells in NHP and crucial for their assessment as cellular therapeutics.

ISOLATION, PURIFICATION, *EX VIVO* EXPANSION AND VALIDATION OF NHP TREG

Details of methods developed for isolation and purification of NHP Tregs from blood or tissues, and for expansion and testing of polyclonal, polyspecific or donor Ag-alloreactive (dar) NHP Treg are summarized in **Table 1**. Thus, *ex vivo* expansion of cynomolgus or rhesus Treg after immunomagnetic bead or/and flow sorting has been well-described in response to either polyclonal stimuli (Gansuvd et al., 2007; Anderson et al., 2008), allogeneic PBMC (Porter et al., 2007) or dendritic cells (DCs) (Moreau et al., 2008), artificial (a)APCs (Weiner et al., 2015; Zhang et al., 2015) or CD40L-stimulated allogeneic B cells (sBc) (Ezzelarab et al., 2020; Alonso-Guallart et al., 2021). Most recent attention has focused on expansion of alloreactive Tregs using various protocols. Thus, Alonso-Guallart et al. (2021) expanded highly suppressive cynomolgus polyspecific Tregs using a combination of CD40L−engineered B cells from 3 to 4 animals representative of diverse MHCs. The expanded Tregs expressed high levels of Foxp3 and Helios, a high incidence of Treg-specific demethylated region demethylation, and markedly suppressed naïve T cell responses *in vitro*. Importantly, these Tregs also expanded after cryopreservation, an important benefit allowing protocol flexibility. Specificity assays confirmed they were suppressive following activation by any APCs whose MHC expression was shared by the CD40L−sBc used during their expansion, proving that they were polyspecific (Alonso-Guallart et al., 2021). Recently, Ezzelarab et al. (2020) also used CD40L−sBc of donor origin to expand cynomolgus darTregs that were adoptively-transferred to transplant recipients.

Weiner et al. (2015) flow-sorted $CD4^+CD25^{hi}$ cells from baboon PBMC, followed by expansion with IL-2, anti-CD3 Ab, aAPCs (transfected with human CD58, CD32, and CD80), and rapamycin, with weekly restimulation. Expanded Treg were cryopreserved for 2 months, after which they were thawed and cultured for 48 h with the same stimulating agents. The purified Treg were expanded > 10,000-fold and exhibited excellent suppressive function. Cryopreservation reduced the cells' suppressive function without changing their phenotype, but increasing levels of reactivation post-thawing improved cell viability and function, with a shift toward greater Treg purity. Thus, both the phenotype and function of the Tregs were preserved or even enhanced by increasing levels of restimulation post-thawing, reinforcing the concept of banking expanded recipient Tregs.

MIGRATORY PROPERTIES

Treg migratory ability is considered key to their *in vivo* homing to lymphoid tissues and sites of inflammation considered critical locations of their immunosuppressive function. While Treg markers are expressed in either accepted or rejected NHP kidney allografts (Haanstra et al., 2007), Treg recruitment to the interstitium of the graft has been implicated in metastable kidney transplant tolerance in rhesus macaques (Torrealba et al., 2004). Numerous studies, including those in transplanted animals (Chen and Bromberg, 2006; Lamarche and Levings, 2018; Mempel and Marangoni, 2019) have shown that the expression of specific chemokine receptors, integrins and selectins is required for Treg activity *in vivo* (Qin et al., 2008; Zhang et al., 2009) and that maximal graft protection occurs when Tregs migrate to both the transplant and the draining lymphoid tissue. Thus, optimization of procedures for generation of highly suppressive NHP Treg needs to include evaluation of the expression of a range of chemokine receptors compatible with their *in vivo* migration to both the allograft and lymphoid tissues. Zhang et al. (2015) reported that *ex vivo*-expanded polyclonal cynomolgus Treg expressed high levels of CXCR3/low levels of CD62L and CCR7, consistent with their potential accumulation at sites of inflammation.

IN VIVO MOBILIZATION AND DEPLETION

The influence of cytokine and other biological agent administration on Treg in NHP has been investigated (**Table 2**). Thus, specific hematopoietic growth factors have been evaluated for their ability to selectively increase Treg *in vivo*, with potential to enhance their therapeutic efficacy, whereas Treg-selective depleting agents enable assessment of the dependency of tolerance-promoting protocols on these cells.

IL-2

Inoculation of low dose IL-2 (10^5–5×10^6 IU/m^2) expands $CD4^+Foxp3^+$ Tregs preferentially due to the cells' expression of the high affinity IL-2 receptor alpha chain (CD25)

TABLE 1 | Timeline of reports of Treg isolation, expansion and suppressive activity in NHP species.

Species	Treg selection (purity)	Cell yield	Expansion method	Expansion rate	Suppressive activity	Reference(s)
Rhesus macaque	T cells from spleen	200 × 10^6 cells per recipient (splenectomized)	Donor splenocytes + anti-CD80/CD86; 13 days	2–4-fold	*In vivo* infusion 13 days post allogeneic kidney transplant; donor-specific inhibition of rejection	Bashuda et al., 2005
Rhesus	MACS (90%) or FACS (98%) $CD4^+$ $CD25^+$	Not indicated	Anti-CD3/CD28 beads + IL-2; 4 weeks	300–2,000-fold	Up to 1:8[a] ratio, suppression of autologous PBMC proliferation	Gansuvd et al., 2007
Rhesus	Anti-CD8 and anti-CD20 Dynabeads, or $CD4^+$ MACS, followed by anti-CD25 MACS (82%)	10% of $CD3^+CD4^+$ T cells	Fresh cells used; no expansion	n/a	Proliferation of Teff in response anti-CD3 or irradiated PBMC decreased at 1:1[a] ratio, however variation between animals	Haanstra et al., 2008
Rhesus	FACS $CD4^+CD25^{hi}$ or $CD4^+CD25^+CD127^-$	10^5 cells/mL blood	Anti-CD3/CD28 beads + IL-2; 4 weeks	Up to 450-fold	CFSE-MLR, up to 1:100[a] ratio; suppression of alloreactive response by responder-specific or third-party Treg	Anderson et al., 2008
Rhesus	MACS $CD4^+CD127^{-/lo}$	7% of $CD4^+$/1.3% of total PBMC; 3.7 × 10^4 cells/ml blood	Immature Mo-DC^+ IL-2^+ IL-15; 14 days, or 10 days followed by 2 days without DC	No expansion	Suppression up to 1:40[a] ratio; donor-specific	Zahorchak et al., 2009
Rhesus	FACS $CD4^+$ CD25- $CD127^-$/lo cells (> 80%)	>or = 10^6	Stimulation with anti-CD3 and anti-CD28 microbeads (1 cell: 2 beads) + IL-2 (2,000 U/ml) ± rapamycin (1,000 nM); re-stimulation at 7 and 14 days; harvested on day 21	210–760-fold	Strong suppression of $CD4^+$ and $CD8^+$ T cell proliferation in CFSE-MLR	Singh K. et al., 2012
Cynomolgus macaque	FACS $CD4^+$ $CD25^+CD127^-$ (> 98%)	0.4% of PBMC	Allo DC (BM-DC/Mo-DC) + IL-2; 7 days	12–25-fold	CFSE-MLR, $PBMC^+$ anti-$CD3/CD28^+$ Treg: 30% inhibition at 1:3[a] ratio. Treg suppressive function stimulated by BM-DC > Mo-DC	Moreau et al., 2008
Cynomolgus (MS model)	MACS $CD4^+$ $CD25^+$ (>90%)	6.4% of T cells	Fresh cells used; no expansion	n/a	Proliferation to anti-CD3/CD28 or CD3/CD46 stimulation impaired during active MS	Ma et al., 2009
Cynomolgus	FACS $CD4^+CD25^{hi}CD127^-$ from PBMC	5% total $CD4^+$ cells	NHP-specific anti-CD2/3/28 microbeads (cell: bead ratio 1:2), rhu IL-2 (1,000 U/ml) and rhu TGF-b; 5 ng/mL); 20 day culture	>80-fold	Strong suppressive effect at ratios up to 1:4 (Treg:Teff) on $CD4^+$ and $CD8^+$ T cell proliferation	Dons et al., 2013
Cynomolgus	FACS $CD4^+CD25^+CD127^-$ (>95%)	4.7 (2–6.9)% of blood $CD4^+$ cells	Artificial (a)APCs (L32) loaded with anti-CD3 (1:1 ratio) for 7–8 days with IL-2 + rapamycin; restimulation with aAPCs on days 7 and 14 without rapamycin; harvested on day 21	1,000-fold	Potent suppression of anti-CD3/CD28-indiced CD4 and CD8 T cell proliferation	Guo et al., 2015; Zhang et al., 2015
Cynomolgus	FACS $CD4^+CD25^{hi}CD127^{lo}$	0.5–1% of total PBMC	Donor Ag alloreactive Tregs stimulated with donor CD40L-activated B cells + IL-2; harvest on day 9 and co-culture with aAPCs (L32 cells) + IL-2 for 2 subsequent rounds of 7 days	10,000-fold	Strong suppression (>polyclonal Tregs) of autologous T cell proliferation in MLR in response to donor APCs.	Ezzelarab et al., 2020
Cynomolgus	FACS $CD4 + CD8^-CD25^{hi}CD127^-$	Top 1% $CD25^+$ cells in the $CD4^+$ $CD8^-CD127^-$ gate	Polyspecific Tregs expanded with a panel of CD40L-stimulated B cells (CD40L−sBc)	10,000-fold after 28 days (4 rounds)	Expanded Tregs suppressive after activation by any APCs whose MHC was shared by CD40L−sBc used during expansion	Alonso-Guallart et al., 2021

(Continued)

TABLE 1 | Continued

Species	Treg selection (purity)	Cell yield	Expansion method	Expansion rate	Suppressive activity	Reference(s)
Baboon	MACSFACS sorting (> 95%) $CD4^+CD25^{hi}$	1.7% spleen, 3.1% lymph node, 1.9% blood T cells; 10×10^4 cells/ml blood	Pig $PBMC^+$ IL-2; 3–4 week + 7–10 days without PBMC	≤ 2,000-fold, depending on IL-2 concentration	1:1[a], strong xenogeneic suppression, donor-specific: 4–10 x less efficient to third-party	Porter et al., 2007
Baboon	$CD4^+CD25^{hi}$FACS-sorted from PBMC (> 95% purity)	1–2% of $CD4^+$ cells	Stimulation with irradiated pig PBMC or anti-CD3/CD28 in cultures supplemented with IL-2 and rapamycin for 4 weeks	200-fold	Suppression of autologous anti-pig T effector cell and B cell proliferation	Singh A. K. et al., 2012
Baboon	FACS $CD4^+CD25^{hi}$ (top 1% of $CD25^+$ cells) from PBMC	10^5 Treg isolated from 20 ml blood	Culture for 26 days with IL-2, anti-CD3 Ab, aAPCs (transfected with human CD58, CD32, and CD80) and rapamycin, with weekly restimulation	> 10,000-fold	Excellent suppressive function,. preserved or even enhanced by increasing amounts of restimulation after thawing	Weiner et al., 2015
Baboon	$CD4^+$ T cells	n/a	Stimulation with tolerogenic, baboon Mo-derived DCs loaded with porcine-specific *in vitro*-transcribed RNA in cocultures supplemented with IL-2 and rapamycin for 10 days	Fivefold within 10 days	Highly suppressive effects on porcine-specific T effector cell proliferation; reduced IFNg/enhanced IL-10/TGFb production	Li et al., 2018

(a)APC, (artificial) antigen-presenting cell; BM-DC, bone-marrow derived dendritic cells; CD40L-sBc, CD40 ligand-stimulated B cells; CFSE-MLR, carboxyfluorescein succinimidyl ester-mixed leukocyte reaction; FACS, fluorescence-activated cell sorting; LN, lymph node; MACS, magnetic-activated cell sorting; Mo-derived DC, monocyte-derived dendritic cells; MS, multiple sclerosis; TGFb, transforming growth factor b.

(Zorn et al., 2006; Sakaguchi et al., 2008; Liao et al., 2013). Aoyama et al. (2012) reported that administration of low-dose IL-2 (1 million international units/m^2 BSA/day) significantly expanded peripheral blood $CD4^+$ and $CD8^+$Tregs in normal cynomolgus macaques, with restricted expansion of non-Tregs. The expanded Tregs were principally $CD45RA^-$ $Foxp3^{hi}$ activated Tregs, with strong immunosuppressive activity *in vitro*.

Exposure of Treg to high IL-2 concentrations during their expansion can render the cells sensitive to "cytokine withdrawal–induced death" (Larsen et al., 2017) following their adoptive transfer. This phenomenon could potentially be alleviated by systemic IL-2 administration. Thus, Furlan et al. (2020) found that, in non-transplanted monkeys, adding low dose IL-2 (1 million units/m^2/day) to rapamycin *in vivo* (target trough level: 5–15 ng/ml) promoted a logarithmic increase in the half-life/persistence of adoptively-transferred, autologous NHP Treg, in effect doubling the number of stable circulating Treg compared with Treg infusion in combination with rapamycin alone.

Yamada et al. (2015) examined the influence of systemic IL-2 on T cell alloreactivity and the maintenance of tolerance to MHC mis-matched kidney allografts induced in cynomolgus macaques via mixed hematopoietic cell chimerism. When given low doses of IL-2 ($0.6–3 \times 10^6$ IU/m^2), animals that had accepted their grafts for 1–10 years in the absence of IS rejected acutely, with reactivation/expansion of alloreactive effector memory T cells. After stopping IL-2 administration, graft rejection was reversed and normal renal function restored.

Hematopoietic Growth Factors

Kean et al. (2011) found that a small molecule antagonist of CXCR4- and CXCL12-mediated chemotaxis (AMD3100; Plerixafor) mobilized circulating lymphocytes in rhesus macaques, with significant increases in Tregs. $CD4^+CD25^{hi}CD127^{lo}FoxP3^+$ Tregs were mobilized efficiently, with increases as great as fourfold in leukapheresis products when compared with G-CSF administration alone. Overall, the results indicated that AMD3100 could mobilize a T cell repertoire that might provide protection against GVHD, and thus be of potential benefit in allogeneic hematopoietic stem cell transplantation. Also, in rhesus macaques, Sasaki et al. (2020) observed that combining GM-CSF and G-CSF administration increased the incidence of Treg in both blood and leukapheresis products. In these studies, the mobilized Treg exhibited a similar range of transcription factors, surface markers and chemokine receptors to Treg in normal monkey peripheral blood Treg. Moreover, when the mobilized Treg were expanded *ex vivo*, they displayed similar capacity to inhibit autologous $CD4^+$ and $CD8^+$ T cell proliferation.

Depletion of Treg

Intravenous injection of cynomolgus monkeys with two doses of an IL-2-diphtheria toxin fusion protein (denileukin diftitox; 8 or 18 μg/kg) resulted in a rapid, but short- duration reduction in circulating resting Tregs ($CD4^+CD45RA^+Foxp3^+$) accompanied by a transient elevation in activated Tregs ($CD4^+CD45RA^-Foxp3^{hi}$), followed by their partial depletion (50–60%) (Yamada et al., 2012a). Wang et al. (2016)

TABLE 2 | Agents used to selectively enhance or deplete Tregs in NHP species.

Agent	Species	Dosage	Reported effect	References
Enhancing agents				
IL-2	Cynomolgus macaque	10^6 IU/m^2/day	Increased peripheral blood CD4 and CD8 Tregs (mainly CD45RA$^-$ Foxp3^{hi})	Aoyama et al., 2012
IL-2	Rhesus macaque	10^6 U/m^2/day in combination with adoptively-transferred autologous Treg and rapamycin	Doubling of peripheral blood Treg compared with Treg infusion and rapamycin alone; stable expression of Treg gene transcripts	Furlan et al., 2020
IL-2	Cynomolgus macaque	0.6–3 × 10^6 IU/m^2	Acute cellular rejection of tolerated renal allografts 1–10 years post-transplant; reactivation and expansion of alloreactive T effector memory cells	Yamada et al., 2015
AMD3100 (Plerixafor),-antagonist of CXCR4 and CXCL12-mediated chemotaxis	Rhesus macaque	1 mg/kg (single dose)	CD4$^+$/CD25^{hi}CD127^{lo}FoxP3$^+$ Tregs mobilized efficiently using AMD3100-containing regimens; up to fourfold enrichment in leukapheresis products compared with use of G-CSF alone	Kean et al., 2011
GM-CSF and G-CSF	Rhesus macaque	GM-CSF (Leukine; 10 μg/kg/day); G-CSF (Neupogen; 10 μg/kg/day)	Significant elevation of Treg in peripheral blood and leukapheresis products	Sasaki et al., 2020
Depleting agents				
IL-2-diphtheria toxin fusion protein	Cynomolgus macaque	Two doses (8 or 18 μg/kg)	Rapid, but short-lived decrease in peripheral blood resting Tregs (CD4$^+$CD45RA$^+$Foxp3$^+$) with a transient increase in activated Tregs (CD4$^+$CD45RA$^-$Foxp3^{hi}), followed by their depletion by50–60%	Yamada et al., 2012b
Anti-human CCR4 immunotoxin	Cynomolgus macaque	25 mg/kg twice a day for four consecutive days, 6 h apart	78–89% CCR4$^+$Foxp3$^+$ Treg reduction in peripheral blood for approx 10 days; 89–96% CCR4$^+$Foxp3$^+$ Treg depletion in lymph nodes	Wang et al., 2016

G(M)-CSF, granulocyte (macrophage)-stimulating factor.

demonstrated that an anti-human CCR4 immunotoxin bound and depleted cynomolgus CCR4$^+$ cells *in vitro*. In addition, they showed that the immunotoxin bound to the CCR4$^+$Foxp3$^+$ monkey Tregs *in vitro*. *In vivo* studies carried out in two naive cynomolgus revealed 78–89% CCR4$^+$Foxp3$^+$ Treg depletion in the peripheral blood that lasted about 10 days. In lymph nodes (LN), 89–96% CCR4$^+$Foxp3$^+$ Tregs were depleted. However, no effect was observed on other immune cell populations, including other CD4$^+$ cells, CD8$^+$ T cells, B cells and NK cells.

INFLUENCE OF IS REGIMENS ON TREG IN ALLOGRAFT RECIPIENTS

The existence of Treg in NHP kidney allografts during their rejection has been considered integral to the rejection response (Haanstra et al., 2007). On the other hand, specific induction of Tregs by donor Ags and intragraft enrichment of Tregs may be key to long-lasting tolerance in NHP induced by transient mixed chimerism (Hotta et al., 2016; Matsunami et al., 2019). Co-stimulation blockade (Co-SB) of the CD28 pathway is deleterious to Treg function, due likely to the importance of the complimentary CTLA4-CD80/86 pathway in Treg development and homeostasis. Thus, administration of CTLA4-Ig (Abatacept) decreases circulating Tregs in MHC-mismatched rhesus renal transplant recipients (Anderson et al., 2019). However, Poirier et al. (2015) found that a novel antagonistic pegylated anti-CD28 Fab' Ab fragment (FR104), together with low dose tacrolimus or rapamycin in a steroid-free treatment regimen, prevented acute allograft rejection and alloAb development, with accumulation of Helios$^-$ Tregs in the blood and graft. Blocking CD28 using a monovalent, non-activating single chain anti-CD28 Fv fragment linked to alpha-1 anti-trypsin (sc28AT) synergized with CNI in cynomolgus kidney and heart transplantation, increasing absolute numbers of peripheral Tregs (Zhang et al., 2018). Kim et al. (2017) found that a novel anti-human CD40L (CD154) domain Ab (dAb) that prolonged rhesus renal allograft survival without thromboembolism, and that synergized with conventional IS to more markedly control graft rejection, increased CD4$^+$CD25$^+$Foxp3$^+$ Treg frequency.

Ezzelarab et al. (2013, 2018) reported that infusion of donor-derived DCreg 1 week before transplant prolonged rhesus renal allograft survival in combination with CD28 Co-SB and rapamycin and promoted maintenance of donor-reactive CD4$^+$CTLA4^{hi} Tregs.

TREG THERAPEUTIC PROTOCOLS IN TRANSPLANT RECIPIENTS

Successful development of a therapeutic protocol based on adoptive transfer of Treg must address: (i) Treg efficacy and the number of cells required to achieve a therapeutic effect; (ii) stability of the suppressive phenotype; (iii) the Treg migratory pattern that assures their optimal regulatory function; (iv) the Ag specificity required for safe/effective control of rejection; (v)

the impact of lymphocyte depletion/concomitant IS therapy on Treg function and durable control of rejection; (vi) conditions permissive to regulation of memory T cell responses. Several NHP studies (**Table 3**) have assessed feasibility, safety and therapeutic efficacy of adoptively-transferred, autologous Treg in transplant recipients. In a direct comparison of the persistence of transferred autologous vs. allogeneic Treg in peripheral blood of immunosuppressed, but not transplanted cynomolgus, we found that autologous Tregs survived much longer than the former (Zhang et al., 2015), a property suggesting more durable potential efficacy of autologous Tregs.

Renal Transplantation

The earliest work (Bashuda et al., 2005) was conducted in rhesus macaques given MHC-mismatched renal allografts. Thus, autologous spleen-derived "suppressive T cells" (approx 10^{7}/kg) that were rendered anergic by co-culture with donor alloAg together with anti-CD80/CD86 mAbs were infused

TABLE 3 | Assessment of adoptive Treg therapy in NHP transplant recipients.

Type of graft (species)	IS therapy/conditioning	Type of Tregs (number/infusions)	Result	References
Kidney (rhesus) *n* = 6	Cyclosporine and post-transplant cyclophosphamide	Autologous splenocyte-derived "suppressive T cells" (approx 10^{7}/kg) rendered anergic following co-culture with donor alloAg and anti-CD80/86 mAbs ($10^{2} \pm 67 \times 10^{6}$ total on day 13 post-transplant)	Donor-specific tolerance in 50% of graft recipients	Bashuda et al., 2005
Kidney (cynomolgus) *n* = 4	ATG and low dose rapamycin	Expanded, donor Ag-specific, host splenocyte–derived $CD4^{+}CD25^{+}$Treg (14 daily infusions; 10^{7} per day)	Graft MST prolonged from 22 to 48.5 days	Ma et al., 2011
Kidney (cynomolgus) (*n* = 5)	Non-myeloablative conditioning and MHC-mismatched BMT. Renal transplantation (from the same BMT donor) conducted 4 months after BMT, with no IS	Expanded polyclonal autologous Treg ($15–53 \times 10^{6}$/infusion) during the 1st week post-transplant (days 0, 2, 5, 7) and day + 50 (total dose: $88–96 \times 10^{6}$/kg)	Two of 5 evaluable recipients of Treg + BMT displayed T cell chimerism up to 335 days post-BMT. In one long-term surviving animal, the delayed kidney graft survived > 294 days without IS, however, non-Treg BMT recipients rejected their delayed kidney grafts within 3–4 weeks	Duran-Struuck et al., 2017
Heart (cynomolgus) *n* = 5	ATG, tacrolimus, anti-IL-6R mAb and tapered rapamycin maintenance IS therapy	Expanded polyclonal Tregs (single or multiple doses (up to a maximum cumulative cell dose of 1.87 billion cells during the early post-transplant period (up to 1 month post-transplant)	Inferior graft function with multiple infusions; elevated incidences of T effector memory cells, increased IFNγ production by host $CD8^{+}$ T cells, enhanced levels of proinflammatory cytokines and anti-donor alloAb	Ezzelarab et al., 2016
Heart (cynomolgus) *n* = 5	ATG, tacrolimus and anti-IL-6R with tacrolimus conversion to rapamycin at 2 weeks (early cell infusion within 3 weeks post-transplant); ATG and tacrolimus with tacrolimus conversion to rapamycin at 2.5 weeks (delayed cell infusion, 6–8 weeks post-transplant)	Expanded donor Ag alloreactive Tregs; $20.5–120 \times 10^{6}$/kg for each of 2–4 infusions	No prolongation of graft survival; loss of regulatory signature and proliferative/survival capacity by transferred Tregs	Ezzelarab et al., 2020
Pig pancreatic islets (xeno; rhesus) *n* = 3	ATG, CVF, anti-CD154 mAb, and rapamycin	Intraportal infusion (x1 or x2) of expanded $CD4^{+}CD25^{hi}CD127^{lo}$ autologous Tregs ($1.87–62.0 \times 10^{6}$) following ATG depletion	Treg infusion associated with more stable and durable normoglycemia	Shin et al., 2015
Pig pancreatic islets (xeno; rhesus) *n* = 2	ATG, CVF, anti-CD154 mAb and rapamycin	As above (ref 65)	Engrafted pig islets rejected by activated T cells following withdrawal of maintenance IS	Shin et al., 2016
Pig skin graft (xeno; baboon) *n* = 4	Splenectomy, TBI, ATG, TI, rapamycin, CVF, GalKO/huCD46/huCD47 transgenic donor pig HSCs, anti-CD40LAb, methylprednisolone	25×10^{6}/kg (8 infusions)	Prolonged donor skin graft acceptance	Stern et al., 2017

ATG, anti-thymocyte globulin; BMT, bone marrow transplantation; CVF, cobra venom factor; Gal KO, α1,3-galactosyltransferase gene-knockout; HSCs, hematopoietic stem cells; hu, human; IS, immunosuppression; MST, mean/median graft survival time; TBI, total body irradiation; TI, thymic irradiation.

once, 13 days post-transplant to splenectomized hosts that receiving brief cyclosporine and cyclophosphamide (Cy) treatment. Grafts survived long-term and donor-specific tolerance was achieved in 50% of recipients (n = 6), but not in controls, confirming the potential of a Treg therapy in NHP organ transplantation. These findings provided the basis for a clinical trial in living donor liver transplantation, in which splenic T cells rendered anergic to donor and administered 13 days post-transplant after Cy, induced operational tolerance in 7/10 recipients (Todo et al., 2016). However, infusion of similarly generated Tregs 12 days post-transplant into living donor kidney transplant patients resulted in high rates of rejection upon subsequent IS drug withdrawal (Koyama et al., 2020).

Ma et al. (2011) reported that *ex vivo*-expanded autologous Treg prolonged renal transplant survival in ATG-treated cynomolgus monkeys. In this study, a much greater number of infusions (fourteen) of expanded, donor alloAg-specific, spleen–derived $CD4^+CD25^+$Treg were infused (10^7 per day). The infusions started at the time of transplantation (i.e., 1 day following a 3-day course of ATG) and were administered together with low-dose rapamycin for 14 days. Thus, kidney graft survival was prolonged significantly, but unlike in the aforementioned study, no grafts survived indefinitely.

Duran-Struuck et al. (2017) reported that recipient Treg co-transfer could enhance bone marrow (BM) engraftment/hematopoietic cell chimerism and prevent cynomolgus kidney allograft rejection. Eight monkeys were subjected to non-myeloablative conditioning and MHC-mismatched BM transplantation (BMT), either with or without infusion of Treg. Transplantation (from the same BM donor) was undertaken 4 months post-BMT, in the absence of IS to evaluate robust, donor-specific tolerance. Five animals received *ex vivo*-expanded polyclonal autologous Treg during the first week post-transplant (days 0, 2, 5, 7) and also on day 50. Transient mixed chimerism, without significant T cell chimerism, was observed in those monkeys given BMT but no Treg (n = 3). By contrast, the 2 of 5 monkeys given Treg + BMT that could be evaluated showed multilineage (including T cell) chimerism up to 335 days after BMT. It was notable that, in the monkey that survived long-term, >90% of donor T cells were found to be new thymic emigrants. In this monkey, the delayed (to 4 months) renal transplant was accepted for >294 days without IS therapy, whereas non-Treg BMT recipients rejected their delayed kidney grafts within 3–4 weeks. Early reactivation of cytomegalovirus (CMV) and anti-viral treatment was linked with early failure of chimerism, irrespective of Treg administration. Overall, these observations reveal that, without early CMV reactivation (and consequent BM-toxic anti-viral therapy), administration of host Treg can promote both prolonged multilineage chimerism and robust donor tolerance.

Heart Transplantation

Addressing the hypothesis that markedly reduced T cell numbers in graft recipients would favor the therapeutic efficacy of adoptively-transferred Tregs, Ezzelarab et al. (2016) infused *ex vivo*-expanded, polyclonal Treg into ATG-treated, MHC-mismatched cynomolgus monkey heart transplant recipients before the homeostatic recovery of effector T cells. Tacrolimus, anti-IL-6R mAb and tapered rapamycin maintenance treatment was also administered. Infusion of Treg in either single or multiple doses during the early post-transplant period (up to a month post-transplant) during which time host T cells were markedly depleted, resulted in poorer heart graft function compared with non-Treg-infused controls. This was accompanied by an increased incidence of circulating effector memory T cells, elevated IFNγ production by $CD8^+$ T cells, and enhanced levels of circulating proinflammatory cytokines and anti-donor alloAb. These observations suggest that Treg infusion early post-transplant period following lymphodepletion should be avoided in human clinical trials. Thus, despite marked but transient increases in circulating Treg relative to effector T cells and the use of so-called "Treg-friendly" agents, environmental conditions and host immune effector mechanisms that prevail under these conditions may negate the potential therapeutic efficacy of the infused transferred Treg.

In a more recent study (Ezzelarab et al., 2020), cynomolgus monkey autologous darTreg were expanded from flow-sorted, circulating Treg using activated donor B lymphocytes and infused after transplant into MHC-mismatched heart graft recipients. While the darTreg selectively suppressed proliferative responses of autologous T cells to donor alloAgs *in vitro*, their adoptive transfer post-transplant failed to prolong graft survival. Early (within 2 weeks post-transplant; under ATG, tacrolimus and anti-IL-6R) or delayed (6–8 weeks post-transplant; under rapamycin) darTreg infusion resulted in a rapid reduction in transferred cells in the circulation. After early or delayed infusion, dye-labeled darTreg could be observed in lymphoid and non-lymphoid tissues and in the heart graft at low percentages (<4% $CD4^+$ T cells). Notably, the infused darTreg displayed reduced expression of Foxp3, CTLA4, Helios, the proliferative marker Ki67, and anti-apoptotic Bcl2, compared with pre-infusion darTreg and endogenous Treg.

Comparison of Doses of darTreg Tested in NHP and Human Allograft Recipients

Doses of darTreg tested in cynomolgus heart allograft recipients following lymphodepletion with ATG (Ezzelarab and Thomson, 2020) were similar to or greater than doses of autologous T cells rendered anergic to donor Ag and infused post-transplant to Cy-treated, renal-allografted monkeys (Bashuda et al., 2005) and that induced donor-specific tolerance. However, they also resemble the single doses of similarly generated Tregs ($14–36 \times 10^6$/kg) infused 12 days post-transplant to living donor kidney transplant patients, including those given Cy, that, by contrast, exhibited high rates of rejection upon subsequent IS drug withdrawal (Koyama et al., 2020). In other clinical studies of darTeg in living donor kidney transplantation, lower doses of darTreg have been targeted (i.e., $2 \times 10^3–2 \times 10^6$/kg or $0.5–10 \times 10^6$/kg, respectively) in small numbers of patients at separate centers in

the ONE Study (Sawitzki et al., 2020) in which overall safety of cell therapy and similar 1-year graft survival compared to a reference standard of care group have been reported. In human living donor liver transplantation, doses of 23.3–14.4 $\times$ 10^6 T cells rendered anergic to donor and administered 13 days post-transplant after Cy induced operational tolerance in 7/10 recipients (Todo et al., 2016). A total of 300–500 $\times$ 10^6 darTreg have been targeted in a liver transplantation IS drug withdrawal study at UCSF (NCT02474199).

TRACKING/MONITORING OF POLYCLONAL OR DARTREGS IN NORMAL NHP OR ORGAN ALLOGRAFT RECIPIENTS

The *in vivo* persistence and migration of adoptively-transferred polyclonal Tregs to secondary lymphoid tissues documented in rodents have been confirmed by NHP studies that have determined the survival, migration, and function of exogenous Tregs. Detection of autologous or allogeneic Tregs infused into normal macaques has been accomplished by direct dye-labeling and subsequent tracking of the Tregs in blood, LNs and spleen at various times after their infusion (Singh et al., 2014; Zhang et al., 2015). Pharmacokinetic analysis of dye-labeled autologous Tregs has revealed an initial rapid period of elimination from the blood between day 0 and 3 post-infusion, after which the transferred Tregs persist at low levels for up to 3 weeks (Zhang et al., 2015). Staining for the cell proliferation marker Ki67 further demonstrates that label dilution as a result of cell proliferation does not contribute to the apparent disappearance of transferred Tregs from blood. Labeled autologous Tregs have been detected in LNs on day 1–2 post-infusion, however analysis on day 6 failed to demonstrate sustained persistence of infused Tregs in secondary lymphoid organs (Zhang et al., 2015).

Concurrent IS therapy markedly increases the survival of adoptively-transferred autologous Tregs in the peripheral blood and LNs. Labeled autologous Tregs persist longer in monkeys given rapamycin and in greater numbers compared to non-immunosuppressed conditions at least 50 days post-infusion (Furlan et al., 2020). Low dose IL-2 together with rapamycin further prolongs the half-life of adoptively-transferred Tregs in NHPs, with detection of labeled cells in the periphery up to 84 days after infusion. These findings highlight a wide variability in the survival of infused Tregs under different conditions, including the use and type of IS, in addition to cell production methods, including cryopreservation.

XENOTRANSPLANTATION

Treg in xenotransplantation has been reviewed (Ezzelarab, 2018). Baboon Treg expanded in response to irradiated pig PBMC for potential use in pig-to-baboon xenotransplantation (Porter et al., 2007) proved strongly suppressive *in vitro* and donor-specific suppression was achieved. Huang et al. (2017) found that baboon autologous Tregs expanded by stimulation with pig PBMC could prevent porcine islet xenograft rejection in NOD-SCID IL-2r$\gamma^{-/-}$ mice reconstituted with baboon PBMC. Prolonged xenograft survival to >100 days correlated with Treg engraftment and intragraft CD39 and Foxp3 gene expression. Shin et al. (2016) transplanted two diabetic rhesus with pancreatic islets isolated from wild-type miniature pigs with IS comprising ATG, cobra venom factor, anti-CD154 mAb and sirolimus. Expanded $CD4^+CD25^{hi}CD127^{lo}$ autologous Tregs were infused during the peri-transplant period, however following withdrawal of maintenance IS, engrafted pig islets were rejected, indicating failure of tolerance induction by the autologous Tregs in this model.

CONCLUSION AND FUTURE STUDIES

NHP have the inherent advantage over rodent or humanized/primatized mouse models of closely resembling the human condition. Endogenous Treg in NHP can be increased or selectively depleted *in vivo*, and both polyclonal and darTreg have been expanded successfully *ex vivo*. In NHP kidney, heart and pancreatic islet transplantation, single or multiple infusions of autologous Treg have yielded results ranging from indefinite, IS-free graft survival to exacerbation of acute rejection, depending on the experimental model and IS regimen. In addition to valuable safety and graft outcome data that help guide clinical trial design, important insights have been gained into the biodistribution, stability and longevity of the transferred cells, and their influence on anti-donor immunity. These findings have drawn attention to the biological and logistical challenges encountered when transitioning testing of Treg therapeutic efficacy in inbred, spf mouse models, usually at very high/unphysiological Treg/effector cell ratios, to more clinically relevant NHP species. Exciting, newly evolving approaches to generation of stable, Ag-specific Tregs (Ferreira et al., 2019; Raffin et al., 2020), include gene editing of bulk T cells (Honaker et al., 2020) and production of CAR Tregs engineered to promote their Ag specificity and homing (MacDonald et al., 2016; Dawson et al., 2019; Hoeppli et al., 2019; Mohseni et al., 2020). Although there are no published reports of these developments as yet in NHP models, testing of such advances in NHP organ transplantation is keenly anticipated.

AUTHOR CONTRIBUTIONS

AT, KS, and ME participated in planning of the manuscript, literature searches, and in the writing of the mini-review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Regulation of Alloantibody Responses

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The control of alloimmunity is essential to the success of organ transplantation. Upon alloantigen encounter, naïve alloreactive T cells not only differentiate into effector cells that can reject the graft, but also into T follicular helper (Tfh) cells that promote the differentiation of alloreactive B cells that produce donor-specific antibodies (DSA). B cells can exacerbate the rejection process through antibody effector functions and/or B cell antigen-presenting functions. These responses can be limited by immune suppressive mechanisms mediated by T regulatory (Treg) cells, T follicular regulatory (Tfr) cells, B regulatory (Breg) cells and a newly described tolerance-induced B (TIB) cell population that has the ability to suppress *de novo* B cells in an antigen-specific manner. Transplantation tolerance following costimulation blockade has revealed mechanisms of tolerance that control alloreactive T cells through intrinsic and extrinsic mechanisms, but also inhibit alloreactive B cells. Thus, the control of both arms of adaptive immunity might result in more robust tolerance, one that may withstand more severe inflammatory challenges. Here, we review new findings on the control of B cells and alloantibody production in the context of transplant rejection and tolerance.

Keywords: Tfh, Tfr, alloantibodies, Bregs, transplantation, tolerant B cells, transplant tolerance

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INTRODUCTION

Transplantation tolerance remains an important goal to reduce side effects associated with lifelong immunosuppressive drugs and to diminish the incidence of chronic rejection that might arise from suboptimal immunosuppression. Both T cell-mediated and antibody-mediated mechanisms have been invoked in acute and chronic rejection, though some controversy exists as to whether the appearance of donor-specific antibodies (DSA) is simply a marker of incompletely suppressed T effector and T follicular helper (Tfh) cell responses, or if DSA independently cause rejection (Chong et al., 2019). Several animal models of transplantation tolerance have been developed, and proof of principle data exist that select patients can develop transplantation tolerance (Brouard et al., 2012; Morris et al., 2015; Miller et al., 2017; Savage et al., 2018). New genetic and flow cytometric tools have increased our ability to probe the mechanisms that underscore tolerance when it is successfully induced, though most of the work has focused on how alloreactive T cells are controlled. Mechanisms of T cell transplantation tolerance appear to parallel endogenous immunological mechanisms ensuring T cell self-tolerance, including clonal deletion, anergy, cell-extrinsic suppression and aborted B cell help. Whereas lack of B cell responses might result from insufficient T cell help, new studies are revealing an active tolerance crosstalk between T and B cells. Indeed, T follicular regulatory (Tfr) cells can dominantly suppress B cell responses, Bregs can

inhibit T cell responses and promote Treg development, and a novel subset of tolerance-induced B (TIB) cell can infectiously inhibit the responses of naïve B cells in an antigen-specific manner. This active tolerization of the alloreactive B cell compartment may be desirable to achieve a more robust transplantation tolerance than that obtained when only the T cell compartment is regulated. This review will focus on new findings involving Tfr cells, Breg cells and alloreactive TIB cells and their roles in the control of alloimmunity and transplant rejection.

T FOLLICULAR REGULATORY (Tfr) CELLS

Tfr Cells in Antibody Regulation

Antibody responses are tightly controlled by the immune system. Tfh cells promote antibody responses by providing costimulatory and cytokine signals to promote B cell effector responses (Crotty, 2019). Humans with mutations in Tfh effector genes as well as mice with genetic deletion of the same genes have severely impaired antibody responses (Crotty, 2019). Tfr cells are a subpopulation of FoxP3^{+} T regulatory cells that express the chemokine receptor CXCR5 and can gain access to the B cell follicle to regulate B cell responses (Fonseca et al., 2019; Sage and Sharpe, 2020; Wing et al., 2020). Although CXCR5 was originally thought to be absolutely essential for the migration of Tfr cells to the B cell follicle, newer data suggest other signals may also be involved (Vanderleyden et al., 2020). Tfr cells have a somewhat overlapping transcriptional program as Tfh and Treg cells, and follow similar differentiation cues as Tfh cells (Hou et al., 2019). The shared transcriptional program between Tfr cells and Tfh/Treg cells has hindered in-depth functional studies.

Although Tfr cells have been studied for over 10 years, the precise functions of these cells have been somewhat controversial. Human correlation studies suggest inverse correlations between Tfr cells (as well as Tfr:Tfh ratio) and antibody responses in settings of vaccination, infection and autoimmunity (Sage and Sharpe, 2016; Deng et al., 2019). In some limited settings, such as Sjogren's syndrome, the Tfr/Tfh ratio may positively correlate with ectopic lymphoid structure formation, suggesting Tfr cells may also act as biomarkers for progressive disease stages (Fonseca et al., 2018). Uncovering the precise roles for Tfr cells in murine models has also been challenging. Early studies utilized adoptive transfer techniques and *in vitro* suppression assays to demonstrate that Tfr cells inhibit antigen specific antibody responses (Wollenberg et al., 2011; Sage et al., 2013, 2016). However, these strategies have technical limitations and lack physiological complexity. Recently, two *in vivo* models have been developed to study Tfr cells: Conditional deletion of Bcl6 in Treg cells (Treg$^{\Delta Bcl6}$) and the Tfr-DTR mouse. The premise behind the Treg$^{\Delta Bcl6}$ model is that the transcription factor Bcl6 may be necessary for most Tfr cell development and eliminating Bcl6 in all Treg populations would restrain Tfr cell differentiation from Treg cells. Although the specificity and potency of Tfr depletion in this model is unclear, a number of studies have demonstrated substantial increases in autoantibodies in Treg$^{\Delta Bcl6}$ mice (Wu et al., 2016; Botta et al., 2017; Fu et al., 2018; Gonzalez-Figueroa et al., 2021). In contrast, Treg$^{\Delta Bcl6}$ mice had minor (if any) increases in foreign-antigen specific antibody responses. Moreover, in some cases such as influenza infection, Treg$^{\Delta Bcl6}$ mice have less influenza-specific antibody, suggesting Tfr cells may actually promote antibody responses in some settings (Lu et al., 2021).

As an alternative approach, we recently developed a Tfr-DTR mouse model in which Tfr cells can be deleted in an inducible manner. Using this mouse, we found that Tfr cells potently suppress germinal center formation to control both autoreactive and vaccine-specific antibody responses (Clement et al., 2019). We also found that Tfr cells potently control antigen-specific, as well as total, IgE responses. Increases in total IgE have subsequently been validated using Treg$^{\Delta Bcl6}$ models (Gonzalez-Figueroa et al., 2021). Therefore, Tfr cells seem to have potent roles in controlling autoantibody responses, and the role of Tfr cells in restraining foreign antigen-specific antibody responses may depend on experimental context.

Tfr cells utilize a number of mechanisms to control B cell responses. The coinhibitory receptor CTLA-4 seems to be a potent mediator of Tfr suppression, and both downregulation of B7–1/B7–2 on B cells, as well as prevention of Tfh-B cell interactions, have been proposed as potential mechanisms (Sage et al., 2014; Wing et al., 2014). Although Tfr cells can produce the inhibitory cytokine IL-10, studies suggest that IL-10 production by Tfr cells may actually promote, not inhibit, B cell responses in some settings (Laidlaw et al., 2017). Mechanisms of Tfr suppression that are not shared with other regulatory cell subsets are less clear. However, a new study has uncovered neuritin as a possible Tfr-specific inhibitory molecule that may restrain B cell responses, although neuritin may suppress IgE/allergic responses more potently than IgG responses (Gonzalez-Figueroa et al., 2021).

Tfr Cells in Transplantation Tolerance

The roles of follicular T cells in controlling solid organ transplant rejection are only beginning to be studied (Mohammed and Sage, 2020). In kidney transplant patients, the frequency of CXCR3^{-} Tfh cells (including Tfh2 and Tfh17 subsets) correlated with donor-specific antibody (DSA) responses and development of antibody-mediated rejection (AMR) (Chen et al., 2017). Similarly, in cardiac transplant patients, CXCR3^{-} Tfh cells were elevated 1 year after transplantation (Wang et al., 2020). In murine cardiac transplant models, Tfh cell expansion occurred prior to DSA responses suggesting Tfh cells may be an inducer (and biomarker) of rejection (La Muraglia et al., 2019). These studies suggest that Tfh cells may be driving AMR. Moreover, the ratio of Tfr to Tfh cells was attenuated in both kidney and cardiac transplant patients when compared to controls, and a decrease in the Tfr to Tfh ratio was associated with rejection in some transplant studies (Chen et al., 2017; Yan et al., 2019; Niu et al., 2020; Wang et al., 2020). These correlation studies suggest that Tfr cells may restrain Tfh and B cell responses to limit generation of pathogenic antibodies and prevent graft rejection, with the caveat that the ratios were quantified among circulating cells whereas regulation takes place in secondary lymphoid organs.

Elucidating the precise roles of Tfh and Tfr cells in controlling AMR after transplantation has been elusive due to a lack of tools. We recently developed a pre-clinical allogeneic kidney transplant model to assess the roles of Tfh and Tfr cells in controlling DSA and AMR. Using a Tfh-DTR model in which Tfh cells were deleted during transplantation we found that Tfh cells were essential for IgG DSA as well as AMR after kidney transplantation (Mohammed et al., 2021). We also performed allogeneic kidney transplantation in Tfr-DTR mice to assess the roles of Tfr cells in controlling AMR. Although we found that Tfr cells can limit IgG DSA using splenocyte alloantigen challenge models, we did not find substantial differences in severity of AMR during kidney transplantation when Tfr cells were deleted (Mohammed et al., 2021). However, one caveat to these experiments is that a full MHC mismatch kidney transplant model was used in which rejection rapidly occurs within 3 weeks. Therefore, it is unclear if it would be possible to detect quicker rejection by eliminating Tfr suppression. Studying the Tfr-DTR mouse in kidney transplant tolerance models will be important to determine if Tfr cells have important roles in promoting B cell tolerance during transplantation. *In vitro*, Tfr suppression of B cells results in epigenetic changes in B cells, diminishing their ability to produce class-switched antibody even after Tfr cells are no longer present (Sage et al., 2016). A somewhat similar phenotype may occur with alloreactive TIB cells acquiring a stable tolerance state during cardiac transplantation tolerance induced with costimulation blockade, although acquisition of tolerance was observed in $Treg^{\Delta Bcl6}$ recipients (Khiew et al., 2020). That Tfr cells may be able to induce a tolerant-like state in B cells is supported by a recent study where tolerance induced by specific donor-recipient MHC combinations during kidney transplantation was mediated by $FoxP3^+$ Treg cell subset(s) (Yang et al., 2020). Although the precise roles for Tfr cells in modulating transplantation tolerance are still being investigated, it is likely that Tfr cells have functional overlap with both Treg and Breg cells. Since Tfr cells differentiate mostly from Treg cells, it is possible that Treg promotion of B cell tolerance may be through a Tfr-dependent mechanism (Aloulou et al., 2016; Maceiras et al., 2017). Moreover, studies suggest that Breg cells may control Tfh cells, such that Tfr cells may synergize with Breg cells to promote tolerance by restraining Tfh-help to B cells (Lin et al., 2019).

REGULATORY B CELLS

Multiple Breg Subsets in Immunity

There is a substantial literature supporting a role for B cells in immune regulation, and numerous regulatory B cell subsets have been implicated (reviewed in Stolp et al., 2014; Alhabbab et al., 2019; Cherukuri et al., 2021a). For humans, these include the $CD24^{hi}CD38^{hi}$ immature transitional B cells (TBs) (Blair et al., 2010), $CD24^{hi}CD27^+$ B10-like B cells (Dilillo et al., 2010), $CD27^+CD38^{hi}$ plasmablast Bregs (Matsumoto et al., 2014), $CD25^{hi}CD71^{hi}CD73^{lo}$ Br1 cells (Van De Veen et al., 2013), $CD39^+CD73^+$ Bregs (Saze et al., 2013), $CD38^+CD1d^+IgM^+CD147^+$ Bregs (Lindner et al., 2013), TIM-1^+ Bregs (Aravena et al., 2017), and induced Bregs (Nouel et al., 2015). Likewise multiple Breg subsets have been reported in mice, including transitional T2 (Mauri et al., 2003), marginal zone (MZ) (Lenert et al., 2005), $CD5^+CD1d^+$ B10 (Kalampokis et al., 2013), TIM-1^+ or $CD9^+$ (Ding et al., 2011; Sun et al., 2015) B cells, as well as LAG-3^+CD138^+ plasmablasts/plasma cells (Shen et al., 2014; Fillatreau, 2018; Lino et al., 2018). The numerous Breg subsets together with the absence of Breg-specific lineage markers have prevented a unifying framework for understanding the origin of these cells.

At present, it appears that Bregs may exist as two distinct subsets; natural Bregs that are similar to thymus-derived natural Tregs and driven by a yet undefined lineage-determining transcription factor instructing their development in the bone marrow; and induced Bregs generated in the periphery from distinct B cell subsets upon encounter with antigen. In support of the former subset, Fillatreau (Fillatreau, 2018, 2019) reported on a novel subset of non-dividing regulatory LAG-3^+CD138^+ plasma cells that develop at steady state, even in germ-free mice, and that require B cell receptor (BCR) signals and Bruton tyrosine kinase (Btk) for their development. Importantly, these cells produce IL-10 within hours after stimulation and are therefore classified as "natural regulatory plasma cells." In contrast, other subsets of Bregs, such as the B10 Bregs require exposure to immunizing antigen, and engagement of BCR, CD40 and/or TLR signaling (Zhang, 2013). An example are Bregs described by Rothstein and colleagues, where a 20–25-fold expansion of TIM-1^+ Bregs across multiple canonical B cell subsets was observed in mice receiving an islet transplant and treated with a low-affinity anti-TIM-1 antibody (Yeung et al., 2015).

Immunomodulatory Cytokines Produced by Bregs

Adding to the complexity of Breg immunobiology, each Breg subset appears capable of suppressing immune responses by shared, as well as distinct, mechanisms of action. The most widely implicated mechanism for Breg-mediated suppression is through production of the anti-inflammatory and immunomodulatory cytokine IL-10. IL-10 produced by B cells may inhibit the differentiation, or induce the apoptosis, of Th1 and Th17 cells while promoting Treg expansion, and alter DC maturation (reviewed in Stolp et al., 2014). In light of the absence of a Breg lineage marker, the expression of IL-10 has been widely used for defining Bregs. In a recent review, Cherukuri et al. (2021a) tabulated IL-10-producing cells within each mouse B cell subset upon a 5 h stimulation with LPS, PMA and ionomycin (LPIM). Only 1.7% of follicular B cells were IL-10-producing, but because they are the major B subset, they represented up to 44% of all B cells producing IL-10. A sizable 23% of MZ B cells were IL-10 producing, and represented 33% of all IL-10-producing B cells. In contrast, plasma cells by far had the highest percentage (74%) of IL-10-producing cells, but only represented 5% of all IL-10-producing B cells. Thus under *in vitro* LPIM stimulation, follicular and MZ B cells represented 77% of all IL-10-producing

cells, although it remains to be determined if follicular and MZ B cells have similar dominant contribution *in vivo*.

Transitional B cells represent 4–10% of $CD19^+$ B cells in healthy adults, and 15–20% of blood B cells in mice. Transitional B cells can be further subdivided into T1, T2 and T3 subsets, and can increase in percentages upon infection and autoimmunity (Giltiay et al., 2019). There is a sizable body of literature correlating the frequency of IL-10-producing transitional (T2-MZ) B cells with transplantation tolerance (Newell et al., 2010; Pallier et al., 2010; Sagoo et al., 2010; Chesneau et al., 2014), leading to the hypothesis that Bregs were being induced and might modulate T cell responses to mediate allograft tolerance. However, a critical examination of the B cell tolerance signature when compared with cells from patients on immunosuppression and controls was subsequently conducted by Rebollo-Mesa et al. (2016). The authors found that the percentage of transitional B cells increased significantly upon steroid withdrawal. Subsequently, azathioprine as well as calcineurin inhibitors were reported to inhibit the levels of transitional IL-10-producing B cells. These findings raised concerns that transitional B cells may not predict nor mediate tolerance and that their elevated levels in tolerant recipients might be a response to the absence of immunosuppression (reviewed in Alhabbab et al., 2019). Nevertheless, the possibility remains that these IL-10-producing transitional B cells might contribute to graft survival as a consequence of their anti-inflammatory properties (reviewed in Alhabbab et al., 2019).

In addition to IL-10, IL-35-producing B cells and plasma cells have been reported to be critical regulators of immunity during autoimmune and infectious disease settings (Shen et al., 2014; Wang et al., 2014). IL-35 is a member of the IL-12 family, and is comprised of Ebi3, which is shared with IL-27, and a p35 subunit, which is shared with IL-12. As a result, the quantification of IL-35-producing B cells is challenging, and attribution of a role for IL-35 using single-chain (Ebi3)-deficient mice is complicated by concomitant loss of IL-27, a cytokine that also has anti-inflammatory and regulatory properties. Similarly, elimination of p35 not only results in loss of IL-35 but also IL-12 (Kourko et al., 2019; Mirlekar et al., 2021). Furthermore, IL-35 is produced by $CD4^+FoxP3^+$Tregs, $CD8^+$ Tregs, activated dendritic cells, and it signals through four receptors, IL-12Rβ2-IL-27Rα, IL-12Rβ2-IL-12Rβ2, IL-12Rβ2-GP130, and GP130-GP130 (Zhang et al., 2019). Thus, the complexity of IL-35 immunobiology requires that multiple controls be included to allow for a definitive demonstration that IL-35 is critical to the function of Bregs.

In the settings of allergy, cancer and autoimmune diseases, the regulatory function of B cells has been reported to be mediated by the potent immunoregulatory cytokine, TGFß (reviewed in Huai et al., 2021). In a model of anti-CD45RB-induced allograft tolerance, Zhao et al. (2010) reported that TGFß produced by B cells was necessary for tolerance, while IL-10 was not, and in fact appeared to promote chronic cardiac vasculopathy. Studies using a tolerance protocol of anti-CD45B and anti-TIM-1 to promote tolerance to islet allografts revealed an expansion of $TIM\text{-}1^+LAP^+$ B cells (Latency-associated peptide, LAP, is non-covalently associated with TGFß, and this complex is either secreted or deposited on the extracellular matrix), and TGFß was necessary for tolerance induction (Lee et al., 2012). Furthermore, TGFß, in combination with indoleamine 2,3-dioxygenase production by B cells, has been reported to regulate T cell proliferation, and promote the induction of TGFß and IL-10-producing Tregs *in vitro* (Nouel et al., 2015). Whether these TGFß-producing regulatory B cells represent a distinct phenotype and developmental lineage from the IL-10-producing Bregs requires further investigation.

Mirroring the observation that $CD4^+$ T cells have different effector and regulatory phenotypes (Hollbacher et al., 2020), B cells can also differentiate into different effector B cell subsets (Beff) producing different pro-inflammatory cytokines capable of enhancing T cell responses independently of antibody production (Lund, 2008; Shen and Fillatreau, 2015). These observations collectively raise the possibility that the ratio of Bregs to Beff may serve as a rheostat for effector T cell responses. In support of this notion, Cherukuri et al. (2014) reported that B cells co-producing TNFα and IL-10 and having a low IL-10/TNFα ratio lacked regulatory function, whereas those having a high IL-10/TNFα ratio effectively suppressed T cell production of IFNγ and TNFα. They further reported that the more immature T1 B cell subset had a significantly higher IL-10/TNFα ratio than the more mature T2 subset, and that a higher IL-10/TNFα ratio in the T1 subset best predicted an absence of T cell-mediated rejection and a better renal graft outcome (Cherukuri et al., 2017, 2021b). Despite its strong predictive value, it is nevertheless unclear whether the balance of IL10 and TNFα in the T1 B cell subset is a biomarker of tolerance or causally inhibits T cell-mediated rejection. T1 B cells are short-lived immature B cells that undergo apoptosis upon BCR engagement, and only a fraction of T1 B cells enter the B cell follicle to access appropriate survival signals and mature into naïve B cells (Chung et al., 2003; Zhou et al., 2020). Thus, where in the secondary lymphoid organs transitional B cells might gain access to T cells to suppress their activation, remains to be more fully explored. Recently, Giltiay et al. (2019) suggested a new perspective on these newly formed B cells, proposing that they function as a first line defense by producing "natural" antibodies and as antigen-presenting cells, based on their distinct BCR repertoire, high expression of activation induced cytidine deaminase (AID), high sensitivity to pathogen-associated molecular patterns (PAMPS) and their ability to produce cytokines. Thus, it is possible that B cells with a transitional phenotype may rapidly differentiate into antigen-presenting cells with the ability to regulate T cell responses through cytokine production.

