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The role of regulatory T cells in controlling inflammatory responses

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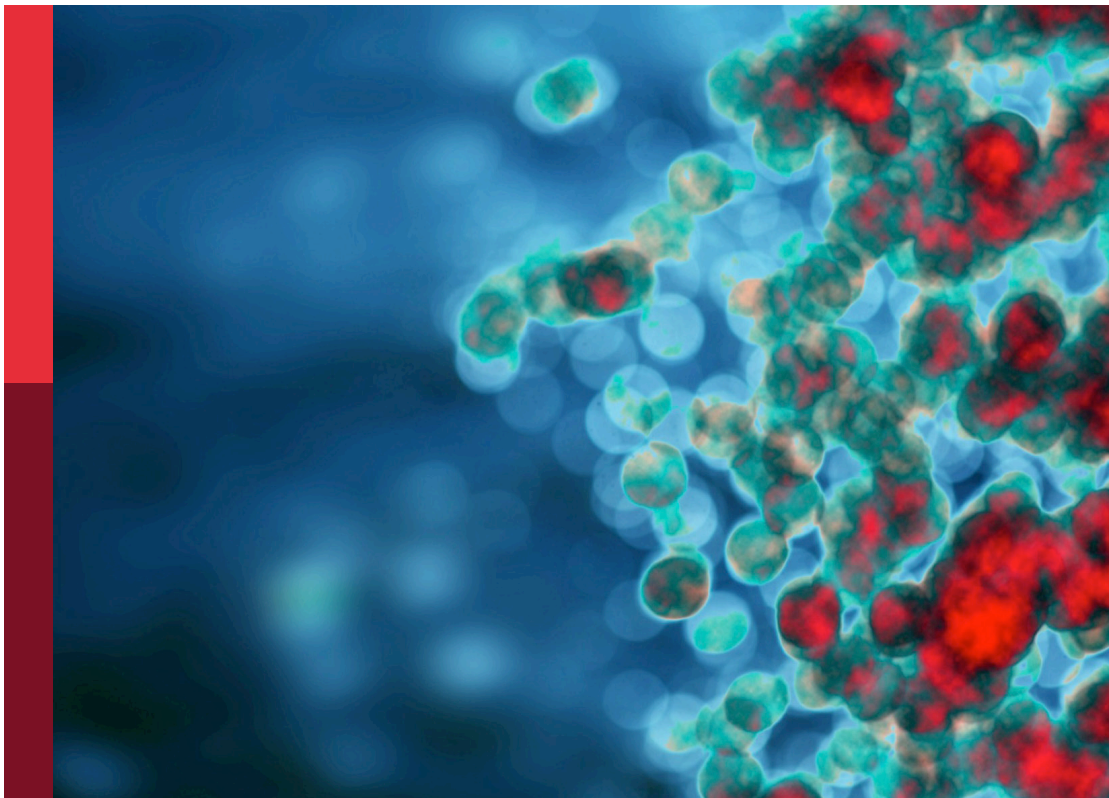

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The role of regulatory T cells in controlling inflammatory responses

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REVIEW
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A Deep Insight Into Regulatory T Cell Metabolism in Renal Disease: Facts and Perspectives

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Kidney disease encompasses a complex set of diseases that can aggravate or start systemic pathophysiological processes through their complex metabolic mechanisms and effects on body homoeostasis. The prevalence of kidney disease has increased dramatically over the last two decades. $CD4^+CD25^+$ regulatory T (Treg) cells that express the transcription factor forkhead box protein 3 (Foxp3) are critical for maintaining immune homeostasis and preventing autoimmune disease and tissue damage caused by excessive or unnecessary immune activation, including autoimmune kidney diseases. Recent studies have highlighted the critical role of metabolic reprogramming in controlling the plasticity, stability, and function of Treg cells. They are also likely to play a vital role in limiting kidney transplant rejection and potentially promoting transplant tolerance. Metabolic pathways, such as mitochondrial function, glycolysis, lipid synthesis, glutaminolysis, and mammalian target of rapamycin (mTOR) activation, are involved in the development of renal diseases by modulating the function and proliferation of Treg cells. Targeting metabolic pathways to alter Treg cells can offer a promising method for renal disease therapy. In this review, we provide a new perspective on the role of Treg cell metabolism in renal diseases by presenting the renal microenvironment、relevant metabolites of Treg cell metabolism, and the role of Treg cell metabolism in various kidney diseases.

Keywords: metabolic pathways, regulatory T cells, renal disease, tissue damage, immune homeostasis

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INTRODUCTION

The kidney is an important organ for excreting metabolic waste and maintaining internal environmental stability and plays an extremely important role in metabolic activities (1) (**Figure 1**). Treg cells are typical $CD4^+$ cells that constitutively express high levels of the interleukin-2 (IL-2) receptor CD25, along with the transcription factor Foxp3, which plays a central role in generating and maintaining Treg cell-specific gene expression by cooperating with other transcription factors, such as runt-related transcription factor 1 (RUNX1) and gata binding protein 3 (GATA3) (2).

Treg cells *in vivo* can be divided into two types (3): thymus Treg cells (tTreg), which mature after positive and negative selection in the thymus and play an immunosuppressive role in peripheral blood and lymphoid tissues; and peripherally induced Treg (pTreg) cells, which originate from T cells after antigenic stimulation and are converted by inhibitory cytokines (**Figure 2**). Treg cells *in vitro* are induced by cytokines and other factors, often referred to as induced $CD4^+$ T regulatory cells (iTreg). *In vitro* and *in vivo*, $CD4^+CD25^+Foxp3^+$ Treg cells inhibited the activation, proliferation, and effector function of a wide range of immune cells, such as $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, and NKT cells. They are indispensable for the maintenance of self-tolerance and immune homeostasis by inhibiting excessive or misdirected immune responses to foreign or autogenous targets (4).

It is important to note that phenotypic differences between tTreg cells and pTreg cells have not been clearly defined, which poses challenges in distinguishing the exact proportions of these two subpopulations in secondary lymphoid organs and non-lymphoid tissues. Studies have shown that neuropilin (Nrp-1) and Helios are highly expressed on tTreg in mice, but not on pTreg/iTreg cells (5, 6). Therefore, some researchers believe that tTreg cells and pTreg cells can be distinguished by Nrp-1 and Helios. However, this hypothesis has been controversial, especially when it comes to the distinction between human tTreg cells and pTreg cells (7, 8). Therefore, in the following review, we tried our best to use accurate classification to describe Treg cells, such as tTreg cells, pTreg cells, and iTreg cells. Where we were unable to distinguish the origins of the Treg cells from the original article, we have described the population studied using 'Treg cell' only.

Recently, increasing evidence has shown that Treg cells can take part in various renal diseases. Treg cells can play a negative regulatory role in kidney diseases and inhibit the immune response through direct cell contact or secretion of inhibitory cytokines (9). At the same time, kidney diseases, in turn, affect the function of Treg cells. For example, the number of Treg cells in patients with IgA nephritis is significantly reduced (10).

It is well known that renal disease is accompanied by significant changes in metabolic patterns (11), such as changes in glucose (12), amino acid (13), and lipid metabolism (14), which are essential for the activation and proliferation of Treg cells. Moreover, the metabolic pattern of Treg cells is also regulated by the metabolic state of nephropathy, and the type of nutrients used by Treg cells in nephropathy changes their differentiation, resulting in alterations in their phenotype and proportion. In addition to nutritional supply, the accumulated byproducts of renal metabolism significantly impair the immunosuppressive function of Treg cells, and the loss or functional deficiency of Treg cells affects the immune homeostasis of the kidney (15–18).

In the following sections, we will introduce renal microenvironment. Treg cell metabolism, the role of Treg cells in various renal diseases, and the importance of abnormalities in various metabolic pathways for the function of Treg cells, and will discuss the factors of abnormal metabolic pathways, which may be the goal of immunotherapy for related renal diseases.

RENAL MICROENVIRONMENT

The kidney is the most important organ in the human urinary system, which undertakes the important mission of filtering metabolic waste, excreting them from the body, and reabsorbing various nutrients (19). The kidney can maintain the body fluid and electrolyte balance by the distal tubule of the collecting duct through the absorption and excretion of various ions (electrolytes) in the body, such as sodium ions, phosphorus ions, calcium ions, and magnesium ions (20), at the same time, discharge the vast majority of metabolic wastes produced by the human body (21), for example, urea nitrogen, creatinine, uric acid, etc., to prevent waste products accumulate in the body, causing various disorders.

The kidney is an organ with important functions and complex structures, which determines that there are many kinds of cells involved in the microenvironment of the kidney, including immune cells and intrinsic cells of the kidney. Cytokines, chemokines, adhesion molecules, and complement secreted by immune cells and intrinsic cells of the kidney in the local immune microenvironment of kidney tissue, which plays a great role in the occurrence of kidney metabolism and injury.

Immune Cells and Intrinsic Cells of the Kidney

Macrophages

Macrophages are classified into M1 macrophages and M2 macrophages (22). In healthy kidney tissue, the main function of macrophages is to phagocytose and digest cell fragments and pathogens in the form of fixed cells or free cells, and to activate lymphocytes or other immune cells to respond to pathogens.

Studies have shown that macrophages play an important role in mediating immunopathology and tissue remodeling in non-renal disease and renal disease (23). In animal models, blocking macrophage recruitment and expression of inflammatory factors can prevent the progression of various kidney diseases (24). At the same time, the damaged kidney produces a large number of macrophages, which continuously infiltrate the kidney and produce pro-inflammatory cytokines, including TNF-α and IL-1β, to induce kidney inflammation (25). In addition,

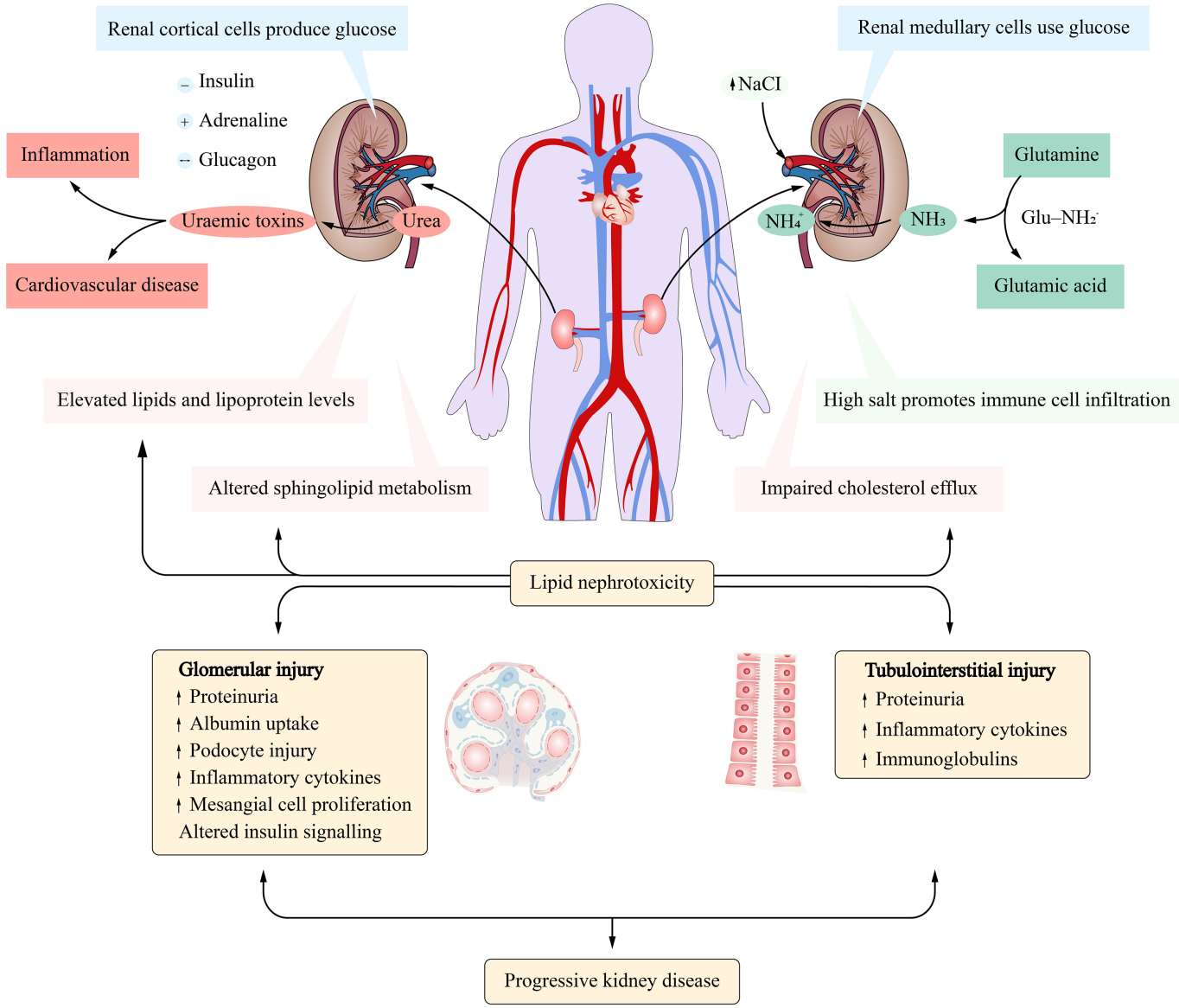

FIGURE 1 | Metabolism of substances in the kidney. Cortical cells undergo gluconeogenesis while medullary cells metabolize glucose. Glutamine is extracted from renal tubular cells and used to produce ammonia (NH3). High levels of urea cause the kidneys to produce uremic toxins. Lipid nephrotoxicity could damage the structure and function of the glomerulus and tubules.

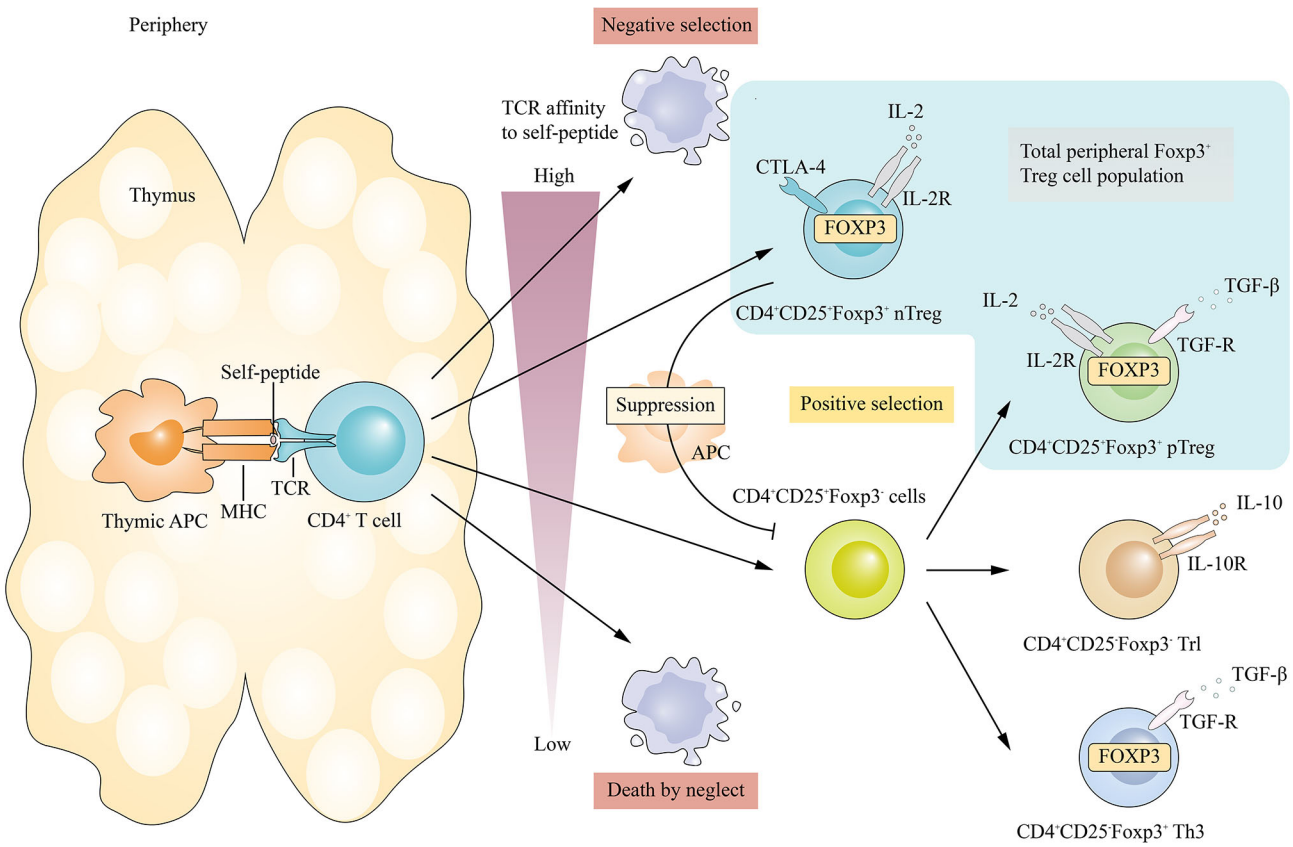

FIGURE 2 | Differentiation of Treg cells *in vivo*. TTreg cell development is initiated by TCR signal transduction. CD4+CD8- thymocytes that bind with high affinity to their self- peptides-MHC complex are positively selected in the thymus. Immature T cells with low affinity for their own peptide-MHC complex are also positively selected and subsequently differentiated into different subtypes, including pTreg, Tr1 and Th3.

macrophages can produce oxygenated nitric oxide Complement components can directly damage renal cells and affect the formation of matrix and blood vessels by expressing matrix metalloproteinases and vasoactive peptides (26).

Mast Cells

Mast cells have long been considered as effector cells for mediated hypersensitivity and inflammatory responses (27). Its role in the kidney has been largely overlooked in comparison to macrophages and other immune cells. In fact, in a healthy kidney, mast cells release cytokines that protect the kidney from immune damage. They also produce chymases, which produce angiotensin II (28).

Studies have shown that chymase expression is proportional to the degree of renal interstitial fibrosis (29, 30). Mast cell infiltration and increased chymase expression are seen in both glomerulonephritis and ischemia-reperfusion-induced renal fibrosis (31, 32). Mast cells can also promote the proliferation of fibroblasts through intercellular interactions (33). But some researchers have come up with evidence to the contrary. They used mast cell deficient rats to induce nephritis with Puromycin aminonucleoside (PAN) (34). After 6 weeks, it was found that the fibrosis degree of the deficient rats was more serious than that of the wild-type rats, and the expression of TGF-β was significantly higher than that of the wild-type rats. *In vitro* experiments showed that heparin, as an important component of mast cells, could inhibit the expression of TGF-β in rat fibroblasts, suggesting that mast cells may reduce the degree of fibrosis through TGF-β -dependent pathways and play a certain protective role in the kidney.

Dendritic Cells

Dendritic cells (DCs) can be divided into plasmacytoid dendritic cells (pDC) and conventional dendritic cells (cDC) (35). Immature DCs have strong migration ability, mature DCs can effectively activate primary T cells, and are in the central link of initiating, regulating, and maintaining immune response (36). It is rarely present in a healthy kidney and can efficiently absorb, process, and present antigens to maintain the stability of the renal internal environment.

Studies have shown that DCs induce and maintain immune responses through migration and maturation in the kidney (37).

DCs, although rarely present in normal kidneys, are significantly increased in chronic kidney disease (CKD) and diabetic nephropathy (DN) (38). A 5/6 nephrectomy model was used to induce renal fibrosis, $CD1a^+CD80^+$DCs was found to accumulate in the renal interstitial from 1 week after modeling and peaked at 12 weeks (39). In another study, galectin 3 protects cisplatin-induced acute kidney injury by promoting TLR-2 dependent activation of the IDO1/Kynurenine acid pathway in renal DCs (40). These two studies suggest that DCs were associated with the severity of interstitial fibrosis.

T Lymphocyte

T cells are characterized by the expression of co-receptor molecules CD4 and CD8 on their cell surface (41). $CD4^+$T cells, also known as T helper cells (Th), recognize antigen/MHC-II complexes on antigen-presenting cells (42) and coordinate the activation of other immune cells, including B cells, macrophages. $CD8^+$ cells, on the other hand, called T cytotoxic cells, recognize antigens/MHC-I complexes and are responsible for killing pathogen-infected cells (43). In healthy kidney tissue, T lymphocytes protect the kidney by performing a variety of biological functions to fight infection.

Th cells are thought to play an important role in kidney disease. Th cells were divided into Th1, Th2, Th17 and Treg cells according to the different cytokines secreted (44). Th1 cells mainly secreted IFN-γ, IL-2, IL-12, and so on. Th2 cells mainly secrete IL-4, IL-5, IL-13, etc (45). Th17 cells mainly secrete IL-17. Treg cells mainly secrete IL-10 and TGF-β (46). In a study of rats with idiopathic nephrotic syndrome, proteinuria and focal segmental glomerular injury were observed at 10 weeks of age. Renal T cell infiltration was detected before proteinuria, Th1 and Th2 cells were increased, and Th2 cells were dominant (46). But exactly what the Th1/Th2 equilibrium theory means is still up in the air. The main problem is that the activity of cytokines and other immune messengers rarely falls into strict Th1 or Th2 patterns, and some immune cells, such as Treg cells, stimulate the Th1/Th2 immune system (47). The imbalance of the Th17/Treg ratio plays a role in tissue inflammation, autoimmune, and various diseases. Recently, researchers have proved that the increased ratio of Th17/Treg cells is related to the progression of CKD (48).

Most $CD8^+$T cells are cytotoxic, which can induce apoptosis through perforin or Fas/FasL pathway, and can also directly stimulate fibroblast proliferation and extracellular matrix production by secreting TGF-β, IL-4, TNF-a, and other factors, thus aggravating kidney injury (49). Depletion of $CD8^+$T cells with antibodies can reduce interstitial dilatation, reduce fibrosis, and alleviate renal parenchymal lesions and renal damage (50). On the contrary, the depletion of $CD4^+$T cells aggravated kidney injury, partly because the decrease of $CD4^+$T cells caused the increase of $CD8^+$T cells (50).

B Lymphocyte

B cells have a variety of functions. In addition to the function of antibody secretion, B cells also have the function of releasing inflammatory cytokines, chemokines, and antigen presentation (51). In healthy kidney tissue, B cells make up a small proportion and, together with other immune cells, maintain the stability of the immune microenvironment of the kidney.

There is growing evidence that B cells play an important role in kidney disease. In lupus nephritis(LN), the researchers treated NZB/W lupus mice with a selective histone deacetylase 6 (HDAC6) inhibitor for 4 weeks and showed that HDAC6 inhibition decreased B-cell activating signaling pathways, resulting in a significant reduction in LN symptoms (52). In a clinical trial on patients with IgA nephropathy, the investigators found that toll-like receptor 7 (TLR7) can activate B cells through the TLR7- GALNT2 axis, which produces high levels of galactose-deficient IgA1 (Gd-IgA1) (53).