Immunoregulation of T Cell Responses by Bregs Through Cognate Interactions

In addition to the production of immunomodulatory cytokines, there is accumulating evidence that cell surface molecules, such as PD-L1 and CD80/86, on Bregs, may engage coinhibitory molecules PD-1 and CTLA-4, respectively, to modulate T cell function (Blair et al., 2010; Siewe et al., 2013). Recently, Hasan et al. (2021) reported that T cell immunoreceptor with Ig and ITIM domains (TIGIT) is expressed on human memory $CD19^+CD24^+CD27^+CD39^+IgD^-IgM^+CD1c^+$ B cells that are

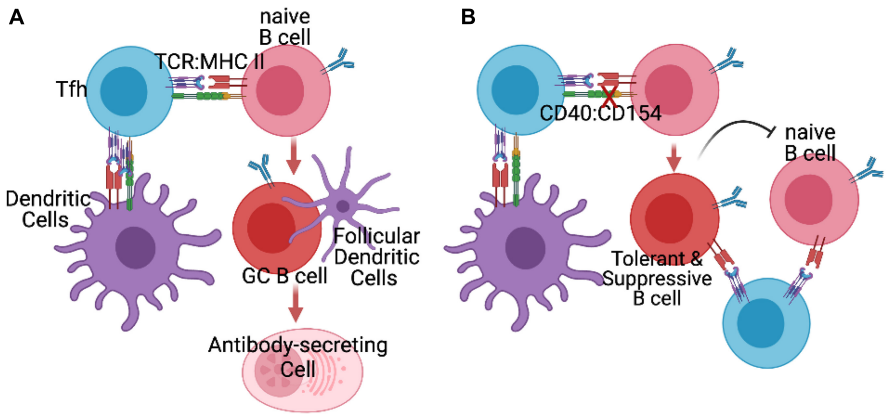

FIGURE 1 | (A) In untreated recipients of allografts, Tfh cells generated by initial encounter with recipient dendritic cells presenting alloantigen engage to provide help to naïve alloreactive B cells, and facilitate their differentiation into germinal center (GC) B cells and antibody-secreting plasmablasts and plasma cells. **(B)** In the presence of blocking anti-CD154 (X), naïve alloreactive B cells fail to receive signals from CD40 and thus differentiate into tolerance-induced B (TIB) cells incapable of differentiating into GC B cells or antibody-secreting cells. Instead these B cells acquire the ability to prevent naïve donor-specific B cells from differentiating into germinal center or antibody-secreting cells, by as yet to be defined mechanisms.

capable of mediating immune regulation *in vitro*. Although these B cells also expressed PD-L1, CD39/CD73 and TIM-1, as well as IL-10 and TGFß1, antagonism of TIGIT or granzyme B reduced their ability to suppress the production of IFNγ and IL-17 by anti-CD3/CD28-stimulated $CD4^+$ T cells. These observations suggest that the engagement of inhibitory receptors by ligands expressed on Breg cells may result in the suppression of $CD4^+$ T cell responses. Additionally, Breg cells were also able to inhibit monocyte-derived dendritic cell activation (CCR7, CD40, CD80/86, CD83, and ICOS-L) and their production of proinflammatory cytokines (IL-12A and IL-6) in response to LPS, thus highlighting a potential indirect mechanism of Breg-mediated control of T cell responses. Consistent with this possibility, Mohib et al. (2020) utilized 2-photon intravital microscopy to demonstrate cognate T:Breg cell interaction at the T:B border of spleens from immunized mice receiving antigen-pulsed Breg cells, and showed that a consequence of this T:Breg interaction is the reduced ability of the "regulated" T cells to interact with DCs. Whether Breg cells exert their regulation mainly within secondary lymphoid organs or are also able to migrate to sites of inflammation to modulate T cell responses, as has been reported for Tregs (Panduro et al., 2016; Campbell and Rudensky, 2020), remains to be clarified.

Immunoregulation of Donor-Specific B Cell Responses by TIB Cells

In models of transplantation tolerance, much of the focus has been on the ability of Bregs to control T cells responses, and indirectly, control T-dependent B cell responses. As a result, other than testing for donor-specific antibody, investigations into the fate of donor-specific B cells have been neglected. We have recently addressed this gap in knowledge by examining the fate of donor-MHC-specific B cells in a mouse model of cardiac allograft tolerance induced by anti-CD154 and donor spleen cells. We showed that donor-specific B cells become dysfunctional and are intrinsically unable to differentiate into germinal center B cells and plasma cells, even upon transfer into non-tolerant mice and exposed to donor-antigen (Khiew et al., 2020; **Figure 1**). The transferred TIB cells were able to sense antigen and respond by increasing expression of CD69 and Glut-1, and augmenting mitochondrial mass and cell size, but exhibited lower proliferation rates and expression of AID, consistent with a cell-intrinsic block after metabolic reprogramming but before entry into germinal centers.

Remarkably, when TIB cells were co-transferred with naïve B cells, they were able to inhibit the naïve B cell responses in a donor-specific manner, though it is not known whether this inhibition involves direct cognate B:B cell interaction or occurs through close by-stander effects as a result of cognate interaction with a donor-specific T cell (**Figure 1**). These observations raise the novel possibility that B cells, upon encounter with alloantigen while being deprived of CD40 signals, become reprogrammed away from memory and plasma cell differentiation into B cells capable of suppressing other naïve B cells; this suppression is associated with thwarted differentiation of naïve B cells into germinal center B cells. It is notable that these TIB cells retain their ability to stimulate naïve antigen-specific T cell expansion and differentiation into Tfh cells, underscoring that such tolerant B cells were not mediating their effects though the overt suppression of T cell responses, as described for Bregs. The mechanism of donor-specific suppression of B cells responses by this unique subset of TIB cells requires further delineation.

CONCLUSION

In addition to antibody secretion, B cells can play many roles in alloimmunity, including antigen presentation to T cells, and suppression of immune responses by Breg cells or donor-specific B cells differentiating into suppressor cells when encountering

alloantigens in the absence of CD40 costimulation. New results suggest that DSA production is not only regulated by the balance between Tfh and Tfr cells, but also by TIB cells that can inhibit *de novo* activation of naïve alloreactive B cells in a donor-specific manner. Moreover, Bregs may also control alloreactive T cell responses, thus potentially reinforcing transplantation tolerance. How Bregs may affect the function and ratio of Tfh and Tfr cells, and how Tfh/Tfr may impact Bregs in alloimmunity, as is being suggested in autoimmunity (reviewed in Ding et al., 2021), remains to be investigated. A better understanding of these interplays in animal models during primary alloimmune responses but also during memory alloimmune responses, as well as investigations into which of these mechanisms are conserved in humans, may help design immunosuppressive regimens that protect the transplants long term, and perhaps facilitate life-long transplantation tolerance.

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REVIEW
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Tissue Tregs and Maintenance of Tissue Homeostasis

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Regulatory T cells (Tregs) specifically expressing Forkhead box P3 (Foxp3) play roles in suppressing the immune response and maintaining immune homeostasis. After maturation in the thymus, Tregs leave the thymus and migrate to lymphoid tissues or non-lymphoid tissues. Increasing evidence indicates that Tregs with unique characteristics also have significant effects on non-lymphoid peripheral tissues. Tissue-resident Tregs, also called tissue Tregs, do not recirculate in the blood or lymphatics and attain a unique phenotype distinct from common Tregs in circulation. This review first summarizes the phenotype, function, and cytokine expression of these Tregs in visceral adipose tissue, skin, muscle, and other tissues. Then, how Tregs are generated, home, and are attracted to and remain resident in the tissue are discussed. Finally, how an increased understanding of these tissue Tregs might guide clinical treatment is discussed.

Keywords: regulatory T cells, tissue Tregs, transcription, immune homeostasis, immune maintenance

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INTRODUCTION

The immune system includes immune organs and their cells and molecules; it has two main immune responses: innate and adaptive immunity. Regulatory T cells (Tregs), usually referred to as $Foxp3^{+}CD4^{+}$ Tregs, are a unique type of immunosuppressive cell in the immune system. Tregs participate in regulating most immune responses and are significant in many physiological processes and diseases, such as immune tolerance, autoimmune diseases, and tumors. It is now clear that Tregs have a strong immune effect in immune tissues, and an effect in maintaining the homeostasis of non-immune tissues (Mueller and Mackay, 2015). The presence of Tregs in visceral adipose tissue (VAT) (Feuerer et al., 2009), muscle (Burzyn et al., 2013), and skin, and the expression and transcription in these cells are different from those in conventional Tregs (Burzyn et al., 2013). The establishment of a tissue-resident immune system makes it possible to respond more quickly to disturbances in local homeostasis during significant diseases, such as infection and foreign matter (Mueller and Mackay, 2015). Therefore, understanding the features of Tregs in tissues is crucial for

determining the regulatory mechanism of maintaining homeostasis and using this type of Treg for treatment (**Table 1**).

THE PHENOTYPES, FACTORS, AND FUNCTION OF TISSUE TREGS

VAT Tregs

Adipose tissue is mainly divided into subcutaneous adipose tissue and VAT (Rosen and Spiegelman, 2014). Adipose tissue in the viscera stores excess energy and affects the body's metabolism (Osborn and Olefsky, 2012). In addition to macrophages and neutrophils, Tregs also located in VAT, or more correctly, in the epididymal adipose depot, mediate cellular immune and metabolic networks, maintain the unique microenvironment of adipose tissue, and show different characteristics from those in lymphoid tissue (Rosen and Spiegelman, 2014).

Visceral adipose tissue Tregs are highly abundant in $CD4^+$ T cell compartments in lean mice; Tregs are located among adipocytes, usually in crown-like structures embracing macrophages (Feuerer et al., 2009). In C57BL/6 (B6) mice, Tregs in the spleen and lymph glands usually remain at approximately 5–15% of $CD4^+$ T cells (Feuerer et al., 2009), while VAT Tregs can reach a peak of 40–80% of $CD4^+$ T cells (Cipolletta et al., 2015). However, age-related $Foxp3^+CD4^+$ Treg accumulation does not happen in other white adipose tissues, because obesity-associated metabolic disorders and inflammation are usually not connected to these sites (Tran et al., 2008). Moreover, unlike lymphoid Tregs, which express a wide variety of T cell receptors (TCRs), VAT Tregs express more restricted TCR lineages (Kolodin et al., 2015). With limited TCR diversity in mice, the α amino acid sequence of the complementary determinant region (CDR3) from VAT Tregs is tautologically generated by various nucleotide sequences, indicating antigen selection (Feuerer et al., 2009). VAT Tregs identify one or more unknown peptide antigens that bind to major histocompatibility (MHC) II on the surfaces of VAT antigen-presenting cells, and this process is vital for their accumulation (Kolodin et al., 2015; Li et al., 2018). More importantly, compared to Tregs in immune organs, VAT Tregs obtain a unique transcriptome, which is involved in homeostasis and function (Feuerer et al., 2009; Cipolletta et al., 2015). Diverse genes have different regulatory roles in these Tregs and common Tregs, including those encoding transcription factors [peroxisome proliferator-activated receptor gamma (PPARγ)] (Cipolletta et al., 2012), chemokine and chemokine receptors [C-C motif chemokine receptor 2 (CCR2) and CCR4], cytokines, and cytokine receptors [interleukin (IL)-10 and IL-1 receptor-like 1 (ST2)] (Feuerer et al., 2009; Cipolletta et al., 2015; Han et al., 2015), as well as an interesting group related to lipid metabolism [low-density lipoprotein receptor(LDLR) and diacylglycerol *O*-acyltransferase (DGAT)] (Cipolletta et al., 2012; DiSpirito et al., 2018). And their increase might reflect additional local adaptation to the lipophilic, hypoxic adipose-tissue environment. Although there is overlap in gene expression between activated and VAT Tregs (cluster of differentiation 44 and nuclear receptor subfamily 4 group A member 1) to a certain degree, a large part of these characteristic genes is not simple activation-related genes (Li et al., 2018). Similar to Foxp3, PPARγ is also required to drive the specific phenotype of VAT Tregs and their accumulation, the activation of TCRs is a necessary and sufficient condition to induce PPARγ expression (Cipolletta et al., 2012; Li et al., 2018). Recent research shows that a $PPAR\gamma^{lo}$ Treg population in the spleen contains precursors of VAT Tregs and produces not only VAT Tregs but also other tissue Tregs, such as liver and skin (Li et al., 2021). The last feature of VAT Tregs is growth factor dependence, especially related to IL-33 and IL-33 receptor ST2 (Han et al., 2015; Kolodin et al., 2015). The homeostasis of VAT Tregs is highly dependent on the IL-33-ST2 axis; total blockade of IL-33 or ST2 results in a severe decrease in these Tregs, while Tregs in immune organs are not affected (Kolodin et al., 2015; Molofsky et al., 2015). In addition, IL-33, which induces the differentiation of Tregs *in vitro*, promotes Tregs accumulation by increasing the expansion and activation of Group 2 innate lymphoid cells (ILC2s) and the interaction of T cell costimulatory factor (ICOS) and ICOS ligand (Molofsky et al., 2015). VAT mesenchymal stromal cells (MSCs) balance immunocyte numbers by secreting IL-33, but they do not encode ST2. VAT Tregs and ILC2 express the highest levels of ST2, and, therefore, VAT Tregs are upregulated *via* MSCs and maintain IL-33-expressing stromal cells in a negative feedback loop (Spallanzani, 2021). Tregs have been reported to participate in tissue repair in many researches. These Tregs often express killer cell lectin-like receptor subfamily G1 (klrg1) and ST2 and are found in almost all non-lymphoid tissues. The transcription factor BATF can drive its phenotypic differentiation, which may be the central factor in inducing Tregs tissue repair (Delacher et al., 2020, 2021).

Visceral adipose tissue Tregs regulate the homeostasis of adipose tissue and metabolism; for mice before age 30–40 weeks, VAT Tregs have obvious insulin-sensitizing effects (Feuerer et al., 2009; Ilan et al., 2010; Eller et al., 2011). The transcription factor Zbtb7b suppresses the expression of the soluble form of ST2 (sST2) by inhibiting NF-κB signaling. Mechanistically, sST2 weakens IL-33 signaling and destroys Treg/ILC2 homeostasis in adipose tissue, thus aggravating obesity-associated insulin resistance in mice (Zhao et al., 2020). The PPARγ agonist pioglitazone (Pio) is a type of insulin sensitizers that prevents the loss of VAT Tregs in the course of obesity. The insulin-sensitizing effect of Pio is more than partly due to its function on VAT Tregs, as it is much less valid in mice fed a high-fat diet that lacks PPARγ (Cipolletta et al., 2012). Despite its accumulation, PPAR-γ is also involved in expressing other essential genes involved in Treg cell differentiation, including *Il2*, *Il10*, signal transducer and activator of transcription 5A, *Il33*, and *Il1rl1*, which are not affected by high-fat feeding (Van Herck et al., 2020). The regulation of VAT Tregs through IL-33 also supports the insulin sensitization of Tregs (Vasanthakumar et al., 2015). Although most studies have demonstrated that VAT Tregs enhance insulin sensitivity in lean mice less than 30–40 weeks, VAT Tregs might accelerate insulin resistance in lean mice more than 50 weeks old (Bapat et al., 2015). In this aging model, the mechanism by which VAT Tregs promote insulin resistance is unclear, although the transforming growth factor (TGF)-β pathway is involved (Bapat et al., 2015). The combination of the agonist Fat1562 (one

TABLE 1 | Location and functions of tissue Tregs.

Tissue	Location	Functions	References
Visceral adipose tissue	In crown-like structures embracing macrophages	Tregs proliferation and differentiation	Kolodin et al., 2015; Molofsky et al., 2015
		Lipid metabolism and glucose uptake	Feuerer et al., 2009; Kolodin et al., 2015; DiSpirito et al., 2018
		Insulin resistance and sensitivity	Cipolletta et al., 2012; Molofsky et al., 2015; Zhao et al., 2020
		Tissue repair	Kolodin et al., 2015; Molofsky et al., 2015; Delacher et al., 2021
		Anti-inflammation	Feuerer et al., 2009
Skin	Near the hair follicles	HF circulation and hair regeneration	Ali et al., 2017
		Immune tolerance	Yamazaki et al., 2014
		Skin maintenance and reparation	Malhotra et al., 2018; Harrison et al., 2019; Shime et al., 2020
		Stem cell differentiation	Nosbaum et al., 2016
		Antifibrosis	Kalekar et al., 2019
Skeletal muscle	In the connective tissue sheath	Tregs proliferation and maintenance	Kuswanto et al., 2016
		Muscle reparation	Burzyn et al., 2013
		Muscle regeneration	Villalta et al., 2014; Kuswanto et al., 2016
		Downregulate Tregs function	Schenk et al., 2011; Gazzerro et al., 2015
Brain	At the site of injury	Foxp3 regulation and proliferation	Gadani et al., 2015; Garg et al., 2019; Ito et al., 2019; Shi et al., 2021
		Tregs metabolism	Weinberg et al., 2019; Yue et al., 2019
		Tissue reparation and nerve recoveries	Guo and Luo, 2020
		Differentiation of oligodendrocyte precursor cells	Dombrowski et al., 2017
Placenta	In the decidua	Decrease in Tregs accumulation	Paolino et al., 2021
		Suppress Th1 and Th17 cells	Li et al., 2017; Zhang et al., 2018
		Inflammation	Li et al., 2017; Zhang et al., 2018

type of surrogate peptide that stimulates clones of VAT Tregs) and anti-tumor necrosis factor (TNF)-α significantly increases the number of VAT Tregs and improves insulin sensitivity in severely obese mice (Fernandes et al., 2020). Androgen also promotes the expansion of the stromal cell population, which produces IL-33, and then recruits Tregs through an inflammatory response (Habrylo and Rosenblum, 2020) depending on the B lymphocyte-induced maturation protein-1 transcription factor (Vasanthakumar et al., 2020). Compared with lymph node Tregs, VAT Tregs release more IL-10, decrease insulin resistance and express TNF-Rs, which may explain why VAT Tregs are more fragile (Feuerer et al., 2009). Moreover, VAT Tregs maintain the balance between anti-inflammatory and pro-inflammatory macrophages, promoting the differentiation of the former and inhibiting the latter (Lynch et al., 2015; Li et al., 2018). TNF-α has been shown to inhibit Glut4-mediated glucose uptake in adipocytes, and this effect can be reversed by IL-10. Since VAT Tregs release abundant IL-10 in adipose tissue, they can suppress inflammatory genes expression, block the downregulation of insulin-dependent cytokines, and reverse the down-regulation of the glucose transporter GLUT4 *via* TNF-α (Feuerer et al., 2009).

Skin Tregs

As the main barrier organ in close contact with the outside world, the skin is composed of the epidermis and dermis. There are some crosstalks between epithelial cells and immune cells, which can balance between anti-inflammatory and pro-inflammatory immune responses. In adult mouse skin, 20–60% of $CD4^+$ T cells are Tregs, and in adult human skin, they comprise about 20% (Scharschmidt et al., 2015). Most mouse Tregs are distributed near the hair follicles (HFs), as are human Tregs (Sanchez Rodriguez et al., 2014; Ali and Rosenblum, 2017). Research shows that mice with dysfunctional Tregs will die of fulminant systemic inflammation at a young age, with obvious dermatitis and hair loss, reflecting the importance of Tregs for inhibiting inflammation (Yang et al., 2015).

The skin Treg bank mainly consists Tregs expressing GATA3, neuropilin 1 (Nrp1), and Helios, which account for 80% of the total Tregs (Wohlfert et al., 2011; Delacher et al., 2017; Malhotra et al., 2018). Skin Tregs have several subsets: $Nrp1^{high}$, $CD39^+CD25^{high}$, $CD39^+$Granzyme B, or $CD39^+$CTLA-4. Tregs resident in the skin displayed high expression of CD25, CD39, and CCR5 (Ikebuchi et al., 2019). Compared with other immune cells, the Notch signaling ligand Jagged1 (Jag1) and mitochondrial protein arginase 2 (Arg2) are mainly expressed in skin Tregs (Ali et al., 2017; Lowe et al., 2019). Mice lacking GATA3 or RORα in Tregs develop type 2 skin inflammation spontaneously or after challenge (Malhotra et al., 2018; Harrison et al., 2019). In a study of patients with psoriasis, it is found that IL-21 up-regulates RORγt expression and down-regulates the expression of Foxp3, increases secretion of IL-17A and IL-22, and finally induces T-helper 17 (Th17) and Tregs imbalance

and promotes inflammation in psoriasis (Shi et al., 2019). Tregs can also promote the repair of skin trauma by the expression of epidermal growth factor receptor (EGFR) (Nosbaum et al., 2016) and are involved in stem cell differentiation in the skin. The lack of EGFR can lead to delayed wound closure and increase accumulation of pro-inflammatory macrophages (Nosbaum et al., 2016). In addition, the tolerance to resident microbiota in the skin depends on Tregs and is connected to the rapid influx of Tregs into the skin during neonatal development (Scharschmidt et al., 2015). In aseptic mice, neonatal skin Tregs decrease by 20% (Scharschmidt et al., 2017). The microbiota promotes the production of HFs derived CCL20, which is the ligand for CCR6 (the skin-homing receptor), promoting the migration of skin Tregs to neonatal skin. Additionally, the maintenance of $CD4^+$ and $CD8^+$ immune cells in mouse skin has been proven to be dependent on IL-7 derived from keratinocytes (Adachi et al., 2015). FuT7, as an enzyme that promotes the binding of E-selectin and P-selectin, is necessary for the optimal transport of Tregs to the skin, and their retention also seems to require IL-7 rather than IL-2 (Gratz et al., 2013). Thrombospondin-1 (TSP-1) served as the barrier preventing blood vessels into the dermis, is now considered an essential factor in inhibiting Th17 and Treg cell differentiation through the interaction with CD47. In mice, using a CD47-binding TSP-1 peptide increases Foxp3 expression and relieves clinical symptoms of Sjögren syndrome-associated dry eye (Rodriguez-Jimenez et al., 2019). In addition to specific cytokines, Treg cells also interact with other skin resident cells to maintain tissue homeostasis. Fibroblasts can preferentially induce CFSE-labeled purified $CD25^{hi}CD4^+$ T cells to proliferate in the skin in a contact-dependent manner (Clark and Kupper, 2007); skin Tregs also inhibit the activation of myofibroblasts, which might suppress excessive scar formation during wound healing (Boothby et al., 2020). Moreover, acute and chronic Treg depletion leads to fibrogenic myofibroblasts accumulation, the upregulation of fibrogenic genes, and the down-regulation of IL-10 production and anti-fibrogenic genes (Kalekar et al., 2019).

As mentioned, skin-resident Tregs are mainly distributed around HFs. HFs cycle between growth arrest (telogen) and activation (anagen), processes that are mediated by hair follicle stem cells (HFSCs) in the bulge region of the HF and Tregs have recently been shown to have effect on HF circulation and hair regeneration (Muller-Rover et al., 2001). In clinical trials, 80% of treated patients was observed successful hair regeneration, which may be linked to increased Tregs accumulation in the lesional scalp skin (Castela et al., 2014). Immunophenotypic analysis showed that the number and activation of Tregs in the skin were closely related to specific stages of the HF cycle (Ali et al., 2017). Tregs show a highly activated phenotype in telogen skin, whereas the spectrum-specific depletion of Tregs leads to a significant decrease in HF regeneration. In terms of mechanism, it has been found that the expression of Jag1 in Tregs promotes the HF cycle by improving the activation and differentiation of HFSCs (Ali and Rosenblum, 2017). Moreover, skin Tregs promote HFSC differentiation by controlling the local inflammatory environment, especially preventing CXCL5 mediated Th17 over secretion and neutrophil responses (Mathur et al., 2019). Ultraviolet B irradiation is a stimulus for the skin and results in many of $Nrp1^+$ Tregs, which is the main reason for skin immune tolerance (Yamazaki et al., 2014). Skin dendritic cells (DCs) have the unique ability to transform the inactive form of sunlight-derived vitamin D into its bioactive metabolite 1,25-(OH)2-D3. Vitamin D3-induced CD141 dermal DCs resident in human skin preferentially expands Tregs that inhibit skin inflammation *in vivo*. Ultraviolet B light can also expand skin Tregs expressing proenkephalin (PENK), an endogenous opioid precursor, and amphiregulin (Areg), thus supporting wound healing (Shime et al., 2020).

Skeletal Muscle Tregs

The basic components of skeletal muscle are myofibres, muscle progenitor cells, commonly known as satellite cells, and fibro/adipogenic progenitors (FAPs). Nerves and blood vessels are among the fibers, and they are bound together and surrounded by connective tissue. Leukocytes comprise a small component based on the histological observation of healthy skeletal muscle and have been neglected for a long time (Martinez et al., 2010; Villalta et al., 2014; Wang et al., 2015). Although there are various cell types, including $CD8^+$ cytotoxic T cells, Tregs, neutrophils, and eosinophils, each population accounts for only a small part of the total number of leukocytes in healthy muscle. Most of the white blood cells in the muscle are located in the connective tissue sheath around the whole muscle or near blood vessels (Brigitte et al., 2010). Similar to satellite cells, resident macrophages are inactive in stable conditions, but active when muscles are used or damaged, which is useful for regeneration (Krippendorf and Riley, 1993). Traditional Tregs also have great effects on muscle, the loss of which influences muscle repair and regeneration (Krippendorf and Riley, 1993).

The difference between skeletal muscle Tregs and lymphoid Tregs is mainly related to their representation, TCR spectrum, and transcriptome (Burzyn et al., 2013). $Foxp3^+CD4^+$ Tregs exist in muscle and expand quickly after mild freezing injury or severe injury caused by the injection of cardiac toxin, reaching 60% of $CD4^+$ T cell subsets. In MDX or dysferlin-knockout mouse models, $Foxp3^+CD4^+$ cell populations are also abundant in muscles, but not in lymphoid organs (Burzyn et al., 2013; Villalta et al., 2014). The proliferation of skeletal muscle DCs is a reaction to acute and chronic injury, and some reports speculate that these skeletal muscle Tregs may respond to local antigens (Burzyn et al., 2013; Kolodin et al., 2015). The clonal duplication of conventional T cells in damaged muscle is also observed, but there is a delay compared to Treg amplification. The expression of $Foxp3^+CD4^+$ Tregs in human and mouse immune organs increases with time (Lages et al., 2008); nonetheless, they are significantly poor in damaged skeletal muscles of old mice (Kuswanto et al., 2016). Data on malnutrition and aging muscle suggest that Tregs attempt to maintain skeletal muscle homeostasis (Burzyn et al., 2013; Villalta et al., 2014). The transcriptome of skeletal muscle Tregs is easily differentiated from that of common Tregs, because the expressions of their genes encoding transcription factors, chemokines and chemokine

receptors are different, such as *Ccr2*, *Il10*, and *Il1rl1* (Burzyn et al., 2013; Cipolletta et al., 2015).

The IL-33-ST2 axis is the crucial regulator of Tregs in skeletal muscle and VAT; the main cells producing high levels of IL-33 are one kind of FAPs in muscles (Kuswanto et al., 2016), which help accumulate Tregs during injury by promoting proliferation and reducing lymphatic outflow, but they do not induce T cell chemotaxis (Kuswanto et al., 2016). The *Il33* transcription level peaks within a few hours after muscle injury, and an increase in the number of IL-33$^+$ FAPs occurs later. Similarly, in aged mice, the levels of *Il33* transcripts and IL-33$^+$ FAPs are decreased; however, when replenishing IL-33, the number of Tregs increases and muscle regeneration is enhanced in aged mice (Kuswanto et al., 2016). Mouse skeletal muscle mesenchymal stromal cells (MmSCs) also link nerves, IL-33, and Tregs; MmSCs that produce IL-33 are not only structurally adjacent to the fiber nerve tracts and sensory neurons, but also encode nerve-related genes and affect Treg accumulation through calcitonin-gene-related peptide (Wang et al., 2020). IL-33 acts on Tregs containing the ST2 receptor encoded by the *Il1rl1* gene; compared to that of Tregs in lymphoid tissue, *Il1rl1* is one of the genes that is upregulated in Tregs isolated from damaged muscle. In ST2-deficient Tregs, *Il1rl1* accumulation is impaired, the clearance efficiency of muscle infiltration is reduced, and muscle regeneration is delayed (Kuswanto et al., 2016). Muscle Tregs express high levels of Areg and support muscle regeneration by acting directly on satellite cells. Areg treatment normalizes the evolution of the muscle transcriptome during muscle repair and promotes myogenic differentiation *in vitro* (Burzyn et al., 2013). Treg loss during muscle regeneration slows down repair, prolonging inflammation, and interfering with the expression of myogenic transcription factors, similar to that with the loss of F4/80$^+$ macrophages (Burzyn et al., 2013). These changes could be partly due to the interruption of macrophage phenotype regulation, which weakens the normal transformation of the macrophage phenotype from M1 to M2. The possible function of IL-10 (Villalta et al., 2014) is mainly due to its high level of expression in Tregs and its currently known effect on myeloid cells in regenerated muscle (Burzyn et al., 2013). Tregs are detected in the skeletal muscle of dystrophin-deficient mice, which is a model of human Duchenne muscular dystrophy (Burzyn et al., 2013; Villalta et al., 2014). In addition, ATP released by necrotic muscle fibers and inflammatory cells inhibits Tregs by activating purinergic P2X receptors; blocking the extracellular ATP-P2X purinergic signaling pathway results in an increase in the functions of Tregs in an inflammatory response and the progression of a malnutrition phenotype (Schenk et al., 2011; Gazzerro et al., 2015).

Brain Tregs

Regulatory T cells play an important role in regulating immune response in the central nervous system (CNS). Tregs deficiency is associated with increased disease progression in Alzheimer's disease, traumatic brain injury, and stroke (Machhi et al., 2020). In the study of experimental autoimmune encephalomyelitis (EAE), it was found that the depletion of Tregs aggravates the disease and prevents recovery (Koutrolos et al., 2014). Unlike other non-lymphoid tissue such as VAT, intestines, or skin, under steady-state conditions, there are virtually no Tregs in the CNS. However, in the event of trauma or inflammation in the CNS (such as hypoxia or stroke), Foxp3$^+$ Tregs will stay in the CNS for a long time and may even establish a resident Treg cell population (Korn et al., 2007; Dombrowski et al., 2017). By analyzing the Treg cells infiltrating the brain after acute cerebral ischemic injury, it was found that these Tregs encode high levels of IL-10, Areg, ST2, and PPARγ, so they seemed to be related to VAT and skeletal muscle Tregs at a transcriptional level (Garg et al., 2019; Ito et al., 2019).

Recent studies have shown that TCR/Irf4 signaling and NF-κB signaling are independent signals needed to establish the transcriptional program in effector Tregs (eTregs), and the transcriptional modifier Blimp1 is the main regulator (Cretney et al., 2011; Vasanthakumar et al., 2017; Rosenbaum et al., 2019). At the mechanistic level, Blimp1 indirectly controls Foxp3 by inhibiting the expression of the methyltransferase DNMT3A in Tregs in inflammation (Garg et al., 2019). The ablation of mitochondrial transcription factor A (TFAM) can lead to a change in the metabolic level in Tregs and decrease the activity of the demethylase Tet enzyme. Then, the demethylation state of conserved non-coding regions 2 (*Cns2*) cannot be maintained, ultimately affecting the stability of Tregs (Weinberg et al., 2019; Yue et al., 2019). In contrast, DNA methyltransferases (DNMT1 and DNMT3A) can methylate *Cns2* (or other CpG islands) to inhibit Foxp3 expression. Given the high expression of ST2 in brain Tregs, which is structurally expressed in the CNS, and that Tregs cannot be generally expanded in the damaged ischemic brain of IL-33/ST2-deficient mice, IL-33 might be an important molecule to replace IL-2 in mediating the survival of brain Tregs after inflammation (Gadani et al., 2015). It has been confirmed that IL-33 can increase the number of Tregs in ischemic brain tissue. The increased Tregs produce Areg and activate EGFR in neurons, which is helpful to improve the prognosis (Guo and Luo, 2020). Another possible influencer of Tregs is the neurotransmitter 5-hydroxytryptamine (5-HT). Its receptor 5-HT7 specifically upregulates Tregs gathered in the ischemic brain. Studies have shown that 5-HT or the inhibition of its uptake can increase the number of Tregs in the brain (Ito et al., 2019). In the toxic demyelination model, Tregs have been shown to support the regeneration of myelin *via* promoting the differentiation of oligodendrocyte precursor cells in the brain by CCN3 (a growth regulatory protein) (Dombrowski et al., 2017). Additionally, brain Tregs upregulate certain CNS-specific genes, such as neuropeptide Y (*NPY*), *PENK*, 5-HT7, and arginine vasopressin receptor (*AVPR1A*) (Ito et al., 2019). Particularly, the increased expression of the EGFR ligand modulin in Tregs recruited by the CNS seems vital for processes associated with nerve recoveries, such as the inhibition of astrocyte proliferation, neuron dysfunction, and neurotoxic gene expression (Ito et al., 2019). The latest research reports that Treg-derived osteopontin plays a role through the integrin receptors on microglia, enhances the repair activity of microglia, thus promotes the formation of oligodendrocytes and the repair of white matter. After a stroke, increasing the number of Tregs by delivering an IL-2:IL-2 antibody complex can improve the

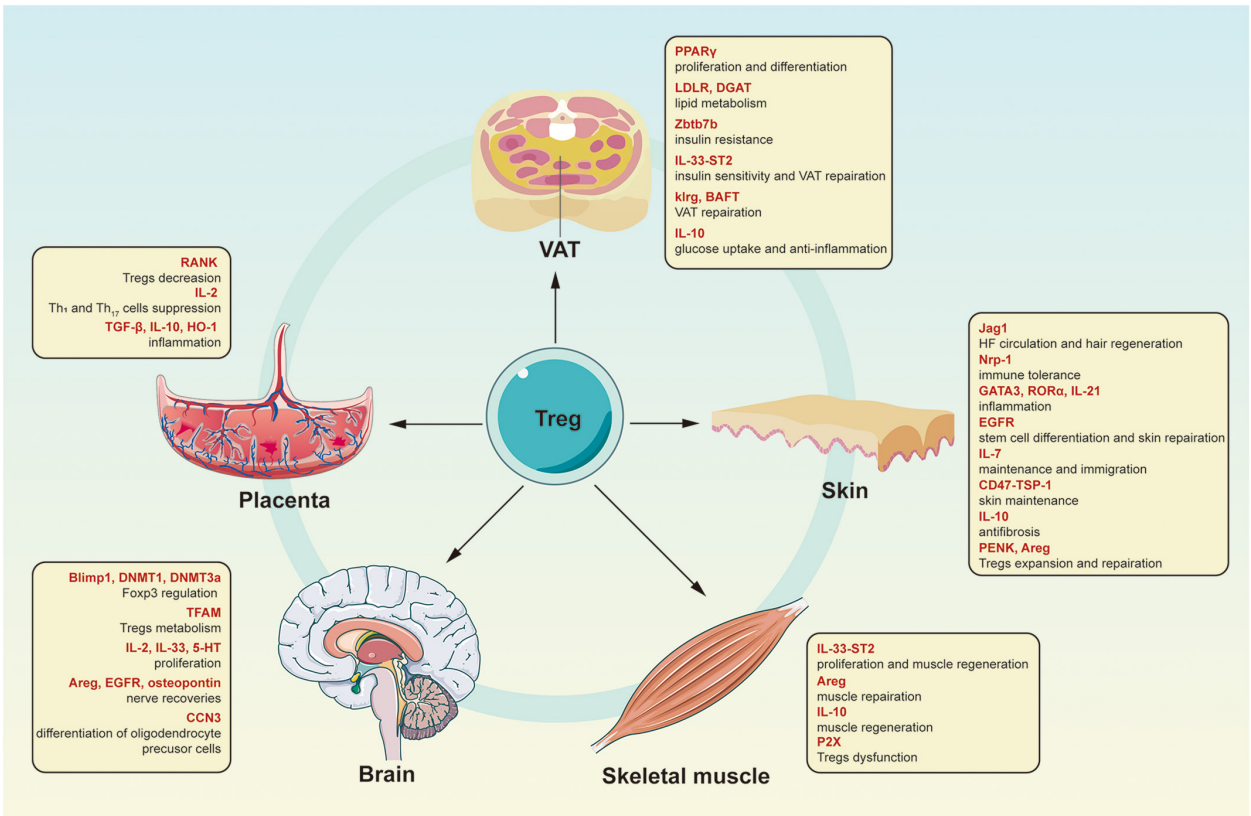

FIGURE 1 | Characteristics of different kinds of tissue Tregs. Tregs are not only critical for affecting immune response, but also for maintaining non-lymphoid tissues homeostasis *via* different cytokines or interacting with other tissue cells. Tregs, regulatory T cells; PPARγ, peroxisome proliferator-activated receptor gamma; LDLR, low-density lipoprotein receptor; DGAT, diacylglycerol *O*-acyltransferase; Zbtb7b, Zinc finger and BTB domain-containing protein 7B; IL-33, interleukin-33; ST2, IL-1 receptor-like 1; klrg1, killer cell lectin-like receptor subfamily G1; BATF, basic leucine zipper ATF-like transcription factor; IL-10, interleukin-10; Jag1, the Notch signaling ligand Jagged1; Nrp1, neuropilin 1; GATA3, GATA binding protein 3; RORα, retinoic acid-related orphan receptor alpha; IL-21, interleukin-21; EGFR, epidermal growth factor receptor; IL-7, interleukin-7; TSP-1, thrombospondin-1; PENK, proenkephalin; Areg, amphiregulin; P2X, purinergic receptor P2X; Blimp1, B lymphocyte-induced maturation protein 1; DNMT1, DNA methyltransferases 1; DNMT3a, DNA methyltransferases 3 alpha; TFAM, mitochondrial transcription factor A; IL-2, interleukin-2; 5-HT, 5-hydroxytryptamine; CCN3, cellular communication network factor 3; RANK, NF-κB ligand; TGF-β, transforming growth factor beta; HO-1, heme oxygenase 1.

integrity of white matter and save neurological function for a long time (Shi et al., 2021).

Other Tissues

Tissue Tregs located in the VAT, skin, and skeletal muscle are the most characteristic Treg communities; however, other tissues also contain Tregs (**Figure 1**). A special Treg population exists in the placenta; Tregs in the decidua of mouse placenta express alloantigens from their father, dependent on *Cns1* (Samstein et al., 2012). When pregnant with offspring of allogeneic (not syngeneic) males, female mice with *Cns1* extension lacking Foxp3 show increased fetal absorption and placental immune cell infiltration (Rowe et al., 2012). At the same time, Tregs in the human placenta seem to be essential to control inflammation in early pregnancy and establish an acceptable decidual environment through its anti-inflammatory effect (Erlebacher, 2013). According to former researches, there are now three distinct decidual $CD4^+$ Treg types in healthy pregnancies with a regulatory phenotype and the ability to suppress T cell responses: $CD25^{hi}Foxp3^+$, $PD1^{hi}IL\text{-}10^+$, and $TIGIT^+Foxp3^{dim}$. The loss of NF-κB ligand (RANK) in the thymus epithelium of mice leads to a decrease in Treg accumulation in the placenta, increasing the possibility of abortion (Paolino et al., 2021). During an absence of Tregs, several crucial biological processes related to embryonic development and cell metabolism are out of balance, which may cause fetal growth disorders and premature delivery in the third trimester of pregnancy (Gomez-Lopez et al., 2020). Tregs suppress the activation and function of T-helper 1 (Th1) and Th17 cells through sequestering IL-2 and other inhibitory mechanisms, and control inflammation by releasing TGF-β, IL-10, and heme oxygenase 1 (HO-1) by interacting with DCs and uterine natural killer cells (Li et al., 2017; Zhang et al., 2018). For example, the activation of invariant natural killer T (iNKT) cells decreased the frequency of decidual Tregs, the production of IL-10 and TGF-β, and suppressive Tregs activity, indicating that iNKT cells may have a role in inflammatory pregnancy loss *via* suppression of decidual Tregs function. The ability of Tregs to transform into T effectors in infection, severe

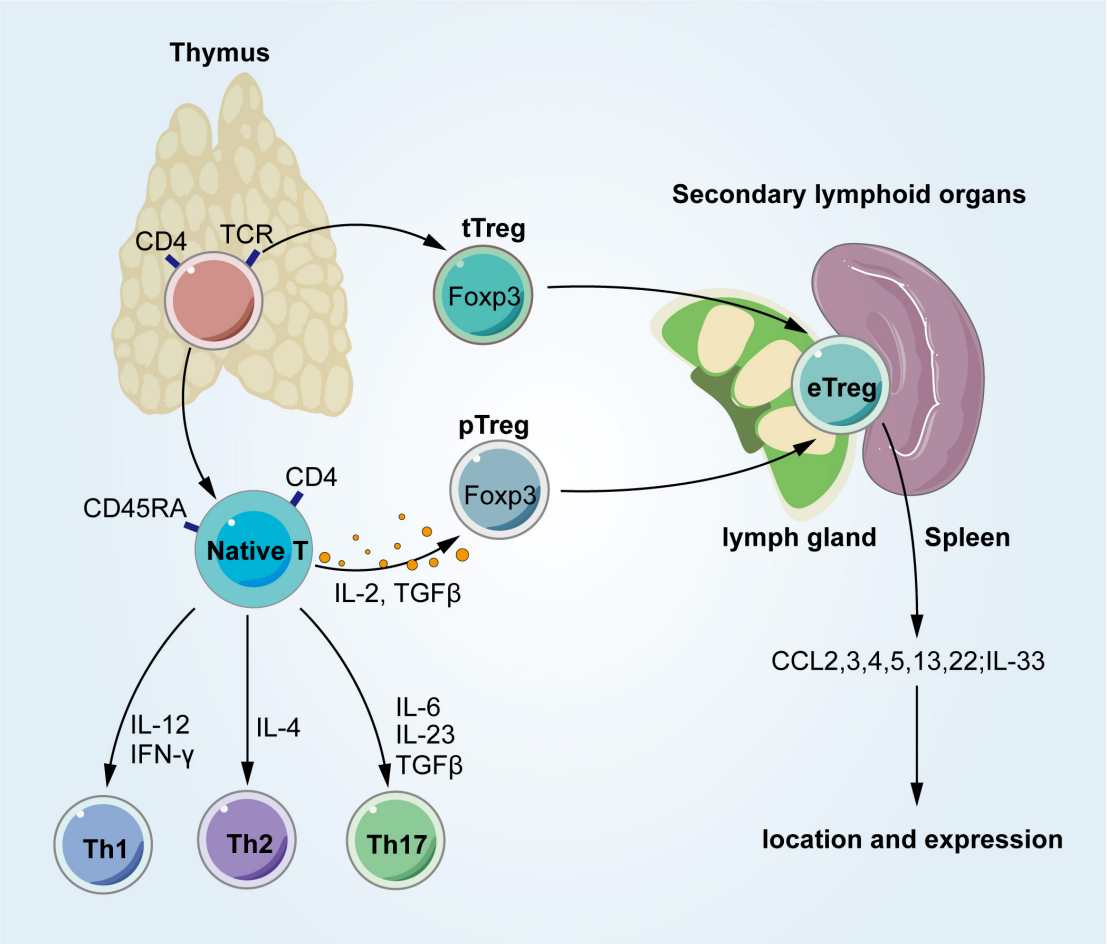

FIGURE 2 | Occurrence and development of tissue Tregs. Derived from the normal thymus, Tregs have two different ways to develop. In the thymus, $CD4^+$ thymocytes turn into tTregs by TCRs. In periphery, Tregs occur with IL-2 and TGF-β. These Tregs stay in SLO, and then migrate into tissues once being stimulated. Tregs, regulatory T cells; tTregs, thymic Tregs; pTregs, peripheral Tregs; TCR, T cell receptors; Th1, T-helper 1; Th2, T-helper 2; Th17, T-helper 17; IFN-γ, interferon-gamma; TGF-β, transforming growth factor beta; IL-12, interleukin-12; IL-4, interleukin-4; IL-6, interleukin-6; IL-2, interleukin-2; IL-23, interleukin-23; CCL, chemokine (C-C motif) ligand.

inflammation, or interruption of fetal development confers the ability to terminate the pregnancy and ensures maternal survival (D'Addio et al., 2011).

OCCURRENCE AND DEVELOPMENT OF TISSUE TREGS

Regulatory T cells develop in the thymus, where there are two different pathways (Josefowicz et al., 2012). Thymic Tregs (tTregs) differentiate into $Foxp3^+$ Tregs in the thymus after TCR-recognized autoantigen binding. In contrast, peripheral Tregs (pTregs) stay away from the thymus as naive $CD4^+$ T cells and differentiate into $Foxp3^+$ Tregs in secondary lymphoid organs (SLOs) after recognizing their homologous antigens (**Figure 2**). Although it is not a strict criterion for differentiation, Helios and the membrane protein Nrp1 expressed in tTregs (not pTregs) may be used to distinguish these Treg subsets (Nutsch et al., 2016). Tregs with activated phenotypes, such as ICOS, glucocorticoid-induced TNFR family related, and IL-10, exist in SLOs and are called effector Tregs (eTregs) (Cretney et al., 2013). Tregs respond to tissue homing signals and then migrate to tissues after stimulating TCRs in SLOs (Wei et al., 2006). eTregs are linked to phenotypic specialization and enhanced migration to tissues, which might represent the intermediate developmental stage of Tregs from the thymus to tissues. Several tissue features have been identified in SLO Tregs, such as a few VAT Treg signatures in spleen Tregs (Vasanthakumar et al., 2015; Li et al., 2018). SLO Tregs also increase the expression of transcription factors related to effector T cells, such as T-box expressed in T cells (T-bet), GATA3, STAT3, and RoRγt (Wang et al., 2011; Wohlfert et al., 2011; Cretney et al., 2013; Schiering et al., 2014; Ohnmacht et al., 2015; Sefik et al., 2015). Although studies using parabiosis show that Tregs are not easily recirculated, they may be continuously supplemented from circulating precursor cells, such as naive Tregs

(Lynch et al., 2015). And regardless of its origin, the tissue tree must reach the appropriate target tissue and survive. A model has been proposed for the development of Tregs that reside in the VAT, colon, and damaged muscle, and has been shown to localize and expand in tissues through a process dependent on IL-33 (Cipolletta et al., 2012; Schiering et al., 2014; Kolodin et al., 2015; Vasanthakumar et al., 2015; Kuswanto et al., 2016). Data show that several components have effects, such as a TCR-MHCII peptide, chemokine-chemokine receptors, and cytokine-cytokine receptor interactions. One of these is G protein-coupled receptor 15, which directs Tregs to the colon lamina propria (Kim et al., 2013). Some of these tissue Treg activities regulate inflammatory cells nearby (Feuerer et al., 2009; Cretney et al., 2013), and they also directly affect the surrounding parenchymal cells (Feuerer et al., 2009; Burzyn et al., 2013; Schiering et al., 2014; Arpaia et al., 2015). Some of the different transcripts in tissue Tregs are dedicated to controlling the underlying pathological process of their release in non-immunologic host tissue cells (Luck et al., 2015).

CONCLUDING REMARKS

Regulatory T cells are essential for maintaining tissue homeostasis. Although existing studies have shown Treg mechanisms in related tissues, the antigenic reactivity and metabolic adaptability of Tregs should be further studied, and whether there are corresponding Tregs in other tissues is also worth exploring. Since tissue Tregs have similar transcription factors with other tissue cells, studying the interaction between them (such as the neuron population) will further promote the understanding of the role of tissue Tregs. Using emerging technologies to further reveal new phenotypes and functions of tissue Tregs provides better guidance and direction for the treatment of the increasing number of chronic tissue diseases and immune deficiency diseases.

AUTHOR CONTRIBUTIONS

QS and JG conceived the manuscript. QS wrote the manuscript, drew the figures and tables, and was a major contributor in writing. JG and JZ contributed to the organization, suggestions on the content, and editing of the manuscript. QW, XL, ZD, and LL revised the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Phenotypic and Functional Diversity in Regulatory T Cells

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The concept that a subset of T cells exists that specifically suppresses immune responses was originally proposed over 50 years ago. It then took the next 30 years to solidify the concept of regulatory T cells (Tregs) into the paradigm we understand today – namely a subset of CD4+ FOXP3+ T-cells that are critical for controlling immune responses to self and commensal or environmental antigens that also play key roles in promoting tissue homeostasis and repair. Expression of the transcription factor FOXP3 is a defining feature of Tregs, while the cytokine IL2 is necessary for robust Treg development and function. While our initial conception of Tregs was as a monomorphic lineage required to suppress all types of immune responses, recent work has demonstrated extensive phenotypic and functional diversity within the Treg population. In this review we address the ontogeny, phenotype, and function of the large number of distinct effector Treg subsets that have been defined over the last 15 years.

Keywords: regulatory T cells, autoimmunity, differentiation, fat, brain, skin, muscle

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THYMIC AND PERIPHERAL TREGS

Tregs develop via two different pathways: differentiation in the thymus from CD4+ thymocytes, or conversion of appropriately stimulated naïve CD4+ T cells in peripheral organs (Josefowicz et al., 2012a). Thymus-derived Tregs (tTregs) typically arise from self-reactive thymocytes. Thymocytes that interact more strongly with self-antigens differentiate into Treg progenitors characterized by increased expression of the TNF receptor superfamily members GITR, OX40 and TNFR2. Increased expression of these TNFR superfamily members renders these Treg progenitors more sensitive to IL-2, a cytokine required for optimal differentiation of mature Tregs in the thymus (Mahmud et al., 2014). In the thymus, one can already see evidence of distinct Treg subsets. Specifically, thymocytes that receive stronger signals via their TCR preferentially differentiate into CD25+ FOXP3- Treg progenitors, while thymocytes that receive somewhat weaker signals preferentially differentiate into CD25-FOXP3^{lo} Treg progenitors. The development of these two distinct Treg progenitors involves distinct signaling pathways and give rise to mature Tregs that have distinct roles in preventing autoimmunity (Owen et al., 2019). Thus, even at the earliest stages of Treg development one can see evidence for Treg diversification.

Peripheral Tregs (pTregs) differentiate from naïve CD4+ T-cells following their egress from the thymus. As pTregs differentiate in response to recognition of non-self-antigens, they are abundant at mucosal surfaces, which are exposed to commensal microorganisms, food, and environmental antigens (Josefowicz et al., 2012b). Mice lacking pTregs develop inflammation and dysbiosis in the gut and lungs (Josefowicz et al., 2012b; Campbell et al., 2018), demonstrating the importance

of this subset for maintaining tolerance at mucosal surfaces. TCR stimulation in the context of signaling by TGF-β and retinoic acids promote pTreg development (Coombes et al., 2007; Yadav et al., 2013). The cell surface receptor NRP1 and the transcription factor HELIOS have both been used to discriminate thymic-derived from peripherally induced Tregs (Thornton et al., 2010; Weiss et al., 2012; Yadav et al., 2012), but this is somewhat controversial (Akimova et al., 2011; Szurek et al., 2015) and may only be true under steady-state conditions in the absence of inflammation (Weiss et al., 2012; Yadav et al., 2012). Regardless of their initial origin, HELIOS-negative Tregs appear to be somewhat less stable with a greater propensity to secrete inflammatory cytokines (Bin Dhuban et al., 2015). Analysis of tTreg and pTreg TCR repertoires demonstrate minimal overlap suggesting that these subsets recognize distinct antigens (Lathrop et al., 2011; Yadav et al., 2013). Both peripheral and thymically induced Tregs have been shown to play complementary roles in preventing autoimmunity (Haribhai et al., 2011). This further extends the concept of specialized roles for distinct Treg subsets.

CENTRAL AND EFFECTOR TREGS

It is well known that conventional T cells undergo further differentiation in peripheral lymphoid organs upon antigen encounter and a variety of distinct effector T cell states have been characterized (Zhu et al., 2010). A similar concept was quickly established for Tregs as well. Tregs can be broadly divided into two subsets: central Tregs (cTregs) and effector Tregs (eTregs) based on their distinct anatomical locations and expression of activation-induced markers (**Figure 1**). cTregs are found primarily in secondary lymphoid organs and express high levels of the lymphoid homing markers, S1PR1, CD62L and CCR7 (Smigiel et al., 2014) and low levels of CD44, a marker of TCR activation. Additionally, cTregs are distinguished by high levels of the transcription factors, TCF1, SATB1, and BACH2 (Zemmour et al., 2018; Miragaia et al., 2019). Signaling through the IL-2R is essential for cTreg survival and maintenance (Malek et al., 2002; Fontenot et al., 2005; Burchill et al., 2007; Soper et al., 2007; Vang et al., 2008). Following TCR activation, cTregs can differentiate into eTregs; this process involves upregulation of activation induced markers, CD44, ICOS, PD-1, HELIOS, and 4-1BB (Miragaia et al., 2019), and concurrent downregulation of lymphoid homing proteins in favor of expression of chemokine receptors and adhesion molecules that enable Treg entry into, and accumulation in, non-lymphoid tissues. eTregs can be found in secondary lymphoid organs and exist in non-lymphoid tissues as tissue Tregs. In contrast to cTreg dependence on IL2, eTreg expression of ICOS is critical for their maintenance (Smigiel et al., 2014). Although TCR signaling is required for the differentiation of cTregs into eTregs (Levine et al., 2014), experiments using Nur77-GFP mice have demonstrated that cTregs express high levels of Nur77 similar to eTregs (Levine et al., 2014; Smigiel et al., 2014). This suggests that cTregs regularly encounter antigen but are precluded from conversion to eTregs. Two recent studies demonstrated that the transcription factor, BACH2, restrains the conversion of cTregs to eTregs by competing with IRF4 for DNA-binding sites and thereby repressing expression of genes involved in eTreg differentiation (Grant et al., 2020; Sidwell et al., 2020). The transcription factors IRF4, BATF, and JunB are expressed highly by eTregs and are critical for their differentiation and maintenance (Cretney et al., 2011; Delacher et al., 2017, 2020; Hayatsu et al., 2017; Koizumi et al., 2018). Specifically, mice with IRF4-deficient Tregs show multi-organ autoimmunity mediated by uncontrolled Th2 immune responses (Zheng et al., 2009). JunB facilitates the binding of IRF4 to a subset of its transcriptional target sites, like CTLA4 and ICOS, and Treg-specific deficiency in JunB results in multiorgan autoimmunity (Koizumi et al., 2018). BATF deficiency causes a reduction in tissue Tregs and their precursor effector Treg populations in the spleen (Delacher et al., 2017, 2020). ChIP-seq studies show that IRF4, BATF, and JunB colocalize near genes upregulated in eTregs, suggesting cooperative regulation of these genes (Koizumi et al., 2018). Another transcription factor, BLIMP-1, cooperates with IRF4 to establish the Treg effector program and promote expression of IL-10 in tissue Tregs (Cretney et al., 2011). In the absence of BLIMP-1, uncontrolled DNMT3A activity leads to increased methylation of the *Foxp3* locus and loss of suppressive activity (Garg et al., 2019). Single-cell ATAC- and single-cell RNA-sequencing of Tregs from lymphoid and non-lymphoid tissues has shown that Tregs undergo progressive, step-wise alterations in chromatin accessibility and gene expression to acquire a tissue Treg phenotype (DiSpirito et al., 2018; Miragaia et al., 2019; Delacher et al., 2020).