Renal Tubular Epithelial Cells

Renal tubule epithelial cells(RTECs) are composed of a single layer of epithelium and have different morphological characteristics and functions according to the position of renal tubules. For example, in the proximal convoluted tubules, the wall is composed of a single layer of cuboidal epithelial cells (54). The lumen is small and irregular and is an important part of tubular reabsorption. The free surface of the cell has a bristle margin, which enlarges the cell surface area and facilitates reabsorption.

RTECs are involved in the occurrence of kidney injury in many aspects. RTECs can be activated by a variety of cytokines, such as IL-1 and TNF-a produced by monocytes (55). IL-17 is a pro-inflammatory cytokine released by activated T cells. *In vitro*, activation of RTECs with IL-17 can promote the production of IL-6, IL-8, and MCP-1 (56). RTECs are not only important sources of cytokines and chemokines but also can produce pro-fibrotic factors, such as TGF-β, PDGF, CTGF, etc (57). In addition, RTECs are important antigen-presenting cells that can interact with T cells and monocytes.

RTECs are also involved in an important process in renal fibrosis called epithelial-mesenchymal transition (EMT) (58). After EMT, morphological and proteomic changes occurred in RTECs. The so-called EMT is the process in which epithelial cells lose their cellular characteristics, such as polarity and intercellular adhesion, gain the ability to migrate and invade, enter the stroma to obtain new phenotypes, and eventually become mesenchymal cells. In renal fibrosis, EMT refers to the transformation of epithelial cells into myofibroblasts, which are the primary source of the extracellular matrix (59). The expression of various proteins, such as TGF-β, MMPs, FSP-1, and vimentin, increased after EMT. In contrast, some proteins, such as e-cadherin and keratin -18, which are the signature proteins of epithelial cells, are also reduced in expression.

Glomerular Mesangial Cells

In healthy renal tissue, glomerular mesangial cells (MCs) only perform the functions of contraction, phagocytosis, and maintenance of normal matrix metabolism. Under pathological conditions, MCs can be transformed from a normal quiescent

phenotype to an active proliferation/secretion phenotype with increased extracellular matrix secretion (60). The activation phenotype of MCs has myofibroblast-like characteristics and is characterized by the expression of A-SMA and ED-A fibronectin. After activation, MCs can release a variety of growth factors, such as TGF-β, CTGF, PDGF, etc. through the autocrine or paracrine form to promote self-proliferation (61). At the same time, MCs can synthesize a large amount of extracellular matrix, and mesangial matrix aggregation is the main pathological feature of glomerulosclerosis (62).

In NZB/WF1 mice, the binding of autoantibodies to MCs leads to the initiation of an inflammatory response, an early-stage marker of glomerulonephritis (63). In an *in vitro* model of lupus nephritis (LN), MCs participate in the inflammatory environment of LN by producing cytokines involved in leukocyte recruitment, activation, and maturation. Treatment of MCs with cytokines or patient serum induces TGF-β1 secretion, suggesting that MCs are also involved in the fibrosis process of LN (64).

Major Metabolites in the Kidney

Urea

Urea is a protein metabolite that is produced in the liver and travels through the blood to the kidneys. Some urea is retained in the blood by glomerular filtration and has the opportunity to be transported to the digestive tract as a nitrogen source for microorganisms, while some urea forms tubule fluid and is reabsorbed by the collecting tube of the kidney and returned to the blood.

Urea transporter is a membrane protein that mediates urea transmembrane transport along a concentration gradient, mainly including urea transporter B(UT-B) and urea transporter A(UT-A) (65). UT-A1 is generally distributed in the apical membrane of collecting duct cells in the renal medullary loop (66), UT-A2 is distributed in the descending branch of the loop of the spinal cord (67), UT-A3 is distributed in collecting duct cells in the renal medullary loop basolateral (68), and UT-B1 is mainly distributed in the descending branches of straight small vessels of the nephron (69, 70).

In mouse kidneys, after the deletion of UT-A1 and UT-A3 genes, urine nitrogen excretion increased significantly. After the deletion of UT-B genes, urea in ascending branches of straight small vessels could not penetrate to descending branches of straight small vessels, and the concentration of urea in inner myelin decreased, leading to a decrease in urea circulation in the kidney (71). In conclusion, UT-B, UT-A1, and UT-A3 play irreplaceable roles in the renal urea cycle.

Aquaporin (AQP) is a membrane protein that regulates the infiltration of water into and out of cells (72). It is embedded in the cell membrane and controls the entry and exit of water molecules. Its mechanism of action is similar to urea transporter. So far, 13 aquaporin subtypes, namely AQP1-AQP12, have been identified in animals, but only AQP3, AQP7, AQP9, and AQP10 have clear permeability to urea, which are collectively referred to as water-glycerin channel (AQGP) protein (73–75). Studies have shown that AQGP can also mediate urea transport (76, 77).

In addition to excreting nitrogen, urea also mediates urine concentration through specific urea transport proteins (78, 79). The establishment of the renal medullary osmotic gradient is a necessary condition for the formation of concentrated urine. The active reabsorption of NaCl in the crude segment of the ascending ramus of the medullary loop is the main driving force for the establishment of the medullary osmotic gradient. Urea and NaCl are the main solutes for the establishment of a medullary osmotic gradient.

Proximal tubules are moderately permeable to urea and can reabsorb up to 50% of filtered urea. The collecting tubes in the distal convoluted tubules, cortex, and outer medullary part of the ascending branch of the loop are almost opaque to urea. As the tubule fluid flows through these areas, the water is reabsorbed by collecting tubes in the cortex and the outer medulla, and the concentration of urea in the tubule fluid increases. The collecting tube in the inner medullary region contains UT-A1 and UT-A3, which are activated by several factors and promote the diffusion of urea into the interstitial fluid in the inner medullary region. Urea can re-enter the medullary loop and be reused with a high concentration in the inner medullary region. Urea in the interstitial fluid of the inner medullary is in equilibrium with urea in the collecting tube so that other substances in the interstitial fluid (such as NaCl) are in equilibrium with other substances in the urine to facilitate urine concentration.

Urea transporters can be mediated by several factors in the renal microenvironment that increases urea transport. In short term rapid regulation, Vasopressin signals through two cAMP-dependent pathways: protein kinase A and cAMP-activated exchange proteins (80), high osmotic pressure signals through increased protein kinase Ca, and intracellular calcium (81), thereby increasing UT-A1 and UT-A3 phosphorylation and urea transport (82–84). Vasopressin increases the abundance of UT-A1 and UT-A3 proteins in long-term regulation (85). In addition, urea transporters are affected by low-protein diets (86, 87), adrenal steroids (86, 88), hypokalemia (86), and acidosis (87).

Ammonia

Renal ammonia metabolism plays an important role in the maintenance of acid-base homeostasis (88). Almost all urinary ammonia is produced in the kidney, and glutamine in the blood flows through the kidney and is broken down into ammonia in the tubular epithelial cells (89). Urinary ammonia is mainly produced by the decomposition of glutamine, and a small amount comes from the catabolism of other amino acids (90).

In proximal tubules, glutamine uptake requires complete metabolism of glutamine through the involvement of root tip Na+ dependent neutral amino acid transporter-1 and basolateral sodium-coupled neutral amino acid transporter-3 (SNAT3) to produce two NH4+ and two HCO3- ions per glutamine (91). The resulting bicarbonate then passes through the basolateral membrane into the blood vessels *via* the electric-sodium coupled bicarbonate cotransporter isoform 1A (NBCE-1A).

Ammonia reabsorption occurs in the ascending part of the medullary loop. Ammonia is reabsorbed as NH4+ mainly

through the transporter NKCC2 and then transported by NHE4, a sodium-hydrogen exchanger on the basolateral membrane (92). NH4+ is a weak acid, and intracellular acidification inhibits ammonia reabsorption (93). Sodium bicarbonate enters cells through electrically neutral sodium-sodium bicarbonate cotransporter subtype 1 (NBCn1) on the basolateral membrane, which appears to buffer intracellular acidification and promote ammonia reabsorption (94).

The collecting tube secretes large amounts of ammonia. The secretion of NH3 is accompanied by the secretion of H+ (95). NH3 secretion seems to be related to the transport of ammonia-specific transporters Rhbg and Rhcg expressed on the collector tube (96, 97). In addition, Na+-K+-ATPase proteins are present on the basolateral side of collecting duct cells, which are involved in ammonia secretion of the intramedullary collecting duct through their ability to transport NH4+ (98).

In summary, ammonia in renal tubular epithelial cells has two pathways: on the one hand, it is discharged into the tubular fluid and excreted in urine; the other is reabsorbed into the blood. NH3 easily passes through the biofilm, while NH4+ does not, so the path of ammonia in the kidney depends on the relative PH of blood and tubular fluid. The PH of blood is generally constant, and therefore actually depends on the PH of the tubule fluid. When the PH value of the tubule fluid is acidic, the NH3 discharged into the tubule fluid combines with H+ to form NH4+ and is discharged with urine. If the PH value of the tubule is high, NH3 is easily reabsorbed into the blood.

Metabolic acidosis can affect ammonia metabolism. During metabolic acidosis, acidosis stimulates the degradation of skeletal muscle protein, which binds to intrahepatic glutamine and increases extrarenal glutamine, leading to increased glutamine flow through the kidneys and increased urinary ammonia production (99, 100). The kidneys remove excess acid from the body by increasing ammonia metabolism (101). Notably, glucocorticoids can modulate ammonia excretion induced by acidosis, possibly by stimulating acidosis-induced extrarenal glutamine increase (102, 103).

Hypokalemia also results in altered ammonia metabolism in the kidneys. Metabolic alkalosis of hypokalemia is often associated with increased bicarbonate production (104). In both adults and children, increased ammonia excretion due to hypokalemia can lead to a negative nitrogen balance and impair health (105).

In addition, a protein diet also regulates ammonia excretion. A high protein diet, especially the intake of sulfur-containing amino acids, lowers PH and promotes ammonia excretion (106). Conversely, a low protein diet reduces ammonia excretion (107).

H_2O, Na+

Water is filtered through the glomerulus and reabsorbed by the renal tubules. The glomerular filtration of protopuria was about 170-180L/d, and the final urine was about 1.5L/d. The reabsorption of water by the kidney can be divided into two forms: passive absorption and active absorption. About 90% of tubule fluid is reabsorbed in renal tubules, and proximal convoluted tubules reabsorb glucose, amino acids, electrolytes, and other substances, and reabsorb water by an osmotic pressure gradient, which is the main form of passive water absorption, accounting for about 80%~90% of water reabsorption (108). The rest are absorbed actively in the medullary loops of renal tubules, distal convoluted tubules, and some collecting tubules, which are regulated by ADH.

There are mainly 8 aquaporins in the kidney, which are AQP1, AQP2, AQP3, AQP4, AQP5, AQP6, AQP7, and AQP11. AQP1 is located at the top of proximal renal tubular epithelial cells, basolateral membrane, and descending branch of the medullary loop. AQP2, AQP3, AQP4, AQP5, and AQP6 are located in the collecting duct, AQP7 is distributed in the brush edge of the proximal convoluted tubule, and AQP11 is located in the endoplasmic reticulum of the proximal tubule cells.

ADH binds with AQP2 in the basement membrane of renal collecting duct epithelial cells to promote the generation of cAMP, activate adenosine cyclase in the perimembrane of tubular cells, increase intracellular cAMP, and then activate protein kinase, phosphorylation of protein located at the luminal surface of the plasma membrane of epithelial cells, and thus increase membrane permeability to water.

It is believed that the water metabolism of the kidney is related to the ball-tube balance. The colloid osmotic pressure in peritubular capillaries can regulate the reabsorption of sodium and water in proximal convoluted tubules. When the glomerular filtration rate (GFR) increases, the filtration excretion fraction (GFR/RPF) also increases. Due to the decrease of protein content in the filtrate, the protein concentration in the blood flowing into the capillaries around the renal tubules increases, and the colloid osmotic pressure in the capillaries also increases, thus promoting the reabsorption of sodium and water in proximal convoluted tubules. In addition, the hydrostatic pressure of peritubular capillaries also regulates the reabsorption of sodium and water in proximal convoluted tubules.

On the other hand, when GFR increased, the amount of Na+ passing through the macula densa also increased, thus increasing the secretion of renin and angiotensin formation in parabulbar cells. Increased angiotensin-2 (AT-2) causes constriction of the entering arterioles, which results in a decrease in GFR and restores the ball-tube balance. Conversely, when GFR decreases, AT-2 production decreases, which causes dilation of the entering arterioles and increase of GFR, and restores the bulbal-tubular balance.

Glucose

Renal regulation of glucose metabolism mainly includes gluconeogenesis, glomerular glucose filtration, and proximal convoluted tubules glucose reabsorption. In the fasting state of normal individuals, the kidney produces 15-55g/d glucose through gluconeogenesis, accounting for about 20%-25% of all endogenous glucose. Renal gluconeogenesis is further increased after eating. Renal gluconeogenesis occurs mainly in the proximal convoluted tubules of the renal cortex and is regulated by insulin and catecholamines (109).

Under physiological conditions, the glomerular filtration of approximately 180g of glucose per day is followed by almost

complete reabsorption in the proximal convoluted tubules, so urine glucose monitoring should be negative. However, when plasma glucose concentrations reach nearly 10.0mmol/L, the renal glucose threshold will be exceeded, resulting in detectable glucose in urine (110).

The renal glucose reabsorption process is completed in the proximal convoluted tubule S1-S3 segment. The Na+ is pumped out of the cells and into the interstitial fluid by the Na+-k–ATP pump located in the basolateral membrane of renal tubules, thereby reducing the concentration of sodium ion in the cells and forming an electrochemical gradient of about -70 mV. Glucose in the tubule fluid is actively transported to renal tubule cells by the sodium glucose cotransporter (SGLT) in a secondary, inverse concentration gradient. Finally, the glucose transporter (GLUT) binds glucose and changes its conformation, and glucose is then returned to the blood *via* facilitated diffusion from renal tubule cells (111).

SGLT belongs to the SLC5 gene family. SGLT1 and SGLT2 play a role in glucose reabsorption, and their differences in expression and the driving force of their cotransport function help to minimize glucose loss from urine. SGLT2, with high volume and low affinity, is mainly distributed in the proximal S1 segment of the proximal convoluted tubules of the kidney and combines actively transported sodium ions and glucose into the blood circulation in a ratio of 1:1, playing a major role in the renal glucose reabsorption function. SGLT1 is mainly distributed in the brush edge of intestinal mucosal epithelial cells and plays an important role in intestinal glucose and galactose absorption. SGLT1 is also expressed in the distal S3 segment of the proximal convoluted tubules of the kidney with a higher affinity than SGLT2. Glucose that is not bound by SGLT2 is responsible for SGLT1, and glucose and Na+ are reabsorbed into the blood in a ratio of 1:2 (109).

Glucose transporter 1 (GLUT1) and GLUT2 are mainly related to the process of renal glucose reabsorption in the GLUT family. GLUT2 is expressed in the basolateral membrane of renal tubular cells in the S1 segment and is responsible for releasing glucose reabsorbed by SGLT2 into the blood through facilitated diffusion. GLUT1 is responsible for the release of glucose from small tubules into the blood at the proximal convoluted tubule S3 segment (112).

Amino Acids

Amino acid metabolism is closely related to the kidney. On the one hand, the absorption, release, metabolism, and excretion of amino acids by the kidney can effectively regulate the level of amino acids in the circulation system and the transport of amino acids between organs. On the other hand, orderly amino acid metabolism is beneficial to regulate renal hemodynamics and protein synthesis, maintain the integrity of renal function, and environmental acid-base balance in the body. Under the normal physiological state, the kidney mainly absorbs glutamine, citrulline, phenylalanine, S-adenosine homocysteine, and proline from the blood, and participates in the synthesis and release of serine, tyrosine, arginine, cysteine, and a small amount of threonine and lysine.

After the kidney absorbs glutamine from the blood, NH4+ and glutamate are mainly metabolized by phosphate-dependent glutamine enzyme, and only a small amount of them metabolized by γ -glutamine transferase in the distal tubules (113). Under the normal physiological state, about 70% OF NH4 + enters the renal vein, and the rest is discharged through urine (114). When PH in the kidney increases, a high concentration of NH4+ inhibits glutaminase activity and increases NH4+ excretion to maintain acid-base balance in the body.

Citrulline is a nitrogenous product of glutamine metabolism in the intestinal tract. Most citrulline is synthesized in the intestinal tract and absorbed by the kidney (115). After the kidney absorbs a large amount of citrulline from the blood, arginine is synthesized and released into the blood with the participation of arginine succinic acid synthase and succinic acid lyase, which accounts for 10-20% of the total plasma arginine (116). Compared with the normal diet, intestinal arginine absorption is reduced in the low-protein diet, resulting in reduced urea synthesis in the liver. At the same time, because citrulline is not taken up by the liver, more citrulline in the low-protein diet enters the kidney to synthesize arginine to maintain normal physiological function.

Arginine can be degraded to guanidine acetic acid and urea, or oxidized by nitric oxide synthase to citrulline and nitric oxide (NO). About 1% of the daily intake of arginine is used to metabolize NO, which is the main source of NO synthesis (117). NO is a small gas molecule that can regulate endothelial cell function and is of great significance in regulating glomerular hemodynamics, maintaining glomerular filtration rate, local vascular tension, and renal blood flow (118, 119).

Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and N(G)-monomethyl-L-arginine (NMMA) are generated from arginine residues after methylation and proteolytic reaction. Both ADMA and NMMA can inhibit the activity of arginine synthase. NMMA is the precursor of ADMA and SDMA, and the content of NMMA is small but the inhibition effect is strongest. The kidney also plays an important role in the clearance of ADMA and SDMA. The clearance of ADMA mainly relies on renal conversion into citrulline and dimethylamine, and the remaining small amount of ADMA is excreted through urine, while SDMA is mainly cleared through renal excretion (120).

S-adenosine homocysteine is a by-product of methionine methyl transfer reaction and a precursor of homocysteine synthesis. The arteriovenous difference of S-ADENosine homocysteine was up to 40%, indicating that the kidney is the main excretion site of S-adenosine homocysteine (121).

Tyrosine, as a non-essential amino acid, can be synthesized by phenylalanine 4-hydroxylase catalyzed by phenylalanine as a substrate in the body. The synthesis process of tyrosine was first discovered in the liver, and it can also be synthesized in the renal cortex in the later study (122). Moreover, tyrosine synthesized by the kidney is the main source of maintaining the level of tyrosine in the circulatory system (123). Glycine is taken up by the kidneys to synthesize serine, which only accounts for 5-7% of the total amount of serine in the body. High arginine is mainly

derived from lysine in the kidney, which can increase intracellular arginine concentration, promote the effective synthesis of NO and improve the dysfunction of endothelial cells and cardiomyocytes (124).

Causes of Renal Microcirculation Disorders

Dietary preferences can cause gastrointestinal microbiota imbalance and translocation, resulting in renal microcirculation disorders. For example, a high-salt diet can induce oxidative stress in the kidney, resulting in increased renal perfusion pressure and immune cell infiltration, thus leading to kidney damage (125, 126). Meanwhile, under high-salt conditions, serum and glucocorticoid-regulated kinase 1 (SGK1)-mediated phosphorylation of forkhead box of transcription factors O1 (FOXO1) and forkhead box of transcription factors O3 (FOXO3) may lead to instability of Foxp3, thus reducing the inhibitory function of Treg cells (127) (**Figure 4B**). A high protein diet leads to increased urea production in the body. Excessive urea will lead to uremia toxin production in the kidney (128), thus changing the integrity of the intestinal barrier, resulting in the migration of intestinal flora into the blood, resulting in inflammation and cardiovascular diseases (129, 130) (**Figure 1**).

When the imbalance and displacement of microbial flora, a stressor, appears, it will stimulate the activation of the stress response mechanism related to metabolism, leading to excessive passive and active absorption of nutrients by the human body. Excessive absorption of nutrients cannot be efficiently and timely metabolized by the body, easy to lead to nutrient metabolism disorder. For example, lipid substances (triglyceride, cholesterol, etc.), glucose, amino acids, etc., and the disorder of metabolism of these intermediates lead to the impairment of the morphology and function of Treg cells (131–133), the blockage of renal capillaries, and then the occurrence of renal microcirculation disorders (134).

Disturbances in the microcirculation of renal resident cells (RTECs and MCs) impair the exchange of cells with external substances, leading to metabolic disturbances in renal cells (135). Due to the tiny blood vessels front-end blood-supply artery atherosclerosis in silt, the various cells of the kidney can't get enough nutrients and energy supply, leading to large proteins, lipids, creatinine, urea, renin, prostaglandins, mineral ions, etc. cannot be effectively out of shipping, and metabolism thus can lead to kidney tissue cell metabolism disorder. For example, renal tissue ischemia and hypoxia activate hypoxia-inducible factor 1α (HIF-1α) and destabilize Foxp3 expression, thus inhibiting Treg cell proliferation (136) (**Figure 4B**).

Treg Cells Metabolism

Cell metabolism is the core of T cell differentiation (137). Resting T cells require little energy production or consumption; however, after activation, their energy demand increases significantly, and they use glucose, amino acids, and fatty acids to meet this requirement (138, 139). An overview of the metabolic pathways is shown in **Figure 3**. Treg cells mainly utilize fatty acid and pyruvate oxidation (mitochondrial oxidative metabolism) to produce energy, which has a different signal and metabolic characteristics from other T cells (139).

Glucose Metabolism in Treg Cells

Glucose is required for the activation and proliferation of Treg cells. They can make use of glycolysis and oxidative phosphorylation for energy production (140). Glycolysis occurs in the cytoplasm by converting glucose to pyruvate (producing two ATP molecules), which is converted to lactate by lactate dehydrogenase A (LDH-A), or to acetyl-CoA by pyruvate dehydrogenase (PDH), which then travels to the mitochondria to participate in the tricarboxylic acid (TCA) cycle, producing ATP (36 molecules) through oxidative phosphorylation (141). Moreover, the pentose phosphate pathway (PPP) that branches from the glycolysis pathway converts glucose-6-phosphate to ribose-5-phosphate for the synthesis of nucleotides.

Foxp3 itself inhibits glycolysis and promotes oxidative phosphorylation (OXPHOS), while Foxp3 deficiency dysregulates mammalian target of rapamycin complex 2 (mTORC2) and promotes glycolysis (132, 142). Upregulation of GLUT1 in Treg cells inhibited Foxp3 expression (142, 143). A study showed that Treg cells had higher levels of C2 and C4-OH carnitine, higher expression of fatty acid transport protein carnitine palmitoyltransferase 1A (CPT1A) and electron transport chain component cytochrome C, and lower levels of GLUT1, a key protein expressed in pyruvate, lactic acid, and glycolysis pathways, suggesting that the energy of Treg cells depends more on oxidative phosphorylation than glycolysis (144). Moreover, some studies have shown that a high glycolysis rate is not conducive to the differentiation of Treg cells. Conversely, inhibition of glycolysis can promote the formation of Treg cells (145, 146). It can be explained because, mechanically, glycolysis requires activation of MYC proto-oncogene (MYC) and Foxp3 binds to the promoter of MYC to inhibit expression of MYC and MYC-dependent transcripts (147).

The glycolytic enzyme enolase 1, relocating from the cytoplasm (where it regulates the glycolysis pathway) to the nucleus, is required for the induction and function of human pTreg cells following suboptimal T cell receptor (TCR) stimulation of T cells in the periphery (138). In the nucleus, enolase 1 binds to the epigenetic promoter region of the Foxp3 gene to inhibit transcription of specific Foxp3 exon-2 (E2) (148) (**Figure 4B**).

As mentioned above, pyruvate can be converted to lactic acid by LDH-A under anaerobic conditions. For example, in ischemic tissue, due to the accumulation of lactic acid caused by ischemia and hypoxia, lactic acid mediates increased HIF-1α production and inhibits pTreg function, which may lose the metabolic advantage of function under low glucose conditions (149). However, in prostate cancer models, lactic acid produced by cancer-associated fibroblasts (CAFs) stimulates Treg proliferation by promoting Foxp3 activation (150). These two contradictory results are currently unclear and may be interpreted that lactic acid increases the number of Treg cells, and the inhibitory ability of Treg cells decreases during Treg proliferation. In addition, lactic acid can also be converted to pyruvate through lactate dehydrogenase B (LDH-B) (151). One study showed that in tumor cells, oxidation of lactic acid to pyruvate changed the ratio of NAD+/NADH, thereby activating

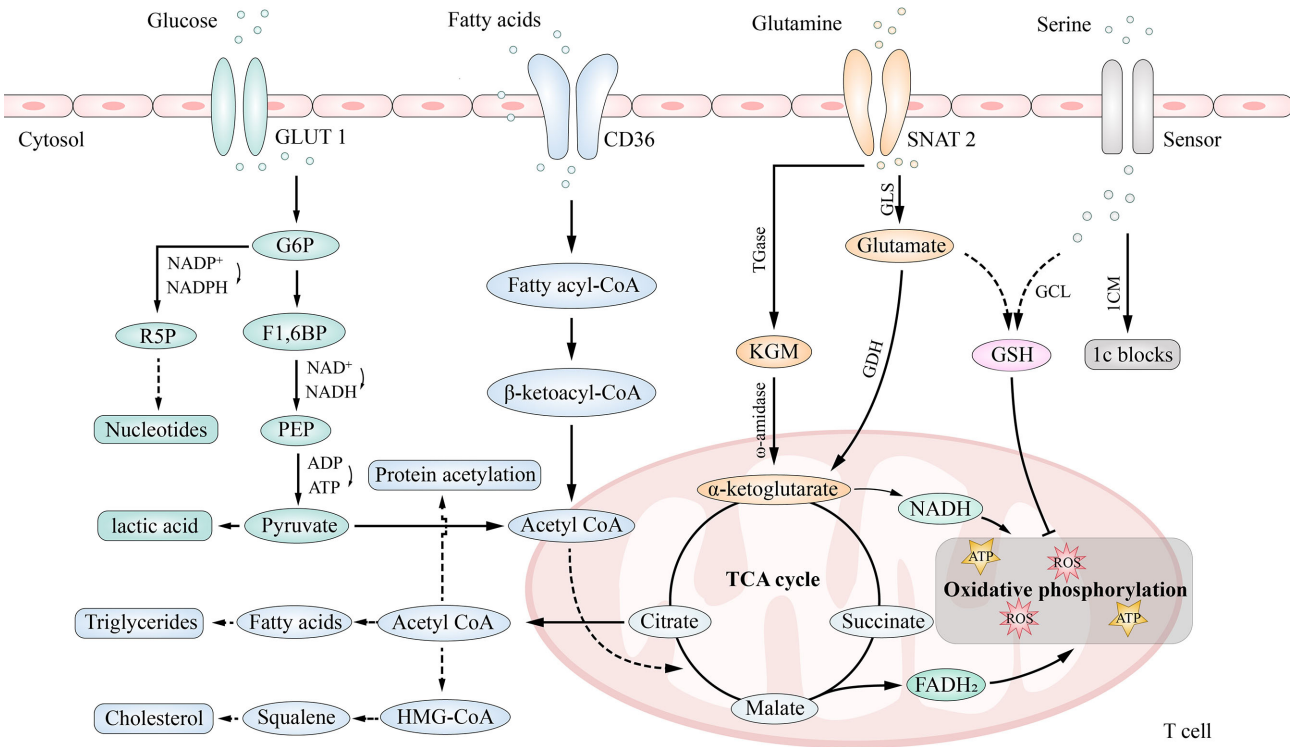

FIGURE 3 | Main metabolic pathways in T cells. T cells generate ATP by glycolysis and oxidative phosphorylation. Pentose phosphate pathway (PPP) is a branch of glycolysis pathway. Cells also generate energy by using glutamine (Gln), which is metabolized by glutaminolysis, and lipids(β-oxidation). Additionally, Serine enters the cell from the extracellular space and then, either enters one-carbon metabolism (1CM), which generating one-carbon (1C) building blocks for anabolism, or produces the ROS scavenger glutathione (GSH).

the Silencing information regulator 2 related enzyme 1/ proliferator-activated receptor γ coactivator-1α (Sirtuin1/PGC-1α) axis of NAD+ dependent deacetylase, enhancing the mitochondrial metabolism and invasion ability of prostate cancer cells (152).

Moreover, glycolysis of Treg cells can be inhibited by the binding of effector molecules on cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed death 1 (PD-1) found on the surface of Treg cells (153). In turn, the inhibition of glycolysis can also suppress the migration of Treg cells, and to meet their glucose demand, Treg cells upregulate insulin receptors (154). Several recent studies have indicated that Treg mobility is regulated by the metabolism of glucose through glycolysis, *via* glucokinase (GCK) activation and phosphoinositide 3-kinase (PI3K)- protein kinase B (Akt) pathways (155).

Fatty Acid Metabolism in Treg Cells

Fatty acids are transformed into acyl-coenzyme A (FA-CoA) in the cytoplasm, and FA-CoA enters mitochondria under the action of carnitine palmityl transferase I (CPT I) and carnitine palmityl transferase II (CPT II) (156). After β-oxidation, acetyl-CoA is formed and enters the tricarboxylic acid cycle. Fatty acid oxidation requires the involvement of four enzymes that produce NADH and xanthine dinucleotide (FADH2), which are used by the electron transport chain to produce ATP (157). Acetyl-CoA in the mitochondria is transported to the cytoplasm through the citrate-pyruvate cycle for the synthesis of fatty acids, triglycerides, cholesterol, and protein acetylation (158). The fatty acid synthesis requires acetyl CoA carboxylase 1 (ACC1) (159), and the cholesterol synthesis requires the participation of acetyl-CoA and hydroxymethylglutaryl-CoA (160).

Acetyl-CoA may promote the acetylation of Foxp3 protein by activating lysine acetyltransferases (KATs) and prevent Foxp3 protein ubiquitination degradation, thus helping to maintain the function of Treg cells (161), while NAD+/NADH activates the deacetylase activity of Sirtuin-1 and inhibits Foxp3 protein (162) (**Figure 4B**). CPT I, a rate-limiting enzyme in fatty acid oxidation (FAO), enhances FAO efficiency, and adenosine monophosphate activated protein kinase (AMPK) induces CPT I expression (163, 164). In fatty acid synthesis (FAS), acetyl-CoA is carboxylated into malonyl-CoA ACC1, the rate-limiting enzyme, and its phosphorylation can inhibit FAS (165). ACC1 inhibits the polarization of Treg cells and inhibition of ACC1 can promote the induction of Treg cells *in vivo* and *in vitro* (166). The inhibition of Fatty acid Binding protein 5 (FABP5) in Treg cells can trigger the release of mitochondrial DNA (mtDNA) and the subsequent cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) dependent type I interferon (IFN) signal transduction, thus inducing the production of the regulatory cytokine interleukin-10 (IL-10) and promoting the

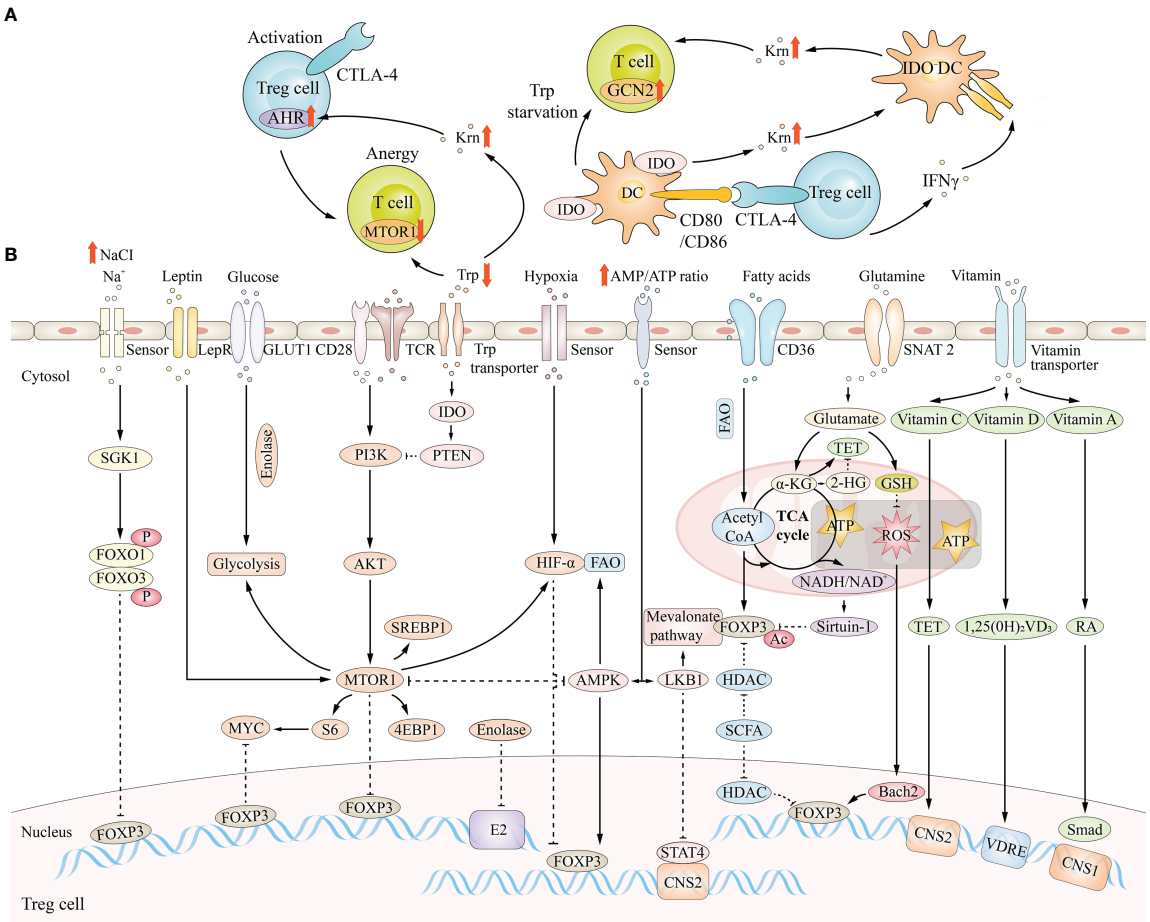

FIGURE 4 | Metabolic regulation in Treg cells. **(A)** Regulatory T cells have a crucial role in establishing an IFN-γ-rich environment that activates Indoleamine 2, 3-dioxygenase (IDO)- and IDO+ dendritic cells (DCs), either by forward signaling to DCs or by direct production of the cytokine. **(B)** Cell-intrinsic metabolic programs and environmental factors that can modulate FOXP3 expression ultimately affect Treg cells fate.

inhibitory activity of Treg cells, suggesting that FABP5 maintains mitochondrial integrity and can regulate the function of Treg cells (167).

TTreg cells and pTreg cells together constitute the Treg cell population *in vivo*, and they have different metabolic characteristics. PTreg cells can utilize both the mevalonate pathway and end products of glycolysis for the synthesis of fatty acids (168). During the induction of Treg cells in the periphery, increased expression of Foxp3 reprograms the cell to exert action in low-glucose environments (169). Therefore, the cell preferentially metabolizes lipids over glucose to maintain the suppressive function of pTreg cells, which maintains peripheral immune tolerance during tissue injury, even under metabolically challenging conditions (e.g., in ischemic tissues) (170). TTreg cells are induced and maintained by exogenous fatty acids (138). For example, Treg cells in adipose tissue express leptin receptors, and the leptin/leptin receptor axis induces an mTOR metabolic state to inhibit Foxp3 expression (171, 172) (**Figure 4B**). In addition, Treg cells in tissues are involved in suppressing inflammation and have regenerative functions in wound healing (173).

Short-chain fatty acids (SCFAs), including acetic, propionic, and butyric acids, are produced by dietary fiber and other undigested carbohydrates in the colon (174). At the molecular level, SCFAs inhibit histone deacetylase (HDAC) in Treg cells in colon tissue and enhance histone acetylation at Foxp3, promoting pTreg cells formation (175). Interestingly, SCFAs-induced IL-10+pTreg cells were not associated with suppression of the immune response in kidney hydronephrosis (C2RD), since the number of Th1 and Th17 cells increased as the number of regulatory T cells increased, suggesting a general increase in SCFAs-induced T-cell response in C2RD (176).

MTOR drives FAS and cholesterol production in Treg cells, while SCFAs can activate the mTOR pathway (177, 178). Studies have shown that the activity of mTOR- S6 kinase (S6K) in SCFAs-induced Treg cells is increased, revealing that SCFAs may regulate Treg cells through the mTOR-S6K pathway (173, 176). Meanwhile, with the activation of the mTOR pathway, SCFAs

also enhance the activity of signal transducers and activators of transcription 3 (STAT3) (176). In addition, impaired Treg homeostasis in mTOR deficient mice was associated with defective lipid biosynthesis (177).

OX40 can also trigger the proliferation of lipid-rich Treg cells in naive mice (168). It has been found that OX40/OX40L signaling occurring in the hepatocellular carcinoma microenvironment may be directly involved in the FAS of Treg cells (179).

In addition, researchers found high expression of peroxisome proliferation-activated receptor gamma (PPARγ) in TUM-Treg cells (Treg cells extracted from the tumor bed) (168). PPARγ is a nuclear factor that controls fatty acid uptake and FAS in adipose tissue (180), and it is believed that excessive FAS induces PPARγ expression.

Amino Acid Metabolism in Treg Cells

Amino acids also play a crucial role in Treg cell regulation. 2-Hydroxyglutarate (2-HG), the metabolite of glutamine, can lead to hypermethylation of the Foxp3 gene locus and then suppress Foxp3 transcription (181); this action further inhibits Treg generation. Tryptophan can produce kynurenine, which is able to combine with the aryl hydrocarbon receptor and then accelerate pTreg generation (182). Moreover, the enzymes that pTreg express participates in the synthesis of amino acids, which play an essential role in the proliferation and function of Treg cells (183, 184). Indoleamine-2,3-dioxygenase (IDO) is expressed on Treg cells and can inhibit mTOR signaling by phosphatase and tensin homolog (PTEN), thus promoting the generation of Treg cells (185). After tryptophan is metabolized by IDO, the metabolite kynurenine will bind to the transcription factor aryl hydrocarbon receptor (AHR), thereby activating Foxp3+ Treg cells (186), and these Treg cells will reverse or inhibit the activity of effector T cells. Kynurenine could also recruit other cell types to the regulatory response, including dendritic cells (DCs), in which the function of IDO is inhibited posttranslationally (187) (**Figure 4A**).

mTOR/AMPK Signal Pathway in Treg Cells

mTOR, a member of the phosphatidylinositol 3-kinase-related kinase family, induces the expression of multiple genes that play a key role in a variety of metabolic processes and is necessary for Treg differentiation, function, and survival (177) The increase in mTOR pathway activity has a negative impact on the generation and function of Treg cells (188) Transient TCR stimulation can induce the PI3K-Akt-mTOR signaling pathway to antagonize the expression of Foxp3 (189).

MTOR consists of the protein complex mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Increased mTORC1 activity is not only a typical characteristic of Th1 cells and Th17 cells differentiation (190) but also has a negative impact on the generation and function of Treg cells (188). The mTORC1 signaling pathway in Treg cells is inhibited by serine/threonine protein phosphatase 2A (PP2A). In the absence of PP2A, the glycolysis and oxidative phosphorylation rates of Treg cells were increased (191), as well as the expression of small subunit 1 (LAT1), a neutral amino acid transporter dependent on mTORC1 activity (177). The increase of glycolysis, oxidative phosphorylation, and LAT1 expression made Treg cells develop into Teff cells. However, whether PP2A has an effect on mTORC2 requires further study (192).

In fact, the relationship between mTORC1 and mTORC2 remains at odds. Aysegul V. Ergen et al. suggested that rapamycin inhibited mTORC1 but not mTORC2 (193). However, Rosner M et al. suggested that chronic treatment with rapamycin also inhibited mTORC2 activity (194). Takahito Kawata et al. argued that mTORC1 negatively regulates mTORC2 (195); Wang et al. believed that mTORC1 maintained mTORC2 activity, while mTORC2 negatively regulated mTORC1 signal activation (196). These differences may be related to researchers' experimental conditions and models, which need further study.

IDO activity reduces local tryptophan availability in the vicinity of Treg cells in the tumor microenvironment (197). A low concentration of tryptophan inhibits mTORC2 through protein kinase and prevents its phosphorylation of Akt, which helps maintain the inhibitory function of Treg cells, suggesting that the MTORC2-Akt signaling pathway has a negative regulatory effect on the differentiation of Treg cells (198).

AMPK can be activated by Treg cells and can inhibit mTORC1, reduce the expression of GLUT1 and promote fatty acid oxidation (199). Liver kinase B1 (Lkb1) is considered to be an AMPK-independent metabolic sensor in Treg cells because it stabilizes the expression of Foxp3 by changing the methylation state of CNS2 (200).

Other Metabolic Pathways in Treg Cells

As mentioned above, SGK1-mediated phosphorylation of FOXO1 and FOXO3 may lead to instability of Foxp3 under high salt conditions, thus reducing the inhibitory function of Treg cells (127). However, in another study, high salt only inhibited the function of tTreg cells and had little effect on TGF-β -induced iTreg cell function (201). This may be due to the different disease models selected by the authors.

Excessive urea will lead to uremia toxin production in the kidney, studies have shown that uremia patients have reduced Treg cells numbers and impaired function (202, 203) However, the specific mechanism of how urea acts on Treg cells remains unclear.

HIF-1α, which is activated either directly by hypoxia or *via* mTORC1, destabilizes Foxp3 expression (204). Moreover, HIF-1α is downregulated by 2-HG through inhibiting the activity of prolyl hydroxylase (205). The vitamin A metabolite RA increases Foxp3 gene expression by maintaining Smad activation (206). Vitamin C, together with Tet methylcytosine dioxygenase, increases Foxp3 expression (207). The vitamin D3 metabolite 1,25(OH)2VD3 stabilizes Foxp3 gene expression by maintaining the state of the VDRE region (208) (**Figure 4B**).

In addition, we summarized some drugs that target metabolic pathways; for example, 2-deoxy-d-glucose (2-DG), a drug that can compete with glucose in binding to hexokinase II (HKII) to inhibit cellular glycolysis activity and regulate the glycolytic pathway, induces Treg cell differentiation and suppression and alleviates the progression of systemic lupus erythematosus (SLE)

in TC mice (209). More drugs and further details are given in **Table 1**. These drugs can change the number and function of Treg cells by targeting their respective metabolic pathways, thus alleviating the progression of diseases.

The Role of Treg Cells in Acute Kidney Injury

Acute kidney injury (AKI) refers to a clinical syndrome in which renal function declines rapidly in a short period caused by a sudden or continuous decline in the glomerular filtration rate (212, 213). AKI has multiple etiologies, with hypovolemia, ischemia-reperfusion injury (IRI), exposure to nephrotoxic agents, and sepsis among the major causes. The immune response mediates the various stages of the occurrence, development, and repair of AKI, and Treg cells play a significant role in the entire developmental stage of AKI (210, 214). Although there is no clinical study of Treg cells in AKI, they have been indicated to protect and repair the kidney after AKI in animal models (215).

Abnormal metabolism in AKI affects the signaling pathways and the extracellular matrix environment, thereby affecting the differentiation of Treg cells. In AKI patients, due to mitochondrial damage and impaired FAO metabolism, as well as decreasing peroxisome proliferation-activated receptor alpha (PPARα) activity and decreasing peroxisome PGC-1 expression, the accumulation of triglycerides in AKI patients result in obvious lipid metabolism abnormalities (216–219). Treg cells express G protein-coupled receptor 43 (GPR43) in mice, which

TABLE 1 | Inhibitors of the metabolic pathways, their influence on Treg cells and disease applied.

Related Mechanism Pathways	Drugs	Pharmacological Effects	Influence on Treg Cells	Other biological Functions	Experimental Subject	Associated Disease	Reference
carbohydrate metabolism	CG-5	Decrease Glut1 expression	Increase Treg cells differentiation	*In vitro*: block glycolysis in CD4+ T cells	Lupus-prone mouse model	SLE	(220)
	2-DG	Compete with glucose in binding to HKII to inhibit cellular glycolysis activity and regulate the glycolytic pathway	Induce Treg cells differentiation and suppression	*In vivo*: dampen Th1 and Th17 cells development	Lewis rats	GBS	(221)
				Decreased ECAR and OCR in TC CD4+ T cells	TC mice	SLE	(210)
	DCA	Inhibit the dephosphorylation and deactivation of PDC to keep PDC active	Increase Treg cells expansion	Inhibit Th17 cells survival and proliferation	C57BL/6J mice	EAE	(222, 223)
	Metho-trexate	Act by competitive inhibition of dihydrofolate reductase to deplete One-carbon metabolism	Increase Treg cells expansion	Deplete purine biosynthesis enzymes	Patients with RA and healthy controls	RA	(224)
Lipid metabolism	Piogli-tazone	Activate PPARγ and high affinity binding to the PPARγ ligand-binding	Induce VAT Treg cells	Decrease the elevated serum levels of both creatinine and CK-MB	C57Bl/6 mice	Obesity	(225, 226)
	Sora-phen A	Lower cellular malonyl CoA, attenuate DNL and the formation of fatty acid elongation products derived from exogenous fatty acids	Induce Treg cells differentiation	*In vivo*: inhibit TH17 cell–associated inflammatory diseases	TACC1 mice	EAE	(211, 227)
	TOFA	Inhibit ACCA to decrease fatty acid synthesis and induce caspase activation	Inhibit Treg cells proliferation	*In vitro*: reduce the MCA38 cell viability in a dose-dependent fashion	Tumor-bearing mice	Tumor	(171, 228)
	Etomo-xir	Bind irreversibly to the catalytic site of CPT-1 to inhibit CPT-1 and up-regulate fatty acid oxidase activity	Abrogate Treg cells development and suppressive function	Reduce the production of pro-inflammatory cytokines in MOG specific T cells and promote their apoptosis	C57BL/6J mice	MS	(229, 230)
Amino acid metabolism	DON	Inhibit glutaminase and glutamine transporters	Promote Treg cells generation and frequency	Decrease IFN-γ production and proliferation in activated CD4+ and CD8+ T cells	C57BL/6 mice	Skin and heart transplantation	(231, 232)
mTOR/AMPK signal pathway	Rapa-mycin	Block mTOR downstream targets, such as p70S6K phosphorylation and activation	Enhance nTreg cells proliferation and function	Suppress proliferation of CD4+ CD25- effector T-cells	Patients with type 1 diabetes and healthy controls	Type 1 diabetes	(233–235)
	Metfor-min	Activate AMPK in liver cells leads to decreased ACC activity, induction of fatty acid oxidation, and inhibition of adipogenic enzyme expression	Induce Treg cells differentiation	Inhibit IL-17, p-STAT3, and p-mTOR expression	C57BL/6 mice	IBD	(236, 237)

2-DG, 2-deoxy-d-glucose; ACC, acetyl-coa carboxylase; CK-MB, creatine kinase-mb; DCA, dichloroacetate; DON, 6-diazo-5-oxo-L-norleucine; EAE, experimental autoimmune encephalomyelitis; ECAR, extracellular acidification rate; GBS, Guillain-Barré syndrome; IBD, inflammatory bowel disease; MS, multiple sclerosis; OCR, oxygen consumption rate; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TOFA, 5-tetradecyl-oxy-2-furoic acid.

when bound to SCFAs results in enhanced differentiation and function (238). Recent work by Field C. et al. demonstrated that inhibiting the lcFA-FAO metabolic pathway may be more favorable as an approach to increasing the suppressive function of Treg cells (167). It is plausible that various intermediates produced during FAO, such as acetyl-CoA, and reduced FADH/NADH, could interfere with Treg cell function through yet unknown mechanisms (167). Mitochondrial dysfunction is also one of the important characteristics of AKI (239); the accumulation of cytochrome C in the mitochondria causes the oxidative respiratory chain to fail to proceed normally, and mitochondrial respiration is weakened, which further affects the metabolism of lactic acid in the kidney tissue, causing metabolic acidosis (240). In this situation, Treg cells can convert lactate to pyruvate. Moreover, Foxp3 can modulate LDH to prevent lactate formation and form pyruvate (241). While lactate may negatively impact T-cell proliferation as a whole, it does not impact Treg cell immunosuppression (242). There are also serious changes in protein metabolism in AKI, and the rapid catabolism of proteins leads to a negative nitrogen balance (243). In a long-term high catabolic state, the activity and metabolism of Treg cells will be affected, resulting in a weakened immune response and anti-infection mechanism (244). Active metabolism in AKI will cause hypoxia, which is associated with increased levels of the HIF-1 complex (245). HIF-1α forms a complex structure with its counterpart HIF-1β, which then binds to specific hypoxic response elements (HREs) (243) to influence Treg cell metabolism/function.