TREG SUBSETS PARALLEL T HELPER CELL SUBSETS

In 1986 Mossman and Coffman proposed that *in vitro* activated T helper cells could be driven to differentiate into two different subsets (Th1 versus Th2) characterized by distinct cytokine expression profiles; this was subsequently expanded to include Th9, Th17, and Tfh (and potentially other) subsets (Mosmann et al., 2005). Importantly, specific transcription factors including T-BET (Th1), GATA3 (Th2), RORγt (Th17), and BCL6 (Tfh) appeared to drive these distinct helper T developmental programs. Subsequently, multiple studies demonstrated that Tregs appeared to differentiate into parallel sets of cells (albeit with different functions from their effector counterparts), distinguished by the same sets of transcription factors (**Figure 1**). The plasticity of Tregs is controlled by cytokines, metabolism, and dietary factors. For example, NaCL has been shown to limit Treg suppressive function and drive them to adopt some Th17-like characteristics (Hernandez et al., 2015; Luo et al., 2019; Yang et al., 2020). The role of metabolic programming in shaping Treg functional plasticity was recently reviewed by Shi and Chi (2019) while the role of dietary factors involved in Treg function was recently reviewed by Arroyo Hornero et al. (2020).

Th1 Tregs

Tregs expressing TBET were the first major Treg subset identified that appeared to parallel the T-helper differentiation paradigm. Koch et al. (2009) identified a population of

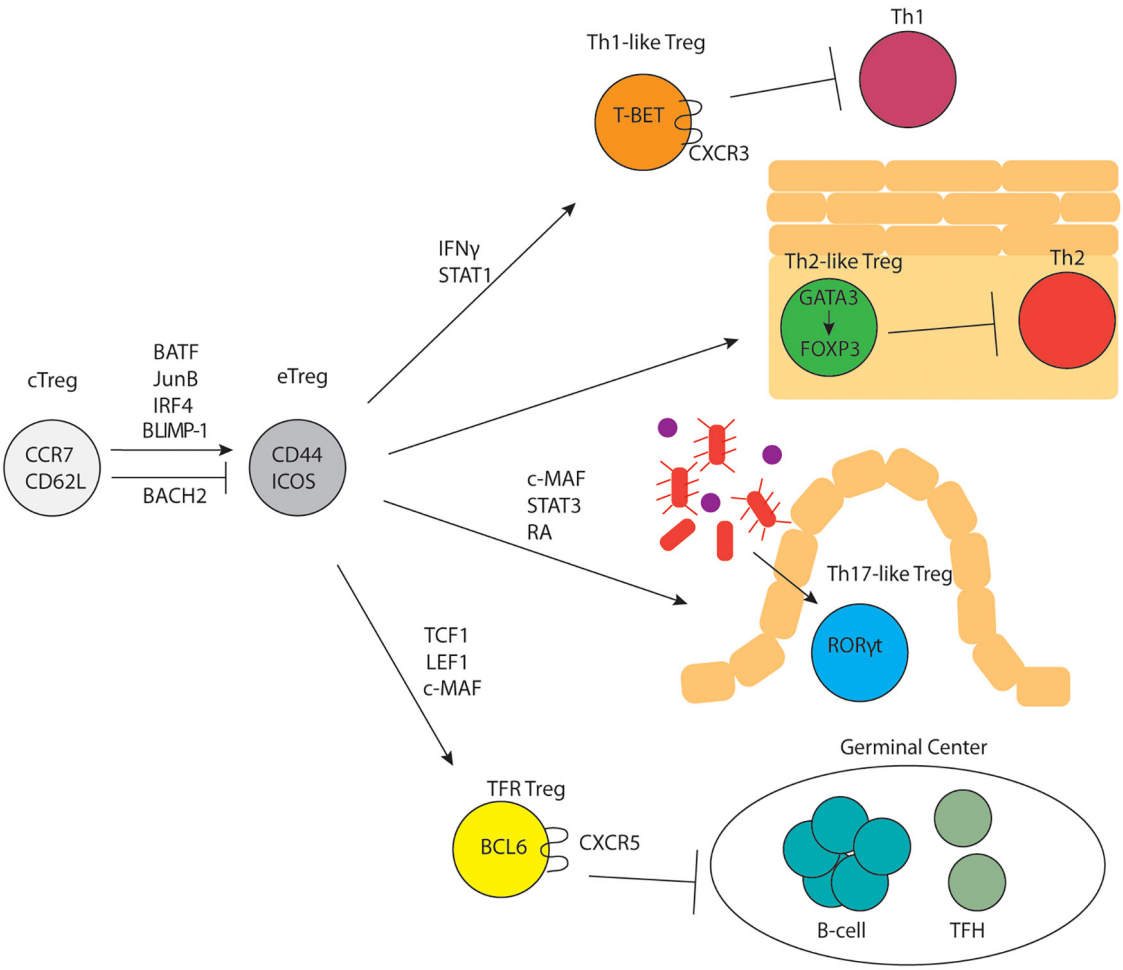

FIGURE 1 | Treg subset differentiation. cTregs differentiate into eTregs in response to TCR signaling and upregulation of specific transcription factors. eTregs undergo differentiation into subsets that parallel CD4+ T-helper cell subsets and control distinct immune responses.

TBET-expressing Tregs in the spleen that expanded during Th1-mediated inflammation (type I inflammation). Similar to Th1 cells, upregulation of TBET in Tregs is dependent on the IFNγ receptor and STAT1. TBET-expressing Tregs are found throughout lymphoid and non-lymphoid tissues and are a stable lineage that maintain TBET expression in perpetuity. TBET-deficient Tregs are unable to expand and control Th1-mediated inflammation due in part to their inability to upregulate the chemokine receptor, CXCR3 (Koch et al., 2009). In support of this idea, deletion of *Foxp3* in TBET-expressing cells or induced deletion of TBET-expressing Tregs leads to uncontrolled Th1 immune responses at steady state (Levine et al., 2017). These results indicated that TBET+ Tregs play a critical role in controlling type I inflammation.

In Th1 cells, TBET promotes the expression of the effector cytokine, IFNγ, which is important for facilitating the type I immune response. Normally, TBET+ Tregs do not complete full Th1 differentiation and do not express IFNγ due to delayed expression of IL12Rβ (Koch et al., 2012). However, IFNγ-producing Tregs have been observed in several pathogenic settings. For example, during highly virulent infection with T. gondii, TBET+ Tregs acquired an effector phenotype and expressed IFNγ (Oldenhove et al., 2009). Tregs that produce IFNγ have been reported in inflammatory diseases including multiple sclerosis (Dominguez-Villar et al., 2011) and type I diabetes (McClymont et al., 2011). In a mouse model of melanoma, intratumoral Nrp1-deficient Tregs produced IFNγ, which enhanced anti-tumor immunity (Overacre-Delgoffe et al., 2017). Mechanisms involved in the development of Th1 Tregs and IFNγ-expressing Tregs were recently reviewed by Kitz and Dominguez-Villar (2017). The functional consequences of IFNγ production by Tregs are not well understood. However, production of Th1 cytokines by Tregs during inflammation is evidence of their phenotypic plasticity in response to environmental cues.

Th2 Tregs

Similar to TBET-expressing Th1 Tregs, another population of Tregs was identified that expresses the canonical Th2 master transcription factor, GATA3. Although detectable in a

number of lymphoid and non-lymphoid tissues, GATA3+ Tregs were initially found at barrier and mucosal surfaces including the skin, gut, and lungs (Wohlfert et al., 2011; Harrison et al., 2019). Initial studies demonstrated that mice with Treg-specific deficiency in IRF4, a transcription factor that is necessary for Th2 differentiation, developed uncontrolled Th2-mediated autoimmunity (Zheng et al., 2009). Subsequent studies determined that GATA3 expression is required for Treg stability and maintenance of *Foxp3* expression (Wang et al., 2011). Mice with Treg-specific deletion of GATA3 develop uncontrolled Th2 immune responses in the skin (Harrison et al., 2019). and a systemic inflammatory disorder later in life characterized by increased IFNγ, IL-4, and IL-17 production by effector T-cells (Wang et al., 2011). Thus, while TBET is required for suppressing Th1 responses, GATA3 appears to be more broadly important for Treg function. In support of this, *Foxp3* expression is reduced in GATA3-deficient Tregs (Wang et al., 2011). Co-immunoprecipitation experiments showed that FOXP3 and GATA3 interact in Tregs. Further, ChIP-qPCR demonstrated that FOXP3 binds to the *Gata3* locus and, conversely, GATA3 binds to the *Foxp3* locus (Rudra et al., 2012). This suggests that FOXP3 and GATA3 act as a complex to regulate gene targets and reciprocally regulate each other's expression.

Recent single-cell RNA-seq experiments have demonstrated the importance of GATA3 in the differentiation of tissue Tregs at multiple sites (Delacher et al., 2017, 2020; Miragaia et al., 2019). GATA3-defined populations of non-lymphoid tissue Tregs are present in the skin, VAT, colon, lung, and liver. In the colon, the IL33R (ST2) is expressed primarily by GATA3-expressing Tregs (Schiering et al., 2014). GATA3 is also expressed in late tissue Treg precursor cells in the spleen. This population co-expresses several type 2 associated genes including *Il1rl1* (IL33R/ST2), *Areg*, *Il10*, and *Irf4*, indicating a role for Th2 programming in the differentiation of a subset of tissue Tregs.

Similar to IFNγ producing Th1 Tregs, there have been reports of pathogenic reprogramming of Th2 Tregs in food allergy (Noval Rivas et al., 2015). Mice bearing a gain-of-function *Il4ra* allele were sensitized and subsequently re-challenged with oral OVA, leading to severe anaphylaxis associated with the development of Tregs expressing IL-4, a Th2-associated cytokine driven by GATA3. In this model, an increase in GATA3+ Tregs was also observed upon allergen challenge. However, the authors did not directly address co-expression of IL-4 and GATA3. Deletion of IL-4 and IL-13 in Tregs protected mice from allergic anaphylaxis, indicating that Treg-produced IL-4 contributed to disease. Thus, while at steady state, Th2 Tregs do not generally produce Th2 cytokines, this has been observed under inflammatory conditions.

Th17 Tregs

Tregs expressing the canonical Th17 transcription factor, RORγt, are highly prevalent in the gut and are important for controlling Th17-mediated immune responses. RORγt+ Tregs constitute approximately 15–30% of Tregs in the small intestine lamina propria and 40–80% in the colonic lamina propria (Sefik et al., 2015; Kim et al., 2016; Yang et al., 2016). RORγt+ Tregs are primarily pTregs induced by commensal microbiota (Kim et al., 2016). However, there is evidence that RORγt+ tTregs are also induced following immunization with the self-protein, myelin oligodendrocyte glycoprotein (MOG) (Kim et al., 2017). In the gut, retinoic acid and the transcription factor c-MAF are critical for the differentiation of RORγt+ Tregs (Ohnmacht et al., 2015; Wheaton et al., 2017; Xu et al., 2018). Initial evidence for the importance of RORγt+ Tregs in controlling Th17 responses came from mice with Treg-specific deficiency in STAT3, a transcription factor critical for expression of RORγt and Th17 differentiation; these mice developed uncontrolled Th17 responses and associated colitis (Chaudhry et al., 2009). In support of this, mice with Treg-specific deletion in RORγt were also more susceptible to colitis (Sefik et al., 2015). In contrast, a separate study demonstrated that mice with RORγt-deficient Tregs were unable to regulate Th2-mediated immune responses in the gut (Ohnmacht et al., 2015). The distinct phenotypes identified in mice with RORγt-deficient Tregs may be attributed to differences in microbiota composition and the anatomical location within the intestine from which cells were harvested and analyzed.

Despite co-opting Th17 features of Th17 programming, Tregs generally do not secrete Th17 cytokines at steady state. However, production of IL-17 by Tregs has been observed in mouse models of asthma and chronic arthritis, and in humans with psoriasis and Crohn's disease (Bovenschen et al., 2011; Hovhannisyan et al., 2011; Komatsu et al., 2014; Sanchez Rodriguez et al., 2014; Massoud et al., 2016). Overexpression of the transcription factor, ID2, has been shown to promote Treg instability and expression of Th17 cytokines upon induction of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (Hwang et al., 2018). The tumor necrosis factor receptor super family (TNFRSF) members, CD27 and OX40, synergize to prevent aberrant expression of IL-17 in Tregs. Mice with CD27- and OX40-deficient Tregs were infected cutaneously with *C. albicans* to stimulate Th17-mediated skin inflammation. In this model, the double knockout Tregs expressed elevated levels RORγt and IL-17 suggestive of uncontrolled Th17-differentiation (Remedios et al., 2018). Thus, this study demonstrated that cell intrinsic expression of CD27 and OX40 by Tregs is required to limit Treg production of IL-17. Expression of the ST2/IL-33R on Tregs is also important for maintenance of Treg stability and resistance to acquisition of a Th17-like phenotype, including expression of RORγt and IL-17. Conversely, Treg expression of the IL-1R promotes instability and a Th17-like phenotype (Alvarez et al., 2019, 2020; Piccirillo, 2020). These studies demonstrated factors that influence Treg conversion to a Th17-like phenotype.

T Follicular Regulatory Cells

T follicular regulatory (Tfr) cells are a subset of Tregs responsible for responding to and controlling the magnitude of germinal center (GC) reactions and subsequent antibody responses (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Similar to T follicular helper (Tfh) cells, which promote GC responses and antibody production, Tfr express BCL6 and CXCR5, allowing them to localize to GCs. The transcription factors TCF1 and LEF1 are required for expression of BCL6

and subsequent Tfr development (Yang et al., 2019). Tfr also express high levels of ICOS, a costimulatory receptor, and PD-1, a coinhibitory receptor. ICOS knockout mice are deficient in Tfr while PD-1 knockout mice have increased Tfr with enhanced suppressive capacity (Sage et al., 2013).

The importance of Tfr in regulating GC responses was initially demonstrated by three separate studies. The first study demonstrated that mice with CXCR5-deficient Tregs, that were unable to enter the germinal center, had increased antigen-specific IgG compared to control mice in response to immunization (Wollenberg et al., 2011). Similar to this, the second study showed that mice with *BCL6*- deficient Tregs experienced higher antigen-specific antibody production in response to immunization (Chung et al., 2011). In contrast, the third report on Treg-depleted Foxp3-DTR mice and Tfr-deficient SAP KO:Foxp3 KO mixed bone marrow chimera mice showed that the absence of Tregs during germinal center reactions lead to decreased antigen specific GC B-cells and outgrowth of antigen non-specific B-cells (Linterman et al., 2011). To more definitively assess the role of Tfr in the germinal center reaction, mice were generated in which CXCR5 expressing Tregs could be inducibly deleted with diphtheria toxin. Using this model, the authors showed that Tfr function early, prior to GC formation, to limit GC B-cell development and antigen-specific antibody production in response to immunization. Tfr also regulated production of auto-antibodies that occur during immunization with foreign antigen and showed that Tfr are critical for controlling house dust mite-specific IgE during an allergic response (Clement et al., 2019).

Recent studies have revealed mechanisms by which Tfr regulate GC reactions and antibody responses. CTLA-4 mediates Tfr suppression of GC responses. Deletion of CTLA-4 on Tregs following their differentiation into Tfr leads to enhanced GC responses (Sage et al., 2014). Intriguingly, production of the neuropeptide, neuritin, by Tregs (likely Tfr) is important for controlling plasma cell differentiation from GC B-cells, limiting development of autoreactive GC-derived plasma cells, and limiting IgE responses (Gonzalez-Figueroa et al., 2021). These effects were attributed to neuritin's effect on B-cells; however, it is possible that Treg derived neuritin is altering the activity of other cell types as well.

Human Treg Subsets

While the majority of studies discussed above were done in mice, analogous subsets of Tregs have been identified in humans. Study of human Tregs and Treg subsets is complicated due to difficulty in accurate identification. A more detailed description of heterogeneity in human Tregs during health and disease can be found in recent reviews by Mohr et al. (2018) and Wing et al. (2019).

Helper Treg Summary

Depending on the inflammatory setting, eTregs can undergo differentiation in parallel to effector CD4 T-cells and adopt limited Th programming. Expression of Th transcription factors allows Tregs functional plasticity to adapt to specific inflammatory environments and regulate the corresponding Th response. Several studies have shown functional deficits in Tregs that are unable to acquire Th programming. Although phenotypes associated with Th subtypes have been used to functionally categorize effector CD4 T-cells, a recent study has questioned this paradigm. Surprisingly, Kiner et al. (2021) has shown that effector CD4 T-cells are not distinguished by their respective Th classes when transcriptomes are analyzed by single-cell RNA-seq. Following Th-biased infections, effector T-cell transcriptomes did not form discrete clusters based on Th signatures. Partitioning of cells was most heavily dictated by the infection type not Th signature. Although this study did not address Treg heterogeneity, future studies should address whether a similar result would be observed for distinct subsets of Tregs responding to these infections.

TISSUE TREGS

In addition to Treg subsets defined by canonical Th transcription factors, there is extensive phenotypic and functional heterogeneity among Tregs that exist in different tissue types. Tissue Tregs are effector Tregs that have entered into and adapted to the specific tissue environment. There they serve to restrain immune responses, maintain tissue homeostasis, and promote tissue repair.

Recent use of single-cell RNA- and single-cell ATAC-seq to analyze Tregs from lymphoid and non-lymphoid tissues have elucidated the process by which circulating Tregs develop into tissue Tregs. Evidence suggests that Tregs acquire tissue Treg programming in a gradual, stepwise process that begins in the spleen and lymph nodes and is dependent on BATF (Hayatsu et al., 2017; Miragaia et al., 2019; Delacher et al., 2020). A Treg population found in the spleen that expresses low levels of the transcription factor, PPARγ, develops into tissue Tregs at multiple sites including skin, VAT, and liver demonstrating that tissue Treg precursors develop in the spleen prior to immigration to their respective tissues (Li et al., 2021a).

Visceral Adipose Tissue Tregs

Early understanding of Tregs suggested their major function was to regulate immune homeostasis and immune response intensity. However, the observation of tissue Tregs in the steady-state challenged this assumption and supported a role outside more general immune homeostasis. First described in 2009, Tregs in the visceral adipose tissue (VAT Treg) represented the earliest example of a tissue-specific Treg subset whose function extends beyond the immune response (Feuerer et al., 2009). VAT Tregs remain one of the purest, and more well understood, examples of a Treg cell subset that adopts a phenotype and performs a function that is specific to their tissue of residency.

Visceral Adipose Tissue Treg Derivation and Differentiation

The developmental scheme for VAT Tregs is well understood. Like most Tregs, the precursors for VAT Tregs also develop within the thymus (Kolodin et al., 2015). Interestingly, the VAT Treg compartment is reconstituted following Treg ablation in

young but not old mice, suggesting that the perinatal thymus is critical for generating VAT Treg cell precursors. VAT Treg cell precursor development in the thymus occurs prior to 3 weeks of age as thymectomy in three-week old mice does not affect the accumulation of Tregs in the VAT (Kolodin et al., 2015). Several studies have suggested differences in thymic selection, and the function of Tregs, that develop in the perinatal versus adult thymus (Yang et al., 2015; Stadinski et al., 2019). However, why the adult mouse cannot replenish the VAT Treg cell compartment is unclear. Antigen presentation or antigen availability in the thymus of different aged mice could alter Treg cell output. This is supported by the observation that thymocytes expressing a transgenic TCR, isolated from VAT Tregs, are preferentially selected in the thymus during the first 2 weeks of life (Li et al., 2018). The endogenous targets of VAT related TCR's have not been identified (Fernandes et al., 2020). However, if expression of the relevant antigen in the thymus is AIRE-dependent, the rapid reduction in AIRE+ mTEC following the perinatal period (Baran-Gale et al., 2020) could make VAT Treg cell selection inefficient in the adult thymus. Identification of the endogenous peptide that VAT Tregs recognize will facilitate a mechanistic understanding of VAT Treg cell selection in the thymus.

Despite developing in the thymus, VAT Treg cell differentiation is characterized as a two-step process that is initiated in secondary lymphoid organs. An important factor upregulated during VAT Treg differentiation is PPARγ, which is highly expressed in VAT Tregs and is critical for recruitment of Tregs to VAT (Cipolletta et al., 2012). However, it was initially unclear if PPARγ was upregulated by Tregs upon entering the VAT or upregulated in VAT Treg cell progenitors in secondary lymphoid organs (Cipolletta et al., 2012). The lack of reagents to easily detect PPARγ motivated the generation of *Pparg-Tomato* reporter mice to isolate and track cells that upregulate PPARγ. A small population of *Pparg-Tomato* positive Tregs can be identified in the spleen, although these Tregs expressed significantly less PPARγ than VAT Tregs (Li et al., 2018). Splenic PPARγlo Tregs do not express the majority of the VAT Treg cell signature but do exhibit an activated phenotype and upregulation of genes important for lipid metabolism. Transfer of PPARγlo Tregs led to more efficient seeding of the VAT compared to PPARγ- Tregs, despite being recovered at similar rates in the spleen, supporting the idea that PPARγlo Tregs are likely the progenitors for VAT Tregs (Li et al., 2018). However, a more recent study found that PPARγlo Tregs are the progenitors for Tregs in several non-lymphoid tissues other than VAT, including the liver and skin (Li et al., 2021a). It is unclear if Tregs seeding all tissues go through this PPARγlo intermediate. No matter the ubiquity of this PPARγlo intermediate in tissue Treg seeding, it clearly supports a two-step process for VAT Treg cell differentiation.

Several important factors required for the differentiation and/or retention of VAT Tregs have been described. A primary factor in VAT Treg cell development and VAT residency is TCR specificity. Though the target of VAT Treg TCRs has not be identified, TCR signaling is important at both steps of VAT Treg differentiation. Upregulation of PPARγ in VAT Treg precursors is significantly inhibited by MHC-II blockade (Li et al., 2018). Multiple observations also suggest VAT Tregs are responding to antigen within VAT. First, clonal expansions of VAT Treg TCRs are suggestive of local expansions that occur once VAT reactive Tregs enter the tissue (Feuerer et al., 2009; Kolodin et al., 2015). Second, Tregs that express a VAT Treg-enriched TCR transgene preferentially enter the VAT and undergo cell division more than their TCR transgenic negative counterparts. However, both populations adopt similar phenotypes within the VAT, a subset of which upregulate *Nr4a1* within the VAT confirming local recognition of VAT antigens (Li et al., 2018). Finally, VAT Tregs closely associate with MHC-II+ APCs in the VAT and MHC-II blockade antagonizes this association (Kolodin et al., 2015). Thus, TCR specificity and signaling are important for VAT Treg cell recruitment and retention. However, it remains unknown what antigen presenting cell (APC) population is required to present antigen in the secondary lymphoid organs and VAT to activate Tregs and what antigen they are presenting.

In addition to TCR signaling, several other factors are also important for VAT Treg cell differentiation. As discussed above, PPARγ is important for seeding the VAT by Tregs (Cipolletta et al., 2012) but is not important for the development of splenic VAT Treg cell precursors, as defined by KLRG1 and the IL-33 receptor, ST2 (Li et al., 2021a). While PPARγ expression is expendable for upregulation of ST2 in secondary lymphoid organs, PPARγ deficient Treg in the VAT do not properly upregulate ST2 (Kolodin et al., 2015). Sensing of IL-33 by ST2 is another important factor for VAT Treg cell phenotype and homeostasis. *Il1rl1* (ST2) knockout Treg have a significant defect in VAT accumulation and the adoption of the GATA3+ and KLRG1+ VAT Treg cell phenotype (Kolodin et al., 2015; Li et al., 2018). Treatment of mice with IL-33 expands the PPARγlo VAT Treg precursor in the spleen and drives expansion of the Treg compartment within the VAT (Kolodin et al., 2015; Li et al., 2018). Interestingly, provision of IL-33 expands VAT Tregs even while blocking TCR stimulation with MHC-II blockade (Kolodin et al., 2015), suggesting that IL-33 production alone can scale the size of the VAT Treg cell compartment. Nevertheless, TCR signaling may still be required for VAT Treg function in the context of IL-33 mediated expansion. Like other Tregs, VAT Tregs expand in response to IL-2 complex treatment (Feuerer et al., 2009). However, the expansion of VAT Tregs to IL-2 complex treatment is weaker than IL-33 treatment (Kolodin et al., 2015; Li et al., 2018) and unlike IL-33, IL-2 stimulation alone does not drive PPARγ upregulation (Li et al., 2018). PPARγ is not the only transcription factor that regulates VAT Treg phenotype. A recent study linked expression of the activated Treg transcription factor BLIMP1 to the VAT Treg phenotype, including expression of ST2 and KLRG1. Loss of BLIMP1 in Tregs reduced Treg production of IL-10 in VAT (Beppu et al., 2021). While coordination of PPARγ expression and IL-33 sensing stabilizes and scales the VAT Treg compartment, questions remain regarding the requirements for VAT Treg functionality.

Visceral Adipose Tissue Treg Function

Visceral Adipose Tissue Tregs are critical regulators of organismal metabolic homeostasis and adapt to metabolic challenges. Visceral Adipose Tissue Tregs play an important

role in regulation of glucose and insulin tolerance. Analysis of various mouse models of obesity revealed a strong negative association between VAT Treg proportions and insulin resistance (Feuerer et al., 2009). Depletion of Tregs in *Foxp3DTR* mice leads to increased insulin concentrations, and augmentation of VAT Tregs with IL-2 complex treatment bolsters insulin sensitivity and glucose clearance (Feuerer et al., 2009). These methods targeted all Tregs and not specifically the VAT Tregs. However, treatment of mice challenged with a high fat diet with the PPARγ agonist pioglitazone does not affect splenic Tregs but increases VAT Treg cell abundance and, coordinately, both glucose clearance and insulin sensitivity. This effect is VAT Treg cell specific as Treg specific knockout of PPARγ mitigated the response to pioglitazone (Cipolletta et al., 2012). However, VAT Tregs appear to reverse their role in metabolic homeostasis in the aged mouse. Depletion of VAT Tregs, via *Foxp3*^{Cre} mediated deletion of *Pparg*, rescued aging induced metabolic dysfunction (Bapat et al., 2015). The reason for these divergent observations in still unclear. However, deletion of PPARγ also reduced Tregs in subcutaneous adipose tissue (Bapat et al., 2015). The function of Tregs in non-VAT adipose depots is uncertain with some studies observing that Tregs promote metabolic homeostasis (Medrikova et al., 2015; Kalin et al., 2017; Fang et al., 2020) while others argue that Tregs promote metabolic disorder (Beppu et al., 2021). Regardless of the directionality, it is clear that adipose Tregs are a key regulator of systemic metabolic homeostasis.

While VAT Tregs are important in maintaining metabolic homeostasis, obesity leads to a diminished VAT Treg compartment. Mice treated with a high fat diet have diminished proportions of VAT Tregs and many of the remaining Treg have downregulated ST2 (Han et al., 2015). A high fat diet triggers increased production of pro-inflammatory cytokines, TNFα and IL-6, and macrophage infiltration in the VAT. Aberrant VAT inflammation can be rescued with IL-33 treatment, which correlated with VAT Treg cell expansion and recovery of ST2 expression (Han et al., 2015). The major producers of IL-33 in VAT are VAT mesenchymal stromal cells. Interestingly, a high fat diet initially decreases production of IL-33 by VAT mesenchymal stromal cells (VmSC), but following 4 months of high fat diet there is significantly more IL-33 producing VmSC in the VAT (Spallanzani et al., 2019). However, provision of IL-33 to mice lacking ST2 in Tregs leads to an increase in IL-33 producing VAT mesenchymal stromal cells (Spallanzani et al., 2019). Collectively, these results are suggestive of a feedback loop whereby VAT Tregs are both scaled by IL-33 but also regulate its production by VAT mesenchymal stromal cells. Importantly, increased production of IL-33 by VAT mesenchymal stromal cells is not sufficient to rescue VAT function in obesity (Spallanzani et al., 2019). However, expression of a soluble form of ST2 (sST2), which functions as a decoy receptor for IL-33, is induced in adipocytes following obesogenic challenge (Zhao et al., 2020). sST2 overproduction may explain the lack of Treg response to the elevated IL-33 produced during obesity. A recent study observed that plasmacytoid dendritic cells (pDC) progressively accumulate in VAT during obesity development (Li et al., 2021b). pDC production of IFNα reduces Treg expansion by inhibiting VAT Treg proliferation and increasing VAT Treg apoptosis (Li et al., 2021b). Several factors outside of homeostatic regulation of IL-33, perhaps increased inflammation or pro-inflammatory cytokines, or sST2 production, may regulate VAT Treg regulation during obesogenic challenges.

Female and male mice have profoundly different VAT Treg compartments and responses to metabolic challenges. A high fat diet induces an expansion of the VAT Treg cell compartment in females but reduces the VAT Treg compartment of males. Further, males on a high fat diet exhibit greater proinflammatory cytokine production and VAT macrophage infiltration than female mice (Pettersson et al., 2012). VAT Treg from female mice are phenotypically distinct from those in male mice, expressing a distinct transcriptome. Female mice have less VAT Tregs that express ST2, KLRG1, IL-10 and CCR2 than their counterparts in male mice (Vasanthakumar et al., 2020). In agreement with these phenotypes, IL-33 producing VAT mesenchymal stromal cells are more prominent in male mice and correlate with increased VAT Treg cell accumulation (Li et al., 2018; Spallanzani et al., 2019; Vasanthakumar et al., 2020). These effects are hormone dependent as androgen receptor deficiency in males and estrogen receptor-α deficiency in females reverse the VAT Treg phenotypes (Vasanthakumar et al., 2020). However, these hormones do not directly target Tregs but instead modulate IL-33 availability from the stromal cell compartment in the VAT (Vasanthakumar et al., 2020). These sex-specific characteristics lead to an improved metabolic response to obesogenic conditions in female mice (Vasanthakumar et al., 2020). An improved response to metabolic challenges appears to be important for healthy pregnancy as well. In agreement with the previous findings of sex hormones on the VAT Treg compartment, pregnancy induces a significant increase in VAT Tregs that is reversed upon *Foxn1-Cre* mediated deletion of RANK (Paolino et al., 2021). RANK deletion, presumably on thymic epithelial cells, caused a reduction in VAT Treg accumulation that antagonized insulin sensitivity in pregnant mice. This phenomenon led to the development of gestational diabetes and impairments in glucose homeostasis of mice gestated in mothers with reduced VAT Treg proportions (Paolino et al., 2021). Thus, sex hormone production, related to either sex or other physiologic processes, leads to strong differences in VAT Treg cell abundance and phenotype that causes differences in adaptation to metabolic challenges. In humans, females have a lower incidence and slower onset of type 2 diabetes than males (Kautzky-Willer et al., 2016), perhaps related to these differences in VAT and VAT Treg biology that have been observed in mice.

Visceral Adipose Tissue plays an important role in organismal metabolic homeostasis and VAT Tregs are crucial for VAT functionality. The metabolic perturbations caused by VAT Treg cell functional deficiency mimic those that contribute to increased incidence of type 2 or gestational diabetes and obesity. Bolstering the VAT Treg cell compartment represents an intriguing possibility to ameliorate these conditions. A recent report identified several mimotope ligands for a VAT Treg cell TCR, although the endogenous ligand remains unidentified. Interestingly, immunization of mice with this VAT Treg cell TCR ligand led to increased VAT Treg accumulation and improved insulin sensitivity in mice challenged with a high fat diet (Fernandes et al., 2020). This promising result suggests that

specific targeting of VAT Tregs in humans may be beneficial for the treatment or prevention of metabolic disorders. However, despite the promise of targeting VAT Tregs in disease, the mechanism by which VAT Tregs function remains elusive. The most likely mechanism by which VAT Treg function is via control of macrophage infiltration and polarization in VAT, which precludes a VAT inflammatory environment and insulin resistance (Kolodin et al., 2015). More specific strategies to target VAT Tregs will facilitate the identification of important factors for VAT Treg regulation of VAT homeostasis.

Muscle Tregs

Much like their Treg complements in VAT, muscle Tregs are a specialized population of Tregs that exhibit a unique TCR repertoire, transcriptome, and function. Despite being less well studied than VAT Tregs, the important mechanisms required for muscle Treg cell function are relatively well understood. Unlike their VAT Treg counterparts, the general function of muscle Tregs, largely in coordinating the tissue repair process by promoting tissue regeneration and reducing inflammation, is shared with other tissues as we will discuss in other areas of this review.

Muscle Treg Derivation and Differentiation

Muscle Tregs, like VAT Tregs, appear to begin their development in the thymus. Muscle Tregs express *Nrp1* and *Helios* (*Ikzf2*), as well as a divergent TCR repertoire from Tconv found in the muscle (Burzyn et al., 2013). Muscle Tregs likely respond to self-antigen present in both the thymus and muscle tissue. In agreement with this, muscle Tregs contain an oligoclonal TCR repertoire that expands upon several types of muscle injury (Burzyn et al., 2013). Impressively, a single TCR clone, or subtle variation thereof, in injured muscle was shared between 11 mice, strongly supporting a dominant antigen driving expansion of the muscle Treg compartment (Burzyn et al., 2013). Further, TCR transgenic Tregs, expressing this publicly shared transgenic TCR, preferentially home to the muscle (Cho et al., 2019).

Like VAT Tregs, muscle Tregs appear to begin their differentiation in secondary lymphoid organs upon receiving TCR stimulation. Amphiregulin (AREG), a defining marker of the muscle Treg signature, is expressed in a small fraction of splenic Tregs that have a clonally expanded TCR repertoire which partially overlaps with the muscle Treg TCR repertoire (Burzyn et al., 2013). The overlap between the AREG+ population in the spleen and muscle strongly suggest an initial priming event in the secondary lymphoid organs that expands muscle specific Tregs. However, two phenomena cloud this interpretation. First, Tregs that express a muscle Treg cell specific transgenic TCR do not exhibit a strong TCR activation signature in the spleen but the TCR activation signature is upregulated when TCR transgenic Tregs arrive in the muscle (Cho et al., 2019). However, the lack of a strong TCR activation signature in the secondary lymphoid organs may just represent that a smaller number of muscle reactive Tregs are engaging cognate antigen in the secondary lymphoid organs versus in the muscle. Second, unlike VAT Tregs, muscle Tregs undergo retrograde trafficking from muscle to secondary lymphoid organs (Kuswanto et al., 2016). Thus, AREG+ splenic Tregs with an overlapping TCR repertoire with muscle Tregs may represent a retrograde trafficking event from fully differentiated muscle Tregs. However, given the ubiquitous activation signature in muscle Tregs (Burzyn et al., 2013), it is likely that their differentiation scheme does involve a priming event in secondary lymphoid organs before trafficking to the muscle where the muscle Treg cell signature is finalized (Cho et al., 2019). Supporting this, muscle Treg cell accumulation following injury is largely dependent on recruitment from secondary lymphoid organs (Kuswanto et al., 2016). TCR specificity is an important signal for muscle Treg cell development from conception in the thymus through initial activation in secondary lymphoid organs and finalization of muscle Treg development in the muscle.

Muscle Tregs share several factors with VAT Treg development although there are some exceptions. One such exception is the independence of muscle Treg accumulation on PPARγ and that PPARγ activation does not benefit the muscle Treg compartment (DiSpirito et al., 2018). It remains undetermined if muscle Treg progenitors go through the same PPARγlo intermediates as other tissue Treg subsets. However, given that PPARγlo tissue Treg precursors are not dependent on PPARγ, this remains a possibility. Nonetheless, like VAT Tregs, muscle Tregs express ST2 (Burzyn et al., 2013) and IL-33 is an important factor for their accumulation and response to injury (Kuswanto et al., 2016). IL-33 availability scales the size of the muscle Treg compartment and is upregulated in both mice and humans in response to muscle damage (Kuswanto et al., 2016). IL-33 is induced upon muscle injury, driving the expansion of muscle Tregs to facilitate muscle repair (Kuswanto et al., 2016). Interestingly, older mice, greater than 6 months of age, have reduced IL-33, both at steady-state and in response to injury (Kuswanto et al., 2016). Mesenchymal stromal cells in the muscle are the primary producers of IL-33 and injury induces their production of IL-33 within hours after injury (Kuswanto et al., 2016). Muscle mesenchymal stromal cells congregate with sensory neurons in the muscle and express a number of neuropeptides and neuropeptide receptors including calcitonin gene-related peptide (CGRP) (Wang et al., 2020). Treatment of mice with CGRP drives muscle IL-33 production and muscle Treg cell accumulation (Wang et al., 2020), suggesting that pain sensing after injury helps bolster the repair program. Upon muscle injury, sensory neurons and muscle mesenchymal stromal cells coordinate to increase IL-33 availability to recruit and expand muscle Tregs to facilitate proper muscle repair and regeneration.

Muscle Treg Function

Muscle Tregs function broadly to promote muscle repair via immune dependent and independent mechanisms. Following injury, muscle Tregs expand peaking at 4 days post-injury and remaining elevated at least 1-month post-injury (Burzyn et al., 2013). Treg cell depletion impairs muscle regeneration due to increased muscle fibrosis and decreased satellite cell differentiation and increases muscle damage markers in a

mouse model of muscular dystrophy (Burzyn et al., 2013). Conversely, bolstering muscle Tregs, either via IL-2 complex (Burzyn et al., 2013) or IL-33 (Kuswanto et al., 2016) treatment, improves muscle damage markers and facilitates superior muscle regeneration. Finally, as discussed above, aged muscle contains fewer Tregs due to reduced IL-33 availability, resulting in a fibrotic response to muscle injury. However, treatment of aged mice with IL-33, which rescues muscle Treg cell proportions, allows for partial restoration of muscle regeneration (Kuswanto et al., 2016). Both gain and loss of function experiments, as well as correlative observations in aged mice, confirm the importance of muscle Tregs in mediating the regeneration of functional muscle tissue following injury.

Muscle injury stimulates an immune response characterized by recruitment of macrophages, peaking at day two post-injury, followed by NK cells, CD4+ and CD8+ T cells by day four (Panduro et al., 2018). Infiltrating macrophages are comprised of two broad subsets – MHCII- and MHCII+. Treg depletion causes an expansion of MHC-II+ macrophages in the muscle along with increased intramuscular IFNγ production from NK cells, CD4+ and CD8+ T cells (Panduro et al., 2018). MHC-II expression on macrophages is important for IFNγ production by NK cells and CD4+ T cells; however, Treg cell expansion is independent of macrophage MHC-II (Panduro et al., 2018). Treatment of mice with recombinant IFNγ partially phenocopies the excessive inflammation and increased fibrosis observed in Treg cell depleted mice (Panduro et al., 2018). Early pro-inflammatory immune responses following injury are necessary to remove debris and damaged cells. However, this response must be shifted later to promote appropriate repair and regeneration. This latter phase of the injury response is promoted by Treg cell expansion and anti-inflammatory macrophage polarization (Panduro et al., 2018). All the mechanisms that Tregs employ to promote this response are unknown but regulation of macrophage polarization and IFNγ production are crucial to muscle regeneration.

Compromised intramuscular Treg cell function also causes immune-independent defects in muscle regeneration and repair. During muscle injury, muscle progenitor cells, termed satellite cells, differentiate into myoblasts and eventually a functional myotube to regenerate functional muscle tissue (Tidball, 2011). Treg cell depletion reduces the capacity of satellite cells to undergo myogenic differentiation (Burzyn et al., 2013). A substantial fraction of muscle Tregs express AREG (Burzyn et al., 2013), the epidermal growth factor receptor ligand critical for tissue repair (Berasain and Avila, 2014). Amphiregulin treatment rescues the myogenic potential of satellite cells and facilitates muscle regeneration during Treg cell depletion (Burzyn et al., 2013). This suggests that AREG production by Tregs is critical for muscle repair and regeneration via immune-independent effects on myogenesis. Indeed, Treg specific-deletion of *Areg* causes defective repair in the lung following influenza induced pathology, another model of inflammatory regeneration (Arpaia et al., 2015). Treg derived AREG has been linked to repair in other tissues including the CNS (Ito et al., 2019) and skin (Nosbaum et al., 2016). While some of the aspects of muscle Treg cell function are generalizable to repair processes in other tissues, such as effector cell activation, macrophage polarization and AREG production, muscle Tregs do contain a unique and clonally expanded TCR repertoire. Further, there are transcriptional features specific to muscle Tregs compared to other identified Treg cell subsets (Munoz-Rojas and Mathis, 2021) – whether these genes confer tissue specific functionality to muscle Tregs remains an open question.

Skin Tregs

The skin contains a high frequency of Tregs. In humans and mice Tregs make up 20–40% of CD4+ T-cells within the skin (Kalekar and Rosenblum, 2019). Tregs in the skin express a strong Th2-biased phenotype where approximately 80% of Tregs express GATA3 (Wohlfert et al., 2011; Harrison et al., 2019). Although GATA3+ Tregs are generally regarded to be of thymic origin, this is not entirely clear as pTregs are able to upregulate GATA3 to the same degree upon antigen exposure (Wohlfert et al., 2011). Deletion of GATA3 in Tregs unleashes Th2 immune responses by commensal microbe-specific T-cells (Harrison et al., 2019). Indeed, it has been shown that the skin is seeded early in life by Tregs that recognize and facilitate tolerance to commensal microbes (Scharschmidt et al., 2015, 2017). A separate study demonstrated the importance of Treg-specific GATA3 expression in control of dermal fibrosis induced by Th2 cytokines (Kalekar et al., 2019). Another study reported that, similar to GATA3, approximately 80% of Tregs from the skin express the transcription factor, RORa (Malhotra et al., 2018), which has a reported role in the development of ILC2s. This study did not report if these RORa+ Tregs co-expressed GATA3. Deletion of RORa in Tregs leads to heightened Th2-mediated allergic inflammation. Overall these reports demonstrate that skin Tregs are poised to restrain Th2-mediated responses. Although GATA3 and RORa have previously been shown to be associated with skin Tregs, new evidence from single-cell ATAC and single-cell RNA sequencing have shown that these transcription factors are expressed broadly in Tregs from many tissue types and their precursors in the lymphoid tissue (Delacher et al., 2017, 2020). By comparison to those in the blood, Tregs from human skin preferentially express the mitochondrial enzyme, arginase 2 (ARG2). Expression of ARG2 promotes accumulation of Tregs within the skin and adoption of a tissue Treg transcriptional signature. In psoriatic skin, ARG2 inhibits mTOR activity, which has been shown to negatively regulate Treg proliferation. *In vitro* experiments indicate that Treg ARG2 can metabolize extracellular arginine to inhibit effector T-cell proliferation (Lowe et al., 2019).

In addition to their immunosuppressive functions, skin Tregs expand following tissue damage and promote wound healing and tissue repair. The majority of skin Tregs express ST2 (Delacher et al., 2020), the receptor for IL-33 that is released by cells in response to stress and damage. In Tregs, signaling through ST2 leads to the production of AREG, a protein with documented tissue repair function (Arpaia et al., 2015). In support of this, a recent study showed that Tregs in the skin expand following UVB irradiation and express AREG and the endogenous opioid precursor, PENK (Shime et al., 2020). Similar to AREG, PENK has been shown to promote wound healing

and tissue repair (Shime et al., 2020). Tregs from wounded skin also express the AREG receptor, EGFR. Deficiency of EGFR in Tregs led to decreased accumulation of Tregs in the wounded skin and reduced wound closure suggesting a potential autocrine role of AREG in Tregs during tissue repair (Nosbaum et al., 2016). In response to acute epithelial injury of the skin, Tregs promote barrier repair by inhibiting the activity of Th17 cells and neutrophils (Mathur et al., 2019). Related to their role in tissue repair, skin Tregs have ascribed importance in facilitating hair follicle stem cell proliferation and differentiation through their expression of Jagged-1 (Ali et al., 2017). More information on the role of Tregs in skin injury can be found in a review by Boothby et al. (2020).

Intestinal Tregs

Intestinal Tregs are important for facilitating tolerance to commensal microbes and environmental antigens. The intestine is regularly exposed to environmental antigens making it an ideal location for the induction of pTregs. Studies attempting to identify the origins of Tregs in the gut were conflicting. Analysis of TCR hybridomas bearing TCRs from murine colonic Tregs demonstrated their reactivity to colonic bacterial isolates. T-cells with retrogenic TCRs from colonic Tregs converted into Tregs in the colon but were unable to undergo diversion to the Treg lineage in the thymus (Lathrop et al., 2011). This suggested that colonic Tregs were pTregs rather than tTregs. However, a conflicting report showed that most colonic Tregs shared TCRs with thymic Tregs indicating a likely thymic origin (Cebula et al., 2013). Both studies agree that TCRs from colonic Tregs recognize microbial antigens.

While the origin of intestinal Tregs is debated, they seem to be composed of at least three populations of Tregs: RORγt+ pTregs, RORγt- pTregs, and GATA3+ tTregs. As mentioned previously, RORγt+ pTregs dominate the Treg population in the colon, while in the small intestine a substantial portion of pTregs do not express RORγt and are dependent on the presence of dietary antigens (Kim et al., 2016). Recent evidence demonstrates that the frequency of RORγt+ Tregs in the colon is stably transmitted from the mother to the progeny. This phenomena is dependent on levels of maternally transferred IgA which coat intestinal microbes and inhibit RORγt+ Treg induction (Ramanan et al., 2020). The enteric nervous system has also been shown to influence intestinal Tregs. Mice deficient in the neuropeptide precursor, TAC1, had increased frequencies of RORγt-expressing Tregs in the colon. Conversely, mice fed capsaicin, which stimulates neurons and release of neuropeptides, had decreased frequencies of RORγt Tregs (Yissachar et al., 2017). Stimulation of enteric neurons by microbes lead to release of neuronal IL-6 which promoted induction of RORγt+ Tregs (Yan et al., 2021). Tregs in the gut express high levels of the immunosuppressive cytokine, IL-10 (Atarashi et al., 2011). In germ free mice, the frequency of IL-10 producing Tregs in the colon, but not the small intestine, is significantly reduced indicating that these are likely pTregs (Atarashi et al., 2011; Ohnmacht et al., 2015). Recent studies have shown that Treg produced IL-10 plays an important role in promoting intestinal stem cell renewal (Biton et al., 2018). A distinct population of Tregs in the colon express high levels of GATA3 and ST2 (Wang et al., 2011; Wohlfert et al., 2011; Schiering et al., 2014). *In vivo* treatment with IL-33 expanded ST2+ colonic Tregs. ST2-deficient Tregs were limited in their ability to accumulate in the colon during inflammation (Schiering et al., 2014). As ST2+ Tregs have associated tissue reparative functions due to their expression of AREG, it has been suggested that ST2+ Tregs in the gut are mediating repair processes during tissue damage.

Single-cell RNA-seq of murine colonic Tregs identified transcriptional heterogeneity that matched previous studies (Miragaia et al., 2019). Three subsets were identified: lymphoid tissue-like Tregs that expressed central Treg markers including *Ccr7, Sell*, and *Tcf7*, non-lymphoid tissue-like Tregs that expressed *Gata3* and *Areg* and correspond to the previously described GATA3+ thymic Treg population, and suppressive Tregs that expressed *Lag3, Il10, Gzmb, and Cxcr3* and resemble the known RORγt+ population. A portion of Tregs within the mesenteric lymph nodes have partially adapted the gene signature characteristic of the colonic Tregs indicating progressive acquisition of this tissue transcriptome from the draining lymph to node to the tissue. Additional information on the roles of intestinal Tregs can be found in reviews by Whibley et al. (2019) and Cosovanu and Neumann (2020).

Tregs in the Central Nervous System

Tregs infiltrating the CNS must navigate the unique compartmentalization and immune privilege of this anatomical space, which shapes their trafficking patterns, activation mechanisms, and tissue-specific functions. Recent investigations have revealed how immunological trafficking occurs between the brain, CSF, meninges, and periphery. The brain parenchyma and spinal cord are surrounded by cerebral spinal fluid (CSF), which is separated from systemic circulation (**Figure 2**). Specialized endothelial cells, surrounded by glial podocytes, form part of the pia mater meningeal layer, are bound by tight junctions, and compose the blood-brain barrier (BBB) (Daneman and Prat, 2015). The BBB, in turn, regulates bidirectional trafficking of cells and metabolic products between the CSF and the parenchyma. CSF ultimately drains into the dural sinus as well as into a separate series of specialized lymphatic vessels lining the dural sinus, before reaching the cervical lymph nodes (Louveau et al., 2015). Immune responses frequently begin in the meninges, which is a uniquely poised checkpoint to support or dampen inflammation in the CNS (Filiano et al., 2015). It is now increasingly understood that the interactions of Tregs with neuronal cells play critical roles in neurological development, neurodegenerative disease, and recovery from cerebrovascular infarction, as well as control of CNS-infiltrating cancers. Therefore, CNS Tregs likely represent a population that shares several properties with tissue Tregs of other spaces, but also harbor unique qualities reflecting specialized non-canonical functions.

In the steady state of healthy adult mice, approximately 2000 CD4 T-cells, including ∼150 Tregs, are found in the brain (Pasciuto et al., 2020). These are scattered at similar densities across most regions of healthy mouse brain, and are also found in similar proportions in rat cerebrum and

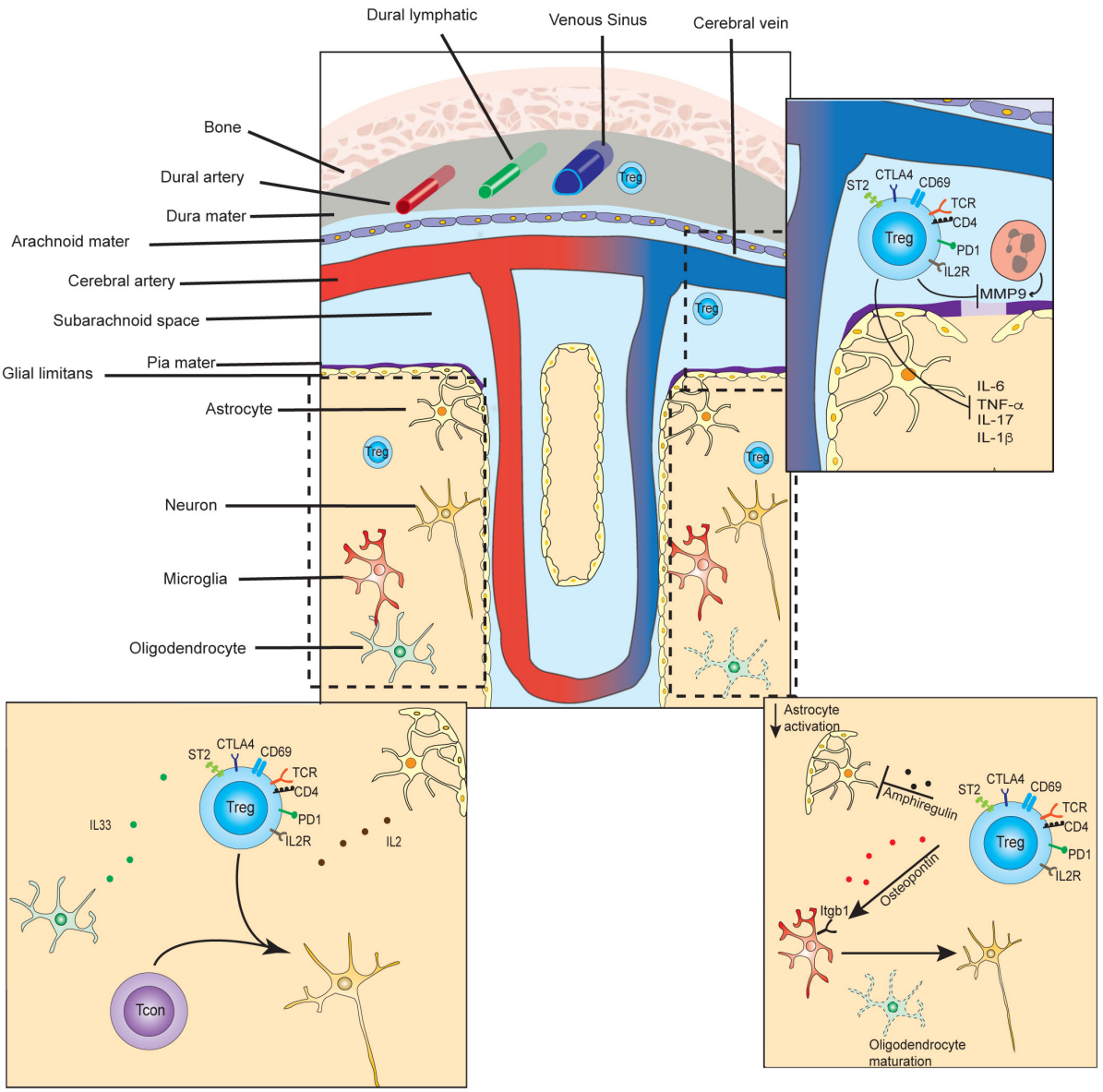

FIGURE 2 | Select functions of Tregs in CNS tissue development, homeostasis, and repair. In the steady-state, small numbers of T-regulatory cells are found transiting the dura and leptomeninges, and persisting in the brain parenchyma. CD4+ T-cells of the parenchyma contribute to microglial maturation and synaptic pruning. (Lower left inset) In the steady state, parenchymal Tregs express markers of long-term residency and are maintained by IL-2 and IL-33, which may be primarily supplied by astrocytes and oligodendrocytes, respectively. (Top right inset) In the subacute or acute period following ischemic stroke, Tregs inhibit matrix metalloproteinase 9 (MMP9) secretion from neutrophils, preventing further breakdown of the blood-brain-barrier, and diminishing inflammation. (Bottom right inset) In the chronic period of ischemic stroke recovery, CNS Tregs suppress astrogliosis via the secretion of amphiregulin, and induce oligodendrocyte maturation via secretion of osteopontin.

biopsies of healthy human brain tissue (Xie et al., 2015). Higher frequencies, and persistent accumulation of Tregs, are seen following inflammation, and following cerebrovascular ischemia (Liesz et al., 2009b). They also increase in the tissues surrounding tumors involving the CNS, with a particularly high frequency of Tregs seen in carcinomas that have metastasized to the brain (Friebel et al., 2020). Compared to peripheral blood Tregs, steady-state brain Tregs are transcriptionally distinct, and express higher levels of activation markers (PD1, CTLA4, ICOS, KLRG1, CD103), and the residency markers ST2 and CD69 (Xie et al., 2015; Pasciuto et al., 2020). During severe inflammation in EAE models, CNS Tregs were also found to be transcriptionally dissimilar as compared to splenic Tregs (Garg et al., 2019). During the chronic phase of stroke recovery Tregs were found to express high levels of IL-10, KLRG1, ST2, and PPARγ, similar to VAT and muscle Tregs (Ito et al., 2019). However, CNS Tregs appeared to also specifically express genes involved in interactions with neural cells including neuropeptide

Y (*Npy*), preproenkephalin (*Penk*), serotonin receptor type 7 (*Htr7*), and arginine vasopressin receptor (*Avpr1a*). Therefore, CNS Tregs are present in low frequencies in the steady state, but increase markedly during inflammation or following cerebrovascular insult, and adopt site-specific transcriptomes and immunophenotypes.

Several studies have now succeeded in delineating critical steps that Tregs undergo while trafficking between the periphery, the meninges, and the parenchyma of the brain. Parabiosis experiments demonstrated that immunophenotypically naïve Tregs only transiently enter the brain parenchyma, while activated Tregs experienced much longer dwell times (∼7 weeks) (Pasciuto et al., 2020). Furthermore, CNS-infiltrating Tregs converted to a CD69+ putative resident population at rates 100-fold higher than that of activated non-Treg CD4s (Pasciuto et al., 2020). Additional experiments found that TCR signaling was critical for both Treg entry and long-term persistence in the CNS. Specifically, usage of Nur77-GFP reporter mice revealed that, in contrast to CD69+ conventional T-cells, CD69+ CNS Tregs expressed high levels of the Nur77-GFP reporter. To formally test whether TCR engagement is required, Tregs bearing a TCR transgene reactive against the neuronal antigen MOG 35-55 (2D2) or the non-self-model antigen OVA (OT-II) were adoptively transferred into wild-type mice. Tregs expressing the 2D2 TCR transgene were enriched in the brain as compared to the periphery, and were preferentially enriched in the CD69+ population. In contrast, OT-II Tregs were undetectable in the same space. In separate work, OTII *Rag-/-* animals also showed no entry of Tregs into the brain during stroke recovery (Ito et al., 2019), although abundant numbers of peripheral endogenous Tregs entered the brain during the same time span. These more recent findings agree with earlier publications, which demonstrated that after the peak of EAE, effector T-cell populations contracted sharply, while regulatory populations were maintained at similar levels, implying a possible long-term resident regulatory population (Korn et al., 2007). Multiple observations therefore demonstrate that Tregs are able to migrate from the periphery to the brain and adopt a resident memory phenotype. This requires peripheral activation but further relies on continuous TCR engagement to retain Tregs and enable long-term residency.