Ischemia–Reperfusion Injury (IRI)

The phenomenon that after blood reperfusion is resumed under certain conditions, some animals or patients have cellular functional metabolic disorders and structural damage that are not reduced but aggravated this is called IRI (246). IRI is a vital cause of AKI and a serious complication secondary to major surgery (247). Endogenous Treg cells can mediate immune responses, reduce the existence of costimulatory molecules after renal IRI, and improve renal IRI (248). Oxidative stress, inflammation, and apoptosis are well-known characteristics of the kidney after AKI (249). *In vitro* and in mice, IRI causes persistent mitochondrial damage and energy loss (250), increased reactive oxygen species (ROS) generation, and decreased ATP synthesis in iTreg/pTreg cells. Upregulation of Treg cell lipogenic genes in the kidneys of IRI mice leads to an associated elevation of lipid deposition (250, 251), indicating the presence of excessive FAS in Treg cells of IRI mice. The tryptophan metabolite kynurenine was increased in plasma and kidney tissues from IRI mice (250, 251). After the elimination of pTreg/iTreg cells in renal IRI mice by anti-CD25 + antibody *in vivo* and *in vitro*, renal injury and inflammation were aggravated, and renal function and mortality continued to deteriorate (252). Adoptive transfer therapy of iTreg cells after IRI can increase the repair rate of mouse kidneys (253). In addition (254), Treg effectively prevents the accumulation of neutrophils and mononuclear phagocytes during renal reperfusion, and its pathway has not been fully understood yet.

Sepsis-Induced Acute Kidney Injury

Severe sepsis can also lead to AKI (255). Although the pathophysiological mechanisms are not fully understood, it is clear that the inflammatory cascade characteristic of sepsis is associated with AKI (256). Different from AKI caused by IR, renal tubular cells in septic AKI are slightly vacuolated and a large number of renal tubular cells undergo apoptosis, without obvious renal tissue necrosis (257). In the septic AKI mouse model, renal tubular cell apoptosis was reduced and renal function was significantly improved after Treg cells were removed, which was completely contrary to the findings in IR mice, since the depletion of Treg cells led to the deterioration of renal function after IR (254). There was no significant change in renal function after IL-10 blockade in IR mice, but in septic AKI, renal function was significantly improved after IL-10 blockade, suggesting that IL-10 reduced the proliferation of Treg cells, thereby improving the survival rate of patients with sepsis (258). The opposite role of Treg cells in septic-induced AKI and IR-induced AKI maybe that Treg cells only play a protective role in aseptic inflammation-mediated AKI, which needs further study to explain.

Cisplatin Nephrotoxicity

Cisplatin, one of the most effective chemotherapeutic drugs, can induce damage in the renal vasculature, which leads to reduced blood flow and ischemic injury in the kidney, thereby inducing an AKI model (259). Cisplatin has been widely used to treat malignant tumors of various organs. It is known that cisplatin concentrates on epithelial cells in the proximal tubule S3 segment (260), where it induces necrotic and apoptotic cell death and is associated with an extensive pro-inflammatory immune response. Salt may ameliorate symptoms (261, 262). Some studies have shown nephrotoxicity in clinical trials of cisplatin chemotherapy (259), and the use of cisplatin is limited because of its nephrotoxicity (263). CD4+CD25+Foxp3+ Treg cells showed a protective effect in the cisplatin nephrotoxicity test in mice (264). Oxidative stress has been considered an essential component that results in cisplatin nephrotoxicity both *in vivo* and *in vitro (*265, 266). Cisplatin aggregates in the mitochondria of renal epithelial cells and disrupts the respiratory chain, resulting in a decrease in ATP production and an increase in ROS production, which cause inhibition of mitochondrial activation (259, 263, 264). Mitochondrial dysfunction and oxidative stress exist in cisplatin-mediated acute renal injury (267), which causes impaired synthesis of Treg cells (268).

With the in-depth understanding of AKI disease and Treg cells, AKI, which was previously thought to have little relationship with immune abnormalities, is partly caused by abnormal metabolism of immune cells, such as Treg cells. However, the immune cells involved in AKI disease are not only Treg cells. What is the proportion of Treg cells interacting with other immune cells in AKI disease? Does targeted Treg therapy for AKI affect other abnormalities in immune cell

function? How do Treg cells affect intrinsic renal immune response in cisplatin-induced AKI? These all require follow-up research.

The Role of Treg Cells Metabolism in CKD

CKD is the chronic process resulting from a variety of kidney diseases, as well as a heterogeneous illness influencing the morphology and function of the kidney (269). A diverse set of components can activate various molecular mechanisms of kidney damage, such as genes, metabolism, autoimmunity, malignancy, toxins, and the environment (270). All these injuries contribute to different categories of vascular, glomerular, and tubulointerstitial renal diseases, which culminate in decreased kidney function and result in CKD syndrome (271).

Tissue damage in CKD is directly or indirectly mediated by the immune system, and the dysfunction of immune cells promotes CKD inflammation and renal fibrosis (272). Treg cells play a protective role in CKD by inhibiting inflammation and immunity, but the number of pTreg cells in the peripheral blood of CKD patients is significantly reduced (273). TGF-β1 is an inducer of Treg cells, which are released after renal cell injury (274). Treg cells can be transformed into Foxp3+IL-17+ T cells under inflammatory conditions in the kidney and then produce a large amount of TGF-β1, leading to CKD inflammation and renal fibrosis (275) (**Figure 5**). A study illustrated that an elevated ratio of Th17 cells and a reduced ratio of Treg cells exist in CKD patients, reflecting that an enhanced Th17/Treg cell rate is related to the progression of CKD and the severity of kidney disease (48).

The state of reduced renal function that results from CKD causes marked alterations in Treg cell metabolism. Typical alterations include increased intracellular ROS (276), high levels of 8-hydroxy-2 deoxyguanosine (8-OHdG) (277), enhanced carbohydrate metabolism (278), and abnormal lipid metabolism (279). ROS stabilize the nuclear factor of an activated T cell (NFAT) in the nucleus and bind to CNS2 to promote Foxp3 expression (280), and interestingly, it can directly inhibit the enzymatic activity of several elements in the cellular respiratory chain, while complex III per se is key to promoting Treg cell suppressive function (242, 281). 8-OHdG (277) is a marker of oxidative DNA stress. Oxidative stress can induce the activation of the PI3K/Akt/mTOR signaling pathway and induce the phosphorylation of mTOR in CKD patients (282), increasing mTOR activation in cells, which negatively affects the protective effect of Treg cells on the kidney. Dietary fiber, a kind of carbohydrate, can be converted into SCFAs, which are a main source of nutrients for Treg cells. Therefore, a high fiber diet can potentially attenuate systemic inflammation and CKD progression (278). Dyslipidemia in CKD patients is largely due to changes in low-density liptein cholesterol (LDL-C) levels (283). Researchers have indicated that proteasome inhibition by ox-LDL leads to CD4+CD25+ Treg apoptosis, affecting the number and suppressive capability of these Treg cells in chronic hemodialysis (HD) patients (284). In addition, in Ldlr−/− mice, Treg cells were found to control very-low-density lipoprotein (VLDL) levels by regulating the lipoprotein binding protein, sortilin 1, protecting against the development of CKD (279).

Diabetic Nephropathy

Diabetic glomerulosclerosis is a leading factor of CKD and end-stage renal disease (ESRD), and an autoimmune renal disease secondary to diabetes mellitus type 1 (T1DM) or diabetes mellitus type 2 (T2DM) (285). Diabetic nephropathy (DN) is characterized by glomerular hypertrophy, basement membrane thickening, the accumulation of extracellular matrix components, and kidney inflammation, which are crucial in promoting the development and progression of DN (286). Recently, the morbidity and mortality of DN have been increasing rapidly worldwide (287–290). Some studies have demonstrated that there is an imbalance in the Treg/Th17 cell ratio in patients with T1DM, which may be related to the progression of microangiopathy (291). Treg cells are correlated with diabetes and DN, and T2DM patients have a low level of Treg cells relative to Th1 or Th17 (292, 293). The increase in the number of Th17 cells leads to an increase in the release of pro-inflammatory factors such as IL-17, which triggers local tissue inflammation and promotes the development of DM complications (291). The level of Treg cells in the peripheral blood of patients with type 2 diabetic nephropathy (T2DN) decreased, and the use of anti-CD25 antibodies to eliminate Treg cells aggravated kidney damage, while adoptive transfer of Treg cells reduced blood sugar and improved diabetic nephropathy (210).

The progression of diabetic nephropathy is also influenced by oxidative stress (294), lipid metabolism (295), and mTOR activation (296). Excessive ROS can damage mitochondria and increase the production of lipid peroxides (297) in Treg cells. Normally, sodium-dependent glucose transporter 2 (SGLT-2)-reabsorbed glucose is utilized by mitochondria to synthesize ATP by oxidative phosphorylation (OXPHOS) (298). However, mitochondrial dysfunction occurs following inhibition of OXPHOS, which results in decreased ATP production (299), and loss of mitochondrial membrane potential ($\Delta\Psi m$) and can ultimately lead to increased ROS from various sites of the electron transport chain (ETC), including complex III, which is key to promoting Treg cell suppressive function (298). Interestingly, Treg-specific knockout of complex III is associated with reduced immunosuppressive capacity and increased DNA methylation status, but it has no relevance to FOXP3 expression (281).

Abnormal metabolism and accumulation of lipids in the kidney play a crucial role in the pathogenesis of DN (300). Abnormalities in lipid metabolism make Treg cells unable to obtain sufficient energy to complete their functions (301). During a state of high ATP consumption, there is a proportional increase in intracellular AMP and HIF-1α (302) (**Figure 5**). The proportional increase in AMP leads to AMPK phosphorylation and activation by liver kinase B1 (Lkb1) (300), which is crucial for Treg cell metabolism and function. Excessive HIF-1α leads to decreased Treg differentiation, as HIF-1α can

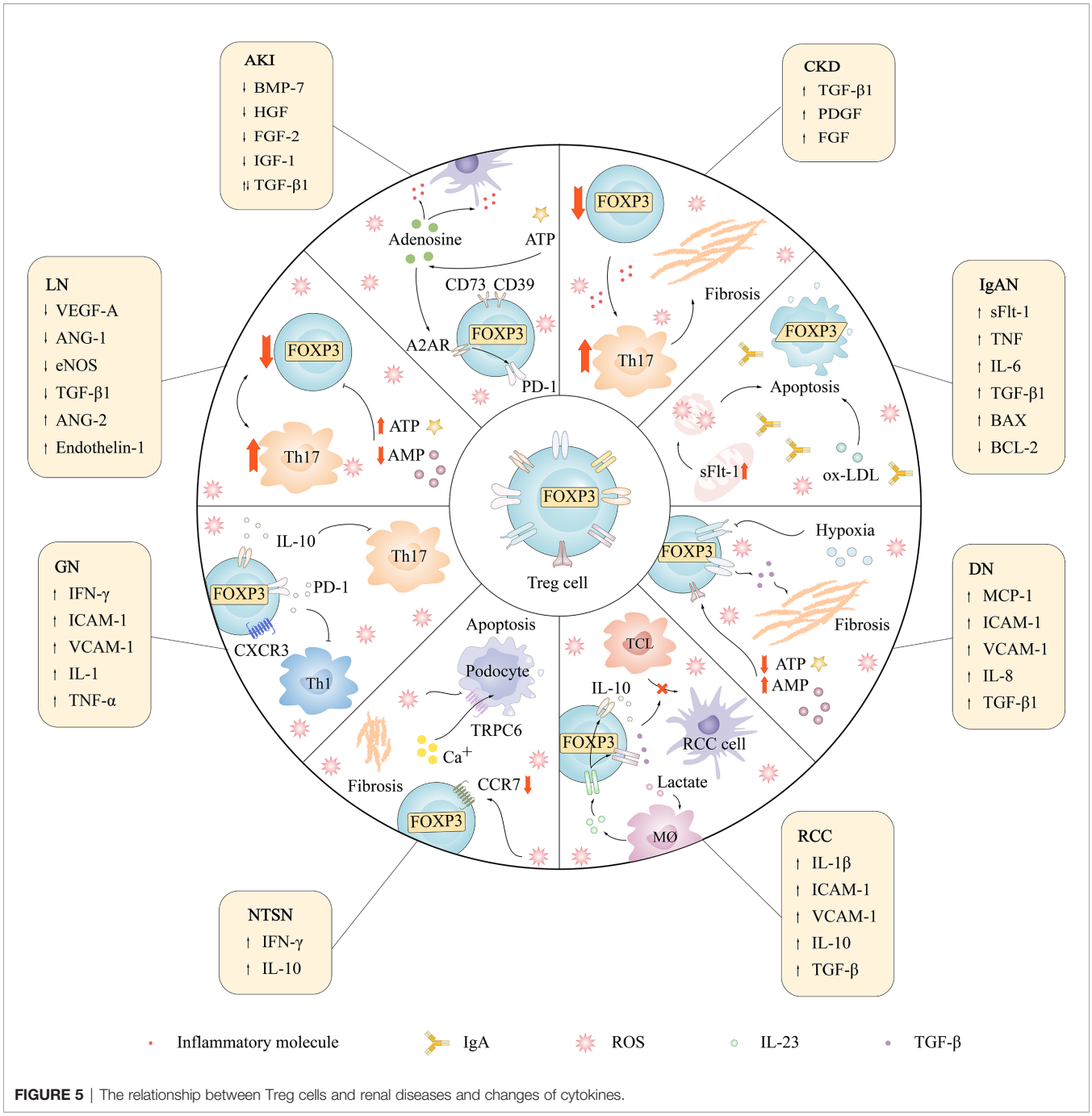

FIGURE 5 | The relationship between Treg cells and renal diseases and changes of cytokines.

promote FOXP3 ubiquitination and subsequent proteasome degradation (136, 146)

Activation of the mTOR pathway is upregulated in renal diseases such as DN (303). The PDK1/Akt/mTORC1 signaling pathway is activated in the glomerular mesangial cells of patients with DN, which induces the high expression of S6K1 and 4EBP1 (304), causing excessive cell proliferation and hypertrophy (305). mTORC1 activity plays an important role in Treg cell activation, function, and increased metabolic demands. mTORC2 is also involved in regulating hypertrophy of mesangial cells induced by high glucose, and inhibition of mTORC2 can reduce the phosphorylation levels of PKC II and Akt, suppress mTORC1 activity, and prevent mesangial cell hypertrophy (306). Some data suggest that mTORC2 inhibition promotes Treg cell activation status, Th2-like differentiation, and immunosuppressive function (142). In addition, the PI3K/AKT/mTORC1 signaling pathway is involved in extracellular matrix (ECM) deposition and tubulointerstitial fibrosis. On the one hand, mTORC1 stimulates the proliferation of fibroblasts and the synthesis of collagen; on the other hand, mTORC1 increases the expression of TGF-β1, which

mediates the development of DN fibrosis (307, 308). Therefore, blocking the mTOR pathway can significantly increase the number of Treg cells, which promotes the improvement of diabetic nephropathy.

Although these studies suggest a possible link between Foxp3+ Treg cells and the progression of CKD disease, however, due to the complex etiology of CKD, the proportion of immune abnormalities is not known at present, and we only describe CKD from the perspective of abnormal metabolism of Treg cells. Further studies are still needed to understand the exact role of Treg in more targeted treatment plans.

The Role of Treg Cell Metabolism in Lupus Nephritis

SLE is a prototypic systemic autoimmune disease, as well as a multisystem heterogeneous disease (309). Immune abnormalities interact with various other factors, leading to a decrease in T lymphocytes and a decline in Treg cell function in SLE (310). LN is an autoimmune disease secondary to SLE, characterized by cell proliferation and immune complex deposition, accompanied by significant clinical manifestations of renal damage (311). Studies have demonstrated that the metabolic patterns of Th17 cells and Treg cells affect the balance of both cell types (312) (**Figure 5**). Th17 cells mainly rely on glycolysis to provide energy (313), while Treg cells mainly rely on fatty acid oxidation pathways to provide energy (314). Inhibition of glycolysis and fatty acid oxidation can promote the development and differentiation of Treg cells, and inhibit the differentiation of Th17 cells (315). Deficient or scarce Treg cells have been found both in murine models of SLE and in human SLE studies (316). Studies have shown that peripheral blood Treg cells decline in number and abnormal Treg cell phenotypes are present in SLE patients (317). Sirolimus has been shown to be an effective retention steroid for the treatment of renal and non-renal manifestations of SLE (318).

Cellular metabolism regulates the differentiation and function of T cells, thus participating in the initiation and progression of SLE inflammation. These characteristics are as follows:

(1) Mitochondrial hyperpolarization: T cells are chronically mobilized, and their mitochondria are hyperpolarized in SLE patients and lupus-prone mice (319, 320); thus, the high mitochondrial transmembrane potential will be expressed in Treg cells from SLE patients (138). The hyperpolarization of mitochondria affects the process of reducing oxygen to water by electron and proton transfer during oxidative phosphorylation, leading to increased oxygen consumption and ROS generation, thereby reducing energy synthesis (320, 321). ROS can oxidize proteins and cause DNA mutations, causing cell damage and cell aging, and excessive ROS will attack the protease complex on the oxidative respiratory chain, leading to mitochondrial dysfunction, reducing ATP production, inhibiting mTORC1, and promoting the differentiation of T cells into Treg cells (319, 320, 322), which is a vital cause of Treg cell functional deterioration. Furthermore, in lupus-susceptible mice, blocking of Rab geranylgeranyl transferase with Rab geranylgeranyl transferase inhibitor (3-PEHPC) could reverse dynamin-related protein 1 (Drp1) consumption, mitochondrial accumulation, and nephritis, confirming that HRES-1/Rab4 regulation of mitochondrial homeostasis is the pathogenesis and potential therapeutic target of SLE (323).

(2) Hyperactivated carbohydrate metabolism: Excessive activation of glucose metabolism leads to the accumulation of energy in T cells (324). Increased ATP content weakens AMP-AMPK signal transduction and then activates mTORC1 in SLE patients (325). The enhancement of mTORC1 activity could inhibit Treg cell activation and function (326).

(3) Enhanced lipid synthesis: Acetyl-CoA is produced by the β oxidation of fatty acids while cholesterol is generated *via* the catalysis of hydroxy methylglutaryl coenzyme A (HMG-CoA) reductase (327). The key enzyme ACC (acetyl-CoA carboxylase) that inhibits the FAS and cholesterol synthesis can also inhibit the expression of Th17 and promote the differentiation of Treg cells (131), thereby reducing the autoimmune response of SLE patients. Studies have shown that the synthesis of lipid rafts (including glycosphingolipids and cholesterol) in SLE patients is increased, and CD4+ T cells from active SLE patients have more lipid raft synthesis than CD4+ T cells from healthy individuals (328), which influences the proliferation and function of Treg cells. In particular, cholesterol biosynthesis was demonstrated mechanistically to be important in promoting Treg cell activation, proliferation, and function (329).

(4) Amino acid dysfunction: Catabolism of the amino acid tryptophan generates metabolic intermediates such as kynurenine that can bind the aryl hydrocarbon receptor on T cells and promote pTreg cell induction (182). The binding of CTLA4 on Treg cells to the costimulatory molecules CD80 and CD86 on antigen-presenting cells (APCs) induces amino acid-consuming enzyme (such as IDO and arginase 1) expression in Treg cells (330). The activities of these enzymes reduce the availability of amino acids (for example, tryptophan, arginine, histidine, and threonine) to surrounding T cells, inhibiting mTOR signaling *via* the lipid phosphatase PTEN and blocking the proliferation of Teff cells, thus promoting Treg cell induction (183).

(5) Highly activated mTOR pathway: High activation of the mTOR pathway may increase protein synthesis, leading to protein accumulation in Treg cells (331), enhancing cell metabolism, promoting the autophagy system of Treg cells, and leading to dysfunction and reduced differentiation of Treg cells (332, 333). A study demonstrated that mTORC2 plays a proinflammatory role in blocking Treg generation in SLE. mTORC2 can activate the Akt signaling pathway and promote glucose metabolism, while Treg cells are mainly powered by FAO (334). Therefore, the activation of mTORC2 will inhibit the development and differentiation of Treg cells, and mTORC2 blockade is important to lineage stabilization and functional maturation of Treg cells except for Treg cell differentiation. Additionally, Treg cells effects

appear to be significantly modulated in humans compared to mice, which may be explained by the fact that blocking mTOR with rapamycin can complete nephritis blocking in several lupus-susceptible strains without affecting Treg cells in mice.

Dietary habits and nutritional factors can regulate Th17/Treg cell balance by affecting T cell metabolism. A balanced diet may help prevent and manage SLE. A low cholesterol diet could improve Th17/Treg cell balance by activating liver x receptor α and β (LXRs), which are nuclear receptors modulating cholesterol metabolism (335). High glucose intake can induce Th17 cells by upregulating mitochondrial ROS in T cells, thus enhancing self-immunity (336). Long-chain fatty acids enhance Th17 cell differentiation, whereas short-chain fatty acids derived from a fiber-rich diet expand Treg cells and reduce IL-17 production (315).

The Role of Treg Cell Metabolism in Other Kidney Diseases

In addition to the three kidney diseases mentioned above, there are also several related to Treg cell metabolisms, such as IgA nephropathy, glomerulonephritis, nephrotoxic serum nephritis, and renal cell carcinoma.

IgA Nephropathy

IgA nephropathy (IgAN) is an autoimmune disease, and its immune pathogenesis is a multilevel process (337). In patients with lgAN, the level of abnormally glycosylated circulating IgA increases, which induces the formation of autoantibodies of IgA and IgG and then forms a circulating immune complex of autoantibodies of IgA and lgG (338). These immune complexes contribute to mesangial cell proliferation and excessive production of extracellular matrix, cytokines, and chemokines, eventually leading to glomerular sclerosis (11, 19, 339–341).

In IgAN patients, serum soluble fms-like tyrosine kinase 1 (sFlt-1) levels are remarkably enhanced, and subsequently, sFlt-1 raises the mitochondrial membrane potential, facilitating mitochondrial-mediated apoptosis (342). A study has shown that patients with less histological injury and proteinuria have higher urinary mtDNA copy numbers, which suggests that mitochondrial damage occurs in the early stage of IgAN (209, 343).

A study showed that patients with IgAN have a higher prevalence of dyslipidemia (319). Excessive ox-LDL content will weaken the immunosuppressive function of Treg cells, and ox-LDL can induce apoptosis of Treg cells by activating P38/MAPK (344), mitochondria (345), and lysosome signaling pathways (**Figure 5**). In addition, ox-LDL can induce cells to produce endogenous ROS (340), which increases the production of lipid peroxides in Treg cells. The damage of oxidized lipids to cells leads to abnormal cell lipid metabolism and impaired exportability, thus inducing apoptosis of Treg cells (341, 346).

Some studies have shown that in rats with IgAN, p-mTOR and phosphorylation of p70 S6 kinase (P-S6K1) are upregulated, which indicates that the mTOR pathway is highly activated and participates in the development of IgAN (343, 347–350).

Glomerulonephritis

GN encompasses a wide variety of kidney diseases (351). Many GNs due to immunologically mediated glomerular damage result in renal dysfunction and proteinuria (352). Treg cells are essential for the autoimmune pathogenesis of GN in the kidney (353), as such, the activation of STAT3 in Treg cells induces the expression of CC chemokine receptor 6 (CCR6) on the cell surface (354) and promotes the transport of Treg cells to the inflammatory area of Th17 cells that also highly express CCR6 through the tight colocalization of CCR6 (355), thereby suppressing the immune response of pathogenic Th17 cells during the GN process. Treg cells can also use CC chemokine receptor 7 (CCR7) expressed on their own surface to migrate to the site of CCR7+ T cell activation and inhibit the activation of T cells (356). Treg cells with defective IL-10Ra expression can significantly reduce the production of IL-10 during GN, while Treg cells can significantly downregulate Th17 cells through IL-10 receptor signal transduction (357) (**Figure 5**). IL-10Ra is a key component that controls the immune function of Th17 cells in the GN process, and a large number of IL-10Ra-deficient T cells differentiate into Th17 cells, which aggravates the condition of GN (349). In addition, IL-6 stimulates Treg cells to produce cells, which have both pro-inflammatory and anti-inflammatory effects (329). ITreg cells can secrete the anti-inflammatory factors IL-10 and IL-35, as well as the pro-inflammatory factor IL-17 (350), and by inhibiting Th2 cells with anti-inflammatory effects, they mediate pro-inflammatory effects in GN (356). Treg cells also inhibit Th1 cells. Nosko et al. showed that Treg cells in which the transcription factor T-bet is activated enhance the ability of Treg cells to downregulate Th1 cell responses by inducing the expression of CXC chemokine receptor type 3 (CXCR3) (358). Studies have also reported that Treg cells inhibit GN driven by the Th1 immune response through the PD-1/programmed cell death-ligand 1 (PD-L1) pathway, and mediate renal protection (359) (**Figure 5**).

The kidney is rich in mitochondria, which meet its high energy demand through the oxidative phosphorylation process (360). Several studies have demonstrated that in glomerulonephritis nephropathy rats, the number of mitochondria in tubular epithelial cells is reduced and cristae structure is destroyed. Albumin and free fatty acid stimulation of cultured human tubular cells *in vitro* increased mitochondrial ROS, which led to mitochondrial damage (361, 362). Generating enough acetyl-CoA to feed into the Krebs cycle and then generating sufficient ATP through the mitochondria is an important process in Treg cells (363). Although the mechanism was not uncovered, the induction of Foxp3 in iTreg cells correlated with increased expression of mitochondria-associated genes (364) Mitochondrial dysfunction can cause abnormal metabolism of Treg cells, and the protective effect of Treg cells on the kidney is limited.

In addition to oxidative stress, GN can also undergo the deposition of lipid-associated molecules, including oxidized cholesterol, apolipoprotein (Apo), and ox-LDL. Oxidative and

helix-related molecules accumulate on the glomerular basement membrane (GBM) along with other molecules (320, 365). ox-LDL can induce apoptosis of Treg cells by activating P38/MAPK (366). AMPK activity together with protein phosphatase 2A (PP2A) restrains the mammalian target of rapamycin complex 1 (mTORC1) signaling, thus promoting Foxp3 expression and the proliferation of Treg cells (333, 367).

In patients with little immune deposition glomerulonephritis, a large amount of the glomerulus and even more cells in the tubulointerstitial area express mTOR (368). Upon Treg cell activation, the increase in mTOR signaling upregulates interferon regulatory Factor 4 (IRF4) (367), which further promotes genes for cellular growth, glycolysis, OXPHOS, and fatty acid metabolism, among others (369). These data suggest that promoting mTORC1 activity can promote the activation and function of Treg cells and support glycolysis and the OXPHOS metabolic pathway (369).

Nephrotoxic Serum Nephritis

Nephrotoxic serum nephritis (NTSN) nephritis is a type of focal segmental glomerulosclerosis (FSGS) that occurs in many kinds of renal disease and ultimately leads to kidney inflammation and fibrosis (370). The histological features of nephrotoxic serum nephritis are the accumulation of macrophages, cholesterol, and cholesteryl esters, as well as the deposition of extracellular matrix in sclerotic glomeruli. The disease is characterized by rapid inflammation and infiltration of leukocytes in the kidneys (371). In a mouse model with NTSN, Treg cells are endogenous immunosuppressive cells that protect kidney tissues from inflammation-mediated damage (372).

Increased mitochondrial ROS generation and mitochondrial oxidative damage are present in the glomeruli of patients with nephrotoxic serum nephritis, inhibiting the protective effect of Treg cells on the kidney (373) so that the expression of CCR7 on Treg cells is downregulated and affects the recruitment of Treg cells to the lymph nodes of NTSN (374). ROS can change the expression of transient receptor potential cation channel, subfamily C, member 6 (TRPC6) protein, or TRPC6 channel activity in kidney cells, thereby regulating Ca2+ signal transduction and mediating podocyte apoptosis (375) (**Figure 5**). Research has shown that the knockout of TRPC6 plays a protective role in NTSN (376). The accumulation of ROS in mitochondria induces mitochondrial dysfunction and apoptosis, eventually causing glomerular disease (377), which includes nephrotoxic serum nephritis. In addition, experiments have shown that the NTSN of CCR7 knockout mice is more serious, and abundant inflammatory cell infiltration was observed (378).

Renal Cell Carcinoma

Renal cell carcinoma (RCC) is one of the most common tumors and arises from the renal parenchyma urinary tubular epithelial system (379). There are many pathological types of renal cell carcinoma, of which clear cell renal cell carcinoma (ccRCC) is the most common (380). Renal cancer cells have a vigorous metabolism competing with immune cells for nutrients, thereby changing the metabolic mode of immune cells, and subsequently affecting their function and differentiation (381). Furthermore, substances produced by renal cancer cells, such as lactic acid and ROS, can cause damage to immune cells and reduce their antitumor effect (382).

Deletion of the von Hippel-Lindau (VHL) tumor suppressor gene in renal cancer cells leads to the accumulation of HIF-1α and an increase in Clut2 expression, which promotes aerobic glycolysis in renal cancer cells and leads to metabolic reprogramming of renal cancer cells (383). This aerobic glycolysis mode of cancer cells is called the "Warburg effect" (382). Hypoxia-mediated expression of HIF-1α selectively upregulates the expression of inhibitory ligands, such as PD-L1, and promotes T cell immunosuppression (384). Such hypoxia-mediated changes also promote Treg cell differentiation and homeostasis (385). The propagation of kidney cancer cells is highly dependent on glycolysis (386), which affects the function of Th17 cells that also rely on glycolysis but does not affect the survival of Treg cells that depend on fatty acid oxidation.

The proliferation of renal cancer cells consumes a lot of glutamine and competes with the surrounding macrophages for glutamine in the extracellular matrix (387). This is related to the promotion of the expression of ASCT2 and SLCIA5 by MYC (388) in renal cancer cells, leading to a large amount of glutamine being transported into the cell, which in turn activates the PI3K-Akt-mTOR signaling pathway and promotes the metabolism of glutamine and the synthesis of protein (389). The metabolic waste products of amide will promote the differentiation of Treg cells (382). In addition, lactate can induce the secretion of IL-23 by macrophages infiltrated by tumor cells (169). IL-23 activates the JAK/STAT pathway of Treg cells, increases the phosphorylation of STAT3, activating the proliferation of Treg cells, promotes the expression of IL-10 and TGF-β, thus inhibiting the killing effect of TCL on renal cancer cells (390, 391) (**Figure 5**).

IDO is overexpressed in a variety of cancers (392–394). IDO activity reduces local tryptophan availability in the proximity of Treg cells (395). A low concentration of tryptophan activates a stress response pathway in Treg cells through the protein kinase general control nonderepressing-2, which inhibits mTORC2 and prevents it from phosphorylating Akt, plus contributes to the maintenance of Treg suppressive function (395–397).

A large amount of lipid accumulation is found in renal cell carcinoma. HIF-1α in renal cell carcinoma inhibits the activity of CPT1 on the outer mitochondrial membrane and prevents the β-oxidation of fatty acids (398), which is important for the differentiation of Treg cells, and its blockade could prevent the accumulation of this immunosuppressive population (199). AMPK in renal cancer cells, a sensor of nutrient deprivation and metabolic stress, is inactivated in the AMPK-GATA3-ECHS1 signaling pathway (399), inhibiting the expression of the transcription factor GATA3 and leading to a decrease in the synthesis of ECHS1. AMPK activation can promote the formation of Treg cells while reducing Th1 and Th17 cells (199), thus, leading to unwanted immune modulation in the context of RCC. In addition, the inactivation of AMPK reduces the activation of adipose triacylglyceride lipase (ATGL) and

inhibits the decomposition of triglycerides into fatty acids, thereby inhibiting the catabolism of fatty acids; at the same time, the inactivation of AMPK weakens the inhibitory effect on acetyl-CoA carboxylase (ACC) and promotes the synthesis of fatty acids. Berod et al. showed that inhibition of ACC1 restrains the differentiation of Th17 cells and promotes the differentiation of anti-inflammatory Foxp3+ Treg cells (400, 401).

CONCLUSION

The immune system searches for pathogens and other danger signals *in vivo* at all times. In recent years, the field linking immunity and metabolism has expanded rapidly (402). In renal diseases, T cells are involved in different abnormal metabolic pathways, such as increased oxidative stress, mitochondrial dysfunction, enhanced glycolysis, abnormal lipid synthesis, glutaminolysis, and highly activated mTOR, which all influence Treg cell proliferation and differentiation.

Inhibition of different metabolic pathways *via* drugs can modify Th17 cells to Treg cells. For example, inhibition of glycolysis (209, 403–405), lipid synthesis (328, 406, 407), and mTOR signaling (211, 408, 409) can control inflammation and alleviate disease activity in lupus mice and SLE patients (**Table 1**). Short-chain fatty acids, which are derived from a fiber-rich diet, can downregulate IL-17 production and amplify Treg cells (410–412). Treg cell metabolism therapy has great potential in many forms of renal diseases. Promoting the proliferation or function of Treg cells by mediating various metabolic pathways are also possible treatments in the future for multifarious diseases that affect the kidney.

It is important to note that many studies involving the immune metabolism of Treg cells have been based on model animal studies (mostly mice) or *in vitro* human cells. Pharmacological or genetic manipulation of metabolic processes in mouse models of human autoimmune diseases offers new opportunities to treat human diseases, but it is not clear how Treg cell immune metabolism is altered in many people with kidney disease. Therefore, we can make inferences based on published articles, but cannot be sure that these experimental results are consistent in animal and human kidney disease. This is a knowledge gap in Treg cell immune metabolism. Treg metabolism may become a target for future treatment of various kidney diseases.

AUTHOR CONTRIBUTIONS

ZH, KM, and HT were involved in the conception of the study. ZH, KM, LS, and QN were involved in writing the article. HL, JZ, XS, YL, MC, and CL critically revised the manuscript. All authors read and approved the final manuscript.

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Epigenetic Control of Regulatory T Cell Stability and Function: Implications for Translation

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FoxP3$^+$ regulatory T (Treg) cells maintain immune homeostasis, promote self-tolerance, and have an emerging role in resolving acute inflammation, providing tissue protection, and repairing tissue damage. Some data suggest that FoxP3$^+$ T cells are plastic, exhibiting susceptibility to losing their function in inflammatory cytokine-rich microenvironments and paradoxically contributing to inflammatory pathology. As a result, plasticity may represent a barrier to Treg cell immunotherapy. Here, we discuss controversies surrounding Treg cell plasticity and explore determinants of Treg cell stability in inflammatory microenvironments, focusing on epigenetic mechanisms that clinical protocols could leverage to enhance efficacy and limit toxicity of Treg cell-based therapeutics.

Keywords: regulatory T cells, plasticity, inflammation, epigenetics, DNA methylation, therapeutics

INTRODUCTION

In health, regulatory T (Treg) cells are essential for maintaining immune homeostasis and promoting self-tolerance. These powerful immuno-modulatory cells, which comprise a subset of CD4$^+$ T cells expressing CD25 (IL-2Rα) and the master transcription factor FoxP3 in humans and mice, suppress immune activation *via* inhibitory cell surface molecules (e.g., CTLA-4 and PD-1) and secretion of anti-inflammatory cytokines (e.g., IL-10 and TGF-β) to dampen pro-inflammatory effector immune cell functions (1–4). Recent data demonstrate that Treg cells also coordinate resolution of inflammation, provide tissue protection, and orchestrate repair of tissue damage, potentially rendering them useful to treat acute inflammation and tissue injury (5–19). Some animal experiments and observations in humans suggest that FoxP3$^+$ T cells can lose their identity and function following exposure to inflammatory cytokines, resulting in loss of the canonical Treg cell transcriptional signature and acquisition of various helper T (Th) cell pro-inflammatory functions (20–25). Hence, the possibility of Treg cell plasticity represents a barrier to incorporating Treg cells into clinical protocols.

Treg cell development in the thymus involves the establishment of a specific epigenetic landscape, which is independent of, but complimentary to, FoxP3 expression and is required for specification of Treg cell lineage identity and function (26–30). Instability of Treg cell identity and function thus results from the loss of FoxP3 expression or changes in the epigenetic landscape. Natural Treg cells (nTreg cells) originate from the thymus with these transcriptional and epigenetic requirements established, persisting as a self-renewing population in the periphery (31, 32). While nTreg cells possess robust immunosuppressive capabilities, they comprise only 5–10% of human peripheral $CD4^+$ T cells, thus requiring prolonged *ex vivo* expansion times (~2–5 weeks) to use them in therapeutic transfer protocols targeting acute inflammation. These long culture times thus limit the practicality of nTreg cells to treat acute inflammatory diseases or to promote tissue protection and repair following an acute injury. As naïve T cells are significantly more abundant than nTreg cells in peripheral blood, high numbers of induced Treg (iTreg) cells—naïve $CD4^+$ T cells in which FoxP3 expression and a Treg cell phenotype have been induced by TGF-β *in vitro*—are rapidly obtainable, presenting a potential alternative to nTreg cells in clinical protocols. Data from murine studies suggest that iTreg cells can be generated within a few days (30, 33–35), possibly facilitating the use of iTreg cells in therapeutic transfer protocols targeting acute inflammation and injury. Induced Treg cells lack nTreg cell-type epigenetic patterns, particularly in DNA methylation, that drive phenotypic stability (27, 29, 30). Thus, defining exploitable epigenetic mechanisms that allow for nTreg cell-level stability in iTreg cells is of particular interest in the pursuit of using iTreg cells as immunotherapy. While minor populations of some immune and non-immune cells can express FoxP3 (36), our review focuses on $FoxP3^+$ T cells.

TREG CELLS AS IMMUNOTHERAPY

The therapeutic goals of using Treg cells to induce self-tolerance and mitigate inflammation are to ameliorate immune dysregulation using minimal or no immunosuppressive pharmacotherapy while allowing proper immune responses to take place during the host response to pathogens (37). Pilot trials of Treg cells as cellular immunotherapy in humans have provided proof-of-concept for their use in diseases of auto-reactivity—including type 1 diabetes, graft-versus-host disease, and organ allo-transplantation—with promising results (38–44). In these studies, nTreg cells were isolated from patients for subsequent re-infusion either after storage or *ex vivo* expansion. Primary strategies of isolation involve obtaining mononuclear cells from leukopheresates, peripheral whole blood, or umbilical cord blood followed by Treg cell sorting using immuno-magnetic systems or flow cytometry cell sorting (45, 46). *Ex vivo* expansion protocols achieve large, pure, and suppressive cell populations while maintaining good manufacturing practice standards (47–50). Clinical trial protocols have infused dosages as high as 5 x 10^9 cells, which typically take 2–5 weeks to generate. To enhance Treg cell purity during expansion, several groups have studied the effect of culture in the presence of the mTOR inhibitor rapamycin, as it selectively promotes growth of $CD4^+CD25^+FoxP3^+$ Treg cells while concomitantly inhibiting $CD4^+CD25^-$ (non-Treg) effector T cells at low doses (50, 51).

Beyond induction of self-tolerance, emerging evidence demonstrates that Treg cells orchestrate resolution of inflammation, provide tissue protection, and coordinate tissue repair following a growing list of acute insults, including lung injury due to pneumonia, muscle injury, dermal injury, and vascular endothelial injury (5–18). The tissue-protective and -reparative properties of Treg cells appear to be the result of specific inflammatory signals, such as the cytokine IL-18 and the alarmin IL-33. Growth factor receptor ligands such as amphiregulin and keratinocyte growth factor may, in part, mediate these tissue-protective and -reparative functions, which are distinct from canonical T cell receptor (TCR) stimulation-dependent Treg cell suppressive functions. Promising data support broadening the use of Treg cells for the treatment of acute inflammation and tissue injury (19). Nevertheless, some lines of evidence suggest that Treg cells can exhibit plasticity in inflamed and damaged microenvironments, resulting in loss of their identity and the potential to gain pro-inflammatory effector functions (22). Discussed below, manipulating epigenetic determinants of Treg cell stability could aid efforts to maintain their beneficial functions in inflamed and damaged tissue microenvironments while limiting the potential for conversion into pathogenic T cells.

EPIGENETIC DETERMINANTS OF TREG CELL DEVELOPMENT AND STABILITY

Epigenetic mechanisms include a set of processes that modify transcriptional patterns without altering the underlying DNA sequence, allowing for heritable changes in gene expression. DNA methylation is a dynamic epigenetic modification mediated by a family of DNA methyltransferases (DNMTs) that add methyl groups to the 5' carbon of cytosine bases to create 5-methylcytosine (5mC), which is associated with chromatin inaccessibility and transcriptional repression (52, 53). The DNMT family member DNMT1 catalyzes maintenance DNA methylation, and ubiquitin-like containing PHD and RING finger domains 1 (UHRF1) recruits DNMT1 to hemi-methylated DNA during DNA replication, serving to maintain DNA methylation patterning in mitotic cells. DNA demethylation occurs either passively during DNA replication or *via* the catalytic activity of the ten-eleven translocation (TET) family of dioxygenases, which oxidize 5mC to 5-hydroxymethylcytosine (5hmC) and other intermediates that ultimately restore unmethylated cytosine at a given position (54). Histone modifications represent another form of dynamic epigenetic alteration to chromatin, which, in combination with the non-catalytic domains of histone-modifying proteins, modulates transcriptional activity (55). For example, enzymes that promote monomethylation of lysine 4 on histone H3

(H3K4me1) and acetylation of lysine 27 on histone H3 (H3K27ac) mark active enhancer elements and promote transcription. Importantly, cellular metabolism provides substrates for epigenetic writers and erasers (e.g., methyltransferases and demethylases) in Treg cells (56). For example, our group determined that the mitochondrial electron transport chain in Treg cells is required to prevent the accumulation of toxic metabolites such as 2-hydroxyglutarate, which inhibits α-ketoglutarate-dependent enzymes such as the TETs (57). We found that loss of mitochondrial electron transport chain complex III in Treg cells results in increased levels of 2-hydroxyglutarate, altered DNA methylation patterning, and impaired Treg cell suppressive function.

The field has now recognized that stable Treg cell phenotype and function depend on a specific epigenetic landscape to maintain lineage-defining Treg cell gene expression, including at the locus encoding FoxP3 (**Table 1**) (26–30). Accordingly, nTreg cells can be distinguished from conventional T cells and iTreg cells by characteristic DNA hypomethylation at the *Foxp3* promoter and additional elements within *Foxp3*-associated enhancer regions, such as the Treg cell-specific demethylated region (TSDR), also known as conserved noncoding DNA sequence 2 (CNS2). How the Treg cell lineage establishes and stabilizes its epigenetic signature remains an active area of investigation (**Figure 1A**). During development in the thymus, TET enzymes and HATs, such as CBP (also known as CREBBP) and p300, are recruited to modify the *Foxp3* locus for induction and maintenance of FoxP3 expression, which is followed by establishment of a Treg cell-specific gene expression profile (60, 61). Epigenetic modification at the *Foxp3* locus involves TET-mediated 5hmC accumulation at the TSDR and other key regions (59). Importantly, in the absence of these epigenetic modifications, Treg cells can lose FoxP3 expression and gain IL-17 expression.

Beyond the *Foxp3* locus, investigators have determined that Treg cell-specific super-enhancers—genomic regions with dense clustering of highly active lineage-defining enhancer elements—regulate *Foxp3* and other Treg cell lineage-defining genes (58). In thymic pre-Treg cells, the genome organizer Satb1 binds Treg cell-specific super-enhancer sites, resulting in chromatin loop formation that allows distal regulatory elements to interact with and recruit transcription factors and epigenetic modifiers to activate and stabilize the Treg cell-defining gene regulatory network. Deletion of Satb1 in double-positive thymocytes results in impaired Treg cell-super-enhancer activation and failure to induce Treg cell signature genes, leading to fatal autoimmunity *in vivo*. These studies also revealed that DNA hypomethylation is a distinguishing feature of the Treg cell-specific super-enhancer landscape in Treg cells. Moreover, our work demonstrated that the Treg cell-specific super-enhancer epigenetic pattern shown to be causally deterministic in mice is also present in Treg cells obtained from the alveolar spaces of patients with severe pneumonia (67). Thus, the Treg cell-specific super-enhancer landscape appears to be a conserved and translationally relevant epigenetic pattern, prompting clinical trials of Treg cell infusions for patients with the acute respiratory distress syndrome due to severe SARS-CoV-2 pneumonia (16, 68, 69).