Further studies have interrogated whether Tregs originate from central (thymus-derived) populations, or from peripheral induction. In both the steady state and at late-stroke recovery timepoints, the majority of brain Tregs express Helios, a putative marker of centrally derived Tregs (Ito et al., 2019). Classic EAE model experiments have also suggested a primary role for thymic-derived Tregs; when Foxp3/GFP- splenocytes were transferred from *Foxp3gfp* KI mice into *RAG-/-* recipients immunized with MOG, the transferred cells were absent from the CNS during peak inflammation, suggesting that peripheral conversion to regulatory status was not occurring (Korn et al., 2007). However some evidence supports the peripheral induction of Tregs in the CNS as well – for example, when OVA peptides were injected into the CSF of OT-II mice preferential accumulation of OT-II cells was observed in the meninges with ongoing polarization to Th1, Th17, or Treg phenotypes, followed by adoption of a resident memory phenotype (CD44+ CD69+) (Rustenhoven et al., 2021). Additionally, pseudotime analysis of T-cell populations in the CSF from patients with brain metastases suggested that naïve T-cells diverged into either Treg subsets or reactive/proliferating T cell states (Rubio-Perez et al., 2021). Conversion to Treg phenotypes in EAE models was previously shown to occur via cell-cell contact between activated T-cells and neurons in a TGFβ-dependent manner (Liu et al., 2006). Studies of TCR repertoire usage by CNS Tregs have revealed further insights into their origins as well as their antigen specificity; in both EAE and *T. gondii* infection models, analysis of TCR usage among regulatory and conventional T cells demonstrated a divergence in TCR repertoires between Tregs and Tcons, suggesting they do not extensively interconvert during peak inflammation (Nguyen et al., 2010; O'Brien et al., 2017). In post-stroke models, TCR repertoire usage of CNS Tregs is significantly limited as compared to splenic Tregs, and skewed toward usage of overlapping TCR clones (Ito et al., 2019), suggesting a distinct antigen-specific subset entered the CNS in this setting. Among patients with brain metastases, TCR analysis revealed that Treg populations had relatively high levels of unexpanded clones, and correspondingly low levels of proliferative marker expression, suggesting that *in situ* Treg expansion was minimal in this disease setting. Overall, the bulk of evidence so far suggests CNS Tregs may be either thymic-derived or peripherally induced and express distinct, relatively limited TCR repertoires, that reflect the influence of antigen binding.

Additional investigations have improved our understanding of the plasticity of CNS Tregs. At the peak of inflammation in EAE models, CNS Tregs were observed to upregulate signature genes of Th1 and Th17 cells but continued to express FOXP3 (Garg et al., 2019). This was due to constitutive expression of BLIMP1 in a STAT1-dependent manner, which antagonized IL-6-directed methylation of key regulatory regions of the *Foxp3* locus. BLIMP1 expression, in turn, has been shown to be maintained by stimulation of the tumor necrosis factor receptor 2 (TNFR2), which is highly expressed in CNS Tregs (Ronin et al., 2021). This is consistent with separate work demonstrating the importance of TNFRSF members at inducing and maintaining effector Tregs, in an NF-kB dependent manner (Vasanthakumar et al., 2017). FOXP3 expression can be downregulated under conditions of significant inflammation; Bailey-Bucktrout et al. (2013) demonstrated that MOG-specific Tregs, when transferred into recipient mice at the peak of EAE, lost FOXP3 expression, and gained effector T-cell properties including the capacity to secrete IFNγ. This was primarily observed in MOG-specific CNS Tregs rather than bulk polyclonal transferred Tregs. Among MOG-specific CNS Tregs, IL-2 complexes could block loss of FOXP3 expression upon transfer. Overall, this implies that CNS Treg identity is not necessarily fixed under severely inflammatory conditions, but influenced by signaling via the TCR, TNFRSF family members, and the availability of antigen and IL-2.

Tregs are known to depend on extrinsically provided IL-2 for maintenance and expansion in non-lymphoid tissues, including the CNS. Curiously, while conventional T-cells contract in the CNS following peak inflammation in EAE models, Tregs are maintained at similar numbers. This suggests they may derive

IL-2 from alternative sources than the rapidly diminishing population of conventional T-cells. Astrocytes may serve this role, as they are known to constitutively express IL-2, and have been shown *in vitro* to function to maintain cerebral Tregs (Xie et al., 2015). IL-2 has been shown to be necessary for Treg expansion following stroke recovery (Ito et al., 2019) and its provision limits EAE-induced disease activity (Rouse et al., 2013; Ito et al., 2019). The therapeutic potential of this is not unrecognized – IL-2/IL-2c complexes, which selectively expand Treg populations, are protective in models of stroke and traumatic brain injury (Boyman et al., 2012; Gao et al., 2017; Zhang et al., 2018).

Maintenance of CNS Tregs is also influenced by IL-33 signaling, similar to other anatomical spaces. IL-33 is expressed widely throughout the healthy brain and is concentrated in white matter due to predominant expression in post-mitotic oligodendrocytes (Yasuoka et al., 2011; Jiang et al., 2012; Gadani et al., 2015; Fairlie-Clarke et al., 2018). ST2, the IL33 receptor, is highly expressed in brain Tregs at steady state. Conditional knockout of ST2 in FOXP3+ cells led to an exacerbation of EAE in mouse models, but did not affect overall cell numbers of CNS-infiltrating Tregs or T-effector cells (Hemmers et al., 2021). Instead, qualitative changes in Treg functionality were implied, reflected by their diminished capacity for suppression of IL-17A-secreting gamma-delta T-cells early in EAE development. ST2 KO CNS Tregs expressed lower levels of NF-kB pathway molecules, suggesting this signaling axis may be critical in CNS Treg maturation and functionality. While knockout of ST2 did not affect numbers of CNS Tregs in EAE models, diminished expansion was observed in ST2- or IL-33-deficient mice following middle cerebral artery occlusion (Ito et al., 2019). This may be explained by Treg-extrinsic effects of IL-33, as IL-33 provision leads to comparable increases in both ST2-sufficient and -deficient Tregs (Hemmers et al., 2021).

Specialized functions of CNS Tregs reflect their interactions with cells comprising the microglia, vasculature, lymphatics, neurons, and meninges. Tregs entering the CNS space have canonical anti-inflammatory roles, as well as contribute to tissue repair following a variety of immune- and non-immune-mediated insults. In mouse models using middle cerebral artery occlusion to induce permanent brain ischemia, multiple findings showed that Tregs affect tissue repair of the ischemic tissue, and ultimately impact neurological outcomes (Liesz et al., 2009b; Li et al., 2014, 2018). Similar findings support a role for Tregs in tissue repair during subacute/chronic phase after spinal injury (Raposo et al., 2014). Tregs accumulated in the infarcted tissue area several days after middle cerebral artery occlusion, and potentiated neurologic recovery (Ito et al., 2019; Shi et al., 2021). At day 14 after infarction, CNS Tregs had transcriptional signatures similar to other tissue Tregs including those present in VAT or injured muscle. However, they uniquely expressed the serotonin receptor type 7 gene, *Htr7*, among other genes. Neurologic recovery was deficient in the absence or blockade of serotonin activity, while serotonin potentiated neurologic restoration. The broad importance of serotonin to immune cell activity and autoimmunity has been reviewed elsewhere (Wan et al., 2020), but recent findings specifically identify a role for serotonin in modifying Treg functionality and frequency in the CNS at late-stroke timepoints (Ito et al., 2019). In middle cerebral artery occlusion models, Tregs contribute to neurologic recovery by suppressing astrogliosis via secretion of AREG. Additionally, Tregs induced oligodendrocyte maturation by secreting osteopontin as well as CCN3, a growth regulatory protein, thereby contributing to re-myelination (Dombrowski et al., 2017; Shi et al., 2021). In contrast to these later effects, other findings suggest Tregs markedly affect neurological recovery from ischemia and other insults during early timepoints as well. In the subacute/acute phase of ischemic stroke, meningeal Tregs inhibited the secretion of matrix metalloproteinase 9 from nearby neutrophils, resulting in better preservation of the blood-brain barrier (Li et al., 2013). This minimized cerebral inflammation and reduced infiltration of peripheral inflammatory cells into infarcted brain tissue. In the periphery, alterations of the frequency and function of Tregs in the spleen and blood are observed in patients following ischemic stroke, and in experimental models, implying that changes in Treg behavior in the periphery may also impinge on neurological outcomes (Offner et al., 2006; Liesz et al., 2009a; Hu et al., 2014; Ruhnau et al., 2016). Further studies are therefore needed to clarify the pleotropic roles that Tregs play in recovery from neurologic injury or ischemic recovery at multiple timepoints.

Interferon Signature Tregs

Recent single-cell RNA-seq studies have identified a unique population of CD4+ conventional T-cells and Tregs that express strong interferon-stimulated gene (ISG) signatures. ISGs are a large family of protein coding genes that are expressed during viral infections and mediate anti-viral immunity. At steady state, a small subset of mature CD4SP thymocytes express a strong ISG signature (Hemmers et al., 2019). The purpose of these thymocytes remains unclear. Following infection with Salmonella or Citrobacter, a small population of effector CD4+ T-cells in the colon express high levels of ISGs (Kiner et al., 2021). Finally, during house dust mite challenge, a subset of lung CD4+ conventional T-cells expressed an ISG-signature (Tibbitt et al., 2019). CD4+ T-cell ISG expression was abrogated upon administration of IFNAR blocking antibody indicating that these cells are receiving signals from type I IFNs. In the skin draining brachial lymph node, a subset of Tregs express high levels of STAT1 and other ISGs (Miragaia et al., 2019). A similar population has been identified in the spleen, lungs, and gut (Lu et al., 2020). The function of these cells is also not known. It is possible that these conventional CD4+ T-cells and Tregs are drawn to an IFN-rich niche due to recognition of IFN-induced antigens and may play a role in tolerance to IFN-driven inflammation. In support of this possibility, one study has shown that Tregs are important for controlling IFNα and associated ISG production in a mouse model of psoriasis (Stockenhuber et al., 2018). Furthermore, a subset of house dust mite (HDM)-reactive CD4+ conventional T-cells and Tregs in humans express an ISG signature and these cells are more frequent in individuals without HDM allergy (Seumois et al., 2020), suggesting their preferential expansion in individuals without HDM allergy. This

observation supports the hypothesis that Tregs bearing the ISG-signature could play a distinct immunosuppressive role during allergic responses. Further studies are needed to understand the function of these unique populations of CD4+ conventional T-cells and Tregs.

FUTURE CONSIDERATIONS

The studies discussed in this review have shed considerable light on the phenotype and function of several Treg subsets. However, further studies are required to understand the developmental timing and factors that drive tissue-specific Treg adaptations and how Treg phenotypes are altered during distinct immune responses. Further, the basic mechanism that licenses Treg activation and eTreg differentiation, required for all subsequent Treg specification, remain incompletely understood. Finally, as we discover more about Treg diversity, it will be important to develop novel tools that will enable precise interrogation of specific subsets and their functions *in vivo*. Currently, many studies make use of *Foxp3-DTR* mice to study Treg function in different contexts. While this is a powerful tool to study the consequences of Treg deletion, it does not allow for specific removal of distinct Treg subsets. Other groups have extensively utilized *Foxp3-CRE* mice crossed to mice with a floxed gene of interest to eliminate expression of that gene from Tregs. While this enables the study of that gene of interest in Tregs, it is possible that eliminating that gene may have broad effects on Treg biology and confound interpretation of functional changes that occur as a result. Finally, *Foxp3*-floxed mice crossed to mice expressing CRE driven by expression of a gene of interest have also been used regularly to eliminate particular Treg subsets. Use of this approach comes with the complication of potentially turning Tregs expressing the gene of interest into conventional CD4+ T-cells that could exert effector functions and preclude interpretation of the function of the subset of interest. While these murine models have provided great insight into Treg function, new tools are needed to isolate and manipulate particular Treg subsets *in vivo*.

The dynamics of Treg heterogeneity during immune perturbations have also been largely unexplored. For example, we do not know how proportions of Treg subsets within tissues change over the course of an infection or injury. scRNA-Seq based approaches should allow us to better characterize Treg subset dynamics during disease and enable better understanding of the function of distinct Treg subsets. Likewise, where tissue Tregs exert their function within a tissue, and which cells they are intimately interacting with remains an open area of exploration. Additionally, aside from expression of Thelper associated markers, there is limited information about how Treg subsets vary in the context of distinct infections and tissue damage or injury. Further studies using single-cell RNA-seq or related approaches should be informative in this context as they allow dissection of heterogeneity within the Treg population and allow identification of potentially novel Treg subsets.

Non-lymphoid tissue Tregs are diverse and express phenotypes that are distinct from those expressed by lymphoid tissues. While several recent single-cell RNA-seq experiments have characterized the transcriptional adaptation of lymphoid Tregs into non-lymphoid tissue Tregs, the factors that drive specific tissue adaptations and the timing of their expression are still not well understood. Thus, it will be important to understand when and how Tregs adapt to specific tissues and how they diverge phenotypically within one tissue.

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LS, DO, and ST wrote the manuscript. MF edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The TNF-α/TNFR2 Pathway: Targeting a Brake to Release the Anti-tumor Immune Response

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Newly discovered anti-cancer immunotherapies, such as immune checkpoint inhibitors and chimeric antigen receptor T cells, focus on spurring the anti-tumor effector T cell (Teff) response. Although such strategies have already demonstrated a sustained beneficial effect in certain malignancies, a substantial proportion of treated patients does not respond. $CD4^+FOXP3^+$ regulatory T cells (Tregs), a suppressive subset of T cells, can impair anti-tumor responses and reduce the efficacy of currently available immunotherapies. An alternative view that has emerged over the last decade proposes to tackle this immune brake by targeting the suppressive action of Tregs on the anti-tumoral response. It was recently demonstrated that the tumor necrosis factor alpha (TNF-α) tumor necrosis factor receptor 2 (TNFR2) is critical for the phenotypic stabilization and suppressive function of human and mouse Tregs. The broad non-specific effects of TNF-α infusion in patients initially led clinicians to abandon this signaling pathway as first-line therapy against neoplasms. Previously unrecognized, TNFR2 has emerged recently as a legitimate target for anti-cancer immune checkpoint therapy. Considering the accumulation of pre-clinical data on the role of TNFR2 and clinical reports of $TNFR2^+$ Tregs and tumor cells in cancer patients, it is now clear that a TNFR2-centered approach could be a viable strategy, once again making the TNF-α pathway a promising anti-cancer target. Here, we review the role of the TNFR2 signaling pathway in tolerance and the equilibrium of T cell responses and its connections with oncogenesis. We analyze recent discoveries concerning the targeting of TNFR2 in cancer, as well as the advantages, limitations, and perspectives of such a strategy.

Keywords: regulatory T cells (Tregs), immunotherapies, cancer, immunosuppression, anti-tumor action

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INTRODUCTION

The last few decades have marked an era of promising advances in the field of cancer therapy. The anti-tumor strategy landscape has shown several fundamental changes of paradigm, switching from cancer cell-centered approaches using chemo- and radiotherapy to strategies focused on the specific features of cells and the tumor microenvironment (TME), with targeted therapies, to therapies that

enable the patient's immune system to fulfill its role of destroying tumor cells. Recently approved anti-CD19-anti-CD3 bispecific monoclonal antibodies (mAb), immune checkpoint inhibitors (ICIs), and adoptive chimeric antigen receptor (CAR)-T cells were developed to enable patients' effector T cells (Teffs) to better recognize and eradicate malignant cells (Waldman et al., 2020). These immunotherapeutic approaches have already demonstrated their efficacy against several malignancies with a poor prognosis, bringing hope to numerous patients.

To date, ICIs that have reached the patients' bedside have focused on releasing effector $CD8^+$ T cells from functional restrictions induced by the tumor and its micro-environment (Topalian et al., 2015; Fesnak et al., 2016; Khalil et al., 2016). However, physiologically, a minor subset of T cells oversees deployment of the adaptative response to protect tissue homeostasis, avoiding outrange proliferation of the Teff population. The crucial role of CD4 + $FOXP3^+$ regulatory T cells (Tregs) in immune tolerance has led to a massive effort to better unveil their origin and suppressive function over the last 50 years (Sakaguchi et al., 2007). In the context of cancer, tumor and TME-associated cells often impair the balance between Teffs and Tregs, with an observed increase in the function or number of Tregs in several types of malignancies, as summarized in **Table 1** (Curiel, 2008, p. 200). Although cytotoxic tumor-infiltrating lymphocytes (TILs) are often associated with favorable clinical outcomes, the relationship between $FOXP3^+$ TIL and the prognosis is less clear. This controversy concerning the role of Tregs in malignancy may arise from various causes, including differences in the methods and markers used to identify Tregs between studies. Furthermore, the fact that the $FOXP3^+$ population is not composed of solely suppressive cells (Tanaka and Sakaguchi, 2017) and that FOXP3 expression in neo-activated human Teff is transitory may also add a level of complexity to interpreting these studies (Saito et al., 2016).

Regardless of the mechanism, the inherent function of Tregs to suppress conventional T cell responses can be acutely detrimental for the immune control of cancer initiation, growth, and dissemination for several cancer types (**Table 1**). In addition, Tregs are likely to interfere with Teff-centered immunotherapies (Binnewies et al., 2018). Although these cells can serve as prognostic factors, it is also necessary to learn how to block this heterogeneous population in patients to overcome its immunosuppressive effects. Initially, mouse models allowed the demonstration of a beneficial effect of Treg depletion on the potency of the anti-tumor response using $FOXP3^{DTR}$ mice or antibodies targeting highly expressed Treg receptors (Klages et al., 2010). Based on these observations, a myriad of potential therapeutic approaches directly targeting Tregs have been described in animal models and several are currently being assessed in clinical trials. In addition, several therapies already in use have a documented, yet still controversial, effect in impairing Tregs, either immunotherapies, such as ipilimumab [anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4)] (Tang et al., 2018, p. 4), or certain chemotherapeutic agents, such as cyclophosphamide and fludarabine (Beyer et al., 2005; Walter et al., 2012). Specific Treg inhibition can refer to the blockade/impairment of: (i) thymic-derived Treg migration to the tumor site or, alternatively, the capacity of naïve T cells to become Tregs on site, (ii) their activation, proliferation, and/or survival, and (iii) their suppressive function. Studies have exploited the chemokine network (mainly the CCR4/CCL22 axis (Ishida et al., 2012; Ni et al., 2015), the IL-2/CD25 pathway (Ross and Cantrell, 2018), and co-stimulatory molecules, such as GITR (Killock, 2019; Zappasodi et al., 2019), inducible T cell costimulatory (ICOS) (Le et al., 2016; Mo et al., 2017; Yap et al., 2018), and OX40 (Aspeslagh et al., 2016).

Despite these emerging approaches to block or deplete Tregs, they still show limitations because of the lack of Treg-specific biomarkers and the potential induction of autoimmunity as a consequence of systemic Treg impairment (Stephens et al., 2001; Kim J. et al., 2009). Additionally, Treg depletion may be followed by their rapid reconstitution, potentially resulting in a higher Treg frequency than their level prior to depletion (Mahnke et al., 2007; Berod et al., 2014). Therefore, developing new tools to circumvent tolerance toward malignant cells is still a priority. In this respect, the type II receptor of tumor necrosis factor alpha (TNF-α) (TNFR2) represents another hope in targeting Tregs, including in the tumoral context (Cohen and Wood, 2017). Due to its particular pattern of expression by cells of the immune system, preferentially immunosuppressive cells, targeting TNFR2 could permit the tuned modulation of both innate and adaptive responses in diverse pathogenic contexts (Salomon et al., 2018; Wajant and Beilhack, 2019). After discussing the role of the TNF-α/TNFR2 in tolerance, we then present and discuss the most recent developments that have led to the consideration of TNFR2 as a new brake to impede the anti-tumor immune response through its beneficial role on Tregs and the associated therapeutic perspectives for this molecule as a novel target for cancer immunotherapy.

THE CRITICAL FUNCTION OF TNF-α/TNFR2 IN TOLERANCE

Comprehensive Overview of the TNF-α Signaling Pathway

Pattern of Expression of TNF-α and Its Receptors

Tumor necrosis factor alpha is currently considered to be one of the most pleiotropic cytokines described in mammals, with roles spanning virtually every biological system beyond its activity in immune system physiology. Indeed, this transmembrane protein is not only expressed by immune cells, such as monocytes/macrophages (including microglia in the nervous system), B cells, activated T and NK cells, but also by a diverse array of non-immune cells, such as fibroblasts, keratinocytes, astrocytes, endothelial cells, epithelial cells, and many cancer cells (Sedger and McDermott, 2014). Transmembrane TNF-α (tmTNF-α) assembles into a homotrimer that is cleaved by the matrix metalloprotease TNF-α-converting enzyme (TACE/ADAM17) releasing a soluble form of the TNF-α (sTNF-α) homotrimer, responsible for the endocrine function of TNF-α (Kriegler et al., 1988; Black et al., 1997; Moss et al., 1997).

TABLE 1 | Detection and clinical association of Tregs in carcinomas (A) and hematological malignancies (B).

(A)

Data	Cancer type(s)	Reference(s)
High Treg tumor infiltrate	pancreas, lung, liver, gastrointestinal tract, breast, ovarian, head and neck	Tanaka and Sakaguchi, 2017
Negative association between high FOXP3$^+$ infiltration and patient survival	melanoma, cervix, breast, kidney	Shang et al., 2015
Diminished ratio of infiltrating CD8$^+$ lymphocytes/FOXP3$^+$ Tregs associated with a poor prognosis	breast, ovarian, gastric, colorectal cancers	Sasada et al., 2003; Curiel et al., 2004; Sato et al., 2005; Bates et al., 2006; Sinicrope et al., 2009
Treg infiltration at each stage of the disease is an indicator to predict disease progression and survival	ovarian	Curiel et al., 2004
High Treg tumor infiltration correlates with a favorable prognosis	breast, ovarian, colorectal, head and neck	Badoual et al., 2006; Leffers et al., 2008; Salama et al., 2009; Correale et al., 2010; Frey et al., 2010; West et al., 2013; Yeong et al., 2017

(B)

Data	Cancer type(s)	Reference(s)
High Treg tumor infiltrate comparable to that of healthy donors (bone marrow and/or PB)	B-ALL, T-ALL, and AML, CLL, CML, MGUS, multiple myeloma	Mittal et al., 2008; D'Arena et al., 2011; Zahran et al., 2014; Wang M. et al., 2018; Niedźwiecki et al., 2019
Elevated percentage of Tregs after completing treatment and achieving remission	AML	Szczepanski et al., 2009
Higher Treg frequencies correlate with more advance disease status	CLL	D'Arena et al., 2011
Lower Treg numbers (i) in patients with chronic phase CML vs. accelerated and blast phases, (ii) in patients with complete molecular remission	CML	Zahran et al., 2014
Higher numbers of Tregs positively correlate with advanced disease stage and serum lactate dehydrogenase (a poor prognosis factor for NHL)	NHL	Mittal et al., 2008
• Low infiltration of FOXP3$^+$ cells in conjunction with high infiltration of cytolytic TIA-1$^+$ cells predicts disease progression and an unfavorable outcome • FOXP3/Granzyme B ratio ≤1 predicts poor failure-free survival and overall survival	cHL	Alvaro et al., 2005; Kelley et al., 2007
Higher Treg numbers predict improved survival, whereas a marked reduction in Tregs is observed on transformation to diffuse large B-cell lymphoma	Follicular lymphoma	Carreras et al., 2006
Association between an increased number of FOXP3$^+$ Tregs with improved survival	CTCL (mycosis fungoides and unspecified CTCL)	Gjerdrum et al., 2007

White indicates data with no prognostic association, red a negative correlation between Treg infiltration and prognosis, and green a positive correlation.
AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; cHL, classical Hodgkin lymphoma; CML, chronic myeloid leukemia; CTCL, cutaneous T cell lymphoma; MGUS, monoclonal gammopathy of undetermined significance; NHL, non-Hodgkin lymphoma.

Both forms can bind to structurally related but functionally distinct receptors: TNFR1 (p55/60), which binds to tmTNF-α, as well as sTNF-α, and TNFR2 (p75/80), which shows higher affinity and is more robustly activated by tmTNF-α than sTNF-α (Grell et al., 1995). TNFR1 is ubiquitously expressed in almost any cell type at a low level, whereas TNFR2 expression is finely regulated and limited to several cell types of the immune system, including CD4$^+$ and CD8$^+$ T cells, but also plays an important role in cells of the vasculature and muscle and brain tissues (Faustman and Davis, 2010; Fischer et al., 2011; Puimège et al., 2014; Pegoretti et al., 2018). A high density of TNFR1 and TNFR2 has been observed on myeloid cells (monocytes, macrophages, and dendritic cells), in which both pathways are interconnected, promoting their activation, proliferation, and survival (Rossol et al., 2007; Maney et al., 2014; Wajant and Siegmund, 2019). Myeloid-derived suppressor cells (MDSCs), an inflammation-induced population, appears to be the one myeloid population that specifically requires TNFR2 for its induction and suppressive functions (Zhao et al., 2012; Hu et al., 2014; Polz et al., 2014; Ham et al., 2015). Finally, the TNF-α/TNFR2 signaling pathway has very recently been shown to be a key regulatory factor for the immunosuppressive effect of mesenchymal stem cells and neural and endothelial progenitor cells (Beldi et al., 2020; Naserian et al., 2020; Shamdani et al., 2020). Overall, these observations support the existence of a TNFR2-dependant network of immunosuppressive cells that have been ignored until recently, that could help broadening the therapeutic landscape.

TNF-α/TNFR2 Signaling Pathways

Tumor necrosis factor receptor 1 and TNFR2 share similar extracellular TNF-α-binding motifs, including the membrane-distant pre-ligand binding assembly domain (PLAD), which is important for the ligand-mediated formation of active receptor complexes (Chan et al., 2000). As both receptors lack intrinsic enzyme activity, they need to recruit cytosolic actors upon ligand binding to initiate intracellular signal transduction. The two

TNF-α receptors differ highly in their intracellular structure, which is responsible for their divergent activity. Simply put, TNFR1 belongs to the family of death domain-containing receptors and is responsible for cell death, whereas TNFR2 is a TNFR-associated factor (TRAF)-interacting receptor, without a death domain, that favors cell activation (Wajant et al., 2003). However, the reality is much more complex, with the addition of the interplay between the intracellular pathways of the two receptors reflecting the broad range of biological actions of TNF-α. The signaling pathways are discussed in detail below and presented in **Figure 1**.

The TNFR1 death domain preferentially interacts with an adaptor protein, called TNFR1-associated death-domain (TRADD) protein (Dempsey et al., 2003). TRADD further recruits another two adaptor proteins, receptor-interacting protein kinase 1 (RIPK1) and TRAF2, thus forming an enzymatic complex signalosome, also known as signaling complex I. One of the main targets of complex I is the enzyme complex IκB kinase (IKK). Phosphorylation of IKK leads, in turn, to the canonical activation of nuclear factor-κB (NF-κB), as well as members of the family of MAP kinases, such as c-Jun kinase (JNK) and p38 MAPK (Natoli et al., 1997; Brinkman et al., 1999). The respective target genes of these proteins allow the organism to respond effectively to environmental changes. The classical NF-κB pathway is activated by a variety of inflammatory signals, resulting in coordinate expression of multiple inflammatory and innate immune genes (Liu et al., 2017). The JNK pathway and p38 signaling are strongly activated by surrounding stress and inflammatory cytokines. They contribute to a broad range of reactions, including inflammation, apoptosis, cell activation/migration/differentiation, cell cycle regulation, cytokine production, and metabolism (Morrison, 2012). Alternatively, signaling complex I can also be internalized and then converted to a death-inducing signaling complex, so-called pro-apoptotic complex II, by adaptor protein Fas-associated protein with death domain (FADD). Complex II includes procaspase 8, which is activated by autocatalytic cleavage, initiating downstream caspase cascades and ultimately inducing cell apoptosis (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004). If caspase 8 is absent or inactivated, kinase-active RIPK1 recruits and activates RIPK3, resulting in the formation of the necrosome and further execution of necroptosis via membrane permeabilization (Vanden Berghe et al., 2014; Grootjans et al., 2017). Caspases and their inhibitors are pivotal elements in deciding the cell's fate after TNF-α/TNFR1 triggering, along with the engagement of NF-κB-mediated anti-apoptotic signaling pathways, which are able to delay the time of death (Schliemann et al., 2011).

In contrast to the well-characterized TNFR1 signaling pathways and their physiological relevance, TNFR2-mediated signaling pathways were uncovered much later. Because of the lack of a death domain, TNFR2 is unable to recruit TRADD protein, but can instead weakly bind to TRAF2 directly (Rothe et al., 1995, p. 2). Under these conditions, TRAF2 induces the non-canonical NF-κB pathway, through the activation of NF-κB-inducing kinase (NIK), which further leads to the phosphorylation of IKKα and the processing of p100, a crucial step in the nuclear translocation of p52/RelB (Borghi et al., 2018). The alternative NF-κB pathway acts quite distinctly from the classical one, for example by being dispensable for the initial activation of naive T cells through TCR signaling but crucial for the *in vivo* generation and maintenance of effector and memory T cells (Sun, 2017). However, upon binding to TRAF2, TNFR2 can also recruit cIAP1/2 proteins, which are involved in TNFR1-mediated NF-κB activation, creating crosstalk between the TNFR pathways. TNFR2 pathway activation consumes the cytosolic pool of the TRAF2-cIAP1/2 complex, limiting its availability for other receptors, including TNFR1. Due to the role of TRAF2 and cIAPs in preventing apoptosis and necroptosis in the context of TNFR1 signaling, TNFR2-mediated deprivation of these molecules is able to enhance TNFR1-induced cell death in macrophages (Siegmund et al., 2016). Finally, another notable adaptor protein, called endothelial/epithelial protein tyrosine kinase (Etk/BMX), has been described that can interact with TNFR2 in the absence of ligand (Pan et al., 2002). TNFR2-mediated Etk phosphorylation is able to partially activate the growth factor receptor VEGFR2, resulting in cell survival and proliferation through PI3K/Akt activation (Fischer et al., 2015, p. 1; Ortí-Casañ et al., 2019).

An amplification loop has been reported for the TNF-α pathway (Zuckerman et al., 1991). TNF-α production is highly inducible, up to 10,000 fold, due to transcriptional upregulation of mRNA production by, among others, the NF-κB transcription factor (Varfolomeev and Vucic, 2018). In addition, TNF-α expression depends on p38-MAPK and JNK signaling at the post-transcriptional level, which act by modulating mRNA stability and translational efficiency (Falvo et al., 2010). As TNF-α binding to the TNFR activates the MAP kinase signaling cascades and transcription factors of the NF-κB family, it induces its own transcription in cells that express its receptors (Wajant and Beilhack, 2019). Furthermore, TNFR-binding can induce reverse signaling in tmTNF-α producing cells (Tartaglia et al., 1993). Such signal transduction results in the activation of NF-κB, this time within the TNF-α-producing cell, leading again to greater TNF-α transcription (Watts et al., 1999; Xin et al., 2006).

TNF-α/TNFR2 in the Control of the T Cell Response

Conventional T Cells

Most described TNF-α-mediated proinflammatory functions are mediated via TNFR1 expression in a multitude of immune cells, including T cells (Mehta et al., 2018). TNFR2 is also found on activated conventional T cells, in which it acts as a co-stimulatory molecule in a unique non-redundant manner, similarly to CD28 co-stimulation (Aspalter et al., 2003; Reiner, 2007). TNFR2 deficiency in knockout mice impairs the proliferative ability of conventional $CD4^+$ and $CD8^+$ cells and decreases their production of TNF-α, IFN-γ, and IL-2 in response to TCR stimulation (Kim and Teh, 2001; Kim et al., 2006, 2008). Intriguingly, TNF-α produced by activated Teffs under inflammatory conditions has been shown to be necessary for the stimulation of Treg expansion and the enhancement of their

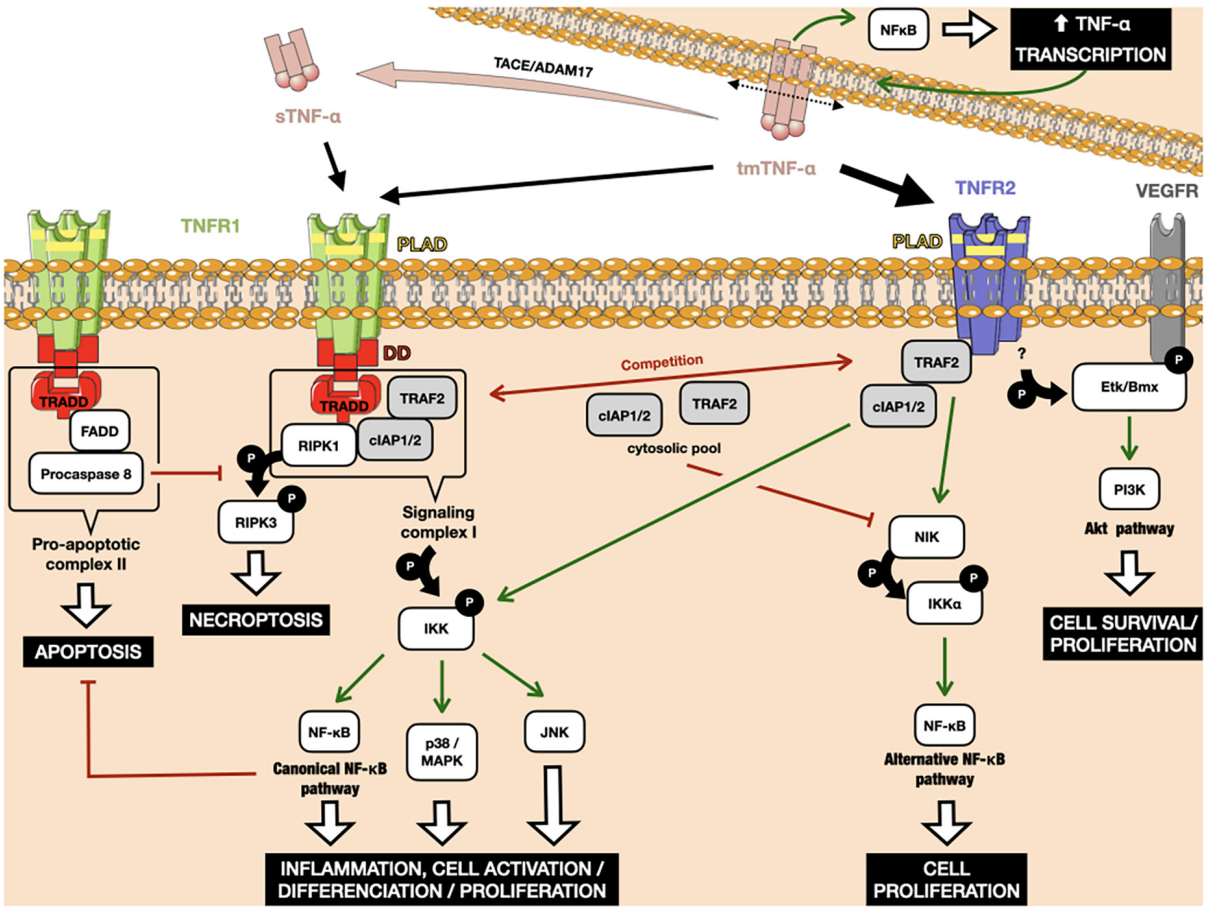

FIGURE 1 | Interplay between the TNFR1 and TNFR2 downstream signaling pathways. TNFR1 can bind to tm-TNF-α, as well as its soluble form after cleavage by the metalloproteinase TACE/ADAM17. TNFR2 binds to tm-TNF-α with higher affinity. Binding of Tm-TNF-α to TNFRs induces a positive retro-control loop on its transcription. TNFR1 activation can lead to apoptosis and necroptosis through interaction between its death domains and the TRADD adaptor. Both TNFR1 and TNFR2 can recruit cIAP1/2, leading to the activation of classical NF-κB. TRAF2 weakly binds to TNFR2, leading to activation of the alternative NF-κB pathway. The amount of cIAP1/2 and TRAF2 in the cytosol is crucial in cells that simultaneously express TNFR1 and TNFR2, as the consumption of these molecular actors after TNFR2 activation leads to the preferential activation of death pathways under TNFR1 rather than those of proliferation and survival. Red arrows indicate inhibition. Green arrows indicate activation. P: phosphorylation.

suppressive function (Chen et al., 2007; Grinberg-Bleyer et al., 2010; Leclerc et al., 2016). TNFR2 is not only essential for optimal Teff proliferation and activation but also the induction of activation-induced cell death (AICD), which terminates the proliferative response, a process that is dependent on the TNFR2 downstream actor TRAF2 (Twu et al., 2011). Consistent with this role of TNFR2, TNFR2$^{-/-}$ CD8^{+} T lymphocytes show high resistance to AICD, leading to the worsening of colonic inflammation (Punit et al., 2015). Thus, TNFR2 signaling is responsible for dual effects in T cells, as its activation stimulates establishment of the effector response while permitting its subsequent regulation through (i) the death of effector cells and (ii) the stimulation of the Treg subpopulation, which also helps to terminate the immune reaction.

Regulatory T Cells

Although TNFR1 expression does not differ between Tregs and other T cells, human Tregs constitutively express high levels of TNFR2 relative to conventional T cells in the steady-state (Fischer et al., 2020). Hence, the use of a target-agnostic phage display screening approach on human Tregs to find antibodies that preferentially bind to them, rather than Teffs, has surprisingly led to only finding candidates that specifically bind to TNFR2 (Williams et al., 2016). Thus, in addition to being a Treg marker, several studies conducted by Chen et al. (2008, 2010a,b) showed that TNFR2 expression by Tregs has important functional implications and defines a maximally suppressive subset of mouse and human Tregs (Chen and Oppenheim, 2011b,c). In particular, it was shown that the level of expression of TNFR2 correlates with the suppressive potential of nTregs (Chen et al., 2007), indicating that the most potent suppressors are highly susceptible to TNFR2 activation. These findings have been confirmed by many other groups since these pioneering findings (Ablamunits et al., 2010; Grinberg-Bleyer et al., 2010; Kleijwegt et al., 2010; Housley et al., 2011; Okubo et al., 2013; Atretkhany et al., 2018). Although the membrane-bound form of TNFR2 can be

either immunosuppressive or immunostimulatory, depending on which cell type expresses it, the function of the soluble form of TNFR2 appears to be consistently immunosuppressive (van Mierlo et al., 2008). Activated Tregs can release high amounts of sTNFR2, which, at least partially, represents an additional immunosuppressive mechanism of Tregs. Interestingly, it appears that these features are not restricted to $CD4^+$ Tregs, as the most potent $CD8^+$ suppressors are also characterized by the expression of TNFR2, although exploratory studies are required to confirm such results about this unrecognized regulatory subset (Ablamunits et al., 2010; Horwitz et al., 2013).

It is now well recognized that TNFR2 also contributes to the expansion of nTregs *in vitro* and *in vivo* (Chen et al., 2007, 2008; Okubo et al., 2013; Chopra et al., 2016; Fischer et al., 2017, 2018, 2019a,b; Padutsch et al., 2019). Recently, TNFR2 ligation was confirmed to enhance cell proliferation through the non-canonical NF-κB pathway in human Tregs, enhancing IL-2-induced cell proliferation (Wang J. et al., 2018). However, in mouse Tregs, the activation of p38 MAPK via the classic NF-κB signal appears to also be important for TNFR2-induced proliferation (He et al., 2018). Much information about the role of TNFR2 in Treg expansion and phenotypic stability has also come from the field of adoptive Treg cell therapy, used to aid tolerance in the context of autoimmunity, organ rejection, and GvHD. A non-commercial agonistic TNFR2 antibody in standard *ex vivo* Treg expansion protocols was shown to confer improved suppressive activity while reducing Treg heterogeneity (Okubo et al., 2013). Furthermore, using the TNFR2-specific mAb MR2-1 as an agonist, TNFR2 signaling promoted the expansion of low-purity MACS-isolated Treg preparations to homogenous Treg populations, stable under further restimulation (He et al., 2016). These studies, in accordance with the above-mentioned *in vitro* and *in vivo* data, demonstrate the role of TNFR2 in the suppressive potency of Tregs and encourage its utilization to improve *ex vivo* Treg expansion methods for clinical applications.

In Autoimmune Settings

Given the above-mentioned function of TNFR2 in Tregs and conventional T cell responses, these observations raise the question of the role of TNFR2 in the interplay between inflammatory and regulatory pathways in human immune pathologies. Initially, data about TNFR2 role in tolerance has come from the field of autoimmune diseases. Interestingly, TNFR2 polymorphisms can be found in patients with several inflammatory and auto-immune diseases, cementing its pivotal link with human immune tolerance (Pierik et al., 2004; Oregón-Romero et al., 2006; Horiuchi et al., 2007; Song et al., 2014). A role for TNF-α in suppressing systemic autoimmune responses has been emphasized by anti-TNF-α therapies for rheumatoid arthritis and inflammatory bowel disease patients. Drug-induced systemic lupus erythematosus symptoms were reported for certain patients after treatment with anti-TNF-α mAbs and soluble TNF-α receptors (Sandborn and Hanauer, 1999; Kollias and Kontoyiannis, 2002; Shakoor et al., 2002).

Although the efficacy of anti-TNF-α treatment requires no further demonstration in autoimmune diseases, it is still a relatively non-specific approach that does not affect solely autoreactive actors. Indeed, as implied by its name, TNF-α inhibition can result in rare but severe occurrences of treatment-induced hematological malignancies (Hansel et al., 2010). The perceived increase in such hematological malignancies has led the World Health Organization classification of tumors to include the category "iatrogenic immunodeficiency-associated lymphoproliferative disease," and the risk of virally transformed tumors is also being closely watched (Campo et al., 2011). Although the risk of anti-TNF-α-induced cancer for autoimmune patients is still debated, it provides a good example of a situation in which promoting tolerance via TNFR2 targeting would presumably be a better strategy than targeting TNF-α. The incredibly wide range of physiological functions dependent on TNF-α/TNFR1 in all body systems, including cancer immunosurveillance, render targeting this pathway highly challenging (Faustman and Davis, 2010, 2013).

THE ROLE OF TNF-α/TNFR2 IN MALIGNANCY

In the Tumor Microenvironment

Although immunosuppressive cells do not have a monopoly on TNFR2 expression in the steady-state, a large number of studies suggest that $TNFR2^+$ Tregs with high suppressive capacity are strongly represented in the TME of cancer patients and sometimes the peripheral blood, comparable to findings in mouse models (Chen et al., 2008). The first remarkable observation concerning TNFR2 in the context of cancer was the decrease in tumor growth and metastasis described in TNFR2 knockout mice, despite the TNFR2 costimulatory function on Teffs (Sasi et al., 2012; Chopra et al., 2013; Ham et al., 2015). TNFR2−deficient mice show reduced infiltration and induction of MDSCs, coinciding with a diminution in the number of Tregs inside the tumors, confirming the requirement of TNFR2 for the participation of these immunosuppressive cells in the TME (Zhao et al., 2012). Furthermore, although the beneficial effects of TNF-α on $CD8^+$ Teffs are mainly mediated through TNFR2 (Zheng et al., 1995; Calzascia et al., 2007), Chen et al. (2010a) showed that the upregulation of TNFR2 on intratumoral Tregs enables them to overcome the greater resistance to suppression of conventional intratumoral $TNFR2^+$ T cells. Overall, these data support the idea of a diverted role of TNFR2 in tumors in favor of a predominant immunosuppressive TME, notwithstanding the relevance of its function in T cells activation mentioned earlier.

In cancer patients, there is now compelling evidence that $TNFR2^+$ Tregs accumulate in TILs in Sézary Syndrome and cervical cancer and tumor ascites in ovarian cancer. This population is also elevated in the peripheral blood of acute myeloblastic leukemia (AML) patients, as well as that of hepatocellular carcinoma, lung, and cervical cancer patients (He et al., 2019). Remarkably, in the peripheral blood samples of lung cancer patients, the expression of TNFR2 on Tregs appears to correlate better with FOXP3 expression than the $CD25^+CD127^-$ combination (Yan et al., 2015, p. 2). Additionally, $TNFR2^+$ Tregs were associated with lymphatic invasion, distant metastases,

and a more advanced clinical stage of lung cancer. This and other observation highlight the pivotal role of TNFR2 expression in Tregs in the context of human cancer, consistent with the conclusions based on mouse models. An updated list of the studies available on TNFR2$^+$ Tregs in mice and humans, and their outlines are provided in **Table 2**. Interestingly, Tregs from the CD8$^+$ T cell subset have been recently found to also constitutively express TNFR2 (Ablamunits et al., 2010; Horwitz et al., 2013). These cells, less studied than their CD4$^+$ counterpart, have also been implicated in the maintenance of pro-tumoral tolerance (Zhang et al., 2018).

Concerning TNFR2$^+$ myeloid cells, the presence of MDSCs has been noted in plasmacytoma, fibrosarcoma, and liver and lung cancer (Sheng et al., 2018). Additionally, the presence of tumor-associated macrophages expressing TNFR2 has been shown to correlate with malignancy grade and metastasis in human triple-negative breast cancer (Frankenberger et al., 2015). Lastly, TNFR2 expression on endothelial cells makes it essential for tumor angiogenesis. In highly vascularized murine lung tumor xenografts tumor growth was inhibited in TNFR2$^{-/-}$ mice, in correlation with decreases in VEGF expression and capillary density, as well as bone marrow-derived endothelial progenitor cell incorporation into the functional capillary network (Sasi et al., 2012).

In Cancer Cells

Apart from its benefits concerning tolerance, another crucial aspect concerning the role of the TNFR2 pathway in improving carcinogenesis relies on its oncogenic features. Indeed, TNFR2-dependant NF-κB activation in epithelial cells induces carcinogenesis and the absence of this mechanism may have taken part as well in the observed impairment of tumor growth in TNFR2$^{-/-}$ mice (Onizawa et al., 2009; Suzuki et al., 2014; Nagaishi et al., 2016). Aberrant expression of TNFR2 on tumor cells has been reported in human hematological malignancies, including Hodgkin lymphoma, cutaneous T cell lymphoma (CTCL), and multiple myeloma, as well as in breast, skin, ovarian, colon, and renal cell cancers (Arnott et al., 2004; Uhlén et al., 2005; Hamilton et al., 2011; Rauert et al., 2011; Nakayama et al., 2014; Ungewickell et al., 2015; Al-Lamki et al., 2016; Williams et al., 2016; Yang et al., 2018). For example, point mutations and genomic gains of the TNFR2 gene (*TNFRSF1B*) enhancing the activation of the non-canonical NF-κB signaling pathway have been described in 18% of patients with recurrent CTCL, for both mycosis fungoides and Sézary syndrome (Ungewickell et al., 2015). Remarkably, recombinant human TNF-α has been shown to increase the quantity of TNFR2 expressed at the surface for a number of tumor epithelial-like cell lines (Medvedev et al., 1996; Alshevskaya et al., 2020). Additionally, as mentioned earlier, the binding of tmTNF-α to TNFR2 can induce reverse signaling, inducing survival via the NF-κB pathway in lymphoma cells, all the more so with soluble TNFR2 which is highly secreted by Tregs in the TME (Zhang et al., 2008). In turn, it appears that TNFR2 overexpression by cancer cells in a model of colon carcinoma (CT26) is associated with a greater presence of TNFR2$^+$ Tregs in draining lymph nodes and four time more sTNFR2 in the peripheral blood (Chen et al., 2018). In colorectal cancer patients, higher sTNFR2 levels are associated with a significant increase in overall mortality (Babic et al., 2016). Thus, TNFR2 is directly involved in uncontrolled tumor expansion, a feature that supplements its previously described role in maintaining an immunosuppressive milieu around malignant cells.

SPECIFIC TNFR2-TARGETING IN CANCER

The Rationale Behind TNFR2 Blockade

Although the qualification of "tumor necrosis" has withstood the test of time, the reality is less straightforward. Due to its plethora of functions through its two receptors, TNF-α is responsible for divergent actions in the context of cancer (Montfort et al., 2019). Although its function through TNFR1 effectively favors the death of cancer cells while promoting the T cell pro-inflammatory response via NF-κB signaling, the activation of TNFR2 on immunosuppressive cells recruited by the tumor could be detrimental for anti-cancer responses. Considering this refined view of TNF-α functions, several studies have focused on sensitizing cancer cells to TNFR1-induced apoptosis, for example by inhibiting survival signals, such as NF-κB, in combined therapy with TNF-α (Wang and Lin, 2008). However, these approaches are, as the historical attempts to use recombinant TNF-α in cancer patients, not specific to cancer cells, with a high risk of off-target effects (Roberts et al., 2011). A more specific approach would be to block TNFR2 to focus the therapy on the immunosuppressive cells that accumulate during carcinogenesis, hoping to:

(i) eliminate the detrimental immunosuppressive TME, including infiltrated TNFR2$^+$ Tregs, to consequently awaken the anti-tumor response,
(ii) while redirecting TNF-α to TNFR1 expressed at the surface of immune effector cells, promoting an inflammatory response.

Furthermore, many tumors appear to start expressing TNFR2 during their transformation or originate from cells that express it in the case of immune cell-derived neoplasms, offering a chance to directly impair tumor evolution by blocking an oncogene.

Pioneering Approaches for TNFR2 Blockade

Based on these compelling assertions, several studies have addressed the feasibility of therapeutic TNFR2 blockade in animal cancer models (**Table 2**.). The group of Faustman has developed two dominant human TNFR2 antagonist mAbs that lock TNFR2 in the form of antiparallel dimmers, preventing further TNF-α binding (Torrey et al., 2017). These compounds were able to kill the patients' Tregs isolated from ovarian cancer ascites more potently than those from healthy donors, supposedly due to the high TNFR2 expression on TME-infiltrating Tregs. Thus, these antagonists can preferentially

TABLE 2 | Role of TNFR2$^+$ Tregs in cancer immunology.

(A)

Tumor type	Mice	Reference(s)
Experimental metastasis	• In TNFR2$^{-/-}$ mice with colon (MC-38) or lung (H-59) carcinoma, metastasis and Tregs accumulation are reduced • In WT mice, treatment with TNFR2 antisense oligodeoxynucleotides inhibits hepatic metastasis • In mice with TNFα- or TNFR2 immune cell-restricted deficiency, melanoma (B16F10-Luc) metastasis to the lung and numbers of Treg infiltration in lungs are decreased	Chopra et al., 2013; Ham et al., 2015

(B)

Tumor type	Mice	Patients	Reference(s)
Lung cancer	• The proportion of TNFR2$^+$ cells in CD4$^+$CD25$^+$ TILs is >70%, higher than in peripheral lymphoid organs	• Increased proportion of TNFR2$^+$ Tregs in PB (>TNFR2$^+$ Teff) • TNFR2$^+$ Tregs positively correlate with lymphatic invasion, distant metastasis, and clinical stage • TNFR2$^+$ Tregs are more proliferative, active, and suppressive	Chen et al., 2008; Yan et al., 2015, p. 2
Hepatocellular carcinoma and colon cancer	• CD103$^+$ Tregs express higher levels of TNFR2 than CD103- Tregs in the spleen and tumor (CT26 and BNL cell model) • Blockade by an anti-TNFR2 mAb or by a soluble TNFR2 fusion protein (sTNFR2-Fc) inhibits TNF-α-induced expansion of CD103$^+$ Tregs *in vitro* • Blockade by sTNFR2-Fc after cyclophosphamide treatment • inhibits tumor growth	• Increased proportion of CD45RA$^-$ FOXP3^{hi} effector Tregs in PB, with high expression of CTLA-4, CCR5, and TNFR2	Chang et al., 2015
Colon cancer	• TNFR2-blocking Ab combined with CpG ODN (TLR9 agonist) reduces the proportion of TIL TNFR2$^+$ Tregs, increases cytotoxic IFN-γ$^+$ CD8$^+$ TILs, and inhibits CT26 growth	• Higher Treg number in colon cancer tissues than in surrounding unaffected mucosa	Williams et al., 2016; Nie et al., 2018

(C)

Tumor type	Patients	Reference(s)
Ovarian cancer	• Higher proportion of CD4$^+$CD25^{hi}TNFR2$^+$ Tregs in tumor ascites • Up-regulation of CD39, CD73, GARP, and TGF-β in this subpopulation • TNFR2$^+$ Tregs dampen local IFN-γ and IL-2 production by Teff (more than blood Tregs)	Govindaraj et al., 2013a; Torrey et al., 2017
Cervical cancer	• PB and TNFR2$^+$Treg TILs significantly elevated in patients with cervical intraepithelial neoplasia and cancer • Circulating s-TNFR1 and s-TNFR2 elevated in cervical cancer patients • Percentage of peripheral TNFR2$^+$Tregs inversely correlates with the clinical stage of cervical cancer	Zhang et al., 2017
AML	• In newly diagnosed patients vs. those in complete remission and healthy controls: (i) higher production of TNF-α by CD4$^+$ T cells (mostly Th17), (ii) higher circulating frequencies of CD4$^+$CD25$^{+/hi}$ Tregs, (iii) higher TNFR2 expression on Tregs, preferentially on CD4$^+$CD25^{hi} Tregs • Most of the patients' Tregs express TNFR2$^+$, have a high migration potential toward the bone marrow, up-regulate CTLA-4 and CD73, and produce more IL-10 and TGF-β. • TNFR2$^+$ Tregs in the PB and bone marrow are selectively decreased after epigenetic therapy with panobinostat (histone deacetylase inhibitor) and azacytidine (demethylating agent) in responder patients in association with a beneficial clinical response • This treatment led to a reduction in TNFR2$^+$ Tregs associated with their down-regulation of FOXP3 and CTLA-4 expression and an increase in IFN-γ and IL-2 production by Teff within the bone marrow	Govindaraj et al., 2014b; Wang M. et al., 2018
Sézary syndrome	• High expression of TNFR2 on Tregs and cancer cells • TNFR2 antagonist killed tumor cells, restored the CD26$^-$ subpopulation, and reduced the number of Tregs and ratio of Tregs/Teff	Torrey et al., 2018

Data found in mouse models (A), both mice and patients (B), or restricted to cancer patients (C). WT: wildtype.

suppress tumor-associated Treg activity with no or only a minor inhibitory effect on regular Tregs in the periphery, permitting the maintenance of immunological homeostasis. On the other hand, TNFR2 antagonistic mAbs were able to directly kill TNFR2-expressing ovarian cancer cell lines *in vitro*. This last observation fosters the hypothesis of the synergistic action of TNFR2 on

Tregs and malignant cells. Importantly, tumor antigens released from dead cancer cells can promote quiescent antitumor immune responses, triggered, in the meantime, by the attenuation of Treg activity. Similar observations were made by the same group in another *in vitro* study in which cancer cells and lymphocytes were isolated from patients with end-stage Sézary syndrome, an interesting scenario for TNFR2 blockade, because, as already mentioned, a portion of these malignant cells show Treg features (Torrey et al., 2018).