The role of maintenance of epigenetic marks in stabilizing lineage identity following the initial establishment of epigenetic patterns at Treg cell-specific super-enhancers and at other important non-coding elements remains unclear (**Figure 1B**). Experimental data suggest that some chromatin organizers necessary for lineage specification are not required for lineage stability. Indeed, deletion of Satb1 in differentiated Treg cells does not lead to any changes in Treg cell numbers or phenotype, indicating that Satb1 is dispensable for Treg cell maintenance (58). In contrast, loss of the chromatin-modifying CoREST repressor complex disrupts FoxP3-driven repression of Th1 cell signature genes encoding T-BET, IL-2, and IFN-γ. Consequently, loss of CoREST results in Treg cell production of IL-2 and IFN-γ, impaired Treg cell function, and enhanced anti-tumor immunity (62).

Maintenance DNA methylation also controls Treg cell stability following FoxP3 induction in nTreg cells. We observed that loss of an epigenetic regulator responsible for maintenance DNA methylation, UHRF1, at the thymic FoxP3$^+$ stage of development in nTreg cells leads to loss of FoxP3 expression and a Scurfy-like phenotype (30). We went on to determine that Treg cell-conditional deletion of UHRF1 results in failure of nTreg cells to persist after FoxP3 induction in the thymus, generating hyperinflammatory ex-FoxP3 cells in which loss of maintenance

TABLE 1 | Selected epigenetic modifiers discussed in the text and their role in Treg cell development and maintenance.

Epigenetic modifier	Mechanism	Role in Treg cells
Satb1	Chromatin organizer	Establishes Treg cell-specific super-enhancer landscape (58)
TET enzymes	DNA demethylases	Induce and maintain expression of *Foxp3* and other loci (59, 60)
CBP and p300	Histone acetyltransferases (H3K27ac)	Induce and maintain expression of *Foxp3* and other loci (61)
CoREST	Epigenetic repressor complex	Represses Th1 cell signature genes (62)
UHRF1	DNA methyltransferase adapter protein	Maintains repressive DNA methylation patterning at Th1 cell signature genes to stabilize the Treg cell lineage (30); promotes proliferative capacity in colonic Treg cells (63); may regulate iTreg cell suppressive function (64)
DNMT1	Maintenance DNA methyltransferase	Required for Treg cell suppressive function (65)
EZH2	Histone methyltransferase (H3K27me3), subunit of PRC2	Deposits repressive chromatin modifications at FoxP3-bound loci (66)

See text for abbreviations.

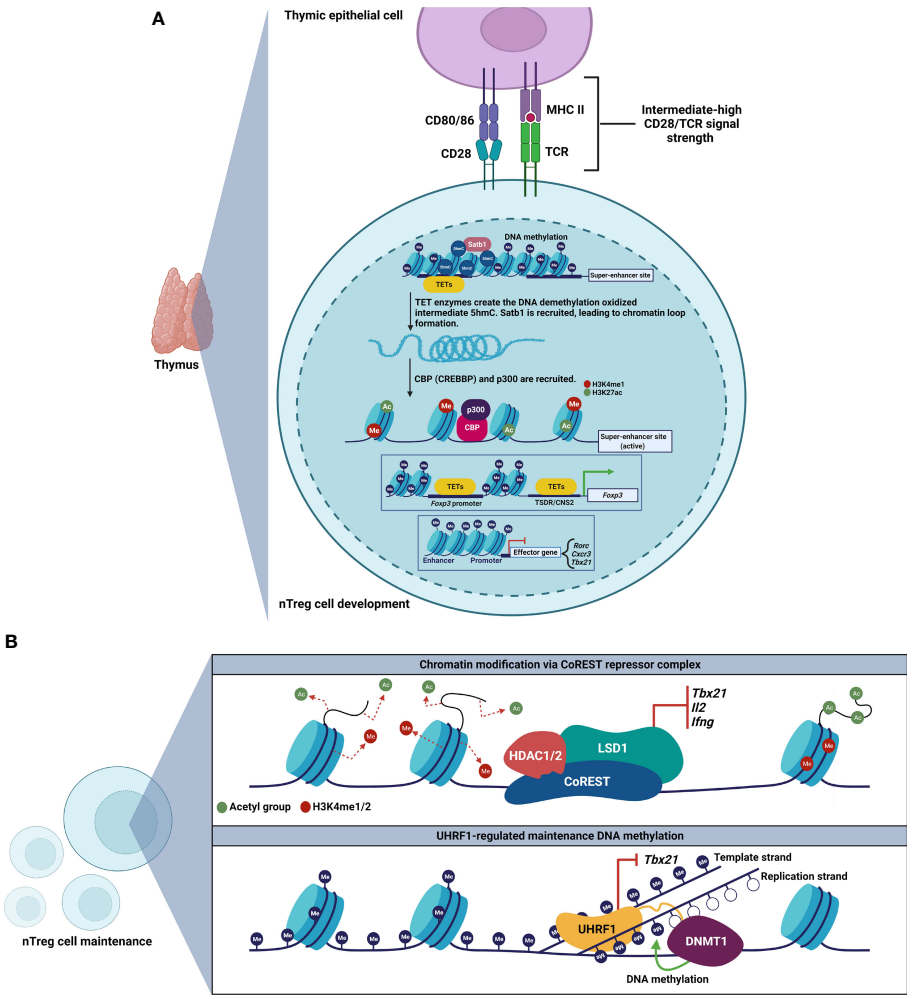

FIGURE 1 | Development and maintenance of Treg cell epigenetic landscapes. **(A)** Thymic Treg cell development involves establishment of a Treg cell-specific super-enhancer landscape at *Foxp3* and other key loci. The chromatin organizer Satb1 establishes a super-enhancer landscape in Treg cells, characterized by active enhancer histone marks, and TET-mediated DNA hypomethylation. Loci encoding effector T cell signature genes are hypermethylated. **(B)** Maintenance of Treg cell epigenetic patterning requires the CoREST repressor complex (top) and the epigenetic regulator UHRF1 (bottom) to repress loci encoding inflammatory genes.

DNA methylation derepresses Th1 cell signature genes, including *Tbx21* (encodes T-BET). Interestingly, UHRF1-deficient ex-FoxP3 cells exhibit downregulation of the TET demethylases and DNA hypermethylation at core Treg cell loci, including *Foxp3*. These observations support a mechanism in which loss of maintenance DNA methylation unleashes a secondary wave of DNA methylation at core Treg cell loci to generate hyperinflammatory, Th1-skewed, ex-FoxP3 cells. Consistent with these observations, others found that constitutive deletion of the maintenance DNA methyltransferase DNMT1, but not the *de novo* methyltransferase DNMT3A, results in diminished numbers and suppressive function of Treg cells (65). Interestingly, DNMT1-deficient Treg cells maintain Treg cell-specific DNA hypomethylation patterns at *Foxp3*, and we determined that UHRF1-deficient Treg cells possess the Treg cell-specific super-enhancer landscape. Additional evidence supports that pan-T cell-specific deficiency of UHRF1 results in defective proliferation and functional maturation of colonic Treg cells (63). Thus, nTreg cells require both a canonical hypomethylation pattern as well as maintenance methylation at loci encoding inflammatory programs to stabilize their lineage identity and function. The role of maintenance DNA methylation in stabilizing iTreg cell identity and function remains less clear. Intriguingly, while we found that UHRF1 is dispensable for induction of FoxP3 expression in iTreg cells (30), others observed augmented suppressive function in iTreg cells generated from UHRF1-deficient naïve $CD4^+$ T cells, even in inflammatory microenvironments (64). As inflammation may drive instability of $FoxP3^+$ T cells, we will explore in the following section how microenvironmental inflammatory signals control T cell plasticity *via* their influence on epigenetic modifiers.

CYTOKINE SIGNALING AND THE EPIGENETICS OF TREG CELL PLASTICITY

Plasticity refers to the capacity of CD4$^+$ T cells to depolarize their specialized functional programs in response to the cytokine milieu of the local microenvironment, resulting in loss of their functional identity and potential for a gained Th-skewed cell phenotype (70). Careful lineage-tracing studies in mice reported the eminent stability of the Treg cell lineage under physiologic and inflammatory conditions (31), and others have argued that the plasticity observed in FoxP3$^+$ T cells in inflammatory or lymphopenic microenvironments results from cellular heterogeneity rather than reprogramming (71). Indeed, minor populations of conventional T cells can transiently express FoxP3 and then differentiate into ex-FoxP3 Th-skewed cells (72, 73). These populations retain the ability to re-express FoxP3 upon activation, a finding correlated with the demethylated status of the TSDR in the conventional T cell population and possibly the Treg cell population.

Nevertheless, several lines of evidence describe plasticity occurring in FoxP3$^+$ T cells to produce ex-FoxP3 cells or FoxP3$^+$ Th-like cells in response to specific signaling events (**Figure 2A**). For example, Th1-like IFN-γ-secreting FoxP3$^+$ T cells exist in patients with relapsing-remitting multiple sclerosis,

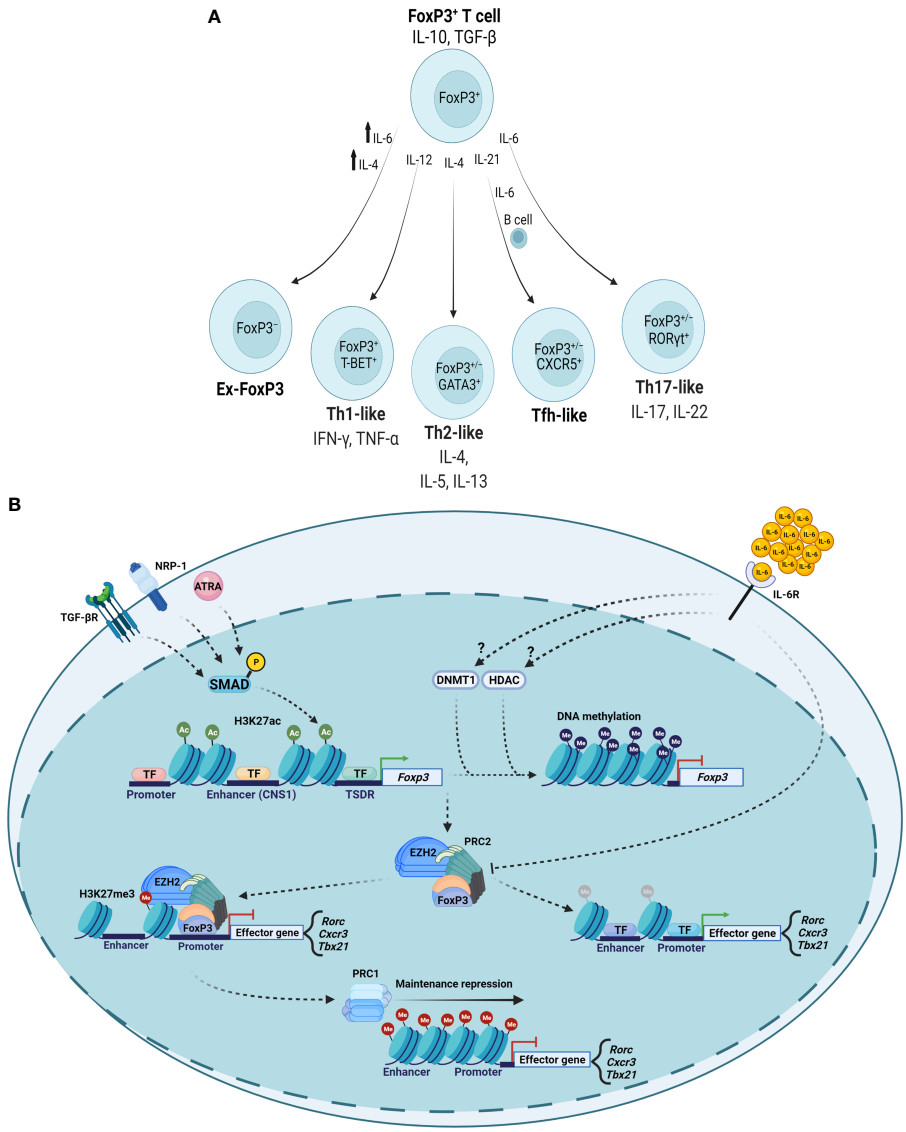

FIGURE 2 | Cytokine-mediated epigenetic reprogramming of FoxP3$^+$ T cell populations. **(A)** Specific cytokine microenvironments can repolarize FoxP3$^+$ T cells with variable effects on FoxP3 expression and Th cell-like phenotypes. **(B)** TGF-β, NRP-1, and ATRA signal to maintain Treg cell-type epigenetic patterns. Inflammatory cytokines such as IL-6 can promote DNMT and HDAC activity to result in loss of *Foxp3* gene expression and modulate PRC complexes to depress loci encoding inflammatory genes. TF, transcription factor.

a finding recapitulated *in vitro* when investigators cultured Treg cells from healthy people in the presence of IL-12 (23). IL-4 signaling promotes the development of ex-FoxP3 Th2-like cells in the setting of chronic helminth infection (25). The presence of an IL-6-, IL-21-, and activated B cell-rich environment in the Peyer's patches of mice results in FoxP3$^+$ T cell transformation into cells with characteristics of follicular Th (Tfh) cells that are capable of promoting germinal center formation (21). Th17 cells express the orphan nuclear receptor RORγt and a characteristic cytokine signature, including IL-17. Regulation of the locus encoding IL-17 *via* reciprocal actions of STAT3 and STAT5 in part determines Th17-Treg cell plasticity, producing FoxP3$^+$IL-17$^+$ or FoxP3$^+$RORγt$^+$ cells (20, 74, 75). Clinically, the joints of patients with rheumatoid arthritis contain FoxP3$^+$IL-17$^+$ cells, which are also present in mice with experimental joint inflammation (24). These FoxP3$^+$IL-17$^+$ cells may represent a transitional cell population moving toward complete loss of FoxP3, as synovial fibroblast-derived IL-6 can cause CD4$^+$FoxP3$^+$ T cells to lose FoxP3 expression and differentiate into Th17 cells in mice with experimental inflammatory arthritis. Further, treatment of patients with rheumatoid arthritis using the IL-6R inhibitor tocilizumab resulted in significant symptomatic benefit along with decreases in circulating Th17 cells and increases in circulating Treg cells (76). Despite expressing RORγt, FoxP3$^+$RORγt$^+$ cells in the intestines of mice demonstrate transcriptional and epigenetic profiles more similar to FoxP3$^+$RORγt$^-$ cells than to FoxP3$^-$RORγt$^+$ cells, including demethylation at Treg cell-characteristic genes encoding FOXP3, CTLA-4, GITR, EOS, and HELIOS. FoxP3$^+$RORγt$^+$ cells retain suppressive function and are more suppressive than FoxP3$^+$RORγt$^-$ cells in a T cell transfer colitis model (77, 78). Collectively, these reports identify plasticity within FoxP3$^+$ T cell populations that is induced and modified by specific cytokine microenvironments.

Data suggest that epigenetic alterations underlie the ability of FoxP3$^+$ T cells to polarize in response to microenvironmental inflammatory signals (**Figure 2B**). For example, some experiments determined that IL-6 can promote DNMT1-mediated DNA methylation and that histone deacetylase (HDAC) activity can destabilize FoxP3$^+$ T cells (79, 80). EZH2 (enhancer of zeste homolog 2) is the enzymatic subunit of polycomb repressive complex 2 (PRC2), which participates in histone methylation to result in transcriptional repression. PRC1 (polycomb repressive complex 1) maintains the repressed transcriptional state, interacting with chromatin by recognizing PRC2-established H3K27me3 marks in an equilibrium with other histone-modifying complexes and repressive DNA methylation (81, 82). In inflammatory microenvironments, FoxP3-containing complexes incorporate EZH2, which deposits repressive chromatin modifications at FoxP3-bound loci (66). Studies of intestinal inflammation in inflammatory bowel disease suggested a disrupted FoxP3-EZH2 physical interaction that investigators recapitulated by treatment with IL-6 (83). In the context of aging (84), we found that cell-autonomous age-related alterations in DNA methylation drive plasticity in FoxP3$^+$ T cells in the inflamed lungs of aged but not young mice during recovery from influenza pneumonia (18, 85). Our studies in aged hosts revealed co-expression of Th1- and Th17-associated transcription factors (T-BET and RORγt) in lung FoxP3$^+$ T cells 60 days following influenza virus infection along with expression of cognate cytokines (IFN-γ and IL-17). In contrast, other signaling events may stabilize Treg cell-type epigenetic patterns. Transcriptional and epigenetic analyses of human Treg cells from inflamed synovial joints compared with peripheral blood in pediatric patients revealed that Treg cells differentiate into effector Treg (eTreg) cells that are suppressive *in vitro* and display increased expression of core Treg cell genes (86). Importantly, epigenetic alterations in active enhancer marks, including H3K4me1 and H3K27ac, explained these transcriptional differences. CD103$^+$ intestinal dendritic cells secrete all-*trans* retinoic acid (ATRA) and TGF-β to induce histone acetylation at the CNS1 region of the *FOXP3* locus to promote FoxP3 expression while restricting Th17 polarization (87). Experimental data suggest that neuropilin-1 (NRP-1) also reinforces the stability of Treg cells in inflammatory environments, as silencing of NRP-1 results in diminished FoxP3 expression with a correlative increase in DNA methylation at the TSDR (88). Collectively, several lines of evidence support that epigenetic mechanisms determine FoxP3$^+$ T cell plasticity in inflammatory environments, prompting consideration of leveraging these mechanisms to promote functional stability in clinical Treg cell transfer protocols.

STABILIZING TREG CELL IMMUNOTHERAPEUTIC FUNCTION *VIA EX VIVO* EPIGENETIC MODIFICATION

Treg cell plasticity may represent an adaptive feature to regulate a given microenvironment. Clinically, however, therapeutic protocols will require a greater understanding of Treg cell plasticity to maximize on-target function and limit unintended toxic inflammation. Recent mouse studies have performed *ex vivo* modifications to induced and natural Treg cells to enhance their stability in the presence of inflammatory cytokines (**Figure 3**). As inhibition of DNMTs or HDACs can induce FoxP3 expression and support Treg cell identity, DNMT or HDAC inhibitors could stabilize nTreg cells in culture before therapeutic infusion (11, 27, 79, 89–92). The DNMT adapter protein UHRF1 also remains a drug target of interest. As noted above, adoptive transfer of iTreg cells generated from UHRF1-deficient naïve CD4$^+$ T cells display enhanced suppressive function in colitis models (64). In contrast, UHRF1 overexpression in T cells causes BCL6 downregulation and decreased Tfh cell differentiation, which may serve as a potential therapeutic target in systemic lupus erythematosus (93). Nevertheless, the field requires further data to determine whether modulation of UHRF1 in mature iTreg cells translates into a more stable, suppressive, and reparative state *in vivo*. Studies of HDAC inhibitors found that they are capable of promoting thymic production of nTreg cells and inducing iTreg cells *ex vivo*, leading to efficacy in attenuating inflammatory bowel disease and

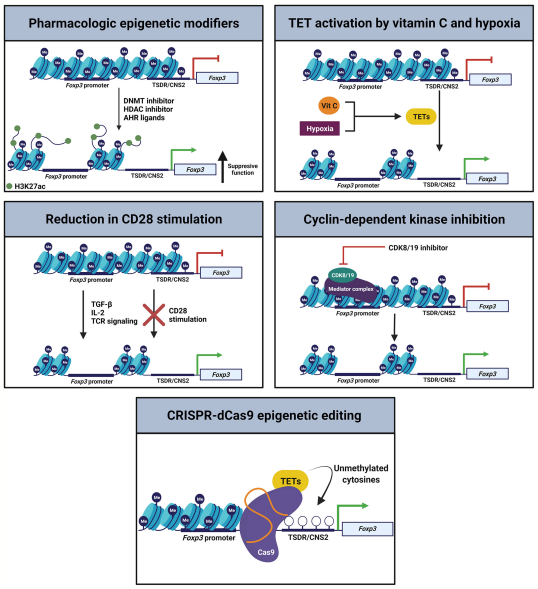

FIGURE 3 | Epigenetic strategies to promote Treg cell stability. Multiple orthogonal pathways could be leveraged during *ex vivo* generation of iTreg cells or expansion of nTreg cells to promote FoxP3 expression and Treg cell stability, enhancing the efficacy of therapeutic transfer.

promoting cardiac allograft tolerance (94). Going forward, selective manipulation of HDAC subclass function may stabilize and promote nTreg and iTreg cell function in clinical trial protocols (95).

The aryl-hydrocarbon receptor (AHR) is a ligand-activated transcription factor that functions in part through interaction with epigenetic regulators, including the mediator complex (96). AHR regulates Treg-Th17 cell plasticity in mice *via* activation by specific ligands. AHR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces functional Treg cells that suppress experimental autoimmune encephalitis. Treatment of mice with TCDD also attenuates delayed hypersensitivity responses, which are associated with induction of Treg cells and suppression of Th17 cells in mesenteric lymph nodes (97). Intriguingly, treatment with AHR ligands such as TCDD or butyrate inhibits pro-inflammatory HDAC classes I and II (97). Hence, generation of iTreg cells or expansion of nTreg cells in the presence of AHR ligands may stabilize the Treg cell epigenetic landscape to maintain their identity following therapeutic transfer.

Several investigations have examined modulation of TET enzyme activity *via* treatment with the TET activator vitamin C (ascorbic acid) or culture under low oxygen conditions to enhance Treg cell induction and stability. Vitamin C facilitates demethylation of the *Foxp3* CNS2 enhancer region in a TET2/3-dependent manner to increase the stability of FoxP3 expression in TGF-β-induced Treg cells (33, 98). Further, culture of iTreg cells under low oxygen (5%) conditions facilitates CNS2 demethylation and stabilization of FoxP3 both *in vitro* and *in vivo*, a finding that correlates with increased TET expression. These post-hypoxia Treg cells exhibit stronger suppressive activity in a colitis model compared with untreated iTreg cells (34), informing potential immunotherapeutic iTreg cell induction protocols. Moreover, activation of TET enzyme activity during *ex vivo* nTreg cell expansion protocols could likewise support their stability and function.

While CD28 is essential for optimal thymic Treg cell development, CD28 is surprisingly dispensable for Treg cell induction or Treg cell-specific gene hypomethylation in the intestines of mice (35, 99). iTreg cell induction *via* TGF-β, IL-2, and TCR agonism in the absence of CD28 signaling induces nTreg cell-type DNA hypomethylation in conventional T cells while hindering skewing toward Th cell phenotypes. Data suggest that CD28 acts *via* the PKC-NF-κB signaling pathway during iTreg cell generation and that inhibition of this pathway enables *de novo* acquisition of nTreg cell-type DNA hypomethylation. Induced Treg cells generated under conditions of absent CD28 stimulation stably express FoxP3 after adoptive transfer and effectively suppress antigen-specific immune responses *in vivo* (35). Thus, potential modifications to standard nTreg cell culture practices or iTreg cell induction protocols include relatively straightforward adjustments such as reducing CD28 stimulation during cellular activation.