TNFR2$^+$ Treg depletion augments the efficacy of chemotherapy in pre-clinical studies (van der Most et al., 2009). In a mouse model, the use of the alkylating agent cyclophosphamide depleted TNFR2$^+$ Tregs by inducing cell death of replicating Tregs co-expressing TNFR2 and the cellular proliferation marker KI-67. However, the re-expansion of Tregs from lymphodepletion can suppress the effective antitumor immunity developed after cyclophosphamide treatment. Interestingly, TNF-α blockade by etanercept inhibits TNFR2$^+$ Treg expansion during recovery from cyclophosphamide-induced lymphodepletion and markedly inhibits the growth of established CT26 tumors in mice, without affecting CD8$^+$ T cell activation (Chang et al., 2015). In the same colon cancer model, as well as in 4T1 breast cancers, the combination of a TNFR2-blocking mAb with an immune stimulator (toll-like receptor agonist) markedly enhanced the antitumor efficacy of immunotherapy by reducing the number of tumor-infiltrating TNFR2$^+$ Tregs and increasing the number of IFN-γ-producing CD8$^+$ cells (Nie et al., 2018). Notably, the antagonistic TNFR2 antibody TR75-54.7 inhibited the growth of mammary carcinoma more efficiently than a CD25 antagonist. In addition, certain pharmacological agents regulate the expression of TNF-α and/or of its receptors. For example, thalidomide and its analogs prevent the surface expression of TNFR2 on activated T cells, which is associated with the inhibition of TNFR2 protein trafficking to the cell membrane (Marriott et al., 2002). Treating AML patients with azacitidine and lenalidomide, a thalidomide derivative, can reduce TNFR2 expression on T cells, as well as the number of TNFR2$^+$ Tregs, *in vivo*, leading to enhanced effector immune function (Govindaraj et al., 2014a).

Impact of TNFR2 Blockade on the Effector T Cell/Regulatory T Cell Equilibrium

A crucial point concerning the pattern of TNFR2 expression is its change under inflammation. Similarly to CD25, conventional T cells upregulate TNFR2 upon TCR activation (Govindaraj et al., 2013b). This raises the question of a potentially deleterious effect on the adaptive response after TNFR2 blockade and, more specifically, on the cytotoxic anti-tumor CD8$^+$ response. To address this question, it is necessary to first understand the role of TNFR2 in Teffs during a resolvable immune response. First, although TNFR2 stimulation in Teffs correlates with a high proliferative capacity and a high capacity to produce effector cytokines, TNFR2-deficient mice still display normal T cell development (Erickson et al., 1994). In addition, the time of exposure to TNF-α strongly shapes the consequences of TNFR2 activation on Teffs. A comprehensive scheme for the role of TNF-α/TNFR2 in Teffs during the natural resolution of inflammation by Tregs can be proposed as follows (Chen and Oppenheim, 2011a):

1) In the steady state, the equilibrium between Treg and Teff activation preserves immune homeostasis (**Figure 2A**).
2) In the early stage of inflammation, activated Teffs up-regulate their TNFR2 expression under TNF-α exposure, increasing their capacity to resist Treg-mediated inhibition, and therefore mount an effective immune response. In addition, a slower Treg response than that of Teffs to TNF-α results in delayed immunosuppressive feedback (**Figure 2B**; Chen et al., 2013).
3) In the later stage of inflammation, chronic exposure to TNF-α leads to impaired production of effector cytokines, caused by the competition of Tregs with Teffs for the co-stimulatory action of TNF-α/TNFR2 (**Figure 2B**; Aspalter et al., 2003; Clark et al., 2005). Importantly, TNFR2 also appears to be necessary for the sensitivity of CD8$^+$ T cells to AICD (Kim E. Y. et al., 2009). At the same time, Treg TNFR2-dependent stimulation enhances their suppressive activity, resulting in the resolution of inflammatory responses and restoration of immune homeostasis.

The pattern of TNFR2 expression suggest that TNF-α can induce a potent immune reaction through monocyte/macrophage activation and T cell co-stimulation. At the same time, its activation launches a delayed immune response controlled through its expression on immunosuppressive cells. In pathological settings, including cancer, this fine balance between TNFR2 dual functions is lost due to changes in the pattern of expression. During carcinogenesis, tumor cells induce multiple modifications in the soluble milieu, including that of TNF-α levels because of its production by TME-cells, such as infiltrating T cells or macrophages, and by the tumor itself (**Figure 2C**; Josephs et al., 2018). They also recruit immunosuppressive cells, which further upregulate their TNFR2 expression under TNF-α exposure. This disturbs the equilibrium of the TNF-α/TNFR2 expression pattern, presumably putting cancer development in the third situation already described, i.e., the impairment of Teff function due to chronic exposure of both Tregs and Teffs to TNF-α. Consistent with this assumption, it was shown that the enhanced protection against lymphoma in TNFR2$^{-/-}$ mice results from the recruitment, activation, AICD resistance, and subsequent prolonged survival at the tumor site of memory TNFR2$^{-/-}$ CD8$^+$ T cell, specific to tumor-associated antigens (Kim J. et al., 2009). Overall, these data suggest a beneficial action of TNFR2 blockade on Teffs anti-tumoral efficacy under chronic TNF-α exposure, despite the co-stimulatory role of TNFR2 in transitory inflammation. However, sufficient data is still lacking to untangle what the real consequences on immunity would be when using a TNFR2 blockade strategy as a therapeutic tool.

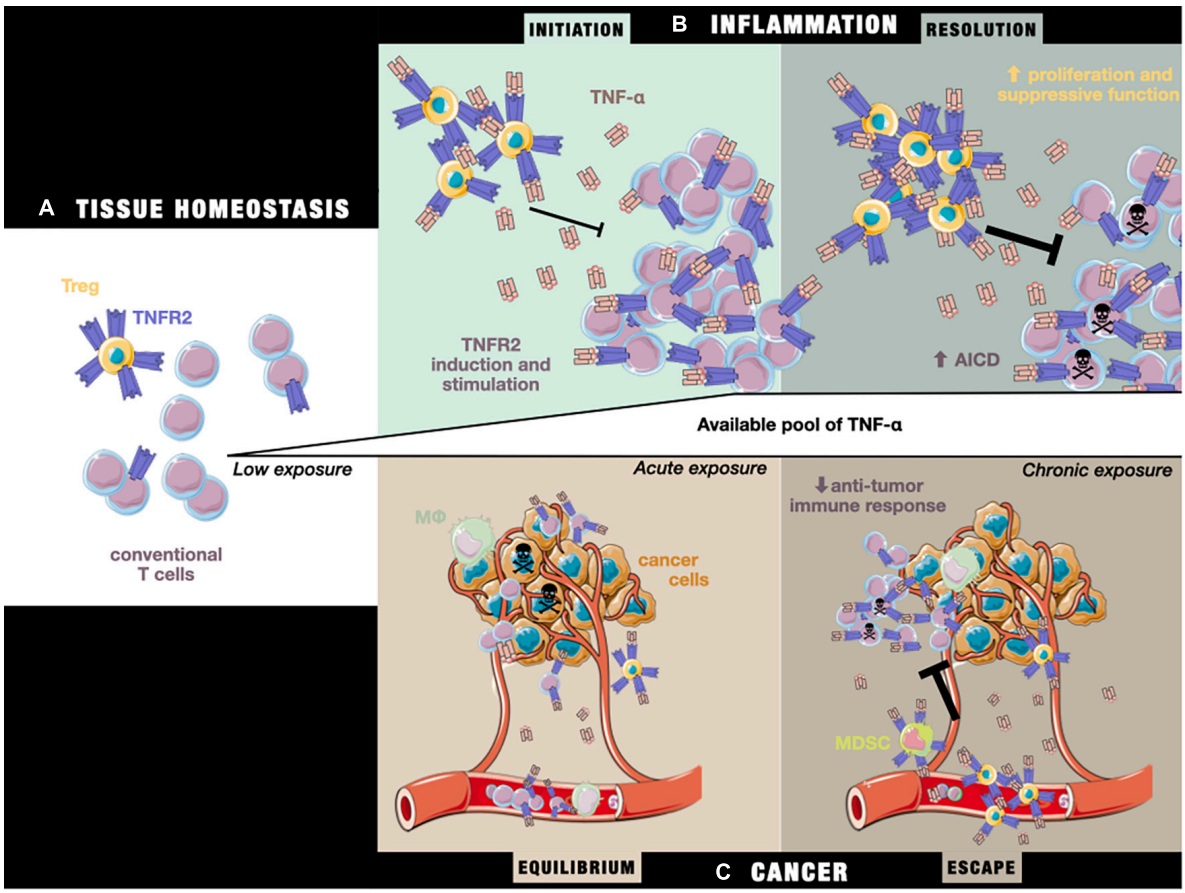

FIGURE 2 | The impact of TNF-α/TNFRs on the T cell equilibrium during **(A)** tissue homeostasis, **(B)** the initiation and resolution of inflammation, and **(C)** cancer equilibrium and escape. **(A)** In the steady state, TNFR2 is more highly expressed by Tregs than Teffs. **(B)** Under inflammatory conditions, exposure to initiation-phase cytokines (including TNF-α) induces higher expression of TNFR2 on T cells and co-stimulation signaling, aiding the T cell response. Tregs are unable to fully repress the Teff response. Later, during the resolution phase, chronic exposure to TNF-α sensitizes Teffs to cell death through TNFR2 activation, while Tregs are able to more potently inhibit Teffs by the stabilization and stimulation of their suppressive functions, leading to restoration of the steady state situation described in panel **(A)**. **(C)** During tumor growth, tumor cells and the TME produce TNF-α, leading to a T cell response against tumor cells by peripheral Teffs that have been attracted to the tumor site. In a state of equilibrium, this allows the elimination of a pool of cancer cells, while others more resistant to the immune response continue to proliferate. Over time, the TME evolves and attracts an increasing number of immunosuppressive TNFR2^{+} cells (including Tregs and MDSCs), leading to suppression of the anti-tumor response, while the chronic exposure of Teffs to TNF-α drives them toward AICD, unleashing tumor growth and allowing tumor cells to escape from immune control.

Activation vs. TNFR2 Blockade

A recent study conducted by a pharmaceutical company evaluated the activity of murine and human anti-TNFR2 antibodies for cancer therapy (Tam et al., 2019). They described the mechanism of action of a novel mouse TNFR2 antibody (Y9) that appears to act through Fc-dependent agonism on conventional T cells, responsible for its antitumor activity in pre-clinical mouse models. In the subcutaneous tumor models they used, with both hematological and carcinoma cell lines, they observed no significant Treg depletion or dysfunction. A second pharmaceutical group screened for TNFR2 binders, a subset of which were found to be agonists for the receptor, leading to NF-κB pathway signaling *in vitro* (Williams et al., 2016). Their TNFR2-specific agonists inhibited tumor growth, enhanced tumor infiltration by CD8^{+} T cells, and increased CD8^{+} T cell IFN-γ synthesis in CT26 syngeneic tumors. In light of our hypothesis in the previous subsection, these results are not inconsistent with the previous results obtained using a TNFR2 antagonist. Indeed, antitumor cells that benefit from TNFα/TNFR2 pathway signaling for their activation can be found in the TME. As cited, they are mainly macrophages and conventional T cells. In a milieu under constant TNF-α saturation, a strategy to target immunosuppressive actors by blocking TNFR2 is probably the better angle of approach because Teffs are susceptible to Treg exhaustion after long TNF-α exposure. However, what about a tumor context in which no TNFR2 signal is delivered because TNF-α is absent? Then, in theory, providing a TNFR2 signal to proinflammatory TME-cells, as well as Tregs, could permit, at least transiently, the triggering of a potent anti-tumor Teff response, resistant to Treg suppression, while preserving tolerance. For instance, TNFR2 agonism could benefit to infiltrating macrophages among TNFR2^{+} cells in the

TME. In the above-mentioned studies of Williams et al. (2016) and Tam et al. (2019) such an effect was not observed. However, in a later study, TNFR2 expressed on macrophages was proven to be sufficient to mediate the antitumor effect of TNF-α, probably by the inhibition of tumor angiogenesis (Zhao et al., 2007). This observation may be another interesting aspect for TNFR2 agonism in cancer, although more data concerning TNFR2, and myeloid cells are still required. Another goal that appears to be potentially obtainable by both TNFR2 agonists and antagonists is the direct impact of the treatment on tumor cells. Although TNFR2 blockade can divert cancer cells from a survival pathway, agonist molecules may cause a domino effect that could sensitize malignant cells to TNFR1-induced cell death by depriving them of cIAP/TRAF (Siegmund et al., 2016).

Due to its wide-ranging functions, TNF-α is also responsible for divergent actions in the context of cancer through its two receptors (Montfort et al., 2019). Far from what was initially thought about its antitumor effect, a growing body of clinical data supports the concept that chronic inflammation promotes tumor development and progression. As a major proinflammatory cytokine, reports have shown TNF-α to be involved in all aspects of carcinogenesis, from cellular transformation to survival, proliferation, invasion, angiogenesis, and metastasis (Wang and Lin, 2008). Hence, in phase I and II clinical trials, two anti-TNF-α antibodies (infliximab and etanercept) achieved prolonged disease stabilization in patients with metastatic breast cancer, recurrent ovarian cancer, or immunotherapy-resistant or refractory renal cell carcinoma (Madhusudan et al., 2004, 2005; Harrison et al., 2007). In the specific situation in which carcinogenesis is proven to be inflammation-driven, including through the proinflammatory effect of TNF-α, Tregs have been demonstrated to have a paradoxical protective effect. Thus, in this case, a strategy to block TNFR2 could be counterproductive by inhibiting the anti-inflammatory function of Tregs. Conversely, the utilization of an agonist to preserve Tregs while activating Teffs and macrophages could be more appropriate. Many examples of tumors for which Tregs may be protective can be cited, including colorectal carcinoma, in which gut microbiota driven-inflammation aids tumor growth (Erdman et al., 2003, 2005; Gounaris et al., 2009; Whiteside, 2012).

THERAPEUTIC PERSPECTIVES

Current State of Clinical-Grade Therapeutic Tools

From the point of view of clinical utility, TNFR2 modulation appears to be an attractive approach in reshaping modern cancer immunotherapy. Targeting TNFR2 in cancer patients now requires clinical grade mAbs or small molecules, agonists, or antagonists, designed to target human TNFR2. Defining the right tool to translate murine findings to patients is a promising but challenging task. The TNF-α/TNFR2 crystal structure is now available, revealing the specific binding between TNF-α and TNFR2 (Mukai et al., 2010). In virtual screening, a library of compounds has been examined to predict their binding poses and affinities (Shaikh et al., 2018). Compounds that resemble the binding pose to the native ligand with better binding affinity will be selected as agonist candidates, together with new blocking mAb candidates, for further research and development in the drug discovery pipeline. As previously mentioned, a few academic groups have also published advances in testing anti-TNFR2 molecules in the context of cancer:

- Torrey et al. (2017, 2018) have developed an antagonist candidate that has proven to be efficient in inhibiting Tregs from patients with ovarian cancer and those with Sézary syndrome.
- Tam et al. (2019) screened for anti-human and anti-mouse TNFR2 agonist antibodies. They reported an antitumor effect on several cancer types using the murine version and found corresponding activity of human agonist TNFR2 antibodies that could be used in patients.
- Encouraging unpublished results were also presented at the AACR annual meetings in 2020 and 2021, regarding advances in both agonists and antagonists targeting TNFR2 and tested in the context of cancer (Mårtensson et al., 2020; Chen et al., 2021).

Although the development of good-manufacturing practice reagents to modulate TNFR2 in patients appears to be on track, finding an efficient a way to prove that mouse results can be translated to strategies for patients is currently less straightforward. *In vitro* testing of anti-TNFR2 tools is challenging as modeling T cell exhaustion or mid-activation of the TCR, to mimic pathologic situations, is complex. Furthermore, most available information concerns isolated Tregs, i.e., mostly Tregs independent of TNF-α exposure. Finally, several groups are trying to assess TNFR2 modulation strategies in human cells *in vivo* using immunodeficient mice. He et al. (2016) were the first to publish preliminary results using a commercialized TNFR2 agonist in a humanized skin allograft model. Tam et al. (2019) tested their agonist candidate in a humanized model of colorectal adenocarcinoma without success. Using patient-derived xenografts, this group then observed an antitumor effect upon TNFR2 activation when combined with an anti-PD-1 relative to PD-1 monotherapy. No TNFR2 inhibition studies in humanized mouse models have yet been published. As for *in vitro* models, testing new immunotherapies using humanized mouse models is challenging, in part because xenogeneic models trigger excessive T cell activation, which does not reflect clinical reality in cancer (Scalea et al., 2012). Further *in vitro* and *in vivo* studies are also needed to better define the intracellular events that follow agonist or antagonist candidate binding to human Tregs and Teffs, as well as to other types of TNFR2-expressing cells to envisage potential off-target effects.

Potential Side Effects

Anti-CTLA-4 and anti-PD-1/PDL-1 mAbs have shown high antitumor efficacy in responding patients. However, a major drawback associated with the use of ICIs agents is the apparition of severe autoimmunity/autoinflammatory symptoms (Young et al., 2018). These therapies are able to

trigger or restore patients' effector responses against tumor cells, which express both tumor-associated antigens and self-antigens (Shimizu et al., 1999). The subsequent over-activation of Teffs against self can lead to diverse organ lesions. In addition, these antibodies can induce deleterious autoimmune effects by binding off-target, for example, diabetes mellitus after PD-L1 treatment due to PD-L1 expression by pancreatic β-cells (Fousteri, 2020). Because such chronic effects deeply affect the patients' quality of life, treatment-safety is a major consideration for the new generation of immunotherapies to come.

One important feature of TNFR2 is its restricted perimeter of expression, raising the hope of limited side effects under therapeutic utilization, contrary to TNF-α or TNFR1 targeting. Because activated T cells upregulate TNFR2, a potential safety concern comes from its inducible expression on Teffs upon TCR stimulation. In addition, co-stimulation through TNFR2 on Teffs could improve their ability to resist Treg-mediated suppression in tumors. Nevertheless, as Tregs in the TME appear to persistently express higher levels of TNFR2 than Teffs, the assumption that this treatment should have a more profound impact on Tregs than Teffs appears to be plausible. Thus, the net outcome of TNFR2 antagonism could favor Teff activation and expansion, triggering the establishment of an effective antitumor immune response. This hypothesis will require further testing, with careful assessment of the impact on the effector response at each step following TNFR2 inhibition, as well as that on pro-inflammatory $TNFR2^{+}$ myeloid cells, which could be impaired by a TNFR2-blockade strategy. Importantly, apart from the immune compartment, TNFR2 is expressed and upregulated in pathological situations, such as ischemia and in endothelial cells and neural tissue at the surface of local macrophages (microglia), as well as other non-neuronal cells (astrocytes) (Fischer et al., 2020). Whether TNFR2 blockade would have off-target effects in these two tissues or non-desired immune effects through myeloid cells will need to be carefully monitored.

In our hands, a blocking mAb against TNFR2 induced a change in phenotype of Tregs when used in a model of allogeneic hematopoietic stem cell transplantation and presumably impaired their suppressive capacity but did not deplete them (Leclerc et al., 2016). The preservation of a viable Treg pool, although less functionally potent, could better prevent autoimmunity than complete Treg depletion. Our observations are consistent with the fact that $TNFR2^{-/-}$ mice do not develop autoimmunity, suggesting that the restriction of TNFR2 on a Treg subset in the steady-state does not impair the capacity of other subpopulations to maintain an immune balance (Vanamee and Faustman, 2017). In cancer, an advantage of TNFR2 is its higher expression among certain tumor-infiltrated Tregs than Tregs in the circulation. The dominant TNFR2 antagonists from the study of Torrey et al. (2017) preferentially suppressed the activity of tumor-associated Tregs but had no or only minor inhibitory effects on regular Tregs in the periphery, which play a crucial role in the maintenance of immunological homeostasis. Importantly, no evidence has been yet reported concerning the transient or long-term nature of the effect observed on Tregs after TNFR2 blockade.

Hopes for Combined Strategies

The clinical use of ICIs has shown limitations in terms of the frequency of responding patients. Anti-PD-1 combinations with chemotherapy or other immunotherapies, such as anti-CTLA-4, have been able to improve efficacy, but often at the expense of substantial increases in toxicity relative to anti-PD-1 alone (Weber et al., 2016; Paz-Ares et al., 2018). As Tregs could be a brake that limits the action of these molecules, the depletion of Tregs or reduction of their suppressive activity are two strategies that could enhance currently available treatments. Tumor killing using chemotherapeutic drugs, irradiation, or ICIs may release self-antigens and tumor-associated antigens and cause local inflammation, possibly resulting in the recruitment of Tregs to tumor tissues, their activation, then hampering ensuing antitumor immune responses. Thus, an anti-Treg strategy could be used with pro-Teff immunotherapies to strongly activate Teffs while avoiding interference from Treg recruitment.

If combinational strategies could improve the therapeutic efficacy of a single agent, they could also possibly enlarge the range of indications compared to monotherapies. Combined approaches are being designed to address the issue of non-infiltrated/cold tumors, for which immunotherapy alone is inefficient (Bonaventura et al., 2019), for instance to normalize vascularization, allowing a better antitumor cells infiltration. In this setting, TNFR2 is a highly interesting candidate since its expression on endothelial cells and immunosuppressive cells makes it essential for both chaotic tumor angiogenesis (Sasi et al., 2012) and the maintenance of an immunosuppressive TME. For these reasons, TNFR2 blockade used in combination with an angiogenesis regulator could permit both the regulation of tumor vascularization, allowing an effective Teff infiltration and a more potent antitumor response.

CONCLUDING REMARKS

Immunotherapy is currently sparking an extraordinary level of energy and enthusiasm among the scientific community and healthcare industry due to its potential for the treatment of solid and hematological malignancies. A better understanding of Treg dysregulation in cancers during the last two decades has opened a new therapeutic window in the field. Clinical strategies to specifically inhibit Tregs without affecting Teffs are still challenging, largely because of phenotypic similarities shared between the two cell subsets. A myriad of tools will undoubtedly be available in the near future, making it possible to increasingly consider the patient's cancer type and immune status. TNFR2 is a versatile target option, of which both activation and blockade could serve as an anti-cancer therapy, probably for distinct therapeutic situations. It is important to stress the inclusion of such an approach in the concept of personalized medicine. Based on our current knowledge, the use of anti-TNFR2 in therapy will have to be context-dependent, with TNF-α levels in the tumor surroundings and the time of exposure to this cytokine (depending on the tumor type and disease stage) being potentially the most crucial factors to consider when using such agents. Future pre-clinical experiments should

probably focus on comparing agonist and antagonist effects in the same model to determine whether the previously discussed assumptions are accurate. Importantly, TNFR2 is not the only target-molecule to offer such a possibility for dual modulation. An entire class of new therapeutics focuses on costimulatory molecules preferentially expressed on Tregs, such as GITR, ICOS, OX40, 4-1BB, and DR3, creating a new potential drug class of "checkpoint stimulators," completing the current available ICI options and allowing new combinations of immunotherapies (Chen and Oppenheim, 2017).

Because of the high toxicity associated with the ICIs currently used in the clinic, there is an ongoing need for new cancer immunotherapies that show promising activity but are also well tolerated. Targeting TNFR2 in monotherapy could spare a functional subset of peripheral Tregs, which offers hope about its capacity to be well tolerated. Nevertheless, the risk of autoimmunity when blocking TNFR2 is considerable, as for other new ICIs candidates, and difficult to evaluate outside of proper clinical trials. The availability of an antagonist and agonist of clinical relevance makes it possible to envisage shortly launching clinical trials in cancer patients. However, the type of tumor to include in these assessments is yet to be precisely determined. Compelling results on patient-derived Tregs are already available for a human TNFR2 antagonist. The presence of $TNFR2^{+}$ Tregs has also been reported in numerous other types of malignancies, which will help in choosing the first cohorts.

AUTHOR CONTRIBUTIONS

AM wrote the first draft of the manuscript. Both authors wrote together the final version of the manuscript.

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Regulatory T Cells Improved the Anti-cirrhosis Activity of Human Amniotic Mesenchymal Stem Cell in the Liver by Regulating the TGF-β-Indoleamine 2,3-Dioxygenase Signaling

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Liver fibrosis is a progression stage of chronic liver disease, while current therapies cannot cure or attune cirrhosis effectively. Human amniotic mesenchymal stromal cell (hAMSC) presented immunoregulatory and tissue repairability of multiple illnesses. Regulatory T cells (Treg) had been proved to be functional in reducing immune cell activity. We showed that co-infusion of hAMSC and Treg prevented mild liver fibrosis comparing with hAMSC or Treg alone group. *In vitro* study indicated that the addition of Treg or the supernatant of Treg improved the hepatocyte growth factor (HGF) secreting and cell differentiation ability of hAMSC. Reduction of TGF-β significantly decreased the HGF secreting and differentiation of hAMSC. Multiple signal neutralizers were added to the culture to understand further the mechanism, which showed that 1-MT, the suppressor of Indoleamine 2,3-dioxygenase (IDO), was involved in the effect of TGF-β in regulating hAMSC. Depletion of TGF-β or IDO signaling successfully abolished the effect of Treg in improving hAMSC's function both *in vitro* and vivo. Finally, our result indicated that Treg improved the function of hAMSC by regulating the TGF-β-IDO signaling and co-infusion of hAMSC and Treg provided a promising approach for treating liver cirrhosis.

Keywords: liver cirrhosis, hAMSC, Treg, IDO, TGF-β

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INTRODUCTION

Liver cirrhosis is unrecoverable progress that may cause liver failure or liver cancer in chronic liver disease. Histopathologically, extensive necrosis of liver cells, nodular regeneration of residual liver cells, connective tissue hyperplasia, and fibrous septum formation are the main features of cirrhosis (Tsochatzis et al., 2014).

Human amniotic mesenchymal stem cell (hAMSC) is isolated from the amniotic membrane (AM) of the full-term human placenta (Li et al., 2020). It has similar characteristics with typical MSC, including fibroblastic morphology, specific surface molecules, and multidirectional differentiation potential. In addition, it has excellent immunomodulatory and paracrine properties (Magatti et al., 2015, 2018; Silini et al., 2017). Human amniotic mesenchymal stem cell shows low

immunogenicity because it does not express human major histocompatibility complex (MHC) or antigens (human leukocyte antigen, HLA) (Evangelista et al., 2008).

$CD4^+$ $Foxp3^+$ regulatory T cell (Treg), a subset of $CD4^+$ T cells, plays a key role in maintaining immune tolerance and immune homeostasis in different tissues. It is mainly composed of natural Treg cell (tTreg cell) and induced Treg cell (pTreg cell) (Yang et al., 2021). Foxp3 is an important marker of Treg cell and a major carrier of differentiation and function of Treg cell. Low expression of Foxp3 leads to the ablation of Treg, which results in inflammatory disease in mice or humans.

Transplantation of hAMSC has been reported to improve liver function in animal models (Kubo et al., 2015; Zhang et al., 2011). However, we found that the therapeutic effect of hAMSC infusion alone in mice with cirrhosis was modest and inconsistent. To improve the therapeutic effect, we tried to adoptive transferring Treg together with hAMSCs into cirrhosis mice. The results showed that the liver function and liver lesions of mice were significantly improved. Mechanism study proved that Treg expressed TGF-β, which activated the Indoleamine 2,3-dioxygenase (IDO) signal of hAMSC and improved hepatocyte growth factor (HGF) secreting of hAMSC. Taken together, our data presented a novel therapeutic strategy of anti-cirrhosis in chronic liver disease.

MATERIALS AND METHODS

Animals

Male mice (C57BL/6) were purchased from Nanjing Medical University. Mice at the age of 6–8 weeks were used. We maintained breeding colonies in our SPF (Specific Pathogen Free) facility. All animals received humane care according to the guidelines for experimental animals and were approved by the Institutional Animal Care and Research Advisory Committee of Nanjing Medical University.

Differentiation of Human Amniotic Mesenchymal Stem Cell

Adipogenic Differentiation

Stem cells were cultured in a 5% CO2 incubator at 37°C. When the cell fusion degree reached 80% to 90%, Stem cells were digested with the TrypLE enzyme. Then the digested stem cells were inoculated in a six-well plate and a 2 mL complete medium was added to each well. After that, the cells were cultured in the incubator of 5% CO2 at 37°C again. Change the fluid every 2–3 days until the cell fusion reaches 100% or overfuses. The growth medium was abandoned and 2 mL PADM (Sciencell 7221) was added to the six-well plate and PADM was changed every 2–3 days. Stem cells were induced for 21–30 days until the lipid droplets became large and round enough and Oil red O staining was performed.

Osteogenesis Differentiation

Stem cells were cultured in a 5% CO2 incubator at 37°C. When the cell fusion degree reached 80% to 90%, Stem cells were digested with the TrypLE enzyme. Then the digested stem cells were inoculated in a six-well plate and a 2 mL complete medium was added to each well. After that, the cells were cultured in the incubator of 5% CO2 at 37°C again. Change the fluid every 2–3 days until the cell fusion reaches 100% or overfuses. The growth medium was abandoned and 2 mL MODM (Sciencell 05465) was added to the six-well plate and PADM was changed every 3–4 days. After 21 days of induction, the cells were stained with alizarin red and monitored according to the morphological changes and the growth of the cells.

Cultivation of the Human Amniotic Mesenchymal Stem Cell

Human amniotic mesenchymal stem cells (hAMSCs) was provided by the Stem Cell Clinical Trial and Research Base of Jiangsu Provincial People's Hospital. The cells were precipitated by centrifugation and resuspended in DMEM/F12 medium containing 10% FBS. The cells were inoculated in a culture flask and cultured in an incubator with 5% CO2 at 37°C. After cultivation for 48 h, replace the culture medium, discard the non-adherent cells and change the culture medium every 2 or 3 days. Add trypsin to digest and observe the cell digestion with an inverted phase-contrast microscope when the cells grow to 80% to 90%. And the cells were subcultured according to the proportion of 1:3.

Co-culture of Tregs and Human Amniotic Mesenchymal Stem Cells *in vitro*

For contact-independent co-culture, Treg and hAMSC were cultured and physically separated using a 0.4-μm porous transwell system. Treg was seeded in the transwell nest and hAMSC was placed in the lower chamber (12-well transwell plate). The ratio between hAMSC and Treg was 1:4. After 72 h, the supernatant of the co-culture system for cytokine measurement was collected. Some experiments added anti-TGF-β monoclonal antibody (0.5 μg/ml) and anti-IL-10 monoclonal antibody (0.5 μg/ml) at the beginning of co-culture. Besides, after co-culture, hAMSC of the lower chamber was collected for injection.

Mouse Model of Liver Fibrosis and Cirrhosis

The 10% volume fraction of carbon tetrachloride was dissolved in olive oil, and the bodyweight of the mice was measured. The mice were injected intraperitoneally according to the dose of 10 μl/g, and the injection frequency was maintained at 3 times per week. In addition, mice were executed at week 4 and week 8 to monitor the progression of liver fibrosis and used after 12 weeks.

Cell Injection

Cells were injected 12–24 hours earlier in beginning of liver fibrosis modeling. Treg and AMSC were mixed and injected into mice through the tail vein. The ratio of of Treg and hAMSC was 1:4. Treg (10^6/30 g) and hAMSC (0.25×10^6/30 g) were suspended in 200 μl PBS and then injected into mice. In some experiments, Treg was co-cultured with hAMSC in a transwell chamber. After 3 days, the lower layer hAMSC was digested and counted, then re-suspended in 200 μl PBS, and injected into mice via caudal vein according to the dose of 0.25×10^6/30 g.

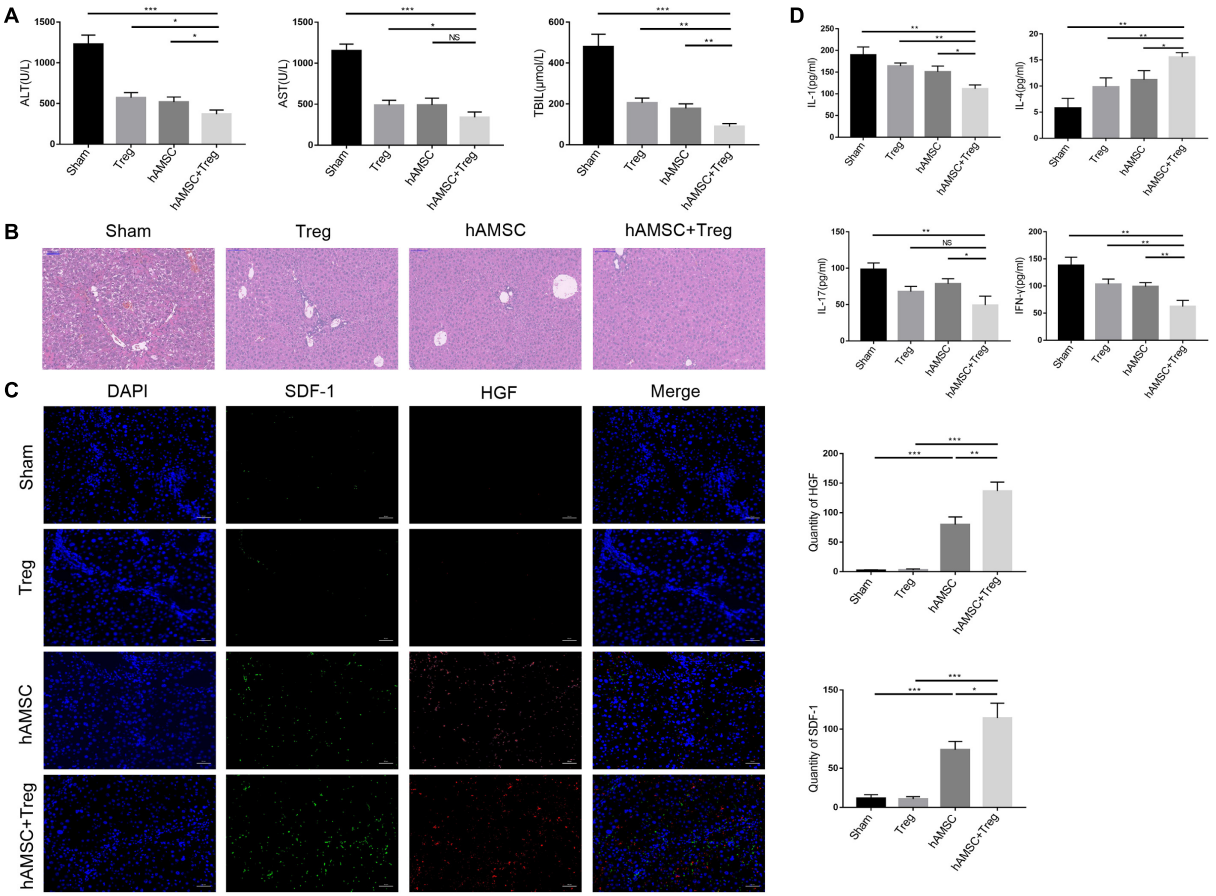

FIGURE 1 | PBS, Treg, hAMSC, and hAMSC + Treg were injected into mice through the tail vein, respectively. **(A)** Level of ALT, AST, and TBIL of four groups. **(B)** Representative images of liver tissue [hematoxylin and eosin (HE) staining] by light microscopy in four groups. **(C)** Representative fluorescent images for SDF-1 and HGF in four groups were visualized by fluorescence microscopy, and DAPI was used to mark nuclei. **(D)** Level of IL-1, IL-4, IL-17, and IFN-γ in sham group, Treg group, hAMSC group, and cotransfer group. The result is representative of three independent experiments. Data were mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The sham group of all experiments were only injected with 200 μ l PBS.

Chemical and Histological Assessment of Liver Injury

Blood samples were collected from the eyes of mice with liver fibrosis. After centrifugation at 12000r for 10 min, the supernatant was measured for ALT, AST, and total bilirubin. The liver tissue was fixed with formalin and then embedded in paraffin. The specimen was cut into 4μm thick, then baked, soaked in xylene, and dehydrated with different concentrations of ethanol. After hematoxylin-eosin (HE) staining, the sections were placed under a light microscope for pathological analysis by the pathologist.

Treg Acquisition

Suspensions of murine leukocytes were obtained from mice lymph nodes and spleens. Naïve $CD4^+$ T cells were then acquired by auto-MACS (Miltenyi, San Diego, CA, United States) according to their $CD4^+/CD62L^+$ surface marker. Then, naïve T cells were activated in 96-well plates with completed media supplemented with anti-CD3/28 beads (Dynal beads, 1:1). For the differentiation of Treg, cells were cultured in the presence of IL-2 (100 U/ml, R&D Systems), TGF-β (5 ng/ml, R&D Systems), and anti-CD3/CD28 beads (Cell: beads = 2:1). The completed media is RPMI supplemented with 10% heat – inactivated FBS.

Cytokine Measurement

HGF and stromal cell-derived factor 1 (SDF-1) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, United States). The supernatant to be detected was added to the orifice plate pre-coated with antibody and incubated for 30 min. The excess samples were washed off, and another primary antibody specific to the test was added and incubated for 30 min. The excess unbound primary antibody was washed off, and the secondary antibody with enzyme was added and incubated for 30 min. The excess unbound secondary antibody was washed off, the enzyme-substrate was added, and the OD value was measured at 450 nm wavelength.

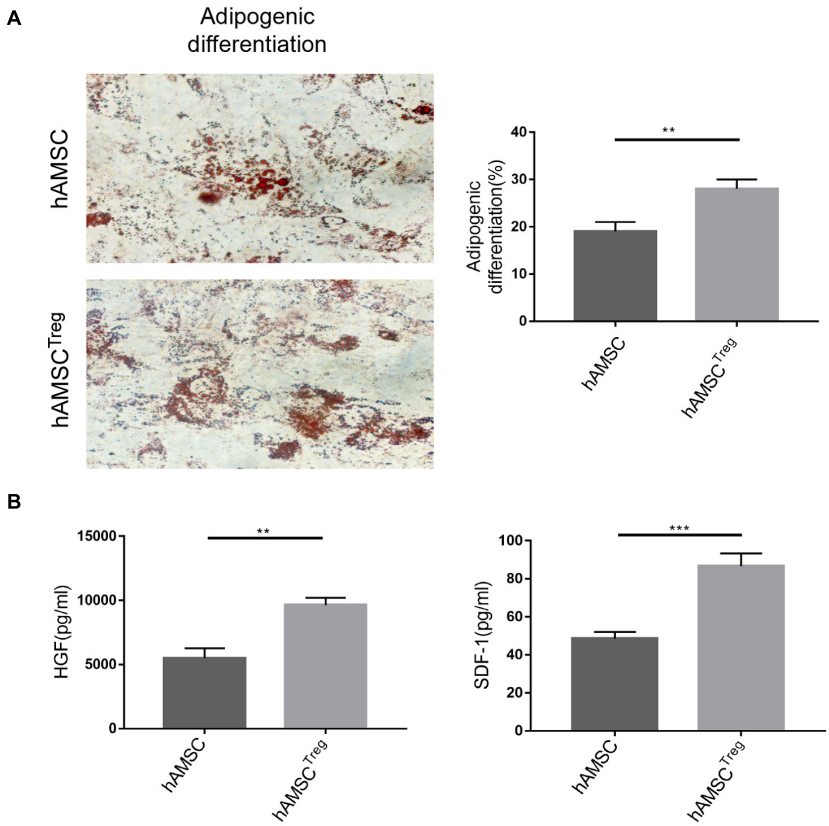

FIGURE 2 | hAMSC was treated with PBS and Treg *in vitro*. **(A)** Representative images of hAMSC's adipogenic differentiation in two groups. **(B)** Level of HGF and SDF-1 in two groups. The result is representative of three independent experiments. Data were mean ± SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

Statistical Analysis

All data were presented as the mean ± SD from at least three independent experiments. Statistical analysis was performed by the Student's *t* test, one-way analysis of variance using GraphPad Prism8.0 software. Probability (P) values ≤ 0.05 were considered statistically significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

Co-transfer of Treg Treatment Enhanced the Anti-cirrhosis Function of Human Amniotic Mesenchymal Stem Cell

We set up four groups (n = 4/group) to evaluate the effect of hAMSC or Treg on cirrhosis in mice. PBS, Treg, hAMSC, and hAMSC + Treg were injected into mice through the tail vein, respectively. The results showed that Treg, hAMSC, and co-transfer group presented effective anti-cirrhosis function, but the co-transfer group is more functional than any other group. Co-transfer of Treg and hAMSC showed lower ALT, AST, and TBIL (**Figure 1A**) and better fibrosis (**Figure 1B**) level compared with the other three groups. HGF and SDF-1 (Stromal cell-derived factor 1) are important liver proteins that have been proved to be expressed by MSC and participated in liver tissue repair (Kim et al., 2014; Wu et al., 2017; Ding et al., 2018; Jin et al., 2018; Mayorga et al., 2018). We used Immunofluorescence and confocal microscopy to observe the expression of SDF-1 and HGF in four groups of liver cirrhosis. It was found that co-transfer of Treg and hAMSC could secrete the most HGF and SDF-1 comparing with hAMSC or Treg alone group, thus limited the damage and reverted the cirrhosis of the liver (**Figure 1C**). Inflammatory factors play a fundamental role in liver cirrhosis. We also tested the expression of cytokines in the liver, which showed that co-transfer of Treg and AMSC most effectively reduced the pro-inflammatory cytokine expression such as IL-1, IL-17, IFN-γ and increased expression of IL-4, comparing with hAMSC group (**Figure 1D**).

Treg Enhanced the Differentiation and Paracrine Function of Human Amniotic Mesenchymal Stem Cell *in vitro*

To further study the effects of Treg on hAMSC, we investigated the differentiation and paracrine function of hAMSC *in vitro*. Astonishingly, we found that after Treg treatment, the differentiation such as the adipogenic ability of hAMSC was enhanced (**Figure 2A**). As a result in **Figure 1** indicated HGF and SDF-1 expression may be the cause for hAMSC's protection, we analyzed the cultured supernatants of two groups through

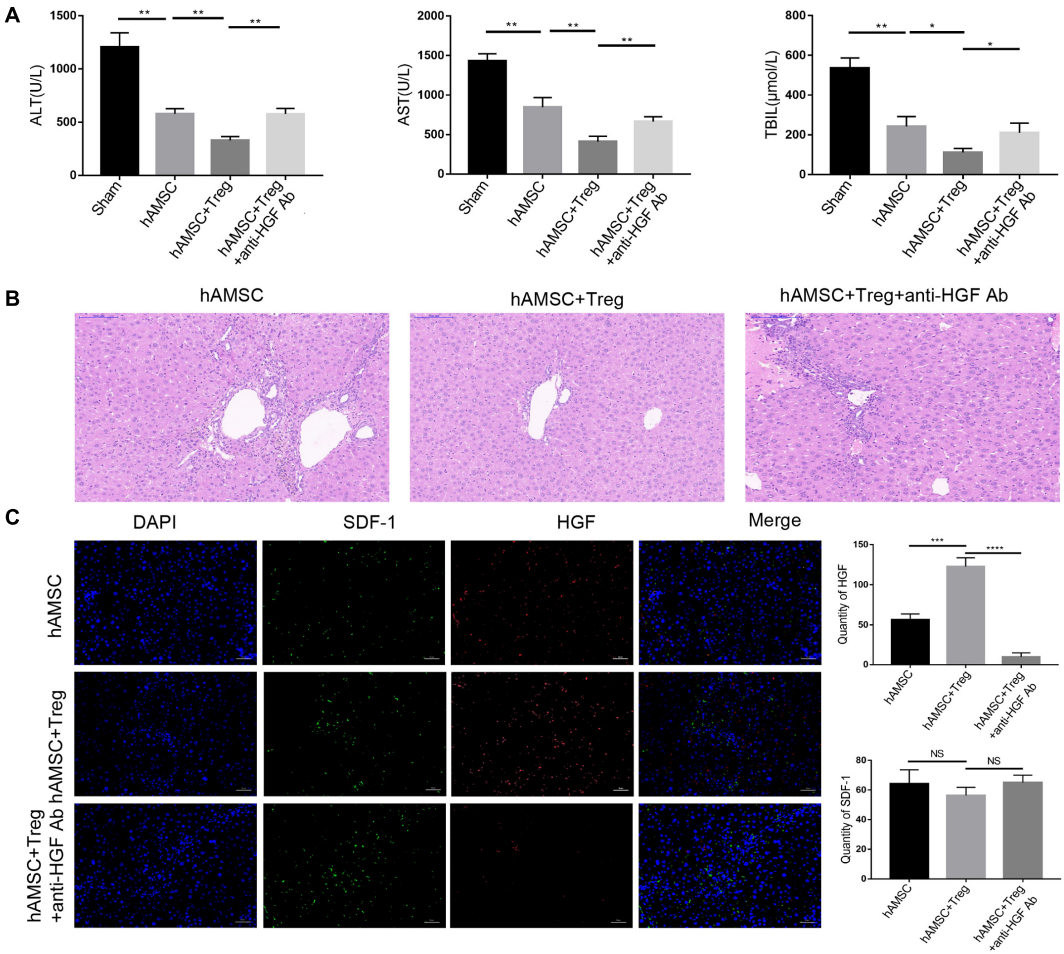

FIGURE 3 | PBS, hAMSC, hAMSC + Treg, and hAMSC + Treg + anti-HGF Ab were injected into mice through the tail vein, respectively. **(A)** Level of ALT, AST, and TBIL of four groups. **(B)** Representative images of liver tissue [hematoxylin and eosin (HE) staining] by light microscopy in three groups. **(C)** Representative fluorescent images for SDF-1 and HGF in three groups were visualized by fluorescence microscopy, and DAPI was used to mark nuclei. The result is representative of three independent experiments. Data were mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ELISA. The results showed that Treg treatment contributed to the upregulation of paracrine production of hAMSC (**Figure 2B**).

Human Amniotic Mesenchymal Stem Cell Secreted Hepatocyte Growth Factor Participated in Resisting Liver Cirrhosis Damage

As HGF is important for liver regenesis, and **Figure 2** had indicated that Treg successfully improved the hAMSC produced HGF. So we used an anti-HGF monoclonal antibody (25 · g/kg) to abolish HGF *in vivo*. Mice were divided into three groups and each group contained four mice. The addition of anti-HGF Ab decreased the protective effect of hAMSC and Treg co-transfer and it significantly up-regulated the liver enzyme levels (**Figure 3A**). And liver biopsy in **Figure 3B** showed that anti-HGF Ab weakened the anti-cirrhosis function of hAMSC. Besides, confocal fluorescence microscopy showed that after the addition of the anti-HGF Ab, the expression of HGF was significantly reduced. At the same time, the expression of SDF-1 did not change significantly (**Figure 3C**), which indicated that HGF participated in repairing liver injury during the protective ability of hAMSC and Treg.

TGF-β Significantly Affected the Activation and Function of Human Amniotic Mesenchymal Stem Cell

As Treg improved the function of hAMSC *in vitro*, we hypothesized that Treg regulated hAMSC in an acellular contact manner. Treg was cultured in a non-cytokine environment for two days. The supernatant was collected and added into the hAMSC culture system (hereafter referred to as $hAMSC^{Treg-S}$). As we expected, HGF and SDF-1 were greatly upregulated with the addition of the Treg's supernatant (**Figure 4A**). TGF-β and IL-10 are the two most important cytokines secreted by Treg (Nono et al., 2020; Bertolini et al., 2021). We added anti-IL-10 monoclonal antibody and anti-TGF-β monoclonal antibody, respectively, and found that only anti-TGF-β Ab

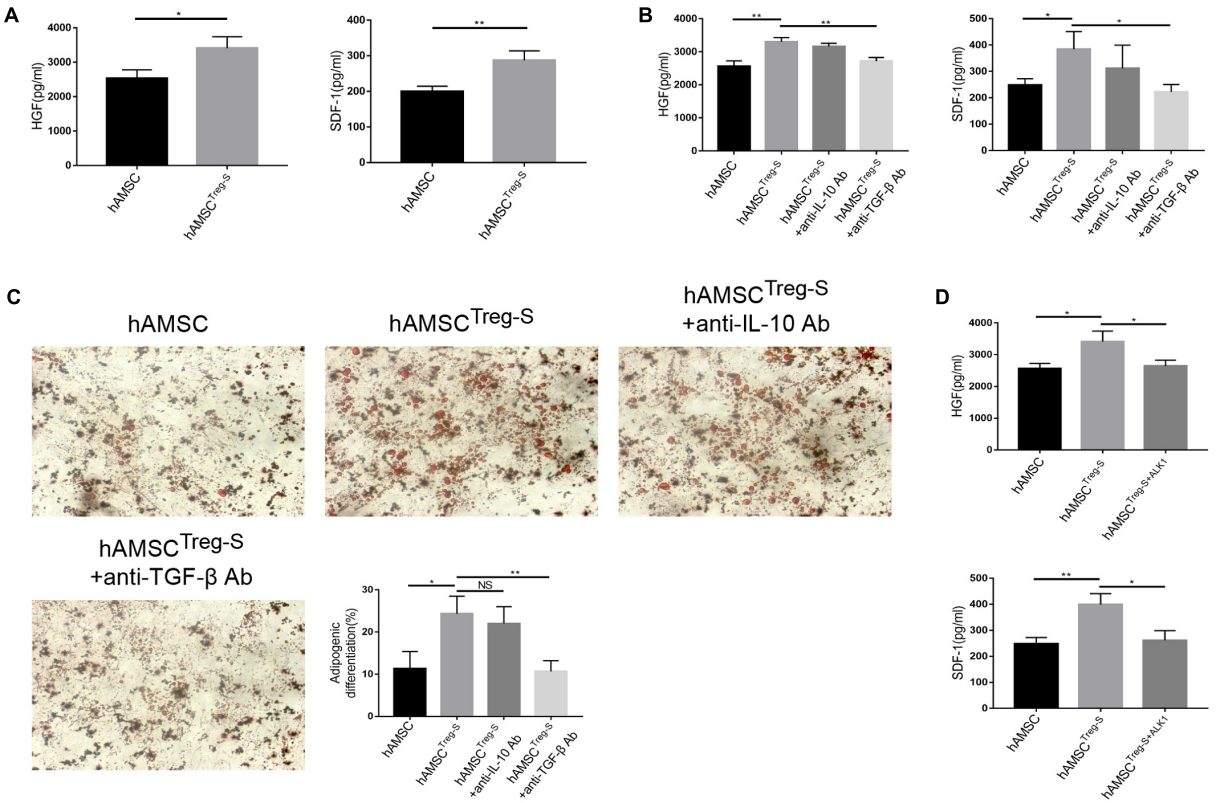

FIGURE 4 | hAMSC was treated with supernatant of Treg, anti-IL-10 Ab and anti-TGF-β Ab *in vitro*. **(A)** Level of HGF and SDF-1 of hAMSC and hAMSC^{Treg-S}. **(B)** Level of HGF and SDF-1 in hAMSC, hAMSC^{Treg-S}, hAMSC^{Treg-S} with anti-IL-10 Ab and hAMSC^{Treg-S} with anti-TGF-β Ab. **(C)** Representative images of hAMSC's adipogenic differentiation in four groups. **(D)** Level of HGF and SDF-1 of hAMSC, hAMSC^{Treg-S}, and hAMSC^{Treg-S} with ALK1. The result is representative of three independent experiments. Data were mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

down-regulated both the HGF and SDF-1 expression *in vitro* (**Figure 4B**). We also tested the cell differentiation, which showed the supernatant of Treg improved the suppressive ability of hAMSC and anti-TGF-β Ab weakened the differentiation ability and decreased the number of adipose cells (**Figure 4C**). Finally, we used ALK1, the neutralizer of the TGF-β type I receptor, to verify the effect of TGF-β on hAMSC (Salmon et al., 2020). Data verified that HGF and SDF-1 secretion by hAMSC^{Treg-S} after ALK1 treatment is significantly reduced (**Figure 4D**).

Indoleamine 2,3-Dioxygenase Signaling Is Involved in Human Amniotic Mesenchymal Stem Cell^{Treg-S} Function and Differentiation Ability

To further understand the mechanism of TGF-β in regulating hAMSC function, multiple signal neutralizers were added to the culture system. Among all the neutralizers, only 1-MT, the suppressor of IDO signaling, reduced HGF expression by hAMSC (**Figure 5A**). After that, we used western blot to prove that the IDO signal was upregulated in TGF-β signaling, and 1-MT successfully down-regulated the IDO expression (**Figures 5B,C**). We also tested the HGF, SDF-1 expression with the presence of 1-MT, which showed that HGF and SDF-1 were also decreased by 1-MT (**Figure 5D**). To further investigate the differentiation ability of hAMSC^{Treg-S}, adipogenic differentiation and osteogenic differentiation were performed. The results showed that both ALK1 and 1-MT significantly reduced the differentiation ability of hAMSC^{Treg-S} either in the adipogenic or osteogenic environment (**Figure 5E**).

Treg Improved the Function of Human Amniotic Mesenchymal Stem Cell in Reducing Liver Cirrhosis Through Regulating Indoleamine 2,3-Dioxygenase Signaling

Finally, we detected the mechanism of Treg treated hAMSC in protecting liver cirrhosis *in vivo*. The murine liver cirrhosis model was established as described above, and hAMSC, Treg pre-treated hAMSC (mixed co-cultivation, hereinafter referred to as hAMSC ^{Treg}) as well as Treg and 1-MT pre-treated hAMSC (hereinafter referred to as hAMSC$^{Treg+1-MT}$) were injected into mice, respectively. Each group contained four mice. The results showed that hAMSC^{Treg} presented a better effect on reducing liver enzymes and total bilirubin. But the effect was recalled after the addition of 1-MT (**Figure 6A**). Hematoxylin-eosin also showed that the addition of 1-MT greatly weakened the

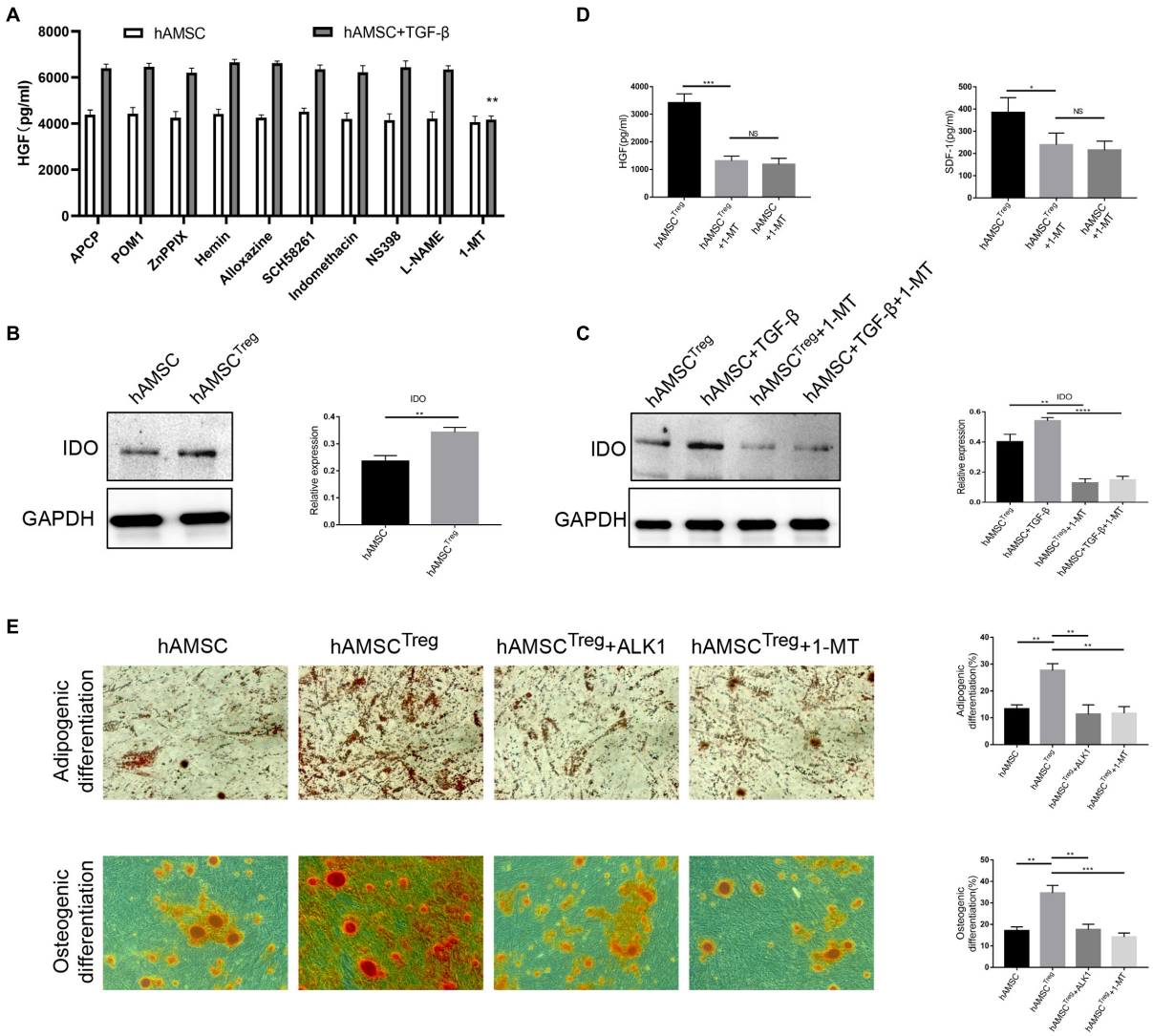

FIGURE 5 | Multiple signal neutralizers were added to the culture system and 1-MT was proved to be effective. **(A)** Level of HGF in hAMSC's supernatant after adding multiple signal neutralizers. APCP: CD73 inhibitor; POM1: CD39 inhibitor; ZnPPIX: HO-1 inhibitor; Hemin: HO-1 inducer; ALLoxazine: adenosine receptor inhibitor; SCH58261: adenosine A2A receptor inhibitor; Indomethacin: COX inhibitor; NS398: COX-2 inhibitor; L-NAME: NOS inhibitor; 1-MT: IDO inhibitor. **(B)** IDO expression of PBS treated hAMSC and hAMSC^{Treg-S} by western blot and the relative expression calculated by IDO versus GAPDH through Image J software. **(C)** IDO expression of hAMSC^{Treg-S}, TGF-β, hAMSC^{Treg-S} with 1-MT, and TGF-β with 1-MT by western blot and the relative expression calculated by IDO versus GAPDH through Image J software. **(D)** Level of HGF and SDF-1 in hAMSC, hAMSC^{Treg-S}, and hAMSC^{Treg-S} with 1-MT. **(E)** Adipogenic and osteogenic differentiation of hAMSC, hAMSC^{Treg-S}, hAMSC^{Treg-S} with ALK1, hAMSC^{Treg-S} with 1-MT. The result is representative of three independent experiments. Data were mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

impact of hAMSC^{Treg} against liver fibrosis (**Figure 6B**). Confocal fluorescence microscopy showed that after the addition of 1-MT, the expression of HGF and SDF-1 in hAMSC^{Treg} were significantly reduced (**Figure 6C**). We also tested the expression of cytokines in the liver, which showed that the addition of 1-MT increased the pro-inflammatory cytokine expression such as IL-1, IL-17, IFN-γ and decreased the expression of IL-4 comparing with hAMSC^{Treg} (**Figure 6D**). To verify that the injected hAMSC did reach the liver and secreted HGF, we labeled the hAMSC and hAMSC^{Treg} with CFSE dye and injected it into the tail vein. The results showed that comparing with hAMSC, more amounts of hAMSC^{Treg} were accumulated in the liver and the expression of HGF also increased under the fluorescence microscope (**Figure 6E**).

DISCUSSION

Mesenchymal stem cells have presented therapeutic potential in various diseases through immunomodulatory and tissue repair functions. Recent studies showed that MSCs are functional in many liver diseases such as liver fibrosis, cirrhosis, and liver failure. In clinical studies registered on www.ClinicalTrials.gov, trials had been taken to treat liver failure, liver transplantation,

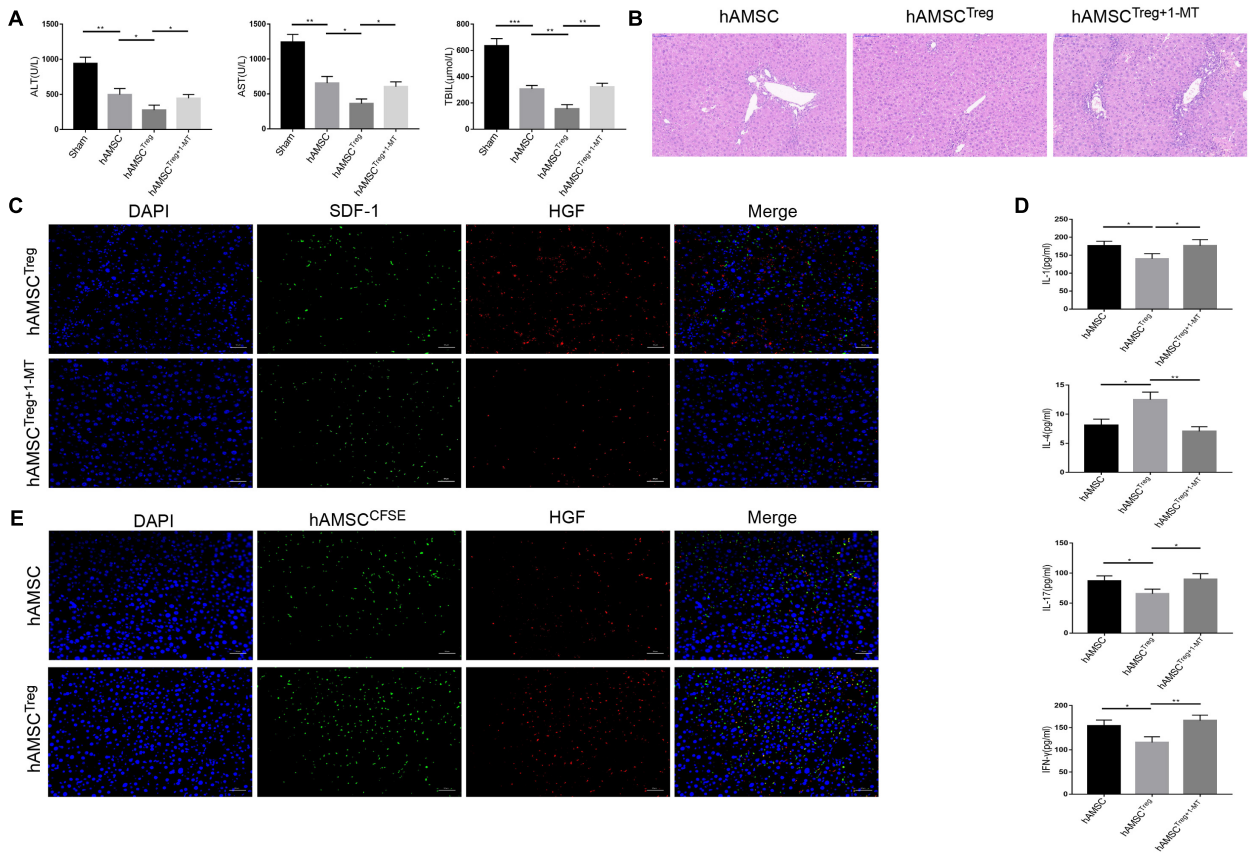

FIGURE 6 | PBS, hAMSC, hAMSC^{Treg}, and hAMSC$^{Treg+1-MT}$ were injected into mice through the tail vein, respectively. **(A)** Level of ALT, AST, and TBIL of four groups. **(B)** Representative images of liver tissue [hematoxylin and eosin (HE) staining] by light microscopy in hAMSC, hAMSC^{Treg}, and hAMSC$^{Treg+1-MT}$ group. **(C)** Representative fluorescent images for SDF-1 and HGF in hAMSC^{Treg} and hAMSC$^{Treg+1-MT}$ group were visualized by fluorescence microscopy, and DAPI was used to mark nuclei. **(D)** Level of IL-1, IL-4, IL-17, and IFN-γ in hAMSC, hAMSC^{Treg}, and hAMSC$^{Treg+1-MT}$ group. **(E)** Representative fluorescent images for hAMSC and HGF were visualized by fluorescence microscopy, and DAPI was used to mark nuclei. The result is representative of three independent experiments. Data were mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

liver fibrosis, autoimmune liver disease, and so on through MSCs transferring. Studies have reported that mesenchymal stem cells secrete various cytokines, chemokines, growth factors, and exosomes, which help indirect and remote tissue repair. It is important that mesenchymal stem cells regulate nutritional factors and indirectly mediate anti-apoptosis, anti-oxidation, anti-fibrosis, angiogenesis and immunosuppressive effects (Wang et al., 2014) and mesenchymal stem cells can synergistically improve liver fibrosis with induced M2 macrophages (Watanabe et al., 2019). However, reports showed limited progress of MSCs in anti-cirrhosis in the liver, and the reason may be related to severe inflammation (Hu et al., 2020). Many inflammatory factors were expressed in the stage of liver cirrhosis, which aggravated the damage during liver cirrhosis. Treg was proved to reduce inflammation in liver disease by suppressing immune status. We thought to use hAMSC to repair tissue while reducing inflammation through reinfusion of Treg.

As we expected, Treg inhibited inflammation during cirrhosis, but surprisingly, improve the function of AMSCs. Co-transfer of Treg downregulated the injury and delayed the cirrhosis process of the liver. Depletion of HGF abolished the treatment effect of co-transfer, indicating that Treg improved the therapeutic impact through improving the cytokine expression ability of hAMSC. *In vitro* experiment surprisingly showed that Treg improved the HGF and SDF-1 expression of hAMSC. As the supernatant of Treg showed a similar result as Treg itself, we hypotheses that Treg expressed pro-inflammatory cytokines such as TGF-β and IL-10 may be the key for Treg's regulation of hAMSC. Using the antibody of TGF-β or IL-10, we found that TGF-β Ab suppressed the upregulation of HGF in supernatant treated hAMSC. Besides, further mechanism studies showed that TGF-β improved hAMSC's function through regulating the IDO signaling.

TGF-β played an important role in maintaining normal human development and homeostasis. It binds to a membrane receptor with a cytoplasmic serine/threonine kinase domain. IDO had been proved to have an indispensable effect on the immune regulation ability of human MSC (Munn and Mellor, 2013; Su et al., 2014). It promoted metabolic immune regulation by catalyzing the oxidative catabolism of the essential amino acid tryptophan (TRP) in the kynurenine (KYN) pathway (Munn and Mellor, 2013). Studies have reported that TGF-β can

up-regulate the expression of IDO in cultured cells *in vitro*. And IDO gene silencing technology can eliminate the increased IDO expression of TGF-β (Fallarino et al., 2005; Pallotta et al., 2011). Studies have also reported that the expression of TGF-β is positively correlated with the expression of IDO in the chorionic villi and decidua tissues of normal pregnant women (Liu et al., 2017). In our research, we found that Treg promoted the IDO signaling pathway of hAMSC by secreting TGF-β, thereby secreting more cytokines such as HGF and SDF-1 and reducing the damage of liver cirrhosis.

MSCs mainly inhibit liver fibrosis by differentiating into hepatocyte-like cells (Dai et al., 2009), secreting nutritional factors, expressing a variety of soluble factors to regulate the proliferation and function of a variety of immune cells, as well as antioxidant activity (Eom et al., 2015). However, as liver cirrhosis is an end-stage organic disease, studies have confirmed that MSCs have a poor therapeutic effect. Lipopolysaccharide (LPS) or endotoxin promotes systemic inflammation by activating TLR-2 and TLR-4 dependent pathways and promoting cytokines in large quantities, thereby accelerating the progression of liver cirrhosis (Wilde and Katsounas, 2019). Treg is functional in suppressing T and other immune cells' activation and thereby inhibiting inflammation. Treg has a comprehensive immune regulation function. First, Treg secretes heterogeneous cytokines, such as IL-10, IL-35, and TGF-β, which inhibit the pro-inflammatory response. Secondly, the cytotoxic T lymphocyte antigen-4(CTLA-4) expressed by Treg cells can impair the maturation of APC. Finally, Treg plays a role through immune checkpoint molecules ICOS and LAG-3 and immunosuppressive metabolites CD39, CD73 and IDO (Sharabi et al., 2018). Although MSCs themselves can suppress immunity, and their functions are not as comprehensive as Treg. More importantly, we discovered for the first time that Treg could improve the function of hAMSC. And TGF-β-IDO signaling may be the possible mechanism, but further research is necessary to analyze.

When selecting the type of MSCs at the beginning of the experiment, we chose hAMSC because of its rich source, limited immunogenicity, and negligible ethical issues (Lobo et al., 2016; Shi et al., 2017; Silini et al., 2017). Human amniotic mesenchymal stem cell has many applications. For example, it can secrete a variety of cytokines to promote angiogenesis and bone formation. Therefore it is expected to be an alternative method for bone tissue regeneration. Our research showed that co-infusion of hAMSC and Treg might provide a promising approach for treating liver cirrhosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC-2008036.

AUTHOR CONTRIBUTIONS

ZD, JRZ, and XM designed the experiments. JG, XL, QS, JL, CY, GH, and JZ performed the experiments, analyzed the data, and wrote the manuscript. YX designed the overall concept, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Butyric Acid Protects Against Renal Ischemia–Reperfusion Injury by Adjusting the Treg/Th17 Balance *via* HO-1/p-STAT3 Signaling

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Immune regulation plays a vital role in ischemia–reperfusion injury (IRI). Butyric acid (BA) has immunomodulatory effects in many diseases, but its immunomodulatory effects during renal IRI are still unclear. Our research shows that BA protected against IRI and significantly improved renal IRI *in vivo*. *In vitro* studies showed that BA inhibits Th17 cell differentiation and induces Treg cell differentiation. Mechanism studies have shown that heme oxygenase 1 (HO-1)/STAT3 signaling pathway was involved in the inhibitory effect of BA on Th17 cell differentiation. HO-1 inhibitors can significantly rescue the BA-mediated inhibition of Th17 cell differentiation. We confirmed that BA promotes the differentiation of Th17 cells into Treg cells by regulating the pathway and reduces renal IRI.

Keywords: butyric acid, renal ischemia–reperfusion injury, Treg, Th17, HO-1, STAT3

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INTRODUCTION

The kidney is a richly perfused organ and is very sensitive to ischemia–reperfusion injury (IRI). Complex physiological processes are involved in renal IRI, such as inflammatory T cells and cytokines, which play an essential role in the process of renal injury (Sakai et al., 2019). Regulatory T cells (Tregs), formerly known as suppressor T cells, are a subpopulation of T lymphocytes that modulate the immune system, maintain tolerance to self-antigens, and abrogate autoimmune disease (Takahashi et al., 1998; Barbi et al., 2014). Tregs express CD4 and CD25 on their surface, and the transcription factor forkhead box p3 (Foxp3) is the critical marker for Treg function (Lu et al., 2017). Previous studies have indicated that a moderate population of Treg is necessary for immune homeostasis (Shevyrev and Tereshchenko, 2019). Although the exact etiology and pathogenesis of renal IRI are not well-defined, current research showed that Tregs protected the kidney against IRI, which were involved in renal tubular epithelial cells, and the imbalance of Th17/Treg is critical in renal IRI processes (Suzuki et al., 2019; Dellepiane et al., 2020). Treg could be the potential treatment in IRI (Brunstein et al., 2016; Lu et al., 2016; Yamamoto et al., 2020). Thus, maintaining Treg stability and function in IRI patients has become a key research direction in immunology.

The microbiome of the intestine and its products could modulate inflammatory reactions by taking part in controlling the activity of Tregs, and the responses can be observed in other

organs, such as the central nervous and airway systems (Ochoa-Reparaz et al., 2009; Trompette et al., 2014). The butyric acid (BA) is the product of indigestible foods fermented by intestinal microbial, which has a small molecular structure and belongs to the short-chain fatty acid (SCFA) family of microbiome products (Maslowski et al., 2009; Furusawa et al., 2013). As is shown in recent works, BA is actively involved in several pathological processes, including autoimmunity, cancer, and neurological disorders (Arpaia et al., 2013; Singh et al., 2014; Stilling et al., 2016). Moreover, according to several recent studies, the BA shows a regulatory effect of Treg induction *in vivo* and *in vitro* (Arpaia et al., 2013; Smith et al., 2013). Therefore, BA may be a potential negative immunoregulative agent that can be used to treat multiple diseases. However, whether BA can inhibit renal IRI has not been explored. Moreover, it is still unclear that how BA mediates immunomodulation.

In the present study, we attempted to use BA to definite the role of BA in renal IRI and explore the possible immunomodulatory mechanism. Our results strongly suggest that BA directly inhibits Th17 cell differentiation and induces Treg cell differentiation to protect renal IRI.

MATERIALS AND METHODS

Cell Acquisition and Culture

Peripheral blood was obtained from volunteers from The Department of Urology of the Third Affiliated Hospital of Soochow University. Human naïve $CD4^+$ T cells ($CD4^+CD25^-CD45RA^+$) were isolated and purified from peripheral blood mononuclear cells (PBMCs) using Ficoll-Hypaque separation (Amersham Biosciences) and the two-step magnetic bead separation method of an autoMACS Pro Separator (Miltenyi Biotechnology Company, Germany).

Naïve $CD4^+$ T cells were induced to $CD4^+$ iTregs with TGF-β (5 ng/ml) (Bio-techne, Abingdon, OX, United Kingdom), recombinant IL-2 (100 IU/ml) (Chiron, Emeryville, CA, United States), and antihuman CD3/CD28 beads (ratio of cells to beads = 1:5) (Life Technology, Carlsbad, CA, United States) in X-Vivo-15 medium (BioWhittaker, Walkersville, MD, United States) containing 10% fetal bovine serum (Cereal Biomedicine) for 6 days. The BA (100 μmol/L) used in cell culture was prepared by dissolving BA sodium powder (Sigma-Aldrich, 303410) in dimethyl sulfoxide (DMSO).

The PC61 α-CD25 antibody (Biolegend Cat#102007) was generated from hybridoma cells (ATCC) in serum-free media (Gibco).

Experimental Animals and Renal Ischemia–Reperfusion Injury Model

We constructed the renal IRI model according to the conventional method (Hosszu et al., 2017). Male C57BL/6 mice (20–25 g) 7–8 weeks of age were purchased from Kawensi Biotech (Changzhou, China). We established the mouse model of renal IRI by clamping the bilateral renal pedicles for 45 min. A warming pad was used to keep body temperature around 37°C during the surgery. After 45 min of ischemia, the needle was withdrawn to allow reperfusion for 1 day. Similar surgical procedures except clamping of the renal pedicles were applied to the sham-operated group. The animal experiments were approved by the Animal Management and Use Committee of Soochow University.

Interventions of Animal Model

Mice were given 100 mM BA in their drinking water. The renal IRI model was established after 6 days. Mice were sacrificed on post-operative day 1. Blood samples were collected from the tail vein to examine creatinine (Cr) and blood urea nitrogen (BUN) levels. Spleens and kidneys from the rats were obtained and fixed in 10% neutral formalin for 48–72 h. The specimens were dehydrated by a graded ethanol series and embedded in paraffin. Kidney tissue sections were subjected to hematoxylin-eosin (HE) staining, and the kidney tubule injury was assessed using the Paller score. Ten non-overlapping visual fields (200× microscope) were randomly selected from each rat and observed under a light microscope. For each field of view, 10 kidney tubules were randomly chosen for quantification. A total of 100 renal tubules were scored, and the higher the score was, the more serious the tubular injury was Paller et al. (1984).

Quantitative Real-Time Polymerase Chain Reaction

We tested the expression of IL-17A, IFN-γ, and IL-10 mRNA in tubules of mice. Total RNA extracted from tissue and cell lysates was further purified using RNeasy Mini Kit (Qiagen, Valencia, CA, United States). We obtained cDNA using Omniscript RT kit (Qiagen, Netherlands). Relative quantification of mRNA expression levels was performed using Absolute QPCR SYBR Green ROX Mix (Thermo Fisher Scientific Inc., Waltham, MA, United States). The relative mRNA levels were assessed (in triplicate) based upon normalization using a reference gene encoding β-actin (Actb).

Western Blotting Assay

The total protein was extracted by radioimmunoassay lysate with 1% protease inhibitor on ice. The protein concentration was measured by BCA protein analysis kit (Thermo Fisher Scientific, United States). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to PVDF membrane. After blocking with 5% fresh milk for 1 h, the membrane was cultured overnight with specific primary antibody at 4°C and then cultured with corresponding secondary antibody for 30 min. Finally, the strips on the film were detected by ECL detection system (Thermo Fisher Scientific, United States) and quantified by Quantity One software (v4.3 in the United States).

Enzyme-Linked Immunosorbent Assay

Enzyme linked immunosorbent assay (ELISA) was performed to detect quantitatively inflammatory cytokine. Blood from the inferior vena cava was left for 20 min and then collected into tubes and centrifuged (2,000 rpm, 15 min,

4°C). Then 3 ml supernatants of kidney homogenates were harvested and stored at −20°C. IL-17A, IFN-γ, and IL-10 were measured by ELISA (R&D Systems) according to the product instruction.

Flow Cytometry

(1) Flow cytometry was used to measure the percentage of Th17 cells. A 250-μl whole blood sample was mixed with 250 μl RPMI-1640 medium without fetal bovine serum to a volume of 500 μl. Individual control and experimental tubes were set up. Then 250 μl diluent, 1 μl PMA (0.1 mg/ml), 5 μl ionomycin (1 mg/ml), and 1 μl monensin (50 mg/ml) were added to each tube, incubated at 37°C for 12 h in a 5% CO_2 incubator. The mixture was centrifuged again and the supernatant was discarded. Then red cells were lysed by adding a 1-ml lysing buffer (FACS lysing solution; Bio Legend Co.). Cells were incubated for 5 min at room temperature. The mixture was centrifuged again and the supernatant again discarded. The cells were resuspended in PBS and transferred into a 1.5-ml Eppendorf tube. The cells were centrifuged at 2,500 rpm for 5 min, and the supernatant was discarded. The wells were washed twice with PBS, added with 1 μl anti-mouse CD3FITC (0.5 mg/ml) and 1 μl anti-rabbit CD8aPE (0.2 mg/ml), and kept at room temperature away from light for 30 min. The wells were washed once with PBS and the supernatant was discarded, then added with 0.5 ml fixatives and protected from light for 20 min for incubation at room temperature. The cells were washed once with 1 ml PBS, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. The cells were added with 1 ml permeabilization wash buffer and protected from light for 20 min for incubation at room temperature, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. After washing with PBS, 1 μl anti-rabbit IL-17AAPC (0.2 mg/ml) was added into experimental tubes and 1 μl APC-lgG1 was added into control tubes. The cells were protected from light for 30 min for incubation at 4°C, washed once with PBS, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. The cells were resuspended in 0.5 ml PBS and analyzed for $CD3^+CD8^-IL\text{-}17^+$ expression by flow cytometry.

(2) Flow cytometry was used to measure the percentage of Treg cells. Individual control and experimental tubes were set up. Then 100 μl whole blood, 1 μl anti-mouse CD4ECD, and 1 μl anti-mouse CD25PE were added to each tube, kept at room temperature away from light for 30 min, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. The cells were washed once with 1 ml PBS, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. Then red cells were lysed by adding a 1-ml lysing buffer (FACS lysing solution; Bio Legend Co.). Cells were incubated for 5 min at room temperature. The cells were added with 0.5 ml Foxp3 fixatives and protected from light for 20 min for incubation at room temperature. The cells were washed once with 1 ml PBS, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. The cells were added with 1 ml permeabilization wash buffer and protected from light for 20 min for incubation at room temperature, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. After washing with PBS, 1 μl anti-mouse Foxp3Percpcy5 was added into experimental tubes and 1 μl Percpcy-lgG2 was added into control tubes. The cells were protected from light for 30 min for incubation at room temperature, washed once with PBS, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. The cells were resuspended in 0.5 ml PBS and analyzed for $CD4^+CD25^+Foxp3^+$ expression by flow cytometry.

Statistical Analysis

Stata software (version 11.0) was used to perform the analysis. Data were expressed as mean ± SD; the differences between groups were analyzed by either the paired *t*-test or ANOVA test (both one-way ANOVA test and two-way ANOVA test). In addition, we use Bonferroni or LSD as a *post hoc* test. *P*-values < 0.05 (two-tailed) were considered statistically significant.

RESULTS

Butyric Acid Treatment Ameliorated Renal Ischemia–Reperfusion Injury

Mice were orally administered BA and DMSO 3 days before surgery as indicated in **Figure 1A**. We tested the BUN and Cr of mice on the first day after surgery and obtained kidney tissue specimens. BA significantly reduced the increase of BUN and Cr in mice caused by IRI, suggesting the protective effect of BA on the renal function of IRI in mice (**Figures 1B,C**). HE staining of kidneys in the control group showed apparent renal tubular damage, renal tubular epithelial cell atrophy, degeneration, necrosis, and extensive lesion range. In the BA treatment group, the renal tubular epithelial injury was significantly improved compared with the control group, the lesion was lighter, and the lesion range was smaller (**Figure 1D**). BA treatment significantly reduced the tubular injury Paller score in IRI mice (**Figure 1E**). All of the aforementioned indicate that BA has a significant effect in alleviating the decline of renal function and tubular damage caused by IRI.

Butyric Acid Regulates Foxp3 Expression, Cell Expansion, and the Function of $CD4^+$ iTregs and Inhibited $IL\text{-}17A^+$ Treg Cell Differentiation *in vitro*

Next, renal cortex homogenate was collected to assay the anti-inflammatory effects of BA. The inflammatory cytokines IL-17A and IL-6 (Proinflammatory factor) were significantly decreased after the treatment of BA in kidney IRI while IL-10 (anti-inflammatory factor) was significantly increased (**Figures 2A–C**). Treg was involved in the process of liver and kidney IRI (Zheng et al., 2018; Luan et al., 2020). Thus, we designed a series of *in vitro* experiments to test the effect of BA on the regulation of iTreg generation. Human naïve $CD4^+CD25^-CD45RA^+$ T cells (purity ≥ 95%) were extracted from peripheral blood samples provided by healthy donors and then induced naïve $CD4^+$ T cells to iTregs by TGF-β, IL-2, and CD3/CD28 beads.

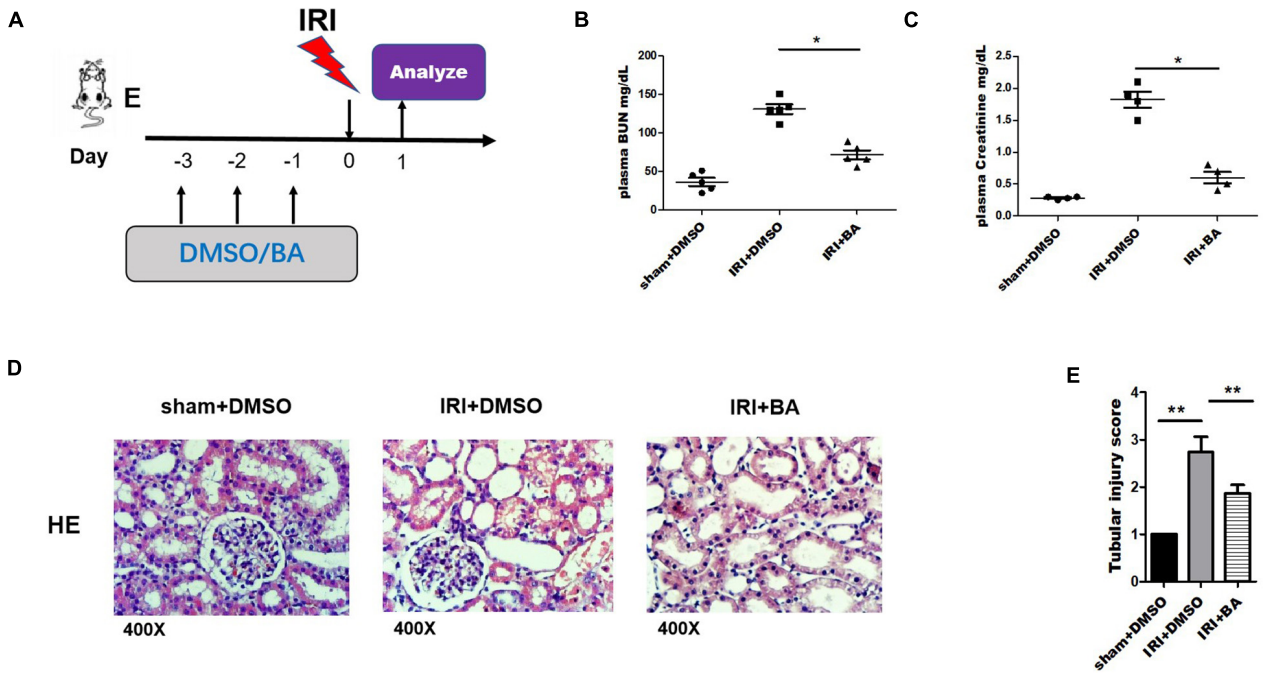

FIGURE 1 | BA treatment ameliorated renal IRI. **(A)** Mice were orally administered BA and DMSO 3 days before surgery. **(B,C)** BUN and Cr of mice on the first day after surgery were tested. **(D)** HE staining of kidneys in the BA treatment group showed alleviate renal tubular damage, renal tubular epithelial cells atrophy, degeneration, necrosis, and extensive lesion range than control group. **(E)** BA treatment significantly reduced the tubular injury Paller score in IRI mice. Sample size = 3 in each group. Data were presented as mean ± standard deviation. N.S. $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

Next, we explored at the cellular level whether BA regulated Treg differentiation *in vitro*. For this reason, $CD4^+$ cells ($CD4^+CD62L^{High}CD44^{Low}$) isolated from C57/B6J mouse spleens were cultured under Treg polarizing conditions with BA or placebo.

The expression of FoxP3 was significantly increased in the cells treated by BA after induction (**Figure 2D**). In addition, compared with the control group, the absolute number of $CD4^+$ iTregs in the BA group was slightly increased on day 8 while the difference was not significant on day 8 (**Figure 2E**). The suppressive ability of BA-$CD4^+$ iTregs was estimated by carboxyfluorescein succinimidyl amino ester (CFSE) coculture assays. The washed $CD4^+$ iTregs were cocultured with CFSE-labeled fresh PBMCs at different ratios (Tregs: PBMCs = 1:1 or 1:8) in the presence of expansion beads. **Figure 2F** shows the expansion of $CD4^+$ effector T cells. Although there was no difference in suppressive ability at 1:1 (Treg:$CD4^+$ effector T cells), BA-$CD4^+$ iTregs showed stronger inhibition at 1:8. Studies have confirmed that there is a significant correlation between pro-inflammatory/anti-inflammatory cytokines and Treg function (Pandiyan and Zhu, 2015; Qiu et al., 2018). These results suggest that BA inhibits the differentiation of naïve $CD4^+$ T cells into other inflammatory cells and partly increases the function of $CD4^+$ iTregs by regulating the expression of cytokines. The demethylation of CpG islands caused by epigenetic regulation of the Foxp3 loci is considered to be an important marker of the stability and functionality of Tregs (Floess et al., 2007). We used bisulfite sequence analysis to detect the methylation status of $CD4^+$ iTregs induced by BA, and compared with the control group, there were fewer methylated CpG sites in BA-$CD4^+$ iTregs (**Figure 2G**). Thus, BA regulates Treg cell differentiation and keeps its regulatory phenotype *in vitro*.

Butyric Acid*BA* Regulates Treg/Th17 Balance by Targeting SOCS3 but Not SOCS1 in Renal Ischemia–Reperfusion Injury

Treg and Th17 cells are critical immune cells and play an essential role in various inflammatory diseases including renal IRI (Dellepiane et al., 2020). Flow cytometry was then used to detect the percentage of Th17 cells in mice blood. The addition of BA significantly reduced the frequency and number of $CD4^+IL\text{-}17A^+$ cells, promoting the inflammatory responses in renal IRI (**Figure 3A**). Our study found that the frequency of Th17 cells in the BA treatment group was significantly reduced, suggesting that BA regulates Treg/Th17 balance and regulates $CD4^+$ T cell differentiation. Suppressor of cytokine signaling family (SOCS1/SOCS3) is necessary for the balance of Treg/Th17 population in different organ diseases (Qu et al., 2017; Wang D. et al., 2018; Kmiolek et al., 2020; Saleh et al., 2020). Therefore, we detected the expression of RORγt, SOCS1, and SOCS3 expression. As indicated in **Figures 3B,C**, the expression of RORγt and SOCS3, but not SOCS1, was decreased after BA

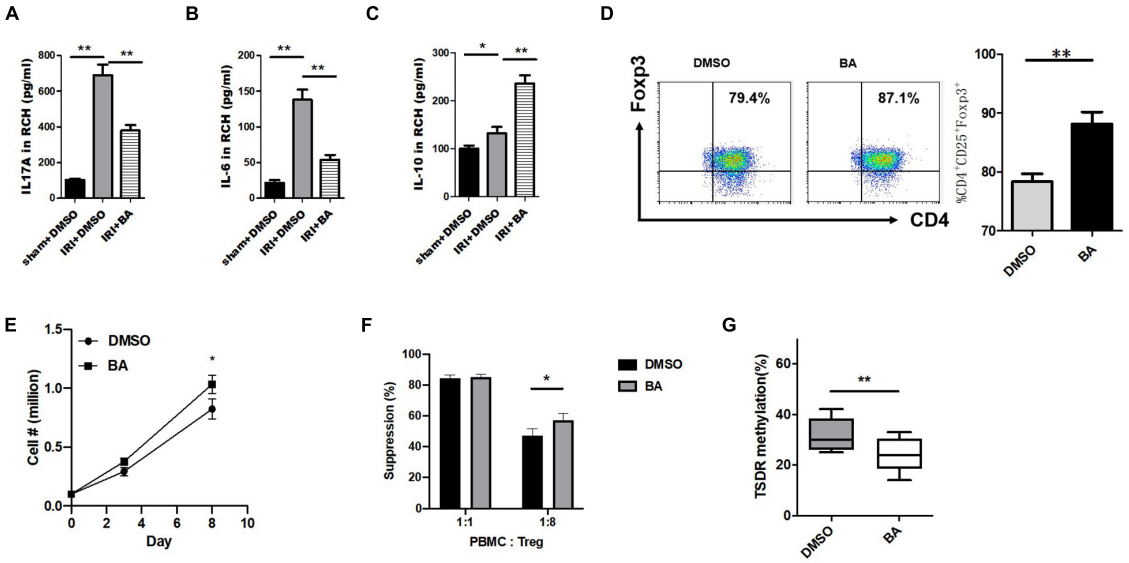

FIGURE 2 | BA regulates Foxp3 expression, cell expansion, and the function of $CD4^+$ iTregs and inhibited IL-17A$^+$ Treg cell differentiation *in vitro*. **(A–C)** Renal cortex homogenate was collected to assay the anti-inflammatory effects of BA. The inflammatory cytokines IL-17A, IL-6 were significantly decreased after the treatment of BA in kidney IRI, while IL-10 was significantly increased. $CD4^+$ cells ($CD4^+CD62L^{High}CD44^{Low}$) isolated from C57/B6J mouse spleens were cultured under Treg polarizing conditions with BA or placebo. The expression of Foxp3 was significantly increased in the cells treated by BA within 8 days after induction. Compared with the control group, the absolute number of $CD4^+$ iTregs in BA group was slightly increased on day 8 while the difference was not significant on day 8. The suppressive ability of BA-$CD4^+$ iTregs was estimated by CFSE coculture assays. Although there was no difference in suppressive ability at 1:1 (Treg:$CD4^+$ effector T cells), BA-$CD4^+$ iTregs showed stronger inhibition at 1:8. We used bisulfite sequence analysis to detect the methylation status of $CD4^+$ iTregs induced by BA. And compared with the control group, there were fewer methylated CpG sites in BA-$CD4^+$ iTregs. Sample size = 3 in each group. Data were presented as mean ± standard deviation. N.S. $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$.

treatment. These results suggest that BA regulates Treg/Th17 balance by targeting SOCS3 but not SOCS1 in renal IRI.

Butyric Acid Protects Against Renal Ischemia–Reperfusion Injury *via* p-STAT3

Foxp3 and RORγt mRNA and JAK2, STAT3, and SOCS3 protein are essential for the balance of Treg/Th17. We further tested the expression of p-JAK2, JAK2, p-STAT3, and STAT3 by western blot. As shown in **Figures 4A,B**, BA treatments attenuated the expression of p-STAT3 but not p-JAK2.

Since BRL52537, a kappa-opioid receptor agonist, could regulate p-STAT3 but not STAT3 expression in cerebral IRI and is involved in kidney injury, we used BRL52537 to assess whether p-STAT3 is necessary for the protective effects of BA (Fang et al., 2013; Golosova et al., 2020). After BRL52537 treatment, the expression of p-STAT3 but not p-JAK2 was increased, which conformed to the p-STAT3 agonist role of BRL52537 (**Figures 4C,D**). The previous studies show that overexpression of STAT-3 would increase the percentages of Th17 and relevantly reduce the rates of Treg cells *in vivo* and *in vitro* (Wang Y. et al., 2018; Liu et al., 2020). We observed similar results after BRL52537 treatment in our results (data not shown). Renal cortex homogenate was collected to assay the inflammatory effects after the increased expression of p-STAT3. **Figures 4E–G** suggests that BRL52537 significantly attenuated the anti-inflammatory effects of BA on renal IRI. Also, HE staining indicated that BRL52537 significantly attenuated the protective effects of BA on renal IRI (**Figure 4H**). Taken together, BA exerts therapeutic and protective effects on renal IRI through p-STAT3.

HO-1/p-STAT3 Signaling Pathway Was Implicated in Treg/Th17 Balance Mediated by Butyric Acid

Butyric acid is an effective activator of Nrf2, while Nrf2/HO-1 signaling pathway is an essential regulator of oxidative stress. The researches show that HO-1 mediates STAT3 pathway in different cells and diseases (Tang et al., 2018; Kuang et al., 2019). Therefore, we decided to identify whether the signaling factor HO-1 was implicated in Treg/Th17 cell differentiation mediated by BA. As shown in **Figures 5A,B**, SnPP, the inhibitor of HO-1, significantly reduced the upregulation of BA-related p-STAT3 expression. In $CD4^+$ T cells under Treg/TH17 polarizing conditions, BA/SnPP co-treatment reversed the immunological balance of Treg/Th17 cells by BA only (**Figure 5C**). Also, the pathological results show that SnPP treatment significantly, but not wholly, reversed the regulating effect of BA (**Figure 5D**). To conclude, HO-1/p-STAT3 signaling pathway was implicated in Treg/Th17 balance mediated by BA in renal IRI.

DISCUSSION

Short-chain fatty acids, such as acetic acid, propionic acid, and BA, are produced by gut microbes during the fermentation of

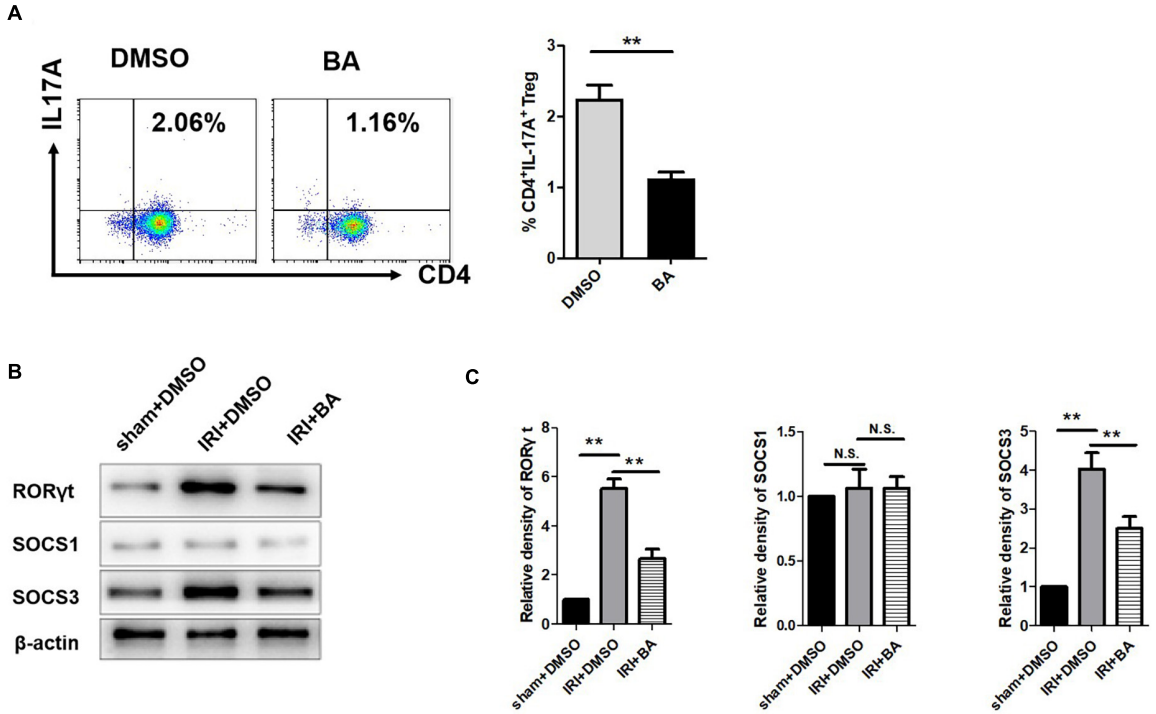

FIGURE 3 | BA regulates Treg/Th17 balance by targeting SOCS3 but not SOCS1 in renal IRI. **(A)** The frequency of Th17 cells in the BA treatment group was significantly reduced. **(B,C)** The expression of RORγt and SOCS3, but not SOCS1 was decreased after BA treatment. Sample size = 3 in each group. Data were presented as mean ± standard deviation. N.S. $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

dietary fibers. BA, a critical SCFA, as a significant energy source for enterocytes, has many physiological functions, including maintaining the balance of intestinal flora and regulating electrolytes and the body-fluid balance. More importantly, BA can inhibit the release of inflammatory cytokines, preserve the integrity of the intestinal mucosal barrier, and improve the function of the intestinal immune system (Hofmanova et al., 2014; Louis et al., 2014). As a histone deacetylase inhibitor (HDACi) (Tan et al., 2014; Cleophas et al., 2016), BA plays a vital role in gene transcription. BA can increase the expression of Fas protein in T cells by inhibiting the acetylation of histones, thus inducing the apoptosis of T cells and inhibiting the inflammatory response (Zimmerman et al., 2012). When inflammatory response occurs, inflammatory cells produced free lipopolysaccharide (LPS), and inflammatory cytokines as well as chaperone protein STUB1. At the same time, FoxP3 degrades after ubiquitination in the presence of chaperone HSP70 (Chen et al., 2013; Laurence et al., 2013). Studies have shown that BA indirectly inhibited the degradation of Foxp3 by inhibiting IL-6 expression (Vinolo et al., 2011). Furusawa et al. (2013), Hoeppli et al. (2015) demonstrated that BA could also act with HDACi on both initial T cells and Treg cells, increasing the acetylation of histone H3 in the conservative non-coding sequence of FoxP3 (CNS-1), which promotes the differentiation of initial T cells into Treg cells on the one hand, and increases the expression of Foxp3 protein in Treg cells on the other hand. Besides, Vinolo et al. (2011), Smith et al. (2013) demonstrated that drinking water containing BA in sterile mice increased the number of Treg cells in the intestinal mucosa lamina propria of mice and promoted Treg cell synthesizing the inflammatory suppressor IL-10. Therefore, BA can promote the differentiation of Treg cells and the expression of FoxP3 and IL-10, thus regulating the inflammatory response.

Th17 and Treg cells, which belong to $CD4^+$ T cells, have gained much attention. The initial $CD4^+$ T cells differentiated into Treg cells induced by TGF-β and differentiated into Th17 cells under the combined action of TGF-β and IL-6 or IL-21. Th17 cells produce IL-17, IL-22, and IL-23, recruited neutrophils, and promoted inflammation at the injection site. On the contrary, after being activated by homologous antigens, Treg cells play a negative immunomodulatory role, mainly secreting anti-inflammatory cytokines such as IL-10 and TGF-β to inhibit the activities of various immune cells, thus suppressing the immune response (Hofmanova et al., 2014). According to many rodent studies, BA increases Tregs and reduces the differentiation of other Th cells (Cao et al., 2018; Luu et al., 2018). As demonstrated by Hu et al. (2018), the development of autoimmune hepatitis can be attenuated through regulation of the imbalance of Treg/Th17 and intestinal barrier function by a high-fiber diet. In addition, inflammatory skin reactions can be mitigated by SCFA sodium BA through inducing Tregs (Schwarz et al., 2017). However, BA also regulates the expression of several cytokines secreted by $CD4^+$ Tregs, which increases the suppressive ability and

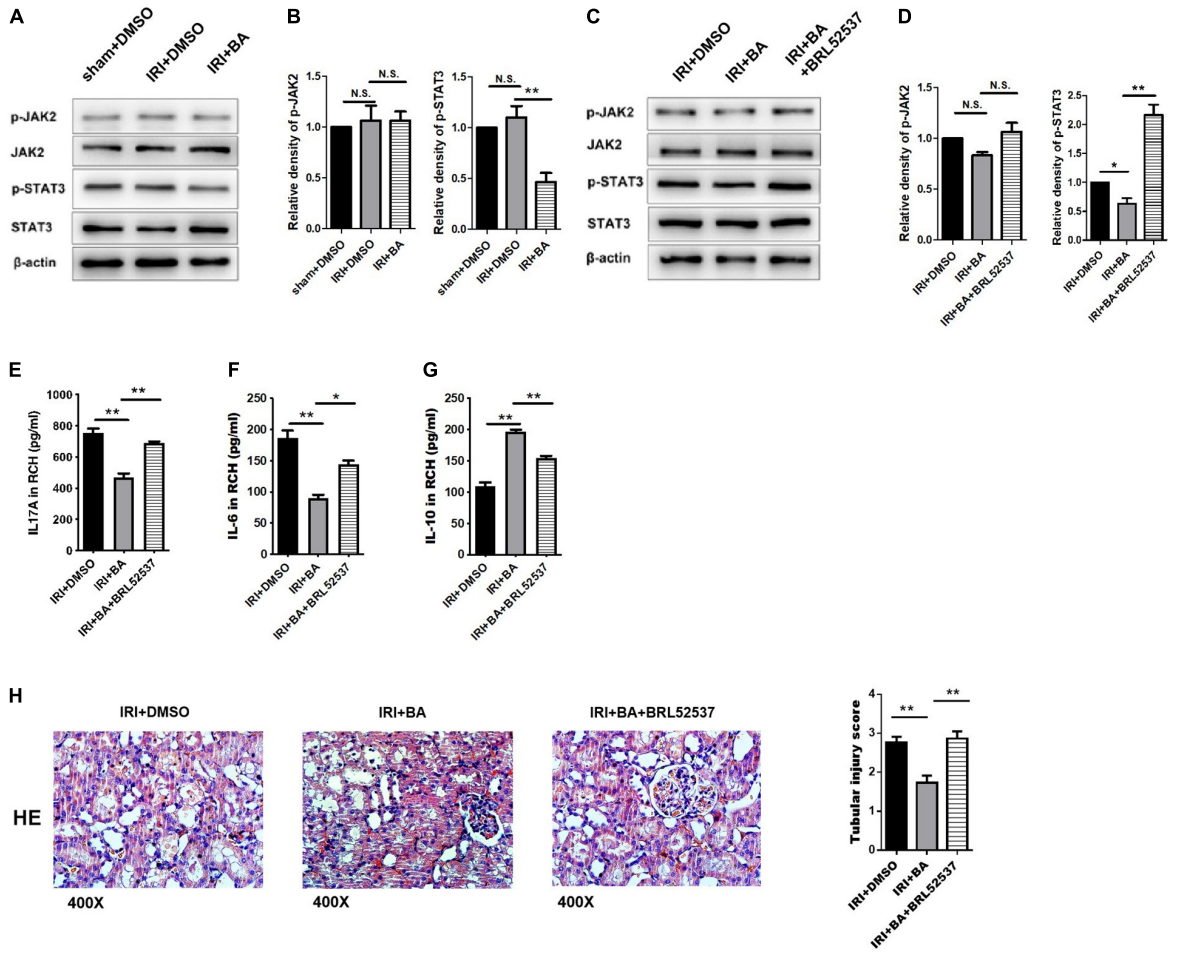

FIGURE 4 | BA protects against renal IRI *via* p-STAT3/SOCS3 signaling. **(A,B)** BA treatments attenuated the expression of p-STAT3 but not p-JAK2. **(C,D)** After BRL52537 treatment, the expression of p-STAT3 but not p-JAK2 was increased. **(E–G)** BRL52537 significantly attenuated the anti-inflammatory effects of BA on renal IRI. **(H)** HE staining indicated that BRL52537 significantly attenuated the protective effects of BA on renal IRI. Sample size = 3 in each group. Data were presented as mean ± standard deviation. N.S. $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

decreases the instability of Tregs. The mechanism underlying these effects will be investigated further and considered for future clinical applications.

Vinolo et al. (2011), Tan et al. (2014) suggested that BA can activate GPRs, especially GPR43 and GPR109a; reduced the synthesis and secretion of pro-inflammatory factors, reactive oxygen species (ROS), and cyclooxygenase (COX2); and inhibited inflammatory responses. Sun and Ye (2012) demonstrated that BA could affect its downstream MEK-ERK pathway *via* activating GPRs, thereby indirectly contributing to the secretion of antimicrobial peptide LL37, ultimately suppressing the inflammatory response. Klampfer et al. (2003) identified that BA inhibited the activity of JAK2 and suppressed IFNγ-induced tyrosine and serine phosphorylation of STAT1, thereby inhibiting the activity of the JAK-STAT pathway and ultimately reducing proinflammatory cytokine synthesis. Many experimental studies have shown that BA can inhibit the release of inflammation-related factors by inhibiting intracellular signaling pathways.

HO-1 is a key star molecule in the process of oxidative stress, and the role of HO-1 in inflammatory response has also been widely recognized (Sun et al., 2021; Wang et al., 2021; Zhang et al., 2021). In recent years, the relationship between HO-1 and immunity has gained some attention (Yan et al., 2019; Van Nguyen et al., 2020). However, whether HO-1 plays an essential role in the immune response to renal IRI remains unclear. Our research shows that HO-1 mediates the protection of BA against renal IRI, and this protective effect is related to the balance of Treg/Th17. We believe that HO-1 mediates the regulation of the Treg/Th17 balance. Similar to our research conclusion, the research of Zhang et al. (2018) showed that HO-1 agonist can increase Tregs in patients with vitiligo. They found that HO-1 restored the function of Tregs by upregulating the expression of IL-10, proved that HO-1 could significantly promote Treg expression in patients with vitiligo, and showed the potential of HO-1 as a therapeutic target for vitiligo Zhang et al. (2018). Another study showed that HO-1 might show anti-inflammatory activity in the mouse model of acute experimental colitis by

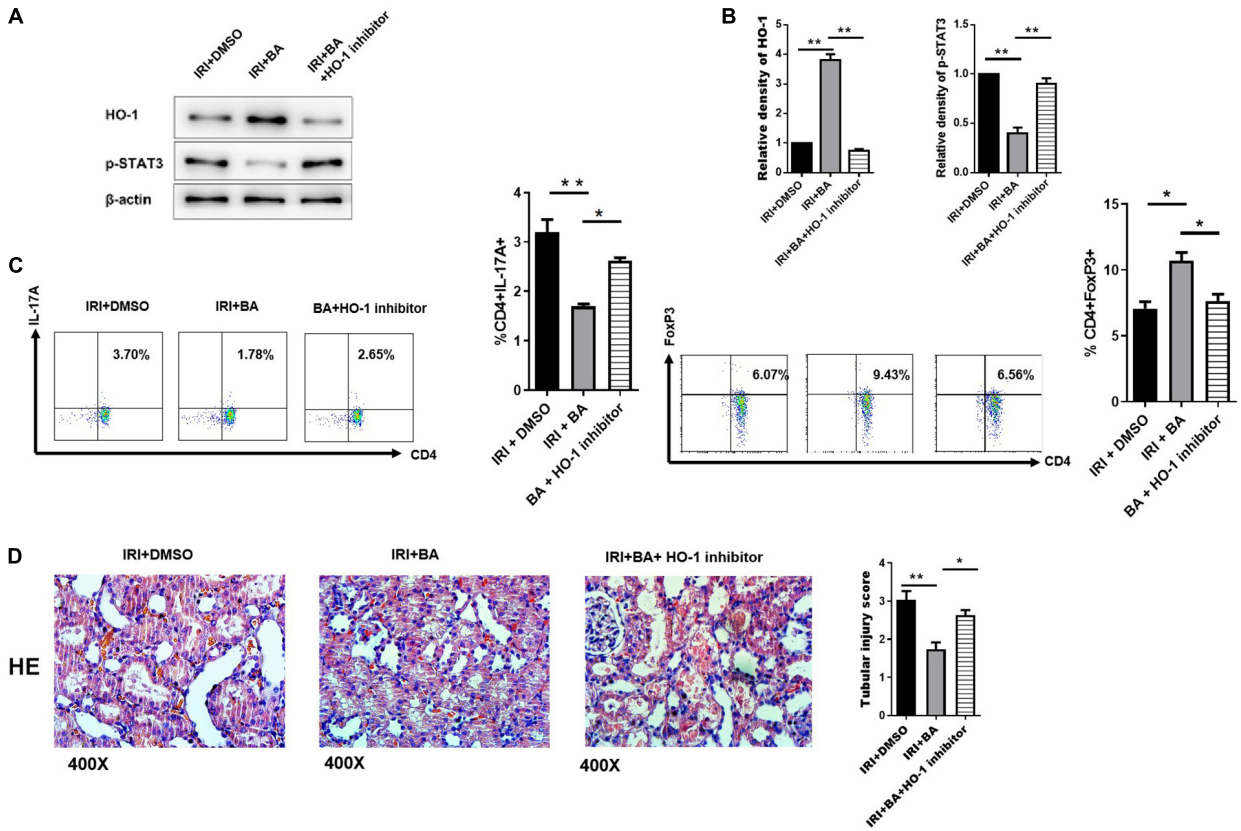

FIGURE 5 | HO-1/p-STAT3 signaling pathway was implicated in Treg/Th17 balance mediated by BA. **(A,B)** SnPP (the inhibitor of HO-1), significantly reduced the up-regulation of BA-related p-STAT3 expression. **(C)** In $CD4^+$ T cells under Treg/TH17 polarizing conditions. BA/Snpp co-treatment reversed the immunological balance of Treg/Th17 cells by BA only. **(D)** HE staining show that SnPP treatment significantly, but not wholly, reversed the regulating effect of BA. Sample size = 3 in each group. Data were presented as mean ± standard deviation. N.S. $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$.

regulating the balance between Th17 and Treg cells, thus providing a new therapeutic target for inflammatory bowel disease (Zhang et al., 2014). Treg/Th17 balance is important for multi-factor regulation. The JAK2-STAT3 signaling pathway is a classic signaling pathway that regulates the differentiation of $CD4^+$ T cells. Influencing the activation of the JAK2-STAT3 signaling pathway can regulate the differentiation of $CD4^+$ T cells, thereby regulating the balance of Treg/Th17. Our research shows that under the intervention of BA, HO-1 significantly affects the activation of the JAK2-STAT3 signaling pathway. HO-1 regulates p-STAT3 to affect the immune-inflammatory response during renal IRI. The research of Lin et al. (2017) corroborates our findings. Their research shows that HO-1 directly binds to STAT3 to control the production of pathogenic Th17 cells during neutrophil airway inflammation. They also creatively clarified HO-1 regulates the site of p-STAT3 (Lin et al., 2017). HO-1 is not only a key molecule of oxidative stress but also participates in the immune response process of a variety of diseases by regulating p-STAT3 (Chiang et al., 2020; Levin-Epstein et al., 2020; Lin et al., 2020). There is a certain link between oxidative stress and immune-inflammatory response that cannot be ignored.