Cyclin-dependent kinase 8 (CDK8) and CDK19 reversibly associate with the mediator complex as well as regulate epigenetic events such as histone modification and chromatin remodeling (100, 101). Inhibition of CDK8 and CDK19 in conventional T cells induces FoxP3 expression and suppressive function independent of TGF-β signaling in antigen-stimulated effector-memory as well as naïve $CD4^+$ and $CD8^+$ T cells (102). Importantly, inflammatory cytokines do not appear to affect the induction of FoxP3 expression following CDK inhibition. These results suggest that CDK8 and CDK19 physiologically repress FoxP3 expression in activated conventional T cells, prompting consideration of targeting CDK8 and CDK19 in *ex vivo* iTreg cell generation or nTreg cell expansion protocols.

Finally, CRISPR-dCas9 epigenome editing systems may be of use to enhance FoxP3 stability during and following Treg cell induction or expansion. Kressler and colleagues demonstrated a transient-transfection CRISPR-dCas9-based epigenetic editing method for the selective de-methylation of the TSDR within the endogenous chromatin environment of a living cell (103). The demethylation marks were durable over weeks, including after expression of the editing complex had ceased. Consistent with prior data, however, successful FoxP3 induction was not associated with a switch to a fully functional Treg cell phenotype, highlighting importance of establishing gene expression and methylation patterns at other key loci in the Treg cell genome.

DISCUSSION

$FoxP3^+$ Treg cells represent a powerful cell type capable of inducing self-tolerance, suppressing over-exuberant immune system activation, promoting resolution of inflammation, and effecting

protection and repair of damaged tissues. Clinical trial protocols have applied Treg cell immunotherapy to disorders of auto- and allo-reactivity as well as to suppress damaging inflammation and hasten recovery from severe pneumonia. Epigenetic mechanisms, particularly those that regulate DNA methylation, control Treg cell lineage identity, stability, and function. Although the Treg cell lineage displays a strong tendency toward stability, many lines of evidence suggest that FoxP3$^+$ T cells can exhibit plasticity in inflammatory microenvironments, with investigators observing both loss of canonical suppressive function and gain of inflammatory effector functions. Going forward, manipulating the epigenetic state of Treg cells *ex vivo* prior to infusion could stabilize their identity and function to enhance clinical efficacy while limiting the potential for off-target effects.

AUTHOR CONTRIBUTIONS

AJ and BS conceptualized the manuscript, wrote the first draft of the manuscript, edited the manuscript, and edited the figures. CR provided essential conceptual input, edited the manuscript, and conceptualized and created the figures. Conceptualization: AJ and BS. Visualization: AJ, CR, and BS. Funding acquisition: BS. Supervision: BS. Writing – original draft: AJ and BS. Writing – review & editing: AJ, CR, and BS. All authors contributed to the article and approved the submitted version.

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

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Neuropilin-1 Identifies a New Subpopulation of TGF-β-Induced Foxp3$^+$ Regulatory T Cells With Potent Suppressive Function and Enhanced Stability During Inflammation

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CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) play a crucial role in preventing autoimmunity and inflammation. There are naturally-derived in the thymus (tTreg), generated extrathymically in the periphery (pTreg), and induced *in vitro* culture (iTreg) with different characteristics of suppressiveness, stability, and plasticity. There is an abundance of published data on neuropilin-1 (Nrp-1) as a tTreg marker, but little data exist on iTreg. The fidelity of Nrp-1 as a tTreg marker and its role in iTreg remains to be explored. This study found that Nrp-1 was expressed by a subset of Foxp3$^+$CD4$^+$T cells in the central and peripheral lymphoid organs in intact mice, as well as in iTreg. Nrp-1$^+$iTreg and Nrp-1$^-$iTreg were adoptively transferred into a T cell-mediated colitis model to determine their ability to suppress inflammation. Differences in gene expression between Nrp-1$^+$ and Nrp-1$^-$iTreg were analyzed by RNA sequencing. We demonstrated that the Nrp-1$^+$ subset of the iTreg exhibited enhanced suppressive function and stability compared to the Nrp-1$^-$ counterpart both *in vivo* and *in vitro*, partly depending on IL-10. We found that Nrp-1 is not an exclusive marker of tTreg, however, it is a biomarker identifying a new subset of iTreg with enhanced suppressive function, implicating a potential for Nrp-1$^+$iTreg cell therapy for autoimmune and inflammatory diseases.

Keywords: regulatory T cells, TGF-β, Foxp3, neuropilin-1, IL-10

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INTRODUCTION

Regulatory T cells (Treg) are crucial for maintaining immune homeostasis, limiting the immune response, and establishing immunological tolerance (1). The transcription factor forkhead box P3 (Foxp3) is a specific marker of Treg. $CD4^+CD25^+Foxp3^+$Tregs are heterogeneous and can be mainly divided into 3 subsets: thymus-derived naturally occurring Treg (tTreg, also called nTreg), peripherally derived Treg (pTreg), and Treg induced *in vitro* with interleukin-2 (IL-2) and transforming growth factor-β (TGF-β) (iTreg) (2, 3). These three subsets have similar phenotypic characteristics and comparable suppressive function against T cell-mediated immune response and diseases. However, they exhibit certain specific differences in mRNA transcripts and protein expression, epigenetic modification, and stability in the inflammatory milieu. Accurately distinguishing them will help to clarify the biological features and contributions of each Treg subset to peripheral tolerance, autoimmunity, and tumor surveillance. The classical Treg surface markers CD25, GITR, CTLA4, and PD-1 are all expressed on these three Treg subsets, making it extremely difficult to distinguish pTreg and iTreg from tTreg using surface markers. However, two reports suggested neuropilin-1 (Nrp-1) as a promising candidate of tTreg specific surface marker (4, 5).

Nrp-1 is a transmembrane glycoprotein previously known to be involved in axon guidance (6) and angiogenesis (7, 8), which was first found on the surface of tTreg in 2004 (9). It was shown that Nrp-1 was expressed by tTreg *in vivo*, but not by pTreg driven by antigenic stimulation or converted under homeostatic conditions in murine models (4, 5). In contrast, the expression of Nrp-1 was found upregulated in TGF-β-induced Treg *in vitro (*4). It was also noticed that Nrp-1 is not a marker of human nature $FOXP3^+$Treg, but can be induced in human blood T cells upon *in vitro* TCR activation (10). Sarris found that Nrp-1 strengthened the contacts between Treg and antigen-presenting cells (11). Similar to Nrp-1, Helios has been reported as a potential marker for tTreg (12–15). iTreg generated from both human and mice $CD4^+$T cells could express Helios (16). Helios is also known as a marker of T cell activation and proliferation (17, 18).

Recently, several literatures described the potential use of iTreg as a therapeutic strategy for autoimmune diseases. One study showed that polyclonal iTreg slowed diabetes progression, prolonging the survival of non-obese diabetic mice (19). Collagen type II-specific iTreg was better than nTreg in suppressing arthritis, partly by inhibiting the development of Th17 cells (20). Furthermore, iTreg could suppress the main features of asthma (21). Although iTreg had a suppressive function, its long efficacy was less acceptable. Thus, it is difficult to identify potent a suppressive iTreg based on surface markers (1).

Our study aimed to systematically explore the role of Nrp-1 and Helios in Treg subsets with a focus on the role of Npr-1 in iTreg function and stability. We found that Nrp-1 and Helios are not exclusive markers of the tTreg subset. The expression of Nrp-1 on the surface of iTreg enables the identification and isolation of an iTreg subset that has superior a suppressive function under inflammatory conditions.

Abbreviations: Foxp3, transcription factor forkhead box P3; Treg, $CD4^+Foxp3^+$ regulatory T cells; Nrp-1, Neuropilin-1; TGF-β, transforming growth factor-β; IL-10, interleukin-10; IL-2, interleukin-2; iTreg, Treg induced *in vitro* with IL-2 and TGF-β; B6, C57BL/6; IRF4, interferon regulatory factor 4; ALK5i, ALK5 (TGFRI) inhibitor.

MATERIAL AND METHODS

Mice

Male C57BL/6 (B6), C57BL/6 thy1.1, B6.129S7-*Rag1*^{tm1Mom}/J ($Rag1^{-/-}$), B6 Foxp3-RFP mice and $CD4^{cre}$ B6 mice were purchased from the Jackson Laboratory. B6 Foxp3-GFP knockin mice were generously provided by Dr. Talil Chatilla (University of California Los Angeles). $CD4^{cre}/Nrp1^{flox/flox}$ B6 mice were generated by crossing the two parent strains at Cornell University. All mice were kept in the specific pathogen-free (SPF) condition. 7-8 weeks age mice were chosen for the experiment.

The Generation of $CD4^+$ Induced Regulatory T (iTreg), Th1, Th2, and Th17 Cells

Naïve splenic $CD4^+CD62L^+CD25^-CD44^{low}$T cells were acquired by negative selection *via* the auto-MACS method (22, 23). Briefly, enriched T cells were first stained with biotin-conjugated anti-CD8a, -CD25, -CD11b, -CD49b, -CD11c, and -B220 mAbs and then washed and combined with anti-biotin microbeads (Miltenyi Biotec, Auburn, CA, USA). After they passed through the MACS separation columns, the negative exports were collected as $CD4^+CD25^-$cells. Subsequently, naive $CD4^+CD25^-CD62L^+CD44^{low}$ T cells were positively selected from the enriched $CD4^+CD25^-$T-cell fraction by the anti-CD62L microbeads. For iTreg differentiation, $0.2x10^6$ cells were cultured in 96-well plates and stimulated with anti-CD3/CD28 microbeads (1 bead per 5 cells, Invitrogen) in the presence of IL-2 (10ng/ml, R&D) with ($CD4^+$iTreg) or without ($CD4_{med}$) TGF-β (2ng/ml) for 3 days. Other $0.2x10^6$ naïve $CD4^+$T cells were cultured with irradiated APCs (1:1 ratio of APCs to Naïve $CD4^+$ T cells) in the presence of 1 μg/ml soluble anti-CD3 and 1 μg/ml anti-CD28, together with different antibodies and cytokines in 96-well plates. For Th1 cell differentiation, 10 ng/ml recombinant murine IL-12 (rm-IL-12, eBioscience) and 10 μg/ml anti-IL-4 (Biolegend) were used. For Th2 cell differentiation, 10 ng/ml rm-IL-4 (R&D Systems), and 10 μg/ml anti-interferon-γ (anti-IFN-γ, Biolegend) were used. For Th17 cell differentiation, 30 ng/ml IL-6 (R&D Systems), 1 ng/ml TGF-β (R&D Systems), 10 μg/ml anti-IFN-γ (Biolegend), and 10 μg/ml anti-IL-4 (Biolegend) were added. RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10mM HEPES (Invitrogen) and 10% heat-inactivated FCS (HyClone Laboratories) was used for all cultures. Cells were harvested and stained with different antibodies.

Flow Cytometry

The following fluorescence conjugated mouse antibodies were used for flow cytometric analysis: From Biolegend: anti-CD4 (GK1.5), CD25 (3C7), CD8 (53-6.7), B220 (RA3-6B2), CD44 (IM7), CD11c (N418), CD11b (M1/70), NK1.1 (PK136), Helios (22F6), IFN-γ (XMG1.2), IL-17a (TC11-18H10.1), IL-4 (11B11), IL-10 (JES5-16E3) IL-10R (1B1.3a); From R&D Systems: Neuropilin−1 (FAB5994A). Cell subset was stained with mAbs and isotype control and analyzed by a FACS Calibur flow cytometer. For intracellular staining, such as IFN-γ and IL-17a, cells were first stained with surface marker CD4, and further fixed, and permeabilized for intracellular staining.

In Vitro Suppression Assays

Freshly isolated $0.2x10^6$ T cells (responder cells) labeled with CFSE were stimulated with anti-CD3 mAb (0.025 μg/mL) and irradiated APCs (30 Gy, 1:1 ratio) for 3 days, with or without iTreg generated as described above. The ratio of Treg/T cells was 1:2-1:16. T-cell proliferation was determined by the CFSE dilution rate after 3 days of culture.

Foxp3(GFP)$^+$Nrp-1$^+$CD4$^+$T Cells Conversion *In Vivo*

When the 90.1$^+$CD4$^+$iTreg were cultured for 3 days and harvested, they were sorted into 90.1$^+$Foxp3-GFP$^+$Nrp-1$^+$CD4$^+$T or 90.1$^+$Foxp3-GFP$^+$Nrp-1$^-$CD4$^+$T cells. Anti-CD3/CD28 microbeads were removed. For *in vivo* conversion, $0.5x10^6$ 90.1$^+$Foxp3-GFP$^+$Nrp-1$^+$CD4$^+$T or 90.1$^+$Foxp3-GFP$^+$Nrp-1$^-$CD4$^+$T cells were adoptively transferred into Rag1$^{-/-}$ mice. 4, 7, and 12 days later, 90.1$^+$CD4$^+$T cells from the spleen were stained for Foxp3-GFP, IFN-γ, and IL-17a.

T Cell-Induced Colitis

Naïve CD4$^+$CD45RB^{hi} T cells were purified (>98%) from spleens of C57BL/6 Foxp3^{gfp} mice *via* FACS sorting (LSR II, BD). Naïve CD4$^+$CD45RB^{hi} T cell suspensions were washed in sterile PBS, and age- and sex-matched C57BL/6 Rag1$^{-/-}$ recipient mice received $4x10^5$ CD4$^+$CD45RB^{hi} T cells by i.p. injection.

For co-transfer experiments, 1:2 mixtures of CD90.1$^+$Foxp3-GFP$^+$Nrp-1$^+$CD4$^+$T or CD90.1$^+$Foxp3-GFP$^-$Nrp-1$^+$CD4$^+$T or CD90.1$^+$Foxp3-GFP$^-$Nrp-1$^-$CD4$^+$T or CD90.1$^+$Foxp3 -GFP$^+$Nrp-1$^-$CD4$^+$T and CD90.2$^+$CD4$^+$CD45RB^{hi} T cells were injected i.p. (total cell number=$4x10^5$) into C57BL/6 Rag1$^{-/-}$. Mice were sacrificed when symptoms of clinical disease (weight loss and/or diarrhea) developed in control groups, 6-8 weeks after cells transfer unless otherwise indicated. Samples of the cecum and proximal, mid, and distal colon were prepared as previously described, and inflammation was graded according to a scoring system (23, 24).

RNA Sequence Analysis

Naïve CD4$^+$T cells were isolated from the spleen of B6 Foxp3-RFP mice. Nrp-1$^+$Foxp3-$^+$iTreg and Nrp-1$^-$Foxp3-$^+$iTreg cells were sorted from iTreg cells on day 3 based on Nrp-1 and Foxp3-RFP expression. Thymus-derived nTreg cells from B6 Foxp3-RFP mice were set as a control. Total RNA was prepared from the above three Treg using the RNeasy Mini Plus Kit (Qiagen). Directional RNA-seq libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs), with initial polyA+ isolation, by the RNA Sequencing Core at Cornell University. Sequencing was performed on Illumina HiSeq 1500, and raw data were processed on the CLC Genomics Workbench v 11.0.1. mRNA profiles were calculated with Cufflinks software and expressed as FPKM (fragments per kilobase of exon model per million mapped fragments). Genes that are significantly altered ($|FC|\geq1$, $P<0.05$) in Nrp-1$^+$ iTreg vs Nrp-1$^-$ iTreg cells, are used for Gene Set Enrichment Analysis (GSEA).

Bisulfite Sequencing

We harvested genomic DNA from Nrp-1$^+$Foxp3-GFP$^+$iTreg and Nrp-1$^-$Foxp3-GFP$^+$iTreg cells using the DNeasy blood & tissue extraction kit (Qiagen) and conducted a bisulfite conversion using an EZ DNA Methylation kit (Zymo Research) following the manufacturer's protocol. Purified bisulfite-treated DNA was amplified by PCR using a pair of primers to mouse Foxp3 TSDR: 5′- AGAGGTTGAAGGAGGAGTATTT -3′ and 5′- ACTATCT ATCCAATTCCCCAAC -3′. The PCR products were purified using ExoSAP-IT PCR Product Cleanup Kit and were sequenced by GeneWIZ company. All sequencing results of the bisulfite converted TSDR region were analyzed on the Bisulfite sequencing data presentation and compilation (BDPC) DNA methylation analysis platform.

Statistics

Data are expressed as Mean ± SEM unless otherwise indicated. Data were analyzed using the unpaired t-tests (Mann-Whitney) or paired t-tests for comparison between two groups or ANOVA for comparison among multiple groups as appropriate. Differences were considered statistically significant when $p<0.05$.

RESULTS

Both Nrp-1 and Helios Were Highly Expressed in iTreg Subset

Current studies debate the value of Nrp-1 and Helios specificity on tTreg. Using Foxp3^{gfp} reporter mice, we systematically investigated their expression profile. Nrp-1 was expressed on CD4$^+$T, DC, and NK cells, which was consistent with similar findings in the reference (25), while Helios was mostly expressed on CD4$^+$T cells (**Supplemental Figure 1**). Among CD4$^+$T cells in the thymus, Nrp-1 was exclusively expressed in CD4$^+$Foxp3$^+$T cells albeit its level was a little lower than that of Helios. However, Helios was also expressed on CD4$^+$Foxp3$^-$T cells (**Supplemental Figure 2**), thus, Nrp-1 would be better to identify tTreg than Helios.

We also studied the expression of Nrp-1 and Helios in the peripheral lymph tissues. As shown in **Supplemental Figures 2A, C**, similar levels of Nrp-1 were observed in CD4$^+$Foxp3$^+$T cells isolated from the lymph node, spleen, and circulating blood compared to the thymus. Helios was also substantially expressed on peripheral CD4$^+$Foxp3$^+$ cells. Given

that peripheral CD4$^+$Foxp3$^+$ Treg could be mixed with tTreg and pTreg, Neither Nrp-1 nor Helios may distinguish nTreg from pTreg cells.

We found that Nrp-1 and Helios were both negative on naïve CD4$^+$T cells. After T cell receptor (TCR) stimulation, 12% of CD4$^+$T cells expressed Helios, but Nrp-1 was lower-expressed on activated CD4$^+$T cells. After treatment with TGF-β, >75% of CD4$^+$T cells expressed Foxp3 that was considered as iTreg. 55% of iTreg expressed Helios and 87% were both Nrp-1$^+$Foxp3$^+$CD4$^+$T (**Figure 1A**). Both Nrp-1 and Helios were hardly expressed on Th1, Th2, and Th17 cells differentiated *in vitro*. Nrp-1 expression increased after 2 days of culture *in vitro*, and was still higher-expressed in iTreg *in vitro* after 13 days (**Figure 1B**).

Nrp-1$^+$ iTreg Was More Stable Than Nrp-1$^-$ iTreg *In Vitro* and *In Vivo*

Given that Nrp-1 was highly expressed on iTreg but not activated CD4$^+$ cells, we chose to determine the biological significance of Nrp-1 expression on iTreg. Foxp3 stability is closely associated with Treg functionality, we therefore compared the stability of two cell populations *in vitro* and *in vivo*. Firstly, iTreg was induced and then the Nrp-1$^+$ and Nrp-1$^-$Foxp3$^+$ subsets were sorted and re-stimulated with TCR and IL-2 *in vitro*. The Foxp3 level of Nrp-1$^+$iTreg was almost maintained from day 4 to day 7, while Nrp-1$^-$iTreg significantly lost Foxp3 on day 7. Neither population produced IL-17A, however, Nrp-1$^-$ but not Nrp-1$^+$iTreg began to produce IFN-γ (**Figure 2A**).

We further evaluated the stability of these two subsets *in vivo*. Both the Nrp-1$^+$ and Nrp-1$^-$ iTreg subsets were adoptively transferred into Rag1$^{-/-}$ mice, and we observed that Nrp-1$^+$ iTreg had a high Foxp3 expression on day 4, which was maintained at 52.3% on day 12 after cell transfer. Conversely, Nrp-1$^-$ iTreg significantly reduced Foxp3 on day 4 and dramatically lost Foxp3 expression between days 7-12 after cell transfer. Few Nrp-1$^+$iTreg produced IFN-γ but not IL-17A; conversely, 25-30% of Nrp-1$^-$ iTreg produced IFN-γ, and 3-4% produced IL-17A (**Figure 2B**).

Nrp-1$^+$ iTreg Displayed Superior Functional Activity *In Vitro* and *In Vivo*

In order to determine the functional significance of Nrp-1 expression on iTreg, we compared the suppressive activity of both iTreg subpopulations. Using a standard *in vitro* suppression system as previously described (26), the suppression exerted by the Nrp-1$^+$iTreg against T cell proliferation was superior to the Nrp-1$^-$ iTreg subset at the ratios (Treg to T effector cells) of 1:2 to 1:16 (**Figure 2C**).

We further developed an *in vivo* colitis model (23) to validate this result. Four CD4$^+$T cell populations including Nrp-1$^+$ and Nrp-1$^-$ iTreg; Nrp-1$^+$GFP$^-$ and Nrp-1$^-$GFP$^-$ cells were co-transferred with naïve CD4$^+$CD45RB^{hi} T cells to Rag1$^{-/-}$ mice. As expected, two GFP$^-$ cell populations failed to suppress colitis, but both iTreg subpopulations displayed suppression. However, Nrp-1$^+$iTreg almost completely suppressed the onset and progression of colitis including weight loss and intestine

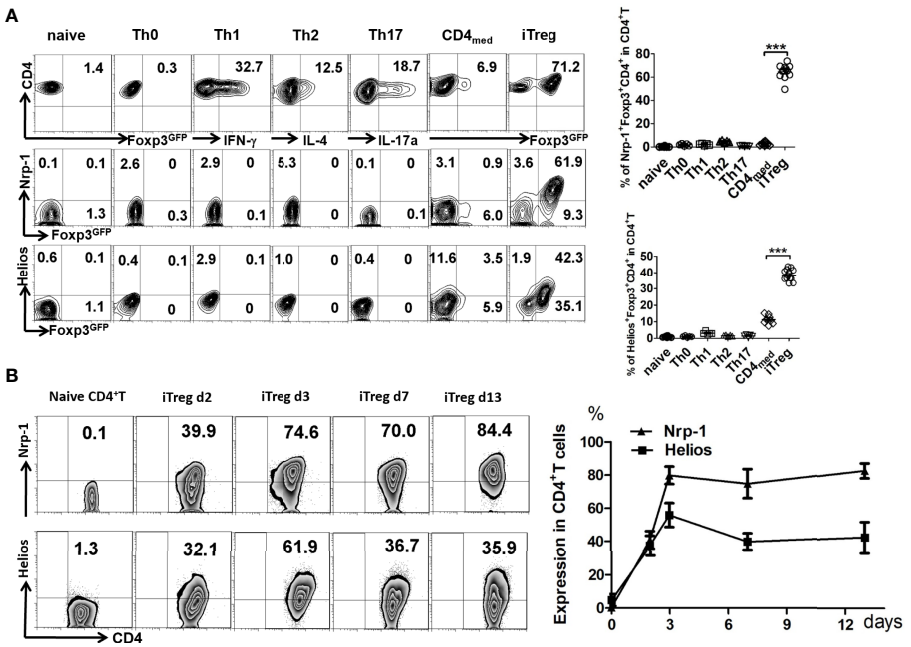

FIGURE 1 | Nrp-1 was highly expressed in induced CD4$^+$ Treg *in vitro*. **(A)** The expression of Nrp-1 and Helios were measured in naïve CD4$^+$T cells, Th0, Th1, Th2, Th17, CD4$_{med}$, or iTreg by flow cytometry. Nrp-1, Helios, and Foxp3-GFP were analyzed together. Representative FACS plots and the summarized data (right) of three separated experiments were shown. (***p<0.001). **(B)** Dynamic Nrp-1 and Helios expression in iTreg cells *in vitro at different days.* Representative data was presented.