Many studies have proved that Treg cells play an essential role in inflammatory bowel disease, transplantation immunity, bronchial asthma, and other diseases (Gibson et al., 2013; Singer et al., 2014; Terhune and Deth, 2014). The most prominent feature of Treg cells is the expression of Foxp3 and the demethylation of the Foxp3 gene locus (Hoeppli et al., 2015). In recent years, experiments have confirmed that by regulating Th17/Treg cell balance, the severity of renal IRI could be alleviated (Gan et al., 2019). Cleophas et al. (2016) also found that in the renal IRI model, IL-2C could regulate Th17/Treg cell balance by increasing Treg expression, thus reducing renal IRI. In the current study, we demonstrated that BA protected against renal IRI by enhancing $CD4^+$ Tregs and keeping the balance of the Treg/Th17 population. *In vitro* experiments showed that BA improved human Treg generation following IL-2 and TGF-β stimulation *via* promoting Foxp3 expression and suppressing T cell expansion.

CONCLUSION

In conclusion, the present study demonstrated that BA directly decreased Th17 cells and increased Treg cells, thus reducing the inflammatory response. Importantly, we further identified that BA protected against renal IRI *via* HO-1/p-STAT3 signaling,

which was implicated in Th17 cell differentiation. Our results provided ideas that BA can inhibit renal IRI by regulating Treg/TH17 cell balance and being a potential therapeutic option for renal IRI after surgery.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Soochow University.

AUTHOR CONTRIBUTIONS

ZC and YJL: Conception and design. MMW, SKY, JS, THJ, and WD: Analysis and interpretation. MMW, SKY, JS, THJ, and WD: Data collection. ZC, YJL, and LHJ: Writing the manuscript. ZWF and JC: Critical revision of the manuscript. ZWF, JC, and YJL: Final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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Strong Expansion of Human Regulatory T Cells for Adoptive Cell Therapy Results in Epigenetic Changes Which May Impact Their Survival and Function

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Adoptive transfer of regulatory T cells (Treg) is a promising new therapeutic option to treat detrimental inflammatory conditions after transplantation and during autoimmune disease. To reach sufficient cell yield for treatment, *ex vivo* isolated autologous or allogenic Tregs need to be expanded extensively *in vitro* during manufacturing of the Treg product. However, repetitive cycles of restimulation and prolonged culture have been shown to impact T cell phenotypes, functionality and fitness. It is therefore critical to scrutinize the molecular changes which occur during T cell product generation, and reexamine current manufacturing practices. We performed genome-wide DNA methylation profiling of cells throughout the manufacturing process of a polyclonal Treg product that has proven safety and hints of therapeutic efficacy in kidney transplant patients. We found progressive DNA methylation changes over the duration of culture, which were donor-independent and reproducible between manufacturing runs. Differentially methylated regions (DMRs) in the final products were significantly enriched at promoters and enhancers of genes implicated in T cell activation. Additionally, significant hypomethylation did also occur in promoters of genes implicated in functional exhaustion in conventional T cells, some of which, however, have been reported to strengthen immunosuppressive effector function in Tregs. At the same time, a set of reported Treg-specific demethylated regions increased methylation levels with culture, indicating a possible destabilization of Treg identity during manufacturing, which was independent of the purity of the starting material. Together, our results indicate that the repetitive TCR-mediated stimulation lead to epigenetic changes that might impact functionality of Treg products in multiple ways, by possibly shifting to an effector Treg phenotype with enhanced functional activity or by risking destabilization of Treg identity

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and impaired TCR activation. Our analyses also illustrate the value of epigenetic profiling for the evaluation of T cell product manufacturing pipelines, which might open new avenues for the improvement of current adoptive Treg therapies with relevance for conventional effector T cell products.

Keywords: regulatory T cells, advanced therapy medicinal products, DNA methylation, biomarker, adoptive cell therapy, good manufacturing practice

INTRODUCTION

T Lymphocytes are one of the most promising effectors for adoptive cellular therapy (ACT) by facilitating target-specific immune interventions, as has recently been impressively demonstrated in clinical practice (Guedan et al., 2019). ACTs comprised of regulatory T cells (Tregs) are currently under extensive clinical testing (Sharabi et al., 2018) to prevent undesired inflammation and immune reactivity during autoimmunity (Bluestone et al., 2015; Elias and Rudensky, 2019; Ryba-Stanislawowska et al., 2019), after solid organ transplantation (Romano et al., 2019; Sawitzki et al., 2020) or during graft-versus-host-disease (GvHD) after hematopoietic stem cell transplantation (Bertaina and Roncarolo, 2019; Elias and Rudensky, 2019; Mancusi et al., 2019). Despite previous concerns of inducing systemic over-suppression and increased risk for tumor formation and infections, studies from different centers including our own have shown a very good safety profile and even hints of efficacy: A recent phase 1/2a clinical trial (The ONE study; Roemhild et al., 2020; Sawitzki et al., 2020) testing Treg products from our facility demonstrated that drug-mediated immunosuppression could successfully be sustainably reduced in most kidney transplant recipients (Roemhild et al., 2020). Still, reduction of immunosuppression was not tolerated by a few patients and complete weaning was not feasible at all, indicating that there is room for improvement in the fitness, function, or engraftment of the Treg products. In addition, other critical challenges remain such as the standardization of manufacturing procedures and of quality control parameters, as well as the high costs of production (Trzonkowski et al., 2015; Raffin et al., 2020).

For Treg products, functional quality assessment remains a major challenge (Trzonkowski et al., 2015; Fuchs et al., 2017), as the only widely used *in vitro* suppression assay (Brusko et al., 2007; Venken et al., 2007) poorly represents the *in vivo* functionality of Tregs (Scheffold et al., 2005; Collison and Vignali, 2011; Wendering et al., 2019). In addition, classical Treg biomarkers (e.g., FOXP3, CD25, and lack of CD127 expression) can also be found on contaminating activated pro-inflammatory conventional T cells (Tran et al., 2007; Wang et al., 2007; Kmieciak et al., 2009). Furthermore, the quality of the final Treg ACT product is highly dependent on the donor and on the purity of the starting material. Until recently, highly pure Treg starting populations from peripheral blood could not be achieved due to the lack of appropriate flow-cytometric sorting devices which would be accepted by most European regulatory authorities. In addition, extensive TCR stimulation-driven *in vitro* expansion of the starting material is required, which poses risks for the outgrowth of contaminating pro-inflammatory T cells (Battaglia et al., 2005, 2006), possible loss of Treg identity (Marek et al., 2011; Bailey-Bucktrout et al., 2013), terminal differentiation, and perhaps functional exhaustion or senescence as reported for conventional T cells (Chou and Effros, 2013; Wherry and Kurachi, 2015; Okuda et al., 2019). Many different protocols for Treg product manufacturing have been developed and tested (Duggleby et al., 2018; Fraser et al., 2018; MacDonald et al., 2019; Alzhrani et al., 2020), but are difficult to compare due to the lack of a standardized quality control procedure (Fuchs et al., 2017). It is therefore of great importance to assess functional or molecular alterations which occur during Treg product generation in order to identify current therapeutic limitations and develop strategies for improvement.

To address this, we characterized manufacturing-induced epigenetic changes at the DNA methylation level during the generation of Treg products at our center. Epigenetic mechanisms define the potential for gene expression at individual loci through chromatin permissibility, and can give insight into both current and future functional states (Youngblood et al., 2013; Durek et al., 2016; Abdelsamed et al., 2017; Delacher et al., 2017, 2021). For T cells in particular, the functional state can be shaped by epigenetic remodeling in response to extracellular signaling cues as was recently shown for exhausted T cells (Pauken et al., 2016; Ghoneim et al., 2017). Therefore, analyzing manufacturing-induced epigenetic changes may be informative for predicting the cellular state and function of the final Treg product. In our analyses, we determined that the manufacturing process introduces wide-spread, reproducible, and progressive changes in the DNA methylome of the Treg products, including at loci important for T cell activation and Treg identity.

MATERIALS AND METHODS

Ethics

All donors donating blood for this study provided written informed consent for their participation. Study procedures were approved by the Ethics Committee of the Charité - Universitätsmedizin Berlin.

Manufacturing of 1st Generation Products

Peripheral blood from healthy donors was used for enrichment of natural regulatory T cells (nTreg). Two consecutive CliniMACS runs, compromising a $CD8^+$ cell depletion

followed by a $CD25^+$ cell selection, were performed according to the manufacturer's instructions (Miltenyi Biotec). Target cell fraction was cultured in complete nTreg-medium, containing X-Vivo 15 medium (Lonza) supplemented with fetal calf serum (FCS, Hyclone), recombinant human IL-2 (Miltenyi Biotec) and Rapamycin (Pfizer). Depending on cell numbers, expansion process of Tregs was performed in 96-well plates and 24-well plates, respectively, for 23 days, using complete nTreg-medium and repetitive stimulation with anti-CD3/CD28 MACSiBead particles (Treg Activation/Expansion Kit, Miltenyi Biotec). Cells were collected for downstream DNA methylation analysis at several time-points during culture.

Manufacturing of 2nd Generation Products

Peripheral blood was obtained from healthy donors, and peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation with Biocoll (Biochrom). The $CD4^+$ $CD25^{high}$ $CD127^-$ Treg cells were sorted by a flow cytometry-based sorting system, the MACSQuant Tyto Cell Sorter (Miltenyi Biotec). Staining was performed using fluorophore-conjugated human anti-CD4, anti-CD25 and anti-CD127 antibodies (all Miltenyi Biotec), achieving purities of over 90%. Treg cells were cultured in a 96-well round-bottom plates (Eppendorf) with complete nTreg-medium compromising X-Vivo 15 medium (Lonza) supplemented with FCS (Biochrom), Penicillin/Streptomycin (Biochrom), Rapamycin (Sigma-Aldrich) and recombinant human IL-2 (Novartis). Treg cells were repetitively stimulated with anti-CD3/CD28 MACSiBead particles (Treg Activation/Expansion Kit, Miltenyi Biotec) for a total of 8 times throughout culture for 21 days.

Isolation and Culture of FACS-Sorted Tregs and $CD4^+$ Memory T Cells

PBMCs were isolated from 50 mL of peripheral blood obtained from healthy donors, by Ficoll-Paque Plus (Thermo Fisher Scientific) density gradient centrifugation followed by erythrocyte lysis using Buffer EL (Qiagen). After CD4 enrichment using magnetic cell separation with the autoMACS instrument (Miltenyi Biotec) using CD4 MicroBeads (Miltenyi Biotec), cells were left overnight at 4°C in basal cell culture medium [RPMI 1640 Medium with GlutaMAX Supplement-10 (Thermo Fisher Scientific), 10% FCS (Corning), 50 nM 2-mercaptoethanol (Thermo Fisher Scientific), 1 mM pyruvate (Biochrom), and 25 mM HEPES (Gibco)]. The following day, $CD4^+$ memory T cells ($CD4^+$ $CD25^+$ $CD127^+$ $CD45RO^+$) and Tregs ($CD4^+$ $CD25^+$ $CD127^-$) were FACS-sorted with a BD FACSAria II SORP (Becton Dickinson). Sorted $CD4^+$ memory T cells and Tregs were initially cultured in 96-well round-bottom plates (Greiner Bio-One) with the aforementioned cell culture media supplemented with 500 IU/mL rhIL-2 (R&D Systems) and 10 nM Rapamycin (STEMCELL Technologies) (day 0). The following day, cells were stimulated with anti-CD3/CD28 MACSiBead particles (Treg Activation/Expansion Kit, Miltenyi Biotec) according to manufacturer's guidelines. Cells were stimulated at the same time points as the 1st and 2nd generation products, and collected for downstream DNA methylation analysis.

Surface Expression Profiling

GMP-compliant Treg products and PBMCs were stained using fluorescently conjugated monoclonal antibodies for CD3 (BV650, clone OKT3), CD4 (PerCP-Cy5.5, clone SK3), CD8 (BV510, clone RPA-T8), CD127 (APC-A700, clone A019D5), and CD25 (APC, clone M-A251) at 4°C for 30 min. Backbone-stained samples were subsequently split to be stained for further 30 min with PE-conjugated antibodies for different human surface antigens: CD5 (clone UCHT2), CD80 (clone 2D10), CD86 (clone IT2.2), CD59 [clone p282 (H19)], CD160 (clone BY55), CD279 (clone EH12.2H7), CD366 (clone F38-2E2), and TIGIT (clone A15153G). All antibodies were purchased from BioLegend. To exclude dead cells, LIVE/DEAD Fixable Blue Dead Cell Stain dye (Thermo Fisher Scientific) was added. Lymphocytes were gated on the basis of the forward scatter (FSC) vs. side scatter (SSC) profile after exclusion of doublets via FSC-Height vs. FSC-Area. Tregs were defined as $CD25^{high}$ $CD127^{low}$ $CD4^+$ $CD8^-$ $CD3^+$ and conventional T cells (Tcon) as $CD25^-$ $CD127^+CD4^+$ $CD8^-$ $CD3^+$. Cells were analyzed on a Cytoflex LX (Beckmann Coulter) flow cytometer. Cytometric raw data were analyzed using Kaluza™ software (Beckman Coulter), mean expression values were visualized using the *nautilus* R package (available from[1]).

Methylation Profiling

Genomic DNA of cell pellets was isolated using AllPrep DNA/RNA Mini Kit (Qiagen) or Zymo's Quick-DNA MicroPREP Kit (Zymo Research), following manufacturer's instructions. DNA concentration was assessed with Qubit dsDNA HS Assay Kit and the Qubit Fluorometer (Molecular Probes/Life Technologies). 200–250 ng of genomic DNA was used as input for bisulfite conversion, which was performed with Zymo's EZ DNA Methylation-Gold Kit (Zymo Research) or Zymo's EZ DNA Methylation Kit (Zymo Research). Methylation analysis was performed with the Infinium MethylationEPIC Kit (Illumina EPIC-8 BeadChip) following the manufacturer's instructions. Bead Chips were imaged on Illumina's Microarray Scanner iScan.

Data Processing and Analysis

All analyses were conducted in the R statistical software (Version > 4.0).

Preprocessing Raw Data

Raw intensity data files (IDAT) from Bead Array scans were preprocessed using *minfi* version 1.36.0. Probes were removed if they did not meet the detection p-value threshold ($p < 0.05$), were found to be cross reactive (Pidsley et al., 2016), or changes in manufacturing process (Infinium MethylationEPIC v1.0 B5 Release Date). Raw data was normalized by quantile normalization, after which M values were extracted. Due to

[1]https://github.com/steschlick/nautilus

sample processing at different institutes, samples were subjected to batch correction using the *ComBat* function from *sva* version 3.38.0 (Leek et al., 2012). Lastly, CpGs within 3 nucleotides away from a SNP/variant was removed with the function *rmSNPandCH* from *DMRcate* version 2.4.1 (Peters et al., 2015).

Differential Methylation Analysis

CpGs on chromosomes X or Y were removed prior to differential methylation analysis. Significant (FDR < 0.05) differentially methylated positions (DMPs) were identified using the *cpg.annotate* function in *DMRcate*, which were then used to identify differentially methylated regions (DMRs) using the *dmrcate* function with lambda = 500 and C = 3. DMRs with a Stouffer's score < 0.05 were kept for further analysis. CpGs and DMRs were annotated to genomic features and genes using *annotatePeak* from the *ChIPseeker* version 1.26.2 (Yu et al., 2015).

Publicly Available Data

Partially Methylated Domains

Bed files containing genomic annotations for PMDs in CD4 central memory (CM), effector memory (EM), effector memory RA (TEMRA), and Tregs were downloaded from Gene Expression Omnibus GSE113405 (Salhab et al., 2018). Due to the heterogeneous starting material of the first generation Tregs, an overlapping core set of PMDs was identified through *findOverlaps* function in *GenomicRanges*.

Whole Genome Bisulfite Sequencing

CpG methylation read counts from WGBS data of HIV-reactive CD8 T cells and CD8 effector memory were downloaded from Gene Expression Omnibus GSE144693 (Abdelsamed et al., 2020). Only CpGs that were covered at least 10X were considered for analysis. *dmrcate* was also used to identify DMRs between exhausted HIV-reactive CD8 T cells and CD8 effector memory cells using the parameters with lambda = 500 and C = 3.

ChromHMM Chromatin States

ChromHMM chromatin states of Treg and CD4 memory cells were downloaded from the NIH Roadmap Epigenomics Project portal https://egg2.wustl.edu/roadmap/web_portal/

Statistical Analysis and Data Visualization

Gene set enrichment analysis was performed with the package *fgsea* version 1.16.0 (Korotkevich et al., 2021). Gene ontology enrichment analysis was performed with *clusterProfiler* version 3.18.1 (Yu et al., 2012). For *T* test statistical analysis, the function *stat_compare_means* from *ggpubr* version 0.4.0 was used[2]. Heatmaps were generated using *ComplexHeatmap* version 2.6.2 (Gu et al., 2016). Alluvial plot was generated with *ggalluvial* version 0.12.3[3]. Density plot margins for PCA plot was generated with *ggExtra* version 0.9[4]. All other plots were generated with *ggplot2* version 3.3.5.

[2]https://cran.r-project.org/web/packages/ggpubr/index.html

[3]http://corybrunson.github.io/ggalluvial/

[4]https://cran.r-project.org/web/packages/ggExtra/index.html

RESULTS

In vitro Expansion During Treg Product Generation Induces Changes in the DNA Methylome

Treg products from three healthy donors were generated twice by two indepenent manufacturing runs. These first generation (1st gen.) Treg products are generated from peripheral blood-derived starting material which is depleted of $CD8^+$ cells and enriched for CD25-expressing cells using the CliniMACS system, yielding a starting populations enriched for $CD8^-$ $CD4^+$ $CD25^+$ Tregs. For generation of the final product, the cells undergo expansion culture for 23 days with repetitive TCR stimulation (using antiCD3/CD28 microbeads, 8 stimulations in total) in the presence of IL2 and Rapamycin. Supplementation of Rapamycin is meant to inhibit the outgrowth of contaminating conventional proinflammatory $CD4^+$ T cells (Battaglia et al., 2005). We harvested samples at the starting (day 0), final (day 23), and relevant additional time points during cell expansion (**Figure 1A**). The samples were subjected to genome-wide methylation analysis using the Illumina Methylation EPIC microarray, which covers a selection of 850,000 CpG methylation sites genome-wide with a special emphasis on regulatory elements such as promoters and enhancers.

Principal component analysis (PCA) of the EPIC array methylation profiles demonstrated that the number of days in culture had a substantial impact on the DNA methylome, as this feature accounted for PC2 (11% of the total variation in the data set) (**Figure 1B**). Inter-donor variances could also be observed and were reflected on PC3 (**Figure 1B**). Importantly, within the same donor, the samples did not separate based on production runs. These observations indicate that there is a progressive change in DNA methylation with prolonged culture, which is reproducible between donors and production runs. We also explored methylation changes at partially methylated domains (PMDs) of the genome, which are large transcriptionally silenced regions characterized by a highly disorganized methylation pattern, a generally reduced DNA methylation level and poor gene content that undergo methylation loss as a result of strong episodes of proliferation (Salhab et al., 2018; Decato et al., 2020). We indeed observed a loss of PMD methylation with increased expansion rate (**Figure 1C**), which is consistent with previous findings that demonstrated that accumulation of cell divisions drive PMD hypomethylation in cancer and aging (Zhou et al., 2018), and during T cell differentiation (Durek et al., 2016).

We next performed differential methylation analysis to identify genetic loci undergoing significant methylation changes during Treg product generation. Increased DNA methylation in promoters, first introns, and enhancers generally correlate with transcriptional downregulation (Varley et al., 2013; Anastasiadi et al., 2018), while hypomethylation correlates with transcriptional activation. In contrast, hypermethylation at gene bodies (mainly on exons) has been reported to correlate with increased transcription (Ball et al., 2009) and increased

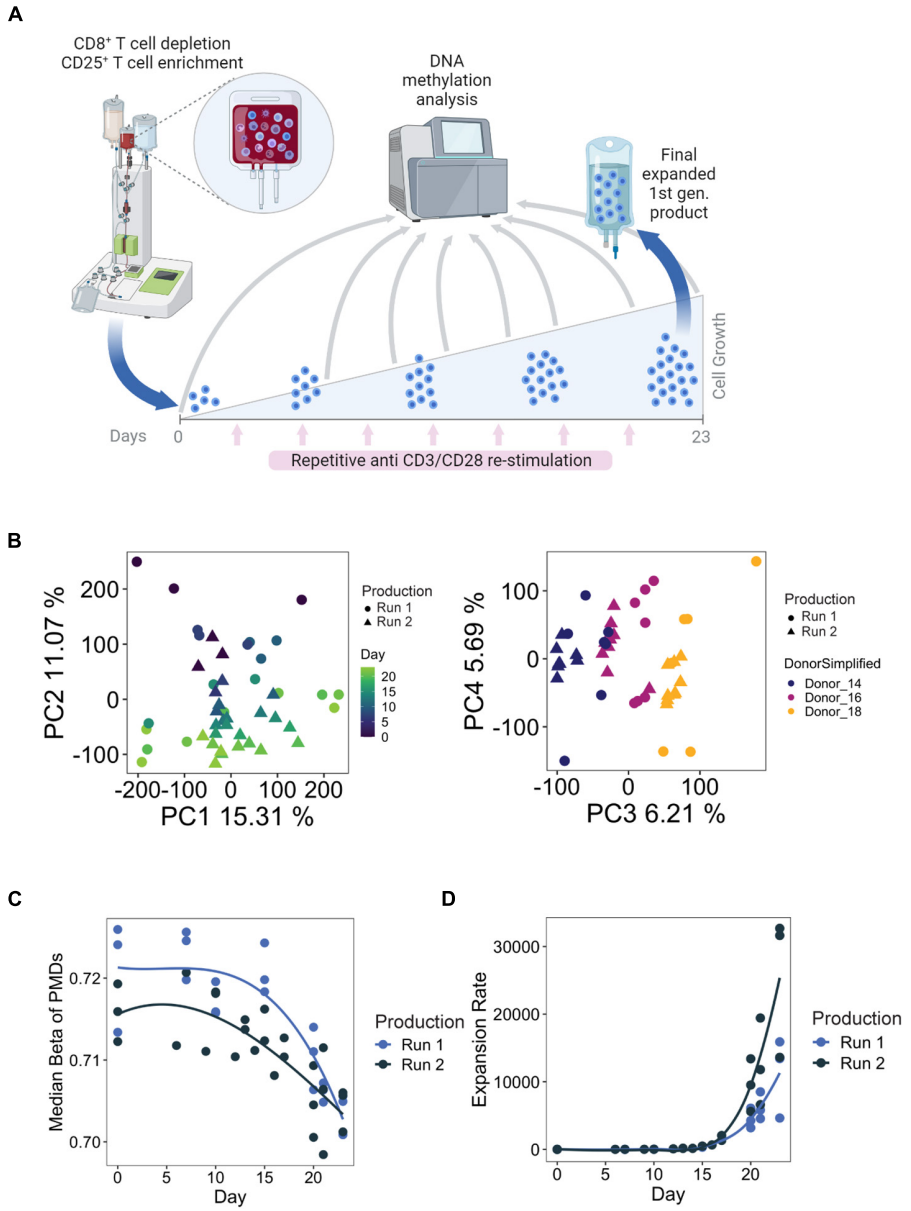

FIGURE 1 | (A) Example manufacturing run for first-generation Treg products. Briefly, Tregs were enriched through CD8$^+$ depletion followed by CD25$^+$ enrichment using the CliniMACS® system from whole blood. Tregs are cultured over the course of 23 days, with 8 total stimulations with anti-CD3/CD28 microbeads. Time points used for DNA methylation analysis are indicated with the gray arrows. Figure created with BioRender.com. **(B)** PCA analysis of samples taken from all available time points from both manufacturing runs. **(C,D)** Median methylation values of partially methylated domains (PMDs) (left) and cumulated expansion rate (right) during Treg product generation.

methylation at alternatively spliced exons has been implicated in differential splicing by mediating exon retention during transcription (Sun et al., 2020).

Although we focus much of our later analyses on differentially methylated regions (DMRs) containing at least 3 CpGs, we also characterized differentially methylated positions (DMPs) of individual CpGs in case biologically important loci were not sufficiently covered by multiple adjacent CpG probes due to microarray design limitations. We detected approximately 40,700 and 30,000 CpGs that were differentially methylated in production Runs 1 and 2, respectively, between the final (day 23) and starting (day 0) products (**Figure 2A**, **Supplementary Table 1**). For both runs, majority of DMPs mapped to introns (38%), followed by distal intergenic regions (29%) and promoters (27%) (**Figure 2B**). Other DMPs mapping to the 5′ or 3′ UTRs, exons, and genomic regions immediately downstream of the gene body (<300 bp) comprised only 5% of all significant sites. Furthermore, promoters and introns consistently had a higher

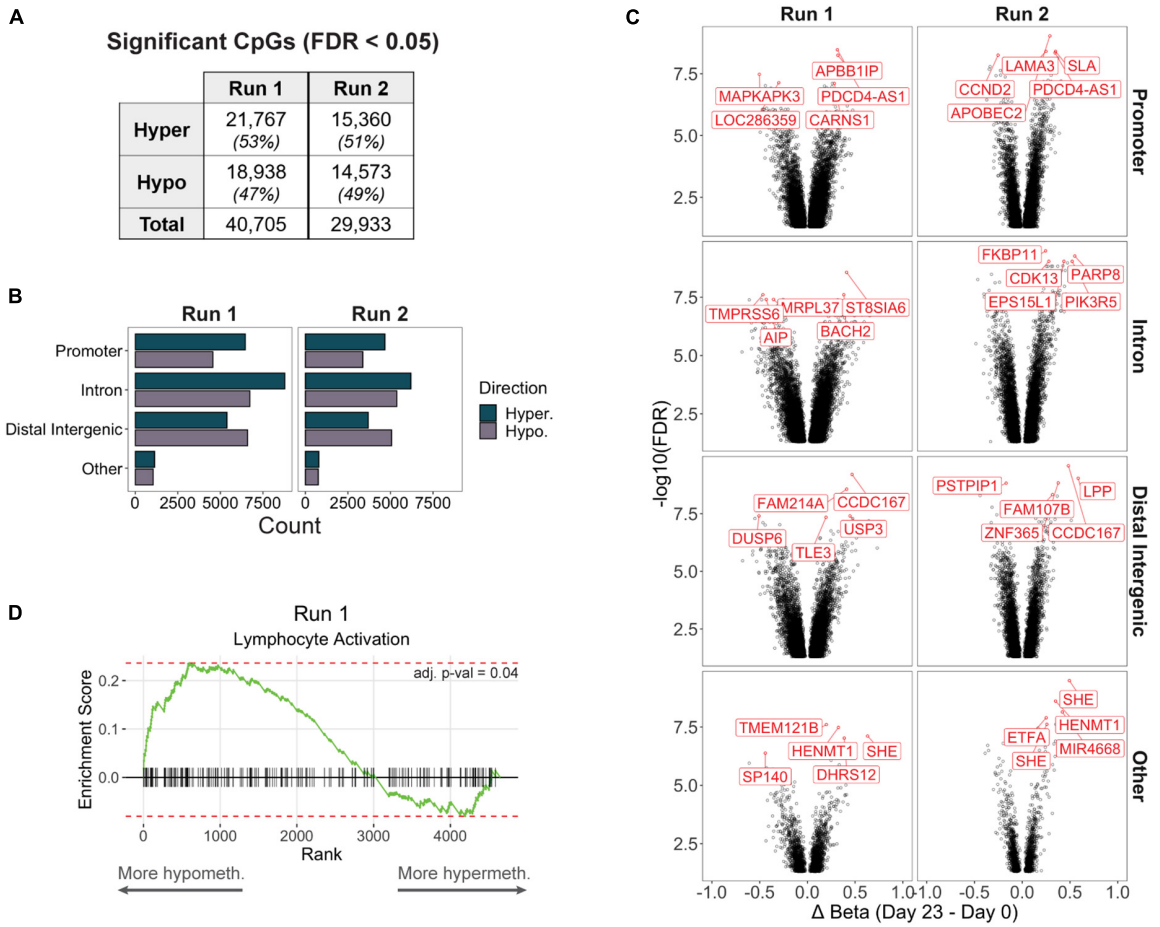

FIGURE 2 | (A) Significantly differentially methylated CpGs (differentially methylated positions, DMPs) between day 0 and day 23 products in each production run. **(B)** Counts of hyper- or hypomethylated DMPs mapping to the promoter, intron, distal intergenic, or other (5′ or 3′ UTRs, exons, or immediate downstream) region of a gene. **(C)** Volcano plots of significant DMPs (FDR < 0.05) stratified by genic annotation. The top 5 most significant DMPs (stratified by genic annotation) are highlighted. **(D)** In Run 1, genes that contained at least 1 promoter DMP but did not contain any differentially methylated regions (DMRs, including at least 3 CpGs) were significantly enriched for the Lymphocyte Activation pathway (adj. *p*-val < 0.05).

number of hypermethylated DMPs, whereas hypomethylated DMPs were more frequent in intergenic regions. Interestingly, a few CpGs were consistently among the top 5 most significant DMPs (stratified by genic annotation) in both production runs, such as a hypermethylated DMP mapping to the promoter of *PDCD4-AS1*, a long non-coding RNA that has been shown to positively regulate *PDCD4*, a tumor suppressor gene that has also been implicated in autoimmune disorders (Hilliard et al., 2006; Jadaliha et al., 2018; **Figure 2C**).

We then performed DMR analysis to identify genomic regions containing adjacent differentially methylated DMPs, and identified 1,753 DMRs associated with 1,515 unique genes in Run 1, and 1,246 DMRs associated with 1,103 genes in Run 2; the remaining 10,852 genes from Run 1 and 9,597 genes from Run 2 did not contain a DMR despite having at least 1 significantly DMP. However, these non-DMR DMPs may still function as important regulators of Treg or T cell biological processes: When we performed gene set enrichment analysis of Run 1 promoter DMP genes that were not represented by any DMRs, we found significant enrichment for genes important for lymphocyte activation (**Figure 2D**).

Consistent Epigenetic Remodeling Occurs at Genes Important for T Cell Activation and Differentiation

We next focused on significant DMRs that were present in both production runs as they would reflect reproducible methylation changes during Treg product generation. We defined "shared DMRs" as production run 1 DMRs overlapping with run 2 DMRs by 50% or more. Furthermore, we were also interested in delineating early epigenetic changes in response to initial activation versus changes occurring only later, the latter resulting from progressive changes due to prolonged culture. We therefore labeled shared DMRs as "Early" (first identified in day 10 vs. day 0 comparison and still significant in day 23 *versus* day 0) or "Late" (first identified in day 23 vs. day 0 in both production runs). As expected, Early DMRs displayed

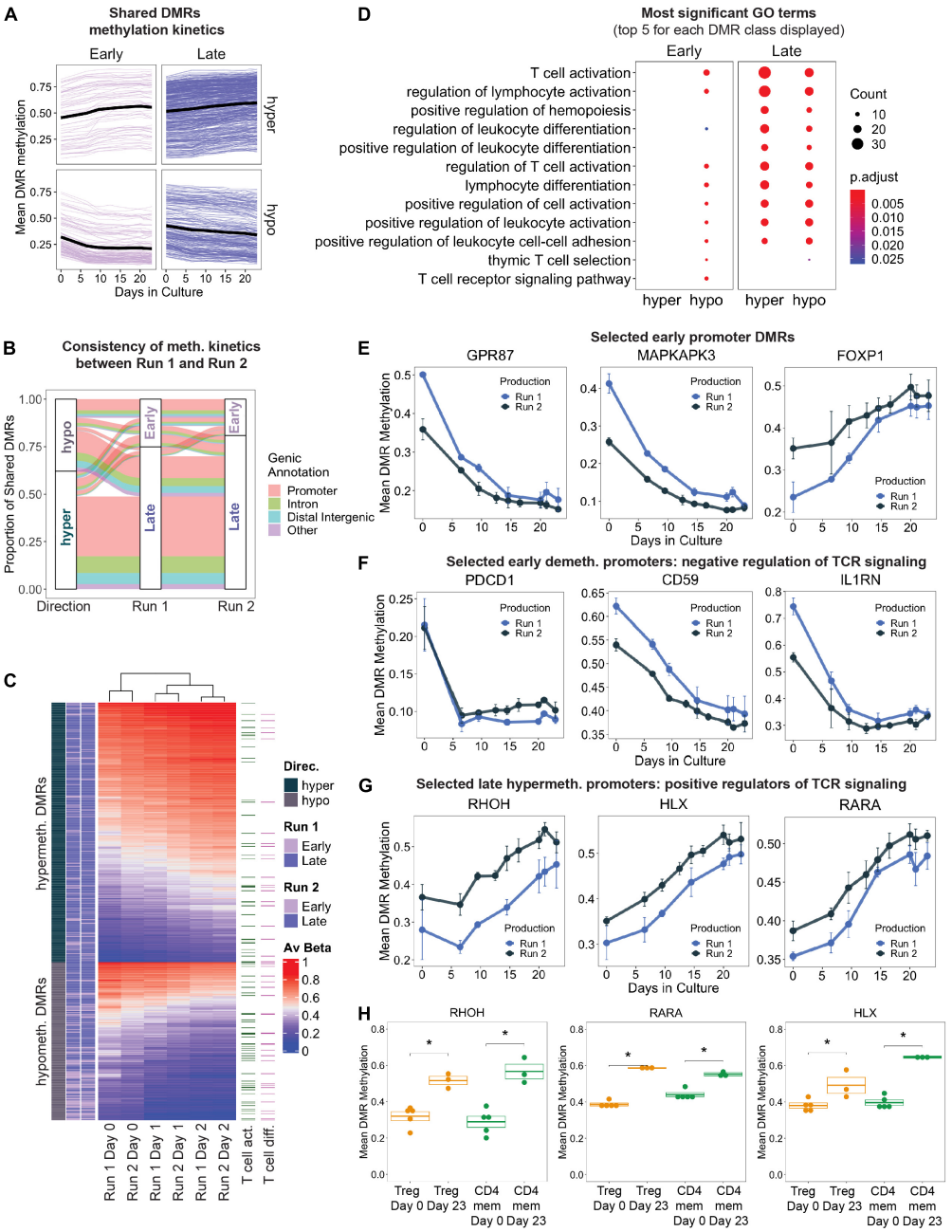

FIGURE 3 | (A) Change in mean DMR methylation of shared DMRs over the 23 days of *in vitro* expansion. Early DMRs were defined as being significantly differentially methylated in day 10 – day 0 (and remained significant in day 23 – day 0), and Late DMRs were only significantly differentially methylated at day 23 – day 0. Each line displays one DMR, bold black line displays the mean. **(B)** Display of all shared DMRs according to their directionality of methylation change (hyper/hypo) and to their Early/Late classification (as in **A**) in production Run1 and 2. Color code indicates genic annotation of the DMR. **(C)** Unsupervised hierarchical clustering of shared DMRs in both production runs, with DMRs mapping to genes implicated in T cell activation and T cell differentiation (GO terms 0042110 and 0030217) highlighted. **(D)** Gene ontology enrichment analysis of shared DMRs that were consistently "Early" or "Late" between both production runs, stratified by directionality. The top 5 GO terms (ranked by adjusted *p*-value) for each DMR class are represented, many of which overlap. Size of dots indicates number of genes associated with GO term, and color indicates adjusted *p*-value. **(E–G)** Level of DNA methylation on selected DMRs in known T cell genes. **(H)** DNA methylation at the promoters of *RHOH*, *HLX*, and *RARA* in FACS-sorted Tregs and memory CD4$^+$ T cells (CD4mem) before (Day 0) and after frequent CD3/CD28 stimulation (Day 23). *denotes $p.\text{val} < 0.05$ (*T* test).

stronger initial methylation change within the first 10 days of culture (indicated by the bend in the methylation time series traces) (**Figure 3A**).

In total, there were 838 shared DMRs (**Supplementary Table 2**), with 62% hypermethylated by the final time point (**Figure 3B**). Although the directionality (hyper- vs.

hypomethylated) of the DMRs was consistent between the two production runs, the methylation kinetics ("Early" or "Late") differed slightly (**Figure 3B**): 16% of shared DMRs had different kinetics between the two runs. This may be a result of variability in expansion rates between the two runs (**Figure 1D**), differences in the proportion of contaminating cell types in the starting day 0 cultures, or simply lack of statistical power. Gene promoters accounted for the majority of all shared DMRs (63%), followed by introns and distal intergenic elements, both of which frequently contain enhancers.

Unsupervised hierarchical clustering based on shared DMR mean methylation values at days 0, 10, and 23 depicted samples of the same time points clustering together regardless of their corresponding production run (**Figure 3C**). Genes implicated in T cell activation and differentiation were present in both hypo- and hypermethylated DMRs. We performed Gene Ontology (GO) enrichment analysis on the shared DMRs that were stratified by directionality and methylation kinetics to evaluate biological processes that may be regulated by epigenetic mechanisms. Genes that were significantly hypomethylated early during manufacturing are implicated in T cell activation, differentiation, adhesion, and other related pathways (**Figure 3D**). Enrichment of these GO terms was maintained even in the late hypomethylated DMRs. Examples of genes undergoing early strong promoter hypomethylation (>15%) include genes important for cell signaling, intracellular signal transduction, and proliferation such as *GPR87* and *MAPKAPK3* (**Figure 3E**; Zhang et al., 2010; Cargnello and Roux, 2011). Genes containing early hypermethylated DMRs did not enrich for any GO term, but still contained interesting candidate genes. We observed strong hypermethylation at the promoter *FOXP1* (**Figure 3E**), perhaps indicating a path toward terminal differentiation of the Treg products as DNA methylation-controlled transcriptional downregulation of *FOXP1* has been shown to correlate with end-differentiated T cells (Goronzy and Weyand, 2012; Durek et al., 2016).

We also observed early DNA hypomethylation of genes reported to negatively modulate TCR-mediated signaling, such as *PDCD1*, *CD59* (Sivasankar et al., 2009), and *IL1RN* (McIntyre et al., 1991; Arend et al., 1998; **Figure 3F**). This may indicate the beginning of a negative feedback loop that is normally part of a physiological response to restrict T cell effector function in order to prevent overshooting of the immune response during antigen encounter. Furthermore, many DMRs mapping to genes important for T cell activation were significantly hypermethylated later in culture (**Figure 3D**). For example, a progressive increase in promoter methylation was detected at genes which positively regulate TCR signaling and T cell differentiation, such as *RHOH* (Wang et al., 2011), *HLX* (Allen et al., 1995), and *RARA* (Hall et al., 2011), suggesting transcriptional inactivation that may occur through repetitive stimulation-induced epigenetic reprogramming (**Figure 3G**). To demonstrate that hypermethylation of these TCR signaling loci occurs independently of starting material purity, we also profiled these promoters' methylation status in FACS sorted primary conventional $CD4^+$ memory T cells (CD4mem) and Tregs which have also been stimulated repeatedly with antiCD3/CD28 beads in similar culture conditions. Indeed, both Tregs and CD4mem at day 23 displayed significantly increased promoter methylation compared to the starting populations (day 0) (**Figure 3H**), indicating that frequent TCR stimulation for 23 days is capable of epigenetically reprogramming and potentially stably downregulating these critical genes.

Genes Upregulated in Chronically Activated T Cells Undergo Significant Methylation and Surface Expression Changes in First Generation Treg Products

We also identified promoter-associated Late DMRs that were significantly and strongly hypomethylated (methylation loss > 15%) at *HAVCR2* (encoding for TIM-3), *TIGIT*, and *CD160* (**Figure 4A**), which are co-inhibitory surface molecules that are upregulated in T cells and Tregs during prolonged antigen encounter. Although upregulation of these co-inhibitory surface molecules is often associated with exhaustion, a hypo-responsive and dysfunctional state in conventional T cells (Tcon) caused by persistent TCR signaling (reviewed in Blank et al., 2019), they are also linked to enhanced immunosuppressive capacity in Tregs (Sakuishi et al., 2013; Fuhrman et al., 2015; Liu et al., 2018).

We were interested in profiling methylation changes at additional genes which have been reported to be transcriptionally upregulated during prolonged antigen exposure. We curated a list of genes by combining T cell dysfunction/exhaustion gene modules from Singer et al. (2016) with genes identified in $CD4^+$ exhausted T cells from Crawford et al. (2014), and checked whether these genes were present in our shared DMR dataset. Indeed, we found several genes known to be upregulated in exhausted T cells which were consistently differentially methylated during Treg product generation, with most of them experiencing strong hypomethylation at their promoters (**Figure 4B**). We confirmed that strong promoter hypomethylation corresponded to upregulation of these chronic activation-induced genes (i.e., HAVCR2/TIM-3, TIGIT, CD160, CD59, CD80, CD86, and CD5), as the final product had higher surface protein expression than either of the *ex vivo* Treg and Tcon populations present in the day 0 starting material (**Supplementary Figure 1**).

Epigenetic changes have also been correlated to the establishment of functional exhaustion, most well studied in the context of tumors and chronic infections (Khan et al., 2019; Scott et al., 2019). In a recent study conducted by Abdelsamed et al. (2020), the authors generated whole genome bisulfite sequencing (WGBS) data from $CD8^+$ effector memory T cells (TEM) derived from healthy donors and patient-derived exhausted PD-1^+ HIV-reactive $CD8^+$ T cells (Texh). We took the opportunity to reanalyze this same WGBS dataset to define DNA methylation features distinguishing chronically activated $CD8^+$ T cells (Texh) from TEM, and determined whether these epigenetic differences were also shared with our first generation products which have undergone repetitive TCR stimulation. Our analysis identified approximately 52,000 genomic regions that

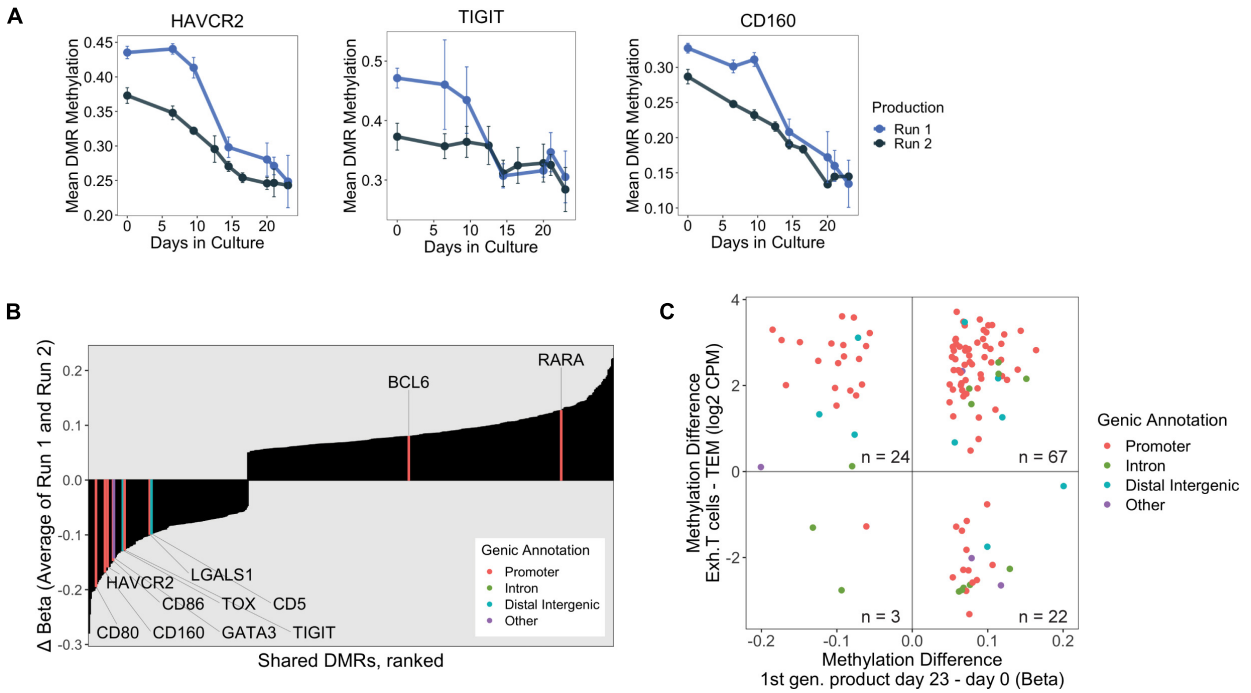

FIGURE 4 | (A) DNA methylation changes over 23 days at the promoters of select exhaustion-associated loci. **(B)** Consistently "Late" shared DMRs were ranked by final change in methylation (Day 23 – Day 0, averaged over both production runs). Genes implicated in exhaustion, taken from publicly available datasets, are highlighted with associated genic annotation. Notably, many of the exhaustion associated genes are among the strongest hypomethylated DMRs. **(C)** Scatter plot displaying shared DMRs which overlapped with ExhDMRs identified from WGBS comparing exhausted CD8 T cells vs. CD8 effector memory T cells. The x-axis depicts methylation difference between day 23 and day 0 products (average of both runs), and y-axis depicts methylation difference between functionally exhausted CD8 T cells (Exh. T cells) and CD8 effector memory T cells (TEM).

were differentially methylated between TEM and exhausted T cells, which we refer to as exhDMRs.

We rationalized that the consistently Late shared DMRs identified in our Treg products would likely contain exhaustion-associated changes because these late changes were likely induced by long-term TCR stimulation. We found that approximately 20% of all Late shared DMRs overlapped with the exhDMRs (116 overlapping DMRs of 585 Late shared DMRs, **Supplementary Table 3**), which is likely an underestimation due to the limited number of CpGs captured by the EPIC microarray. Of the overlapping DMRs, 91 were significantly hypermethylated in exhausted T cells compared to TEM, of which 74% were consistently hypermethylated in the final Treg products (day 23) compared to day 0 (**Figure 4C**). A vast majority (85%) of these hypermethylated DMRs also mapped to promoters of genes (highlighted red in **Figure 4C**), suggesting that a set of hypermethylated exhaustion-associated genes are similarly transcriptionally silenced by epigenetic mechanisms during *in vitro* manufacturing of Treg products.

Treg-Specific Demethylated Loci Are Progressively Hypermethylated With Culture

Preservation of Treg immunosuppressive function and identity is a primary concern for the development of adoptive Treg therapies, as persistent TCR-mediated activation has been shown to correlate with downregulation of the lineage-defining transcription factor FOXP3, although perhaps only selectively in memory ($CD45RA^-$) Tregs (Hoffmann et al., 2009; Arroyo Hornero et al., 2017; Guo et al., 2019). The downregulation of FOXP3 is linked to the hypermethylation of an intronic enhancer in the FOXP3 gene, the so-called FOXP3 Treg-Specific Demethylated Region (FOXP3-TSDR), which is used as an epigenetic marker to distinguish *de facto* Tregs from closely related conventional $CD4^+$ memory T cells (CD4mem) due to its highly selective demethylated status only in Tregs (Huehn et al., 2009). The consequential gain of FOXP3-TSDR methylation in response to persistent TCR stimulation demonstrates the need to profile additional culture-induced epigenetic changes at other Treg-specific loci, as these changes may be predictive of impaired Treg immunosuppressive function.

Recent WGBS analysis comparing primary conventional $CD4^+$ T cell and Treg subsets identified an additional 33 Treg-selective demethylated regions (Treg-DRs; Ohkura et al., 2020). Unsurprisingly, several of these Treg-DRs map to transcription factors important for Treg stability, mediators of IL-2 signaling, and immunosuppressive surface molecules, among other genes. We asked whether these Treg-DRs gained methylation in response to repetitive CD3/CD28 stimulation during the manufacturing of the Treg products, which may indicate TCR activation-induced destabilization of the Treg

identity. As additional positive and negative controls, we included EPIC array data from FACS-sorted and expanded primary Tregs and CD4mem T cells (n = 3). In addition, we had the chance to analyze the final time point of second-generation Treg products, which were generated through GMP-compliant FACS and expanded with the same protocol as the first generation products (n = 4; see Methods).

We first selected all CpGs from our microarray data which overlapped with the 33 Treg-DRs identified from Ohkura et al. (**Supplementary Table 4**). As validation, we compared DNA methylation between primary Tregs and CD4mem at day 0, and confirmed that nearly all selected CpGs were significantly hypomethylated in Tregs (**Figure 5A**), with CpGs mapping to intronic parts of the *FOXP3* gene body displaying the greatest absolute difference. Additionally, these Treg-DR CpGs map to strong, active regulatory regions (e.g., active transcription start sites, genic enhancers that correlate most strongly with gene expression, and additional active enhancers) identified in Tregs (**Figure 5B**; Roadmap Epigenomics et al., 2015). Strikingly, at the identical genomic location in CD4mem cells, most of these regions were identified to be transcriptionally repressed (e.g., weak enhancer, weak transcription, repressed polycomb region, and quiescent region), further emphasizing that these Treg-DRs are important regulatory features distinguishing Treg identity from that of a CD4mem. We observed that the methylation values of the starting material (day 0) of the first generation products were consistently in between sorted primary CD4mem and Tregs (**Figure 5C**), confirming that MACS-mediated CD8 depletion followed by CD25 enrichment is not sufficient to obtain a pure Treg starting population.

We were next interested in assessing culture-induced changes in Treg identity and performed a PCA with these selected CpGs to determine how all time points of expanded primary CD4mem T cells, Treg, and first- or second-generation products clustered relative to each other. We found strong separation between CD4mem and Tregs along PC1 (76% of total variation) at any time point, with final first generation products (indicated by highest cumulative expansion rates) clustering more closely with CD4mem cells than final second-generation products (**Figure 5D**). Indeed, unsupervised hierarchical clustering confirmed that highly expanded final first generation products clustered with the CD4mem cells (**Figure 5E**), with the selected CpGs showing hypermethylated values relative to primary Tregs. However, primary Tregs also exhibited significantly lower expansion rates, which may partially explain the discrepancy in methylation status of these CpGs.

While the observed similarities between the final first generation Treg products and expanded CD4mem T cells might partially stem from the relative impurity of the MACS-enriched starting material, we found indications that the culture process alone can destabilize the epigenetic identity in primary FACS-sorted Tregs: Although expanded primary Tregs had lower Treg-DR methylation relative to CD4mem T cells at day 23, we were keenly aware that a subset of these selected methylation sites gained methylation with days in culture, approaching similar methylation levels as CD4mem T cells. For example, in both the first generation Treg products and primary Tregs, the selected CpGs within the *IKZF2* (HELIOS) locus showed progressive gain in methylation throughout culture (**Figure 5F**).

We therefore assessed whether *in vitro* expansion resulted in an epigenetic switch of Treg-DRs in the primary FACS-sorted Tregs. We performed a linear regression on all 31 Treg-DR CpGs that were significantly demethylated in Tregs relative to CD4mem at day 0, and used log10 expansion rates as the dependent variable instead of days in culture to adjust for possible donor-specific differences in day-to-day proliferation behavior. Strikingly, several CpGs mapping to *FOXP3* and *TNFRSF1B* (TNFR2) showed very little change in methylation with increased expansion rate (**Figure 5G**) indicating a stable demethylated behavior even under strong proliferative conditions. Many of the remaining CpGs, however, displayed significantly higher R^2 values (> 0.4) which indicates strong correlation between hypermethylation of indicated Treg-DR CpGs and cumulative expansion rate of the population. A similar correlation for many of the same sites could not be observed in the CD4mem controls. This expansion-induced increase in methylation on genes essential for Treg function (e.g., *IKZF2*, *IKZF4*, *IL2RA*), are likely to impact the functional behavior of the Tregs and are of general concern for any Treg product, irrespective of the purity of the starting material.

DISCUSSION

While adoptive Treg therapy is undoubtedly one of the most promising approaches for intervening excessive inflammatory conditions, standardization of the manufacturing process and quality control remain particularly challenging. As current options for *in vitro* functional testing and phenotype validation are limited, the quality control measures are highly variable between manufacturing sites (Fuchs et al., 2017). In our analyses, we found several lines of evidence that *in vitro* expansion induced by repetitive TCR stimulation leads to progressive remodeling of the DNA methylome, which may impact transcriptional regulation of genes important for Treg function and identity.

Firstly, we observed progressive promoter hypomethylation (activation) of genes known to downregulate T cell activation (e.g., *PDCD1, CD59*, and *IL1RN*) with concomitant promoter hypermethylation (deactivation) of genes that positively regulate TCR signaling (e.g., *RHOH, HLX, RARA*). These methylation changes may indicate that the final Treg product have suboptimal TCR signaling, where an optimally activated state might be more desired.

Secondly, we detected strong promoter hypomethylation in genes implicated in conventional T cell exhaustion, such as *HAVCR2* (TIM-3), *TIGIT*, *LGALS1*, *CD5*, and others, which corresponded to an increase in protein expression on the cell surface. Although these genes have been shown to promote conventional T cell dysfunction during cases of chronic viral infection (Khan et al., 2019), cancer pathogenesis (Scott et al., 2019), and tonic signaling in CAR-T cells (Weber et al., 2021), they also play an active role in enhancing Treg immunosuppressive function (Ukena et al., 2011; Sakuishi et al., 2013; Kurtulus et al., 2015; Sprouse et al., 2018) and hence,

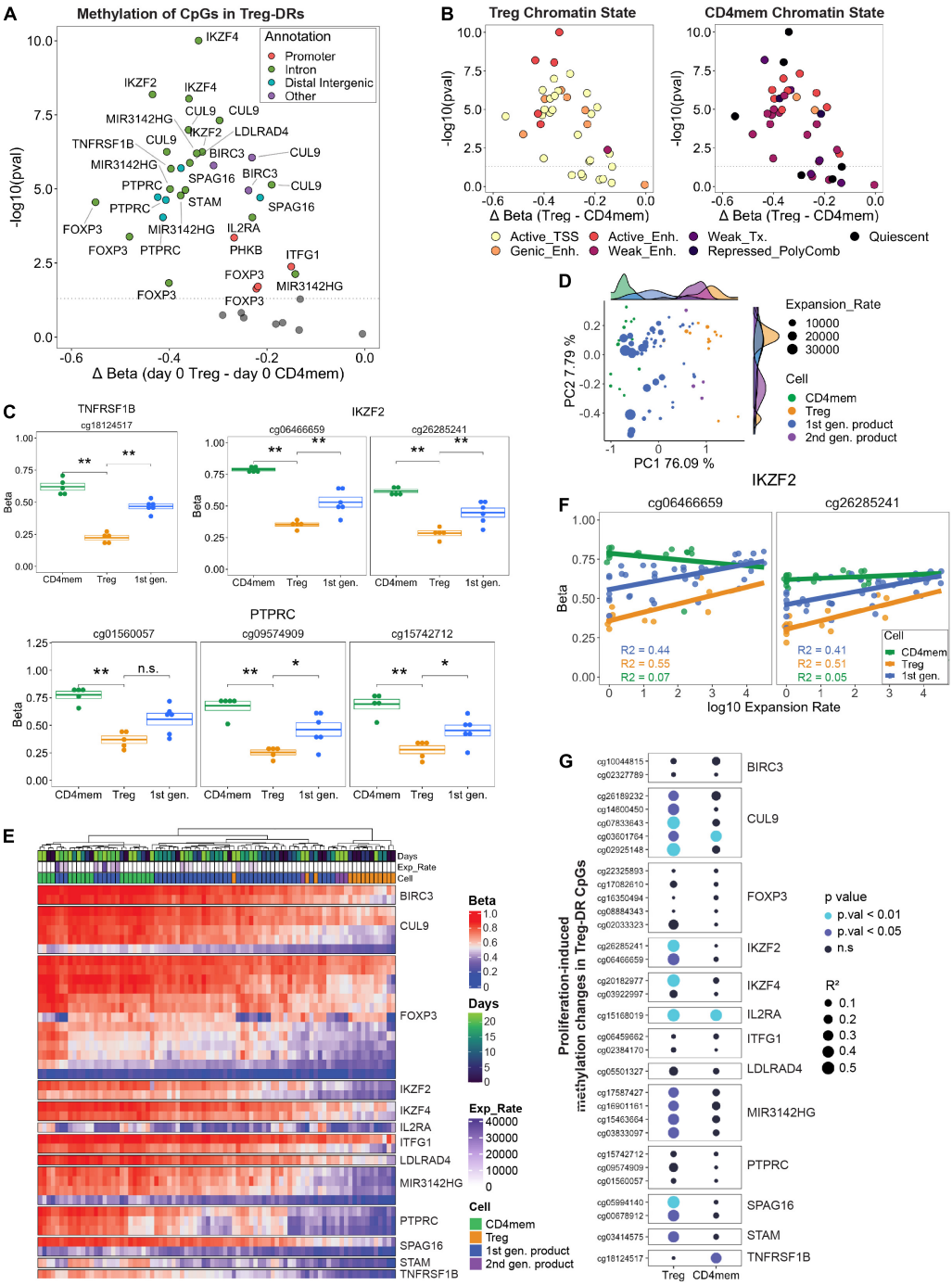

FIGURE 5 | (A) FACS-sorted Tregs and CD4 memory T cells (Day 0) were analyzed for CpG methylation at Treg specific demethylated regions (Treg-DRs). Significantly differentially methylated CpGs (*p*. val < 0.05, Student's *t*-test) were color-coded by genic annotation. **(B)** CpGs in Treg-DRs plotted with the same axes as A, but color-coded by chromatin state annotation (TSS = transcription start site; Enh. = enhancer; Tx = transcription). **(C)** Methylation levels (Beta) of selected Treg-DRs in Treg, CD4mem and Treg products on day 0. *denotes *p*.val < 0.05, **p.val < 0.01, ns = not significant (*T* test). **(D)** PCA analysis of day 0 and day 23 1st generation products, FACS-sorted Tregs and CD4mem as well as day 23 2nd generation products. Histograms at the top and right display representation on PC1 and PC2, respectively, of the sample distribution for each experimental group. **(E)** Unsupervised hierarchical clustering of all samples based on methylation levels of Treg-DRs CpGs. **(F)** DNA methylation change of the *IKZF2* Treg-DR in CD4mem, Tregs and 1st gen Treg product in relation to the expansion rate. R^2 values display the degree of correlation (see **G**). **(G)** Results of linear regression analysis of all differentially methylated Treg-DRs on day 0 (see A) calculating the degree of correlation between gain of methylation and log10 expansion rate. Higher R^2 value (indicated by size of the dot) indicates greater correlation. *P*-values are indicated by color.

might be beneficial for treatment efficacy. This enhanced effector function may come at a cost, as highly suppressive TIM-3$^+$ Tregs have impaired survival rates *in vivo*, notably during allograft response during transplant settings (Gupta et al., 2012; Banerjee et al., 2020).

Lastly, we observed progressive hypermethylation of essential Treg-specific demethylated regions (Treg-DRs; Ohkura et al., 2020) during product generation, indicating a destabilization of the Treg identity. Indeed, Tregs have been reported to lose their immunosuppressive phenotype under unfavorable conditions (Marek et al., 2011; Bailey-Bucktrout et al., 2013). In our study, we found significant DNA hypermethylation in a Treg-specific enhancer within the *IKZF2* gene. *IKZF2* encodes for the transcription factor HELIOS, a member of the Ikaros family that has been shown to be a critical regulator of immunosuppressive function. Loss of HELIOS expression has been linked to conversion of Tregs into T effector cells under proinflammatory conditions (Nakagawa et al., 2016). Importantly, the partial remethylation of Treg-DRs also occurred in primary FACS-sorted Tregs and therefore is a concern for improved second-generation Treg products, too, which undergo GMP-compliant FACS sorting for isolation of the starting material.

Although we focused our study on DMRs in transcriptional regulatory elements such as promoters and enhancers, we also observed changes outside regulatory elements, such as in partially methylated domains (PMDs), which largely localize to transcriptionally silent heterochromatic areas (Lister et al., 2011; Hon et al., 2012). While the functional impact of PMD hypomethylation in Tregs remains to be determined, reports have indicated that aberrant PMD hypomethylation may be correlated with cellular senescence and dysfunction (Lister et al., 2011; Schellenberg et al., 2011; Salhab et al., 2018).

Taken together, our study demonstrates that strong *in vitro* expansion leads to epigenetic remodeling at loci important for Treg function and identity during Treg ACT manufacturing, which may ultimately impact long-term persistence or therapeutic efficacy of the product. Whether alternative protocols requiring fewer cycles of TCR-mediated restimulations (MacDonald et al., 2019) or alternative modes of T cell activation (Hippen et al., 2011; He et al., 2017) mitigate the observed epigenetic changes remain to be seen. Prior to our study, characterization of global epigenetic changes throughout Treg or other T cell ACT manufacturing were lacking. However, studies exploiting other *in vitro* and *in vivo* models of frequent T cell activation have reported extensive changes to epigenetic features beyond DNA methylation. For instance, tonic signaling in CAR-T cells results in dynamic genome-wide changes to chromatin accessibility (Gennert et al., 2021), which was similarly found in antigen-specific CD8 T cells responding to chronic viral infections in mice (Sen et al., 2016). Both models demonstrated a marked increase in chromatin accessibility at loci encoding for inhibitory, exhaustion-associated surface markers. Although the effect of long-term frequent stimulation of T cells on histone post-translational modifications is unknown, dynamic changes to both activating and repressive histone modifications can be detected during early activation and differentiation of T cells (Russ et al., 2014; LaMere et al., 2016; LaMere et al., 2017). Due to the clear role of epigenetic regulatory mechanisms on T cell function (and dysfunction), we hope to advocate for the inclusion of epigenetic profiling during manufacturing and quality control assessment of final T cell ACT products, which may uncover opportunities for improving current therapies.

DATA AVAILABILITY STATEMENT

All DNA methylation data is made available on Gene Expression Omnibus (GEO) Repository (GSE185854).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Charité - Universitätsmedizin Berlin. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KO performed and coordinated all bioinformatical DNA methylation analyses with contributions from DH and ASa and generated figures with contributions by ASc. KO, ASc, and GG generated DNA methylation profiling data. SS and MS generated and analyzed protein expression data. GZ, LA, DK, and AR generated Treg products under supervision of PR. KO, DH, and JP designed the study, lead the data analysis, and interpretation with contributions by JW, H-DV, MS-H, and PR wrote the manuscript. JP supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.751590/full#supplementary-material

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Freezing Medium Containing 5% DMSO Enhances the Cell Viability and Recovery Rate After Cryopreservation of Regulatory T Cell Products *ex vivo* and *in vivo*

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Cell therapies have significant therapeutic potential in diverse fields including regenerative medicine, transplantation tolerance, and autoimmunity. Within these fields, regulatory T cells (Treg) have been deployed to ameliorate aberrant immune responses with great success. However, translation of the cryopreservation strategies employed for other cell therapy products, such as effector T cell therapies, to Treg therapies has been challenging. The lack of an optimized cryopreservation strategy for Treg products presents a substantial obstacle to their broader application, particularly as administration of fresh cells limits the window available for sterility and functional assessment. In this study, we aimed to develop an optimized cryopreservation strategy for our CD4+CD25+Foxp3+ Treg clinical product. We investigate the effect of synthetic or organic cryoprotectants including different concentrations of DMSO on Treg recovery, viability, phenotype, cytokine production, suppressive capacity, and *in vivo* survival following GMP-compliant manufacture. We additionally assess the effect of adding the extracellular cryoprotectant polyethylene glycol (PEG), or priming cellular expression of heat shock proteins as strategies to improve viability. We find that cryopreservation in serum-free freezing medium supplemented with 10% human serum albumin and 5% DMSO facilitates improved Treg recovery and functionality and supports a reduced DMSO concentration in Treg cryopreservation protocols. This strategy may be easily incorporated into clinical manufacture protocols for future studies.

Keywords: cell therapy, regulatory T cells (Tregs), cryopreservation, freeze-thawing, freezing medium, cell recovery rate, cell viability

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INTRODUCTION

Adoptive and regulatory T cell (Treg) therapies offer promising new options for the treatment of various clinical indications originating from a compromised immune system (Bluestone and Tang, 2018; Raffin et al., 2019; Roemhild et al., 2020; Waldmann, 2021). Academic institutions recognized this potential early and were crucial in their development, moving these therapies through the clinical trial phases toward market authorization for this new class of "Advanced Therapy Medicinal Products" (ATMPs).

Regarding clinical translation, all these innovative approaches face the same regulatory and logistical challenges, some of which are new and still rapidly changing (Hickson et al., 2021). Regulatory requirements or their interpretation vary between major markets, such as North America and Europe, especially in the early clinical phases, but also between countries within Europe. Many requirements such as purity, potency, safety, and stability of ATMPs known from conventional drug manufacturing must be redefined and taken into consideration. Stability is particularly important if the products cannot be used immediately after manufacture. This is not only a major logistical problem, but also a safety issue, as microbiological testing can take up to 14 days, depending on the applied test. In addition to the safety aspect, product stability and storage also have a major impact on subsequent commercialization. The ability to manufacture "off the shelf" products helps to reduce manufacturing costs drastically thereby increasing the access to such therapies (Abou-El-Enein et al., 2016, 2017a,b; Fritsche et al., 2020).

The cell therapy based treatment of diseases after solid organ transplantation (SOT) or hematopoietic stem cell transplantation (HSCT) in conjunction with virus-specific effector T cell (Teff) products, often consisting of a mixture of CD4+ and CD8+ T cells, has rapidly evolved. These originated from autologous cell products with simple virus specificity to multi-virus specific allogeneic therapy options becoming accessible due to good outcomes with few side effects (Gerdemann et al., 2012). This is partly due to the amenability of these T cell products to freeze-thawing and cryostorage. In recent years, the potential of Tregs has been increasingly recognized, based on good tolerability and promising clinical data (Roemhild et al., 2020). For cryopreservation of cell products, such as Teff cells, a freezing medium based on 10% dimethyl-sulphoxide (DMSO) and 10% serum is commonly used. This standard medium did not yield satisfactory results for clinical grade Tregs in our hands.

Cryopreservation is a highly challenging process for cell therapy products that can substantially compromise their quality and clinical efficacy (Galipeau, 2013; Moll et al., 2014, 2016). Standard cryoprotectants such as DMSO, a low-molecular-weight agent that penetrates the cell membrane by forming pores and thus prevents intracellular ice formation by reducing the water content inside the cell (Mazur, 1984), can comprise concentration-dependent cell toxicity (Verheijen et al., 2019). Extracellular cytoprotective agents such as polyethylene glycol (PEG) have also been described (Moll et al., 2016). These have a higher molecular weight and reduce ice formation outside the cell by breaking the hydrogen bonds between molecules through spatial separation (Towey and Dougan, 2012). Interestingly, a number of cell types can protect themselves from thermal, oxidative, and osmotic stress by synthesizing so-called heat shock proteins (HSP; Lindquist and Craig, 1988). Heat shock proteins assume a wide variety of functions in this context, with anti-apoptotic, antioxidant and cytoprotective effects having been described (Concannon et al., 2003; Sharp et al., 2013; Shaik et al., 2017).

Here, we use a manufacturing protocol based on the GMP-compliant production of our natural Treg (nTreg) cell product, as recently described in more detail in the report of our clinical study (Roemhild et al., 2020). Using freeze-thawing experiments, we assess cell product viability, the expression of characteristic cell surface markers (e.g., CD4/CD25/Foxp3), and cytokine secretion after stimulation. In addition to other tests usually employed for product release, such as sterility, endotoxin content, and mycoplasma testing, these parameters are of critical importance. We also assess the effects of lowering the DMSO concentration in the freezing medium on the above-mentioned parameters and critically, the product recovery rate post thawing, which is a decisive parameter, since it determines the number of viable cells available for therapy. For this purpose, freezing media with 5 and 10% DMSO content as well as a DMSO and serum-free synthetic cryoprotectant were compared. In addition, the optimization potential of a combination of intra- and extracellular cryoprotectant was investigated. Furthermore, *in vivo* survival of our fresh and thawed nTreg product was evaluated using an immunodeficient mouse model. Finally we tested the suppressive capacity of our Treg cell product after thawing. To our best knowledge, this is the first report on the effects of inducing cellular protective mechanisms in the context of cryopreservation of Treg cell products to achieve a storable product with reproducible and valid quality characteristics. The experimental design is shown in **Figure 1A**.

MATERIALS AND METHODS

For all experiments, 50 ml peripheral blood were collected from healthy volunteers or immunosuppressed patients after SOT. Peripheral blood mononuclear cells (PBMCs) were isolated by lymphopreparation density gradient centrifugation. The Charité Universitätsmedizin Berlin ethics committee and institutional review board (Hickson et al., 2021) approved the study protocol and all blood donors provided written informed consent.

Manufacture of Natural Treg Cells

For isolation of CD8-CD25+ cells, PBMCs were isolated by density gradient centrifugation and MACS technology (Miltenyi Biotech) was used to deplete the CD8 cells from the PBMC fraction and then to enrich the CD25+ cells from the CD8-depleted cell fraction. CD4+CD25+ cells were first cultured in 96-hole round-bottom plates in X-Vivo 15 (Lonza) culture medium supplemented with 10% FBS (Biochrom), interleukin-2 (Novartis, 500 IU/ml), and rapamycin (Pfizer, 100 nM) at 37°C and 5% CO2. Polyclonal expansion was achieved by repetitive stimulation with anti-CD3/CD28 beads (MACS GMP

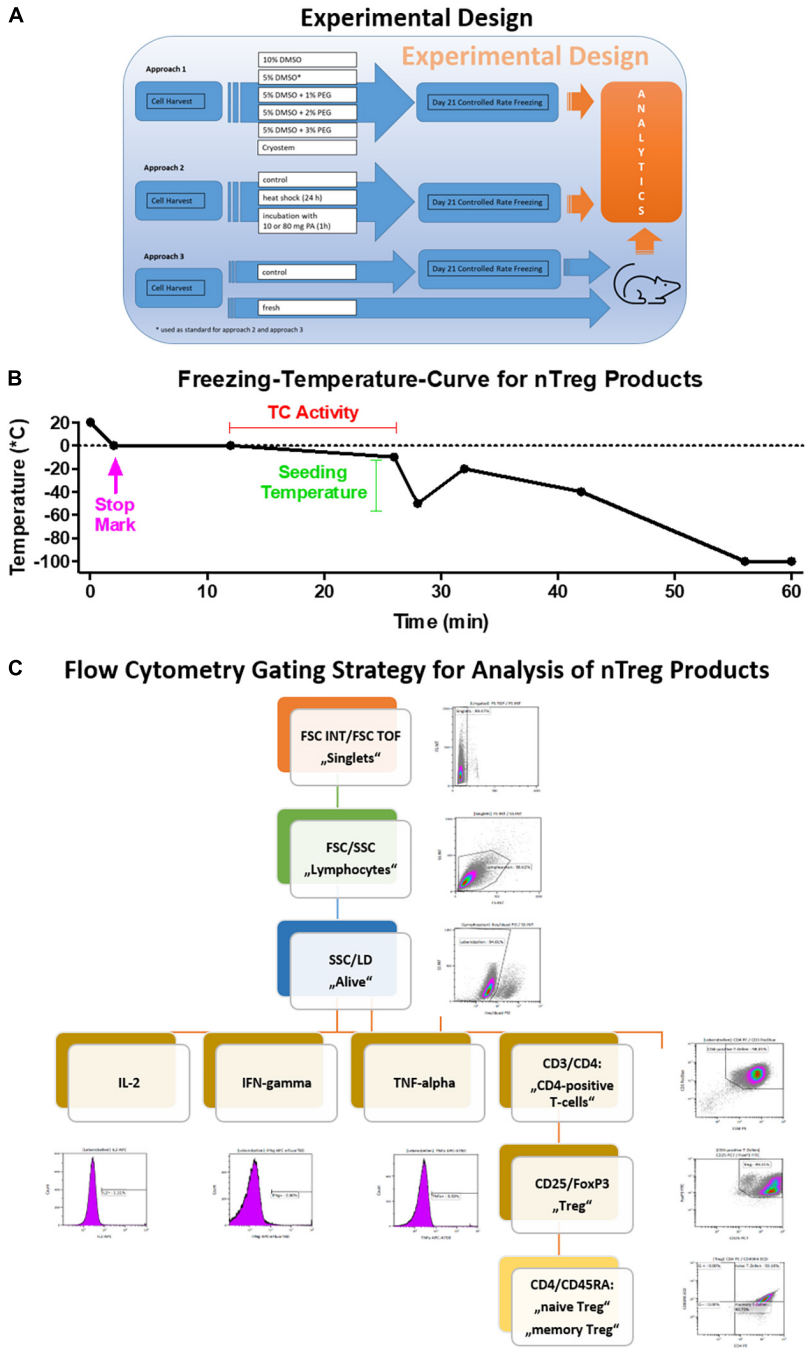

FIGURE 1 | Freezing curve and flow cytometry gating strategy. **(A)** Experimental design and **(B)** Programmed freezing curve as shown in **Tables 1**, **2** [Stage Temp. (°C) Duration Heating Stop Seeding Temp. control] and **(C)** Gating scheme used for the analysis of the FACS data. The scheme ensures the exclusion of doublets and dead cells. It identifies lymphocytes first and uses the marker CD3 for the detection of CD4+ cells. The latter are further analyzed for their expression of the Treg relevant markers CD25 and Foxp3. The amount of cytokine producing cells is gated on all living lymphocytes.

ExpAct Treg Beads; Miltenyi Biotec) over a period of 21 days (Roemhild et al., 2020).

Depending on the cell density cells were transferred to 24-hole flat-bottom plates or for shortening the expansion time and to be closer to a GMP compliant "automated" and (semi)closed system to G-Rex bioreactors (Wilson Wolf) and continued to be cultured until harvest. For harvest, the cells were resuspended, washed several times, and expansion beads added during culture were depleted using MACS technology. In the first set of experiments to determine the DMSO concentration in the freezing medium and to optimize the medium with PEG, the Treg cell products were then cryopreserved directly. In

the second set of experiments to optimize the cryopreservation strategy by activating cellular protective mechanisms, additional cell products were prepared. Cells were either bead depleted 1 day before harvest and cryopreservation and then incubated with two different concentrations of paeoniflorin (PA; Sigma) for 24 h, or exposed to a heat shock of 42°C for 1 h in a heating block on the day of harvest and cryopreservation.

Composition of the Freezing Media

The composition of the different freezing media tested is shown in **Table 1**. Except for the Cryostem medium, which is synthetic and to be used without the addition of DMSO, the freezing medium consisted of the components dimethyl sulfoxide (DMSO), human serum albumin (HSA), and sodium chloride solution (NaCl). Shown are the media compositions of the investigated approaches, including the volumes of individual components calculated on 1.5 ml freezing medium. Moreover, the concentrations of polyethylene glycol (PEG) investigated in the optimization experiment as an additional supplement are given.

Cryopreservation of Natural Treg Cells

All equipment/materials and buffers used for cryopreservation were precooled to 4°C. Subsequently, the washed and bead-depleted cells were divided into aliquots with identical cell concentrations. Depending on the experimental procedure, the cell pellet was resuspended in the appropriate cryopreservation medium and pipetted into cryotubes (1.5 ml). The cryotubes were then placed in the freezing machine (previously precooled to 4°C for 30 min). The Consarctic freezing machine with Biofreeze software then lowered the chamber temperature to −100°C in a controlled manner (duration approximately 55 min) (**Figure 1B**). During the freezing process, both the chamber temperature and the temperature of a reference tube filled only with the freezing medium were detected and recorded. After reaching a chamber temperature of −100°C, the samples were immediately transferred to a nitrogen tank and stored at <−150°C in the vapor phase until analysis.

Thawing of Cryopreserved Natural Treg Cells and Analysis of Cell Number and Viability

To thaw the cryopreserved cells, a 50 ml tube was prepared with chilled (4–8°C) PBS (Biochrom) buffer containing 10% FBS. A cryotube containing 1.5 ml of cell suspension was then removed from the nitrogen storage container and swirled in a temperature-controlled water bath (37°C). Immediately after reaching a liquid phase, the cells were transferred into the prepared 50 ml tube under a sterile bench using a transfer pipette. After swirling the tube several times, the cells were pelleted in a precooled centrifuge (4°C) at 300 g for 10 min. Subsequently, the supernatant was removed and the pellet was resuspended in 15 ml PBS containing 10% FBS for further analyses. Dead and viable cells were determined using the CASY Cell Counter (OLS Life Science) based on the resistance measurement principle, as reported previously (Moll et al., 2014).

Analysis of Treg Cell Phenotype and Effector Cytokine Production by FACS

Following harvesting or thawing of the nTreg cells, cell numbers were adjusted to 10×10^6 cells/ml in stimulation-free medium (X-Vivo 15 with 10% FBS) and 500 µl of the cell suspension were pipetted into one FACS tube each and incubated at 37°C and 5% CO2 overnight. The next day, one of the two mixtures was stimulated with a PMA (Sigma Aldrich, 5 g/ml)-Inomycin (Sigma Aldric, 2.5 g/ml) solution and both tubes were incubated again for 1.5 h at 37°C and 5% CO2. Subsequently, 1 µl of Brefeldin-A (Sigma Aldrich) and 500 µl of stimulation-free medium were pipetted into each tube. This was followed by another incubation step for 4.5 h. After incubation, the cells were washed with cold PBS, centrifuged (10 min, 300 g, 4°C) and the cell number of both tubes was adjusted to 5×10^6 cells/ml, and 100 µl of cell suspension from each of the two preparations was pipetted into three new FACS tubes each for antibody staining. This was followed by the addition of the anti-CD25 (Beckman Coulter, PC7), CD45RA (Beckman Coulter, ECD) and Live/Dead (Life Technologies, aqua 405 nm) antibody. After incubation of the samples for 30 min at 2–8°C, 500 µl of cold FixPerm buffer (eBioscience) was added to each tube. This was followed by centrifugation (10 min, 300 g, 4°C) and addition of FoxP3 (BD, FITC), CD4 (Beckman Coulter, PE), CD3 (Beckman Coulter, PacBlue), IFNγ (eBioscience, APC eFluor780), and IL-2 (BD, APC) antibodies onto the broken pellet. After a 30 min incubation at room temperature (RT) and another centrifugation step (10 min, 300 g, 4°C), the supernatant was discarded and pellets resuspended with 400 µl Perm buffer (eBioscience). This was followed by flow cytometric measurement using the Navios flow cytometer (Beckman Coulter). The flow cytometry gating is shown in **Figure 1C**.

TABLE 1 | Composition of freezing media.

Approach	DMSO (%v/v)	DMSO (µl)	HSA (% v/v)	HSA (µl)	NaCl (% v/v)	NaCl (µl)	PEG (% v/v)	PEG (µl)
1	5	75	10	750	85	675	–	–
2	10	150	10	750	80	600	–	–
3	Cryostem							
4	5	75	10	750	84	625	1	50
5	5	75	10	750	82	525	3	150
6	5	75	10	750	80	425	5	250

The values are given as volume percent and calculated in µl per vial (assuming 1.5 ml content). Composition one and two served as standard for the two different main approaches.

TABLE 2 | Temperature ramps underlying the programmed freezing curve.

Stage	Temp. (°C)	Duration (Wendering, #6)	Heating	Seeding	Temperature control
1	0.0	3.0	Off		
2	0.0	10.0	Off		
3	−10.0	14.0	Off		active
4	−50.0	0.5	Off	active	
5	−20.0	5.0	On		
6	−35.0	10.0	Off		
7	−100.0	13.0	Off		
9	−100.0	2.0	Off		

Within each ramp, the temperature is maintained for specified time before the program adjusts the temperature of the freezing chamber to the next stage. In stage 5, the heat generated by recrystallization during ice formation is counteracted for a short time (30 s) with a jump to lower temperature (−50°C).

Treg Suppression Assay

Suppressive potency was examined as previously described (Wendering et al., 2019) CFSE-labeled autologous PBMCs were cultivated alone or in the prescence of different numbers of thawed corresponding Tregs. Seven PBMC Treg ratios ranging from 1:2 to 32:1 were analysed. αCD3/28 microbeads (Treg Suppression Inspector, Miltenyi Biotech) were applied for cell stimulation at a cell bead ratio of 1:1 (adjusted to the total cell number per well). After incubation at 37°C and 5% CO2 for 96 h cells were stained for live dead differentiation and CD3 surface antigen. Cell proliferation and its suppression was investigated by CFSE dilution. The percentage of proliferation suppression was calculated by relating the percentage of proliferating PBMCs in the prescence and absence of Tregs. Data were acquired on the Fortessa flow cytometer (BD) and analysed with FlowJo software (Treestar). Results are summarized in **Figure 3C** while FACS plots and calculation equation can be found in the **Supplementary Figures 1,2**.

In vivo Analysis

Freshly expanded nTreg were manufactured at GMP facility in Berlin, transported in G-Rex (Wilson Wolf) culture chambers by overnight courier to the project partner (RESTORE) in United Kingdom and processed immediately upon arrival. Freshly expanded nTreg were transported in G-Rex (Wilson Wolf) culture chambers by overnight courier and processed immediately upon arrival. Cells were recovered into 50 mL Corning tubes and washed. Anti-CD3/CD28 beads (MACS GMP ExpAct Treg Beads; Miltenyi Biotec) were magnetically depleted (LS columns, Miltenyi Biotec). nTreg cryopreserved in 0.9% sodium chloride supplemented with 5% DMSO and 10% human serum albumin were transported by overnight courier in a temperature-controlled container on dry ice and, on arrival, were immediately transferred to a liquid nitrogen tank and stored at <-150°C in vapor phase. Thawing was achieved by gentle submersion in a pre-warmed 37°C water bath. On reaching a liquid phase, cells were transferred to a 50 mL Corning containing 30 μL DNAse and slowly diluted by intermittent swirling with pre-warmed (37°C) pure RPMI. Cells were centrifuged at 500 g for 5 min at room temperature and resuspended in pure RPMI. Viable cells were identified by 0.05% trypan blue exclusion with a haemocytometer under light microscopy. Immediately following recovery or thawing, nTreg were stained with violet proliferation dye (VPD, BD Biosciences) at a concentration of 1 μM (1 μL of 1 mM stock per mL) for 10 min in a 37°C water bath. 5×10^6 VPD-labeled nTreg were injected into the peritoneal cavity of immunodeficient BALB/c Rag2$^{-/-}$cγ$^{-/-}$ mice. On day 6, cells were recovered by lavage of the peritoneal cavity with 10 mL room-temperature PBS. The following antibodies (fluorophore, manufacturer, clone) were used to stain recovered cells: anti-mouse CD45 (PE-eFluor^{TM} 610, Invitrogen, 30-F11) anti-human CD4 (APC, BD Pharmingen, 7137857), anti-human CD8 (APC-Cy7, BD Biosciences, SK1), anti-human CD25 (PE-Cy7, BD Biosciences, M-A251), anti-human CD3 (FITC, BioLegend, UCHT1). Dead cells were excluded with 7-AAD viability dye (Invitrogen, lot 2115592) Fluorescence was quantified by flow cytometry (BD FACSCanto, 3-laser).

RESULTS

Recovery Rate and Cell Viability Benefit From Low DMSO Concentration in the Freezing Medium

Freezing media based on 10% DMSO and 10% serum are commonly used for cryopreservation of T cell products (Moll et al., 2016). We compared the standard freezing medium containing 10% DMSO with a freezing medium containing only 5% DMSO and synthetic Cryostem in a first set of experiments (**Table 1**). The recovery rate, as well as the viability of two Treg products were analyzed before freezing (fresh), immediately after thawing (0 h) and 1 day after thawing (24 h) (**Figure 2A** left panel). The cryopreservation process (**Figure 1B**) was associated with a substantial cell loss, indicated by poor recovery rates 0 h after thawing of 75–58% and 24 h after thawing of 48–20%. The viability of nTregs immediately after thawing was similar to the fresh cells before freezing (fresh 95 vs. 93–95% thawed) but decreased after an incubation of 24 h under culture conditions (5% DMSO: 78%, 10% DMSO: 60%, Cryostem: 76%). Freezing medium with 5% DMSO was superior to that with 10% DMSO content as well as the synthetic freezing medium in both recovery rate and viability.

The Combination of DMSO and PEG as Freeze Protection Did Not Improve Recovery or Viability, While Treg Marker Expression Slightly Declined but Cytokine Secretion Was Not Affected

To optimize the cryopreservation medium, the superior medium composition containing 5% DMSO was supplemented with three different concentrations of PEG (1, 3, and 5%) in the second part of the experiment (**Table 1** and **Figure 2A** right panel). However, these combinations of intracellular (DMSO) and extracellular (PEG) freeze protection did not improve recovery rate or cell viability compared to the medium with 5% DMSO without PEG addition. After a cryopreservation period of 35 days in the vapor phase of liquid nitrogen, clinical grade nTreg cell

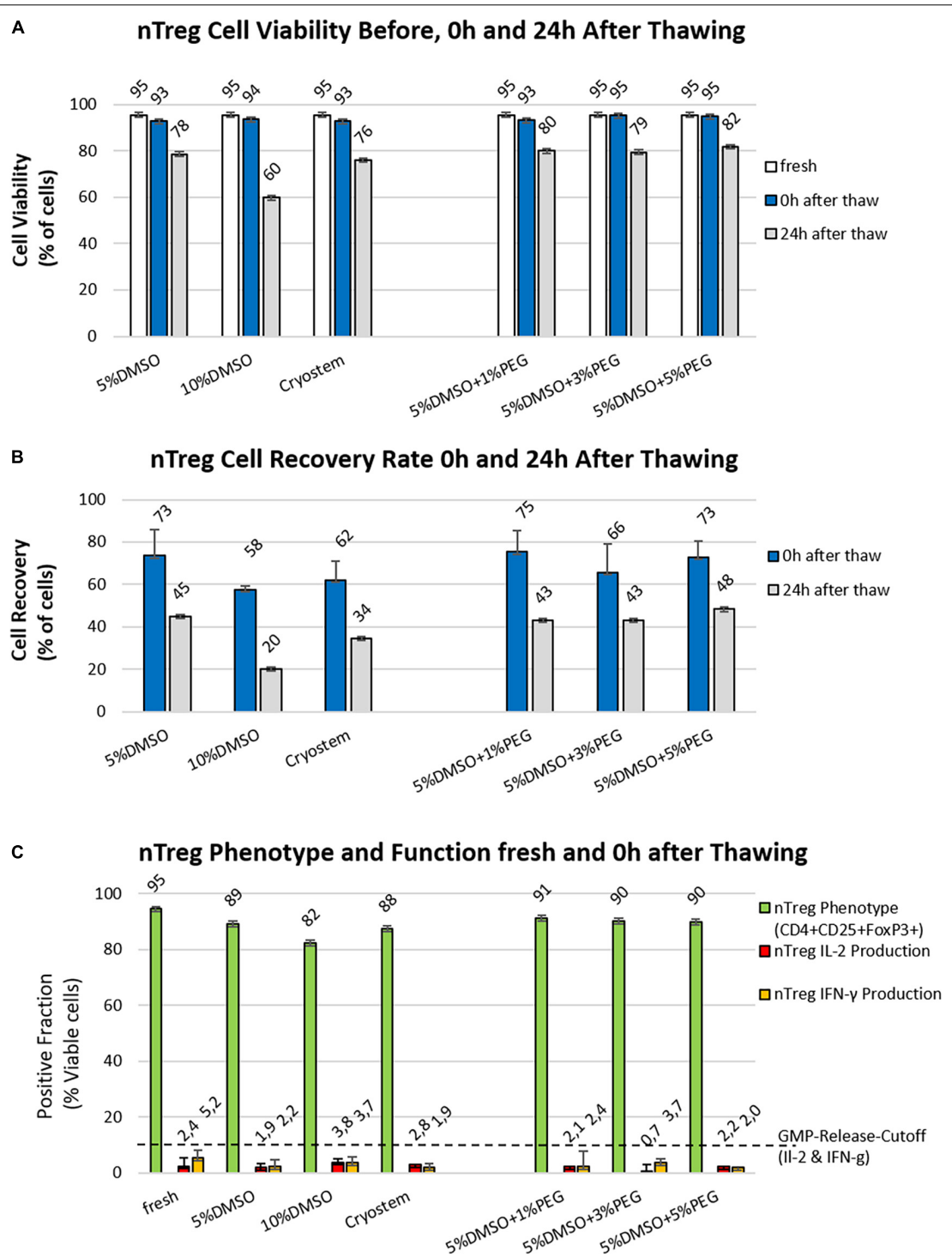

FIGURE 2 | Optimizing nTreg cell viability and recovery post thawing. **(A,B)** Different readout parameters before freezing (white) and at 0 h (blue bars) or 24 h post thawing (gray bars) are shown for the six freezing media as listed in **Table 1** (n = 2 cell products produced under GMP-like conditions). **(A)** Viability (%): There is only a small difference in viability between fresh cells and all the other preparations directly after thawing, but strong decreases are found at 24 h depending on the approach, freezing medium with 10% DMSO content performed the worst, while addition of PEG brings only minimal improvement in viability at 24 h compared to the freezing medium with 5% DMSO content; **(B)** Cell Recovery (%): Analysis at 24 h time point gives a more informative readout on quantitative differences and best performance with 5% DMSO; and **(C)** Phenotypic and Functional Analysis: Shown are the results of phenotyping as well as cytokine secreting cells before freezing (fresh) and after cryopreservation (thawed) for the investigated 6 different freezing media for the two initial nTreg cell products. The green bars show the percentage of Treg cells (% CD4+CD25+Foxp3+), the red bars represent the percentage of interleukin 2 (IL2)-producing cells, and the yellow bars show the percentage of interferon gamma (IFN-γ)-secreting cells both expressed as % positive cells.

products from the two donors were thawed in a water bath at 37°C and compared with the cell product before freezing with respect to their phenotype (CD4+CD25+FoxP3+ cells of all CD4+ cells) and cytokine secretion (IL-2 and IFNγ) (**Figure 2B**), which serve as relevant release criteria for our clinical grade nTreg products. Duplicates of these cell products were stimulated with PMA/Iono or remained unstimulated and flow cytometry analysis was performed according to the gating scheme shown in **Figure 1C**. Compared to the fresh-from-culture-derived cells, the proportion of CD4+ and CD4+CD25+FoxP3+ within the "live-gated cells" decreased the most in the conventional freeze-thawing approach with 10% DMSO standard (95 vs. 82%). For the other approaches, the proportion decreased less (88 to 91%). The freezing medium with 5% DMSO content contained 89% CD4+CD25Foxp3+ cells after thawing. The proportion of cytokine-secreting cells did not change, which indicates no significant impact of cryopreservation on functional nTreg parameters (fresh vs. thawed IL2: 2.4 vs. 0.7–3.8% and IFNγ: 5.2 vs. 1.9–3.7%).

Activation of Cellular Protective Mechanisms Before Cryopreservation Only Slightly Improved Cell Viability After Thawing With Only Minor Changes in Phenotype and Cytokine Secretion

Adding another cryoprotectant to the freezing medium did not yield an improvement in the first optimization attempt (**Figure 2C**). Thus, two additional nTreg products were manufactured to investigate the effect of inducing cellular heat shock mechanisms though a 24 h incubation with different concentrations of paeoniflorin (PA; 10 μg and 80 μg) on day 20 or a 1 h heat shock in a heating block at 42°C on day 21 (**Figure 3A**). The best performing freezing medium (5% DMSO content) was chosen as reference. With recovery rates comparable to the first set of experiments (data not shown), incubation with PA prior to cryopreservation increased cell viability immediately after thawing by a mean of 5.42% (10 μg) and 4.59% (80 μg), respectively. Heat shock increased this value by a mean of 3.11% compared to the control approach with pure freezing medium. The percentage of CD4+CD25+Foxp3+ cells was not substantially affected by cryopreservation compared with the reference value (5% DMSO without additives) (98 vs. 97%). For cellular protection induced by PA or heat shock, this percentage decreased by 3% (10 μg PA) and 8% (heat shock). Cytokine secretion increased slightly compared with fresh Treg cells for IL-2 (6–7%) and IFNγ (4–5%) in all approaches (**Figure 3B**). In comparison with the first two Treg products, the stable phenotype was confirmed when 5% DMSO was used as a cryoprotectant. The percentage of cytokine-secreting cells shows an opposite tendency. The percentage of cytokine secreting cells was slightly increased by cryopreservation compared to the comparative value before freezing. IL-2 producing cells increased from about 2% (fresh) to about 9% (after thawing) and the proportion of IFNγ-secreting cells increased from 1.5% (fresh) to approximately 6.5% (after thawing). All data from cryopreserved mixtures were in a similar range (IL-2: ±1.2%, IFNγ: ± 2.6%). Overall, however, the additional activation of cellular protective mechanisms does not seem to confer any advantage over the standard freezing medium supplemented with 5% DMSO.

Freezing Medium With a Content of 5% DMSO Maintains the Suppressive Potential of Treg Cell Products After Cryopreservation

In vitro analysis of the suppressive capacity revealed that freezing medium with 5% DMSO content enables strong suppressive activity of cryopreserved Treg products after thawing. A 4-day proliferation assay showed that growth of effector cells is inhibited by approximately 60% (with a maximum of 67% at a ratio of 1:2) up to a ratio of 8 PBMC to 1 Treg cell. **Figure 3C** further shows that even at a ratio of 16 to 1, cell suppression is still approximately 43%.

In vivo Survival of Natural Treg Is Comparable Between Freshly Produced and Cryopreserved Natural Treg Products

To ensure that freeze-thawing does not compromise *in vivo* survival, freshly expanded and cryopreserved nTreg were injected *via* the intraperitoneal route into immunodeficient BALB/c $Rag2^{-/-}c\gamma^{-/-}$ mice (5×10^6/mouse) and recovered after 5 days (**Figure 4A**). We first assessed cell recovery of fresh or frozen cell products following overnight transportation and bead depletion. Cell viability was 91.3% (95% CI 90.0 – 92.7) compared with 92.8% viability following thawing (**Figure 4B**, left panel). Following cell transfer into mice, a mean of 16.9×10^4 fresh and 8.8×10^4 thawed nTreg were recovered after 5 days with no statistically significant difference between the groups ($p = 0.074$, **Figure 4B**, right panel). Within the human lymphocyte (mCD45-CD3+) population the phenotype between freshly expanded and thawed nTreg was assessed by expression of CD4 and CD25 (**Figure 4C**, left and middle panels). A mean of 99.7% fresh and 91.7% thawed nTreg expressed CD4. Expression of CD25 (IL2RA) within the CD4 population was also uniformly high across both populations with no statistically significant difference between the groups ($p = 0.13$). Finally, proliferation was assessed by categorizing proliferating and non-proliferating CD3+CD4+ lymphocytes based upon dilution of violet proliferation dye. Within the CD3+CD4+ population a mean of 88.2% fresh and 86.0% thawed nTreg underwent at least one division *in vivo* (**Figure 4C**, right panel) with no significant difference between the groups ($p = 0.72$).

DISCUSSION

Optimizing cryostorage and freeze-thawing procedures is an essential task for developing successful cell therapy products that can be incorporated into clinical practice (Galipeau, 2013; Moll et al., 2014, 2016). Here, we studied these procedures on four nTreg cell products prepared using our group's GMP grade manufacturing process, which we have also applied to nTreg cell

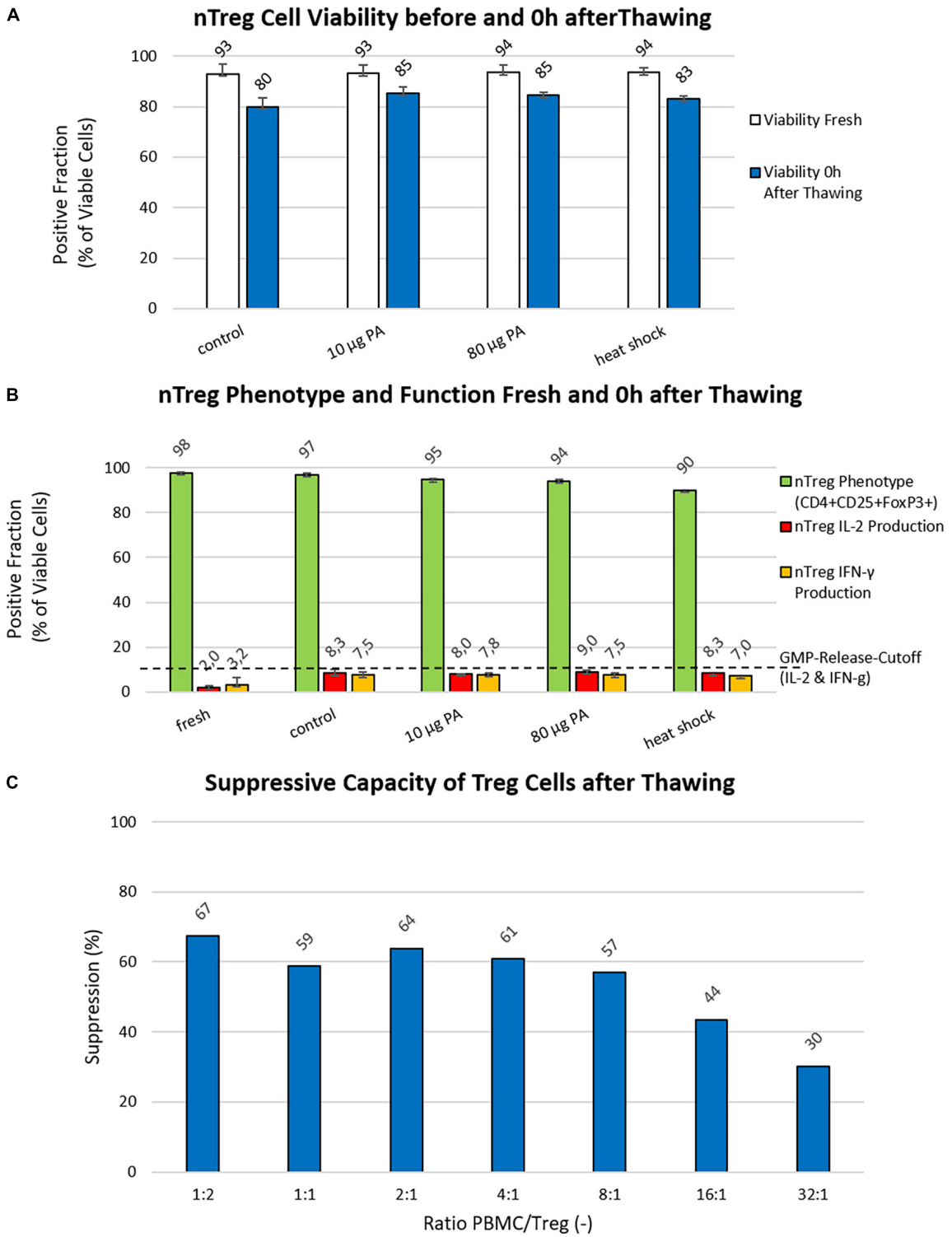

FIGURE 3 | Analysis of Cryo-/Cyto-protective pathways and suppressive potency. **(A)** Viability (%): The viability of nTregs before freezing (white bars) and immediately after thawing/0 h (blue bars) is shown for the control approach (freezing medium with 5% DMSO) as well as the investigated approaches with heat shock or paeoniflorin (PA; 10 μg and 80 μg) induction of the cell's own protective mechanisms. Data were collected using two generated Treg products from two different donors. **(B)** nTreg Phenotype and Function (%): The nTreg phenotype (green bars) and function assessed as the percentage of IL-2 (red bars) and IFN-g (yellow bars) cytokine secreting cells before freezing and after thawing of the two investigated nTreg cell products are shown. Depicted are the data of the cells before freezing, the control approach with 5% DMSO in the freezing medium (Control), the heat shock, and treatment with paeoniflorin (PA; 10 μg and 80 μg) induction. **(C)** Suppression Assay: The suppressive capacity of different PBMCs to Treg ratios are shown, starting with 1:2 PBMC:Treg down to 32:1.

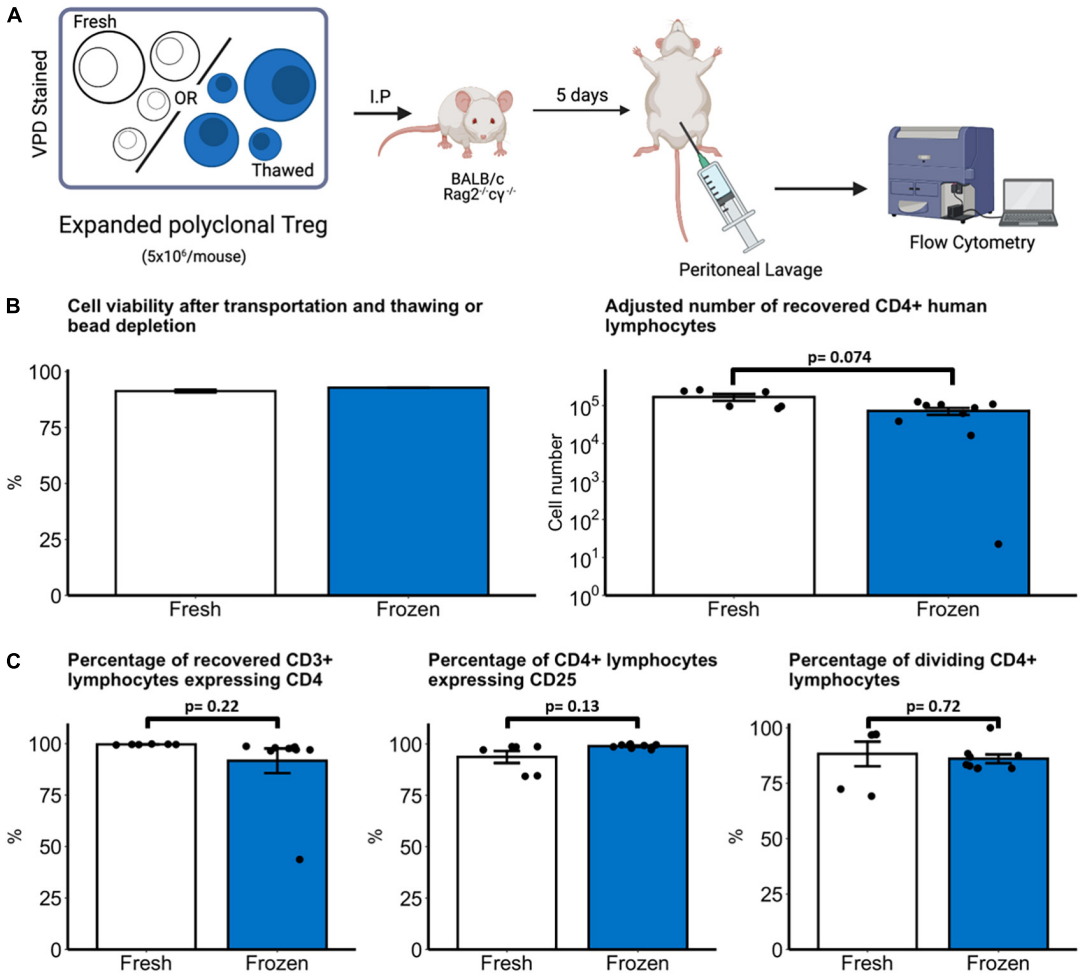

FIGURE 4 | *In vivo* survival of fresh and thawed nTreg. **(A)** Experimental schematic, created with biorender.com. Freshly expanded or cryopreserved nTreg were recovered and stained with violet proliferation dye (VPD). Before injection into mice cells were characterized for Treg markers. VPD staining ensured the traceability to Treg-positive cells for later investigations. Immunodeficient BALB/c Rag2$^{-/-}$cγ$^{-/-}$ mice received 5 × 10^6 VPD-labeled nTreg from one of two donors which were recovered after 5 days by peritoneal lavage **(B)**. Cell viability was quantified by light microscopy with 0.05% Trypan Blue dead-cell exclusion after transport and bead depletion (fresh nTreg) or transport and thawing (frozen nTreg). The number of nTreg recovered from lavage after 5 day incubation was quantified by flow cytometry. **(C)** Phenotype and proliferation of recovered human (mCD45$^-$CD3$^+$) lymphocytes were quantified by expression of CD4 and CD25. Division was defined as lymphocytes with VPD staining intensity less than the maximally stained (undivided) peak. Each point represents a separate mouse with n = 2 Treg donors. Data are represented as mean ± SEM and statistical significance determined using unpaired t-tests.

product generation during the ONE Study (Roemhild et al., 2020; Sawitzki et al., 2020). Our approach is based on the collection of a small volume of peripheral blood. To obtain sufficient cells for clinical application, the cells are subsequently expanded. To address the difficulties and limitations of freezing, expansion of thawed cells has been described (Golab et al., 2013), among others. Since from a regulatory point of view the expansion of frozen cells results in a new product to be released (EU GMP Guideline), this procedure is not suitable to be part of a manufacturing process. We first demonstrated that the cell recovery rate with all cryopreserved products, using 10% DMSO in freezing medium or synthetic Cryostem medium, was within the range of published data with approximately 60% recovery directly after thawing (Hippen et al., 2011; Golab et al., 2013; MacDonald et al., 2019). By using only 5% DMSO we were able to substantially increase the recovery rate directly (58 vs. 73%) and at 24 h (20 vs. 45%), as well as cell viability 24 h after thawing (60 vs. 78%).

When analyzing cell viability and recovery after thawing of therapeutic cells, the chosen analysis time point post-thawing has a major impact on the result, typically resulting in a further decline in cell viability and recovery 1 day post thawing (cells recovered in culture) compared with the viability obtained directly after thawing (Moll et al., 2014, 2016). Thus, when conducting this analysis after 24 h, the values for our analyzed nTregs dropped even further, suggesting irreversible cell damage due to cryopreservation or freeze-thawing (Christenson et al., 2012). Viability of recovered cells varied between 95% (first series of experiments) and 80% (second series of experiments) for the four nTreg products analyzed.

This was not clear from other previous publications, which usually do not distinguish between viability and recovery rate, or offset both values against each other (MacDonald et al., 2019). The viability of nTreg cells decreased further 24 h after thawing, highlighting the negative influence of known factors such as DMSO cell toxicity or osmotic shock in the context of cryopreservation (Elkord, 2009; MacDonald et al., 2019). The standard freezing medium widely used for cryopreservation of a wide variety of cell types is based on a DMSO content of 10% (Broxmeyer et al., 2003). We have found that lowering the DMSO concentration had a positive effect on the result of Treg cryopreservation. The same has been reported for cryopreservation of cord blood cells (Hayakawa et al., 2010). Among others, a possible explanation might be a mitigation of the induction of drastic changes in human cellular processes by DMSO (Verheijen et al., 2019). In addition, Treg cells are known to have a different metabolic profile compared with Teff cells (Macintyre et al., 2014; Gerriets et al., 2015; Angelin et al., 2017; Hashimoto et al., 2020). Thus, one can speculate whether Treg cells, due to their different metabolism, are more susceptible to toxic effects when metabolizing DMSO. This could explain why, despite the improvement by 5% DMSO-containing medium, the results are 30% below the recovery of our CMV- or EBV-specific Teff products (data not shown).

To further improve the cryopreservation results of our expanded Treg cells, we first assessed the effects of supplementation of the cryoprotective effect of DMSO as a penetrating agent with polyethylene glycol (PEG) as an extracellular cryoprotectant. However, considering future commercialization of production, the results obtained are disproportionate to the added effort, due to the waxy consistency of pure PEG, which greatly impairs handling. In the next round of experiments, we tested whether activation of cellular protective mechanisms improves the cryopreservation process through a mechanism of action which has already been demonstrated to be beneficial in stem cell cryopreservation (Shaik et al., 2017). Expression of the protective heat shock proteins or heat shock factors was induced either thermally (heat shock at 42°C for 1 h) or by incubation with paeoniflorin (Sawitzki et al., 2020) before cryopreservation. This increased the viability directly after thawing by around 5% compared to cryomedium with 5% DMSO. Nevertheless, this in turn was offset by a considerable additional process-technical effort.

The scientific literature proves that Treg cell products with suppressive properties are generated when appropriate isolation techniques and culture medium containing rapamycin are applied. However, applying a proliferation assay, we also clearly demonstrated/confirmed/verified that the functional suppressive potency of the cell products is not negatively affected by cryopreservation or freeze-thawing with 5% DMSO-containing freezing medium. This is in line with various publications showing that the suppressive activity of Tregs is not significantly affected by the freeze-thaw processes (e.g., Nadig et al., 2010).

Our comparision of freshly expanded and cryopreserved nTreg recovery and phenotype, however, supports the application of cryopreserved nTreg products. In our mouse model, there were no differences in recovery, phenotype, or proliferation between fresh or thawed nTreg. We did observe one outlier animal within the thawed group from which few human cells were recovered, reducing the mean values of both recovered lymphocytes and the proportion of CD3+ lymphocytes expressing CD4+ within the frozen group. This may be related to technical factors which are unclear, such as failure to inject cells correctly into the peritoneum. The number of nTreg recovered by lavage is also in keeping with our previous experience with this model (Zaitsu et al., 2017). In clinical studies, Treg dosage is closely related to the eventual number of circulating Tregs (Harden et al., 2021), which is ultimately crucial for a therapeutic outcome (Tang and Lee, 2012). The clear practical advantages of ATMP cryopreservation for transportation and administration, in conjunction with the comparable *in vivo* survival data demonstrated here, supports the validity of cryopreservation and thawing as an approach to delivering nTreg cell therapy.

There are some limitations to this study. As experiments were conducted under GMP-like conditions with the associated high costs and other practical limitations, the number of nTreg products that could be studied here was limited (n = 2–4 depending on the experiment), thus more subtle changes over a large sample size have not been explored here. A consequence of this limitation is that no valid calculation of a correlation is possible. A number of other factors would be helpful to investigate in the future. From our perspective and in analogy to the developments observed with other cellular therapeutics (Galipeau, 2013; Moll et al., 2014, 2016, 2020; Hoogduijn et al., 2016), understanding the influence of cryopreservation on clinical grade nTreg products is critical for increasing their availability and safety. More in-depth studies are therefore required to assess metabolic characteristics of Tregs in the context of cryopreservation. In addition, knowledge surrounding applied procedures for freezing (e.g., metabolic reduction by cooling) and thawing (e.g., "uncontrolled" in a 37°C water bath) is crucial. While this study provides important reassurance regarding the use of thawed cryopreserved Treg cells, further work must focus on optimising all other procedures in the freeze-thaw process to ensure the maximum number of viable and functional cells eventually reaches the patient.

CONCLUSION

In summary, we here demonstrate that reducing the amount of DMSO in the freezing medium from 10 to 5% improves the cell recovery rate and viability without negatively affecting the suppressive potential or our release criteria, such as relevant phenotypic markers or cytokine secretion profiles of our GMP Treg cell products. Furthermore, we investigated the combination of an intracellular and extracellular cryoprotectant and, to our knowledge, for the first time, the induction of cellular cyto-/cryo-protective mechanisms for clinical grade nTreg products. Unfortunately, no obvious larger optimization potential for cryopreservation has emerged from these pilot study approaches. Nonetheless, these studies involved substantial resources and time, due to simulation of the approaches within a GMP environment. Currently, the hurdles of unsatisfactory

cryopreservation of nTregs still stand in the way of broader availability, the establishment of "off the shelf" approaches, and successful commercialization. Although the administration of fresh products is currently accepted by regulatory agencies in early-stage trials and clinical therapeutic results are promising, improved performance of clinical-grade nTreg products in cryostorage and freeze-thawing approaches would greatly facilitate their broader use. Although the focus of the many academically driven approaches in the cell therapy field is often on the isolation and expansion of specific cell populations, storage and clinical handling is an important central component of the manufacturing process. Thus, future efforts on cell therapy optimization, for nTregs and other cellular therapeutics alike, such as optimal storage, clinical handling and delivery to patients, should be an integral part of their development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by all mouse experiments were performed using protocols approved by the Committee on Animal Care and Ethical Review at the University of Oxford and in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and under PPL number P8869535A.

AUTHOR CONTRIBUTIONS

DK, NO, OM, FI, GM, PR, and AR contributed in writing, review and editing, and conceptualization and supervision. DK, OM, HH, GZ, MS, CB, IM, MH, JH, and AR contributed in experiments and investigation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.750286/full#supplementary-material

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