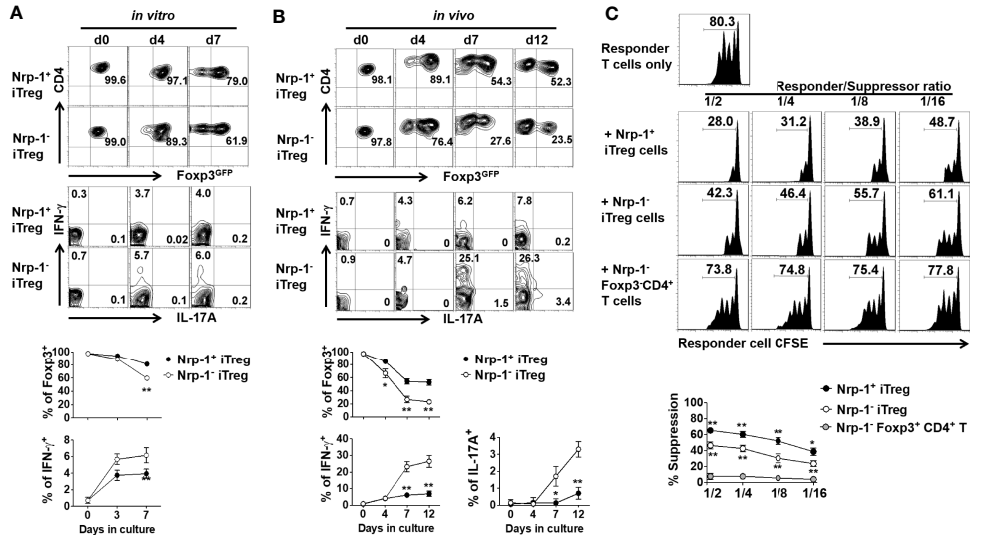

FIGURE 2 | Foxp3-GFP$^+$Nrp-1$^+$iTreg was more stable *in vitro* and *in vivo with* a prior suppression *in vitro*. **(A)** Foxp3-GFP$^+$Nrp-1$^+$iTreg and Foxp3-GFP$^+$Nrp-1$^-$iTreg were sorted from iTreg, and then cultured with CD3CD28 beads (cells/beads 1/5)+rhIL-2 (10ng/ml) for several days *in vitro*. Cells were harvested at indicated days; Foxp3-GFP, IFN-γ, and IL-17a were measured on GFP$^+$Nrp-1$^+$iTreg and GFP$^+$Nrp-1$^-$iTreg by FACS. Representative FACS plots and the summarized data of three separated experiments were shown. **(B)** GFP$^+$Nrp-1$^+$iTreg and GFP$^+$Nrp-1$^-$iTreg were transferred into Rag1$^{-/-}$ mice. Mice were sacrificed, spleen cells were harvested at indicated days; Foxp3-GFP, IFN-γ, and IL-17a were measured on GFP$^+$Nrp-1$^+$iTreg and GFP$^+$Nrp-1$^-$iTreg by FACS. Representative FACS plots and the summarized data of three separated experiments were shown. The data indicated Mean ± SEM of two separate experiments (n=6) (*p<0.05, **p<0.01). **(C)** Freshly isolated T cells (responder cells) labeled with CFSE were stimulated with anti-CD3 mAb (0.025 μg/mL) and irradiated APCs (30 Gy, 1:1 ratio) for 3 days, with or without Foxp3GFP$^+$Nrp-1$^+$iTreg or Foxp3GFP$^+$Nrp-1$^-$iTreg or Foxp3GFP$^-$Nrp-1$^-$CD4$^+$T cells. (The ratio of Treg: T cells was 1:2-1:16) T-cell proliferation was determined by the CFSE dilution rate after 3 days of culture. The data indicate the Mean ± SEM of 3 separated experiments (*p<0.05, **p<0.01).

inflammation pathology with a significantly better effect than Nrp-1$^-$iTreg (**Figures 3A–C**). Accordingly, neither GFP$^-$ cells suppressed Th1 and Th17 development, while both iTreg subsets significantly suppressed the development of two pathogenic cells (**Figure 3D**). Consistently, Nrp-1$^+$iTreg had a more potent ability to suppress Th1/Th17 cells in colitis (**Figure 3D**). Eight weeks after transfer with naïve CD4$^+$CD45RB^{hi} T cells into Rag1$^{-/-}$ mice, Nrp-1$^+$iTreg itself maintained relatively higher Foxp3 expression than Nrp-1$^-$iTreg. Furthermore, Nrp-1$^+$iTreg had lower expression of IFN-γ and IL-17a (**Figure 3E**). It suggested that Nrp-1$^+$iTreg suppressed the T cell-mediated intestinal inflammation with stronger stability in the inflammatory milieu *in vivo*.

Nrp1$^+$ iTreg and Nrp1$^-$ iTreg Were Distinct Types by RNA Sequence Analysis

Next, we performed an RNA sequence analysis. The principal component analysis revealed that nTreg, Nrp1$^+$ iTreg, and Nrp1$^-$ iTreg were distinct types (**Figure 4A**). Different gene numbers are detected with more than 2 FC in Nrp1$^+$ iTreg or Nrp1$^-$ iTreg *vs.* nTreg. Nrp1$^+$ iTreg and Nrp1$^-$ iTreg gene numbers are shared or distinct (**Figure 4B**). We identified the top 50 significantly altered genes (FC≥2) in Nrp1$^+$ iTreg vs Nrp1$^-$ iTreg. Interestingly, interferon regulatory factor 4 (IRF4) and TGFB1 were increased in the Nrp-1$^+$iTreg, while the signal transducer and activator of transcription 3 (STAT3), and interleukin 17 Receptor A (IL17RA) were highly expressed in Nrp-1$^-$iTreg (**Figures 4C, D**).

IL-10 May Account for the Superior Functional Activity of Nrp-1$^+$ iTreg *In Vitro*

The inhibitory effect of Treg is partly dependent on IL-10. We also tested IL-10 and IL-10R expression in iTreg. In the resting state, Nrp-1$^+$iTreg and Nrp-1$^-$iTreg lowly expressed IL-10 (not shown). After resting for 4 days and reculturing with IL-2 or IL-27 that can promote the IL-10 expression in CD4$^+$T cells (27), IL-10 and IL-10R were higher in Nrp-1$^+$iTreg than in Nrp-1$^-$iTreg (**Figures 5A, B**). IL-10R expression was also much higher in resting Nrp-1$^+$iTreg than in Nrp-1$^-$iTreg (**Figure 5B**).

We further generated CD4^{cre}/Nrp1$^{flox/flox}$ mice to conditional knockout Nrp-1 (Nrp-1 CKO) in CD4$^+$T cells. CD4^{cre} mice were set as wild-type (WT) control. We found that Foxp3 expression was equivalent in Nrp-1 CKO iTreg and WT iTreg (**Figure 5C**). However, IL-10 mRNA expression was much higher in WT iTreg than that in Nrp-1 CKO iTreg (**Figure 5D**), Nrp-1 CKO iTreg had an inferior functional activity *in vitro* (**Figure 5E**). Furthermore, blocking experiments demonstrated that anti-IL-10 antibody impaired the suppression of Nrp-1$^+$iTreg against the proliferation of CD8$^+$T cells *in vitro* (**Figure 5G**).

DISCUSSION

Two important works found that Nrp-1 is expressed at high levels on most nTreg; in contrast, its expression levels in mucosa-generated iTreg and other noninflammatory iTreg were lower (4,

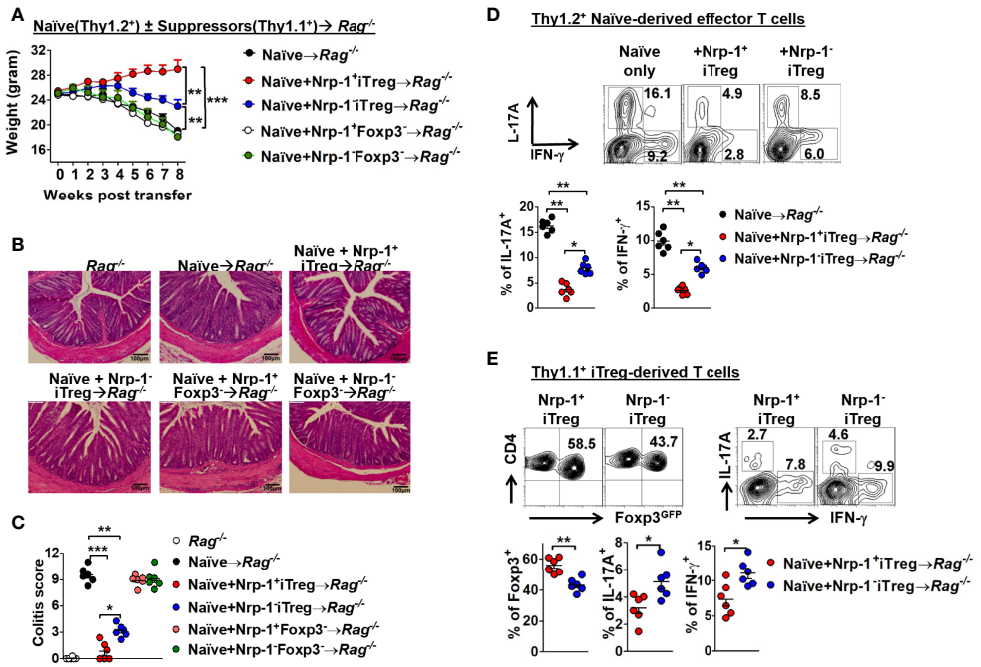

FIGURE 3 | Foxp3-GFP^{+}Nrp-1^{+}iTreg had prior suppression than Foxp3-GFP^{+}Nrp-1^{-}iTreg *in vivo*. C57BL/6 Rag1$^{-/-}$ mice were transferred with 4x10^{5} CD4^{+}CD25^{-} CD45RB^{hi} T cells from C57BL/6 WT mice. Thy1.1 GFP^{+}Nrp-1^{+}iTreg or GFP^{+}Nrp-1^{-}iTreg or GFP^{-}Nrp-1^{+}CD4^{+}T or GFP^{-}Nrp-1^{-}CD4^{+}T cells were co-transferred with congenetic CD4^{+}CD25^{-}CD45RB^{hi} T cells into rag1$^{-/-}$ mice. **(A)** Representative weight loss curve, shown as a percentage of initial weight. **(B, C)** Intestinal inflammation scores for the colon. **(D)** IFN-γ and IL-17a were detected in CD4^{+}T cell derived from Thy1.2^{+} naïve T cells in the mesenteric lymph node (MLN). **(E)** Foxp3-GFP, IFN-γ, and IL-17a were detected in GFP^{+}Nrp-1^{+}iTreg or GFP^{+}Nrp-1^{-}iTreg from MLN; data were summarized (below). The data indicate the Mean ± SEM of 3 separated experiments (n=6 mice/group) (*p<0.05, **p<0.01, ***p<0.001).

5). Abundant Nrp-1-expressing Treg was found within tumors and inflamed tissues (4). We showed that Nrp-1 was higher-expressed in CD4^{+}Foxp3^{+}T cells from the lymph node, spleen, and blood, while relative lower-expressed in CD4^{+}Foxp3^{+}T cells from the thymus. This will raise a question of why Nrp-1 is selectively higher-expressed in peripheral lymphoid organs, but not really in thymus nTreg. A recent research found that Nrp-1 could not identify the nTreg of intrathymic origin by analyzing the T cell repertoire (28). Nrp-1 was abundant among Tregs in peripheral and mesenteric lymph nodes as well as the colon. Some induced Tregs mixed in peripheral CD4^{+}Foxp3^{+}T cells may be one answer to this question: Is Nrp-1 a marker for induced Treg?

We demonstrated that Nrp-1 expression increased after 2 days of culture *in vitro*, and was still higher-expressed in TGF-β-iTreg (cultured in the presence of TGF-β, TCR, and IL-2) *in vitro* days 13; however, Nrp-1 was lower-expressed in CD4$_{med}$T cells (presence of TCR and IL-2). Weiss, et al. confirmed that Nrp-1 was upregulated in TGF-β-induced Treg *in vitro*, but this was thought of as a transient phenomenon. They induced iTreg cultured with plate-bound anti-CD3 and anti-CD28, IL-2, and TGF-β, and cells were transferred to new wells on day 3. Nrp-1 expression was reduced after the withdrawal of TGF-β (4). They demonstrated the TGF-β mediated control on Nrp-1 expression *via* differentiation of T helper cells using different TGF-β conditions as well as by using TGF-β receptor II conditional knockout mice (4). We further found that the TGF-β signal inhibitor (ALK5i) shut down the Nrp-1 expression in iTreg *in vitro* (**Supplemental Figure 3**). Another group reported that Nrp-1 was lower expressed on iTreg *in vivo* and *in vitro* (5); they generated iTreg using CD4^{+}CD25^{-}Foxp3^{-}T cells stimulated *in vitro* with anti-CD3 plus anti-CD28 or with irradiated spleen cells in the presence of TGF-β. Meanwhile, IL-2 was not added in their protocol (5). We induced iTreg using IL-2, TGF-β, and anti-CD3/CD28 beads, which can supply instant TCR signals to allow iTreg to survive. IL-2 signaling was important for Nrp-1 expression in Treg (29). It suggested TGF-β can promote the expression of Nrp-1 in iTreg based on TCR and IL-2 signaling. Recent studies demonstrated that TGF-β can also promote the expression of Nrp-1 in lung type II innate lymphoid cells (ILC2) (30). TGF-β can induce the expression of transcription factor SP1 (31), which can upregulate Nrp-1 expression by binding to the Nrp-1 promoter (32). We therefore speculate that Nrp-1 expression in iTreg may be under the control of TGF-β through SP1.

Herein, we demonstrated that Nrp-1^{+}iTreg is more stable *in vivo* and *in vitro*, even in an inflammatory state, resistant to conversion to Th1 and Th17 cells, gaining a strong suppressive activity. The DNA demethylation of conserved element within the Foxp3 locus named TSDR (Treg-specific demethylated region) was related to the stability of Treg cells. This region of nTreg is in a non-methylated state, but highly methylated in

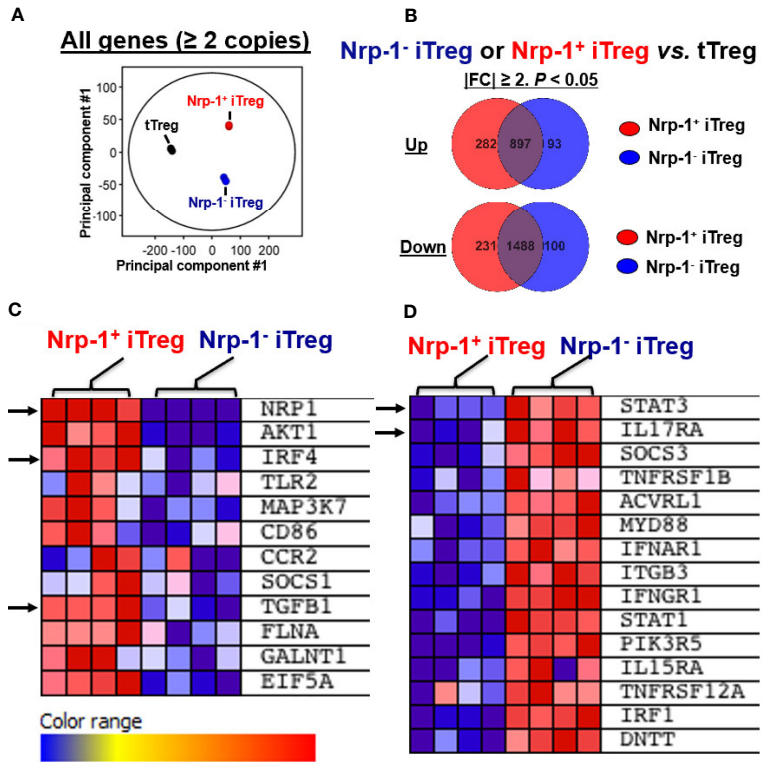

FIGURE 4 | Nrp1$^+$iTreg and Nrp1$^-$iTreg are distinct by RNA sequence analysis. **(A)** Principal component analysis revealed that nTreg (3 replicates), Nrp1$^+$iTreg (4 replicates), and Nrp1$^-$iTreg (4 replicates) are distinct. **(B)** Different gene numbers are demonstrated with more than 2 fold changes (FC) in Nrp1$^+$iTreg or Nrp1$^-$iTreg *vs.* nTreg. Nrp1$^+$iTreg and Nrp1$^-$iTreg gene numbers are shared or distinct by Mann-Whitney unpaired analysis (p<0.05). **(C, D)** some significantly altered genes (|FC|≥1, p<0.05) in Nrp1$^+$iTreg vs Nrp1$^-$iTreg were shown by Gene Set Enrichment Analysis (GSEA).

iTreg (33). The methylation levels were detected by primers designed for the TSDR region, and we found that Nrp-1$^+$iTreg and Nrp-1$^-$iTreg had a similar DNA methylation (**Figure 5F**), suggesting that the methylation of Foxp3 did not determine the stability of Nrp-1$^+$iTreg. The semaphorin-4a(Sema4a)/Nrp-1 interaction recruited phosphatase and tensin homolog (PTEN) and suppressed Akt, consequently maintaining nTreg stability (34). However, this was unknown in iTreg.

Previous studies have confirmed that TGF-β promotes downstream SMAD2/3 phosphorylation and Foxp3 expression through its receptors (TβRI and TβRII), thus maintaining iTreg stability. Nrp-1 was similar to TGF-β receptors TβRI and TβRII. Nrp-1 has an affinity for two receptors, acting as a co-receptor for TGF-β to enhance the TGF-β signaling (35). Chuckran, et al. found that Nrp-1 can promote hepatocellular inflammation and fibrosis *via* the TGF-β-mediated SMAD signaling pathway (25). We found that TGF-β1 mRNA expression was increased in Nrp-1$^+$iTreg by RNAseq analysis. This may create a positive loop, in which Nrp-1$^+$iTreg displays higher TGF-β signaling, further supporting the Nrp-1 function. Nrp-1 may further promote the stability of iTreg *via* the TGF-β-mediated SMAD signaling. On the other hand, Nrp-1$^-$iTreg lacks the positive loop mechanism and responds to TGF-β without the enhancement. Mechanistically, the Nrp-1 may serve as a coordinator, which is also important for TGF-β-induced iTreg.

It was reported that iTreg is resistant to Th17 conversion by IL-6 under inflammatory state, because IL-2 and TGF-β downregulate IL-6 receptor expression and IL-6 signaling (26). Our RNAseq analysis demonstrated that IL17RA and STAT3 (downstream of IL-6 signaling), which was related to Th17 cell differentiation, were lower-expressed in Nrp-1$^+$iTreg. This suggested that Nrp-1$^+$iTreg may exhibit non-plasticity in an inflammatory milieu.

Furthermore, we found that Nrp-1$^+$iTreg displayed superior suppression against T cell proliferation *in vitro*. Nrp-1$^+$iTreg could completely prevent colitis development, while Nrp-1$^-$iTreg exerted only half control of colitis disease. Thus, Nrp-1$^+$iTreg is a crucial subset with high suppressive activity and stability. We further found that IL-10 and IL-10R were expressed higher in Nrp-1$^+$iTreg than in Nrp-1$^-$iTreg. We considered that Nrp-1$^+$iTreg had a suppressive function partly depending on IL-10 signaling.

Schmitt EG confirmed that iTreg controlled inflammation by producing IL-10 (36, 37). IL-10 deficiency impairs Nrp-1$^+$nTreg function, promotes Th1 and Th17 response (38). One group found that the CD4$^+$Nrp-1$^+$T cells express greater amounts of IL-10 and show suppressive function. Sema3A, the binding receptor

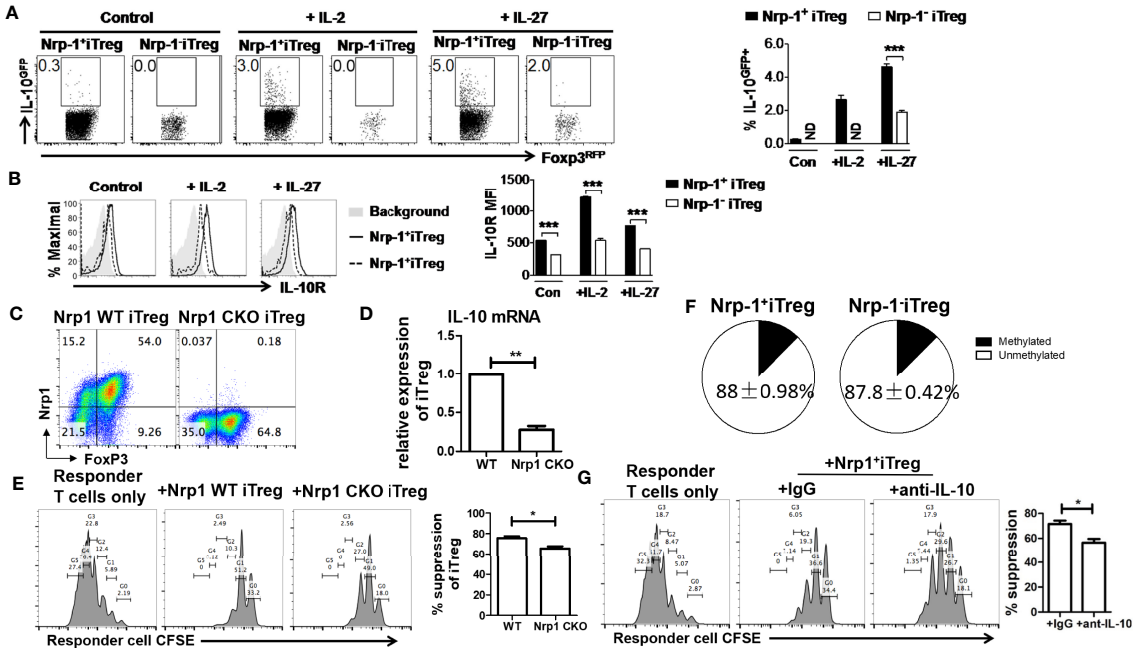

FIGURE 5 | IL-10 may be account for a superior functional activity of Nrp-1$^+$ iTreg *in vitro*. **(A, B)** After resting for 4 days, Nrp-1$^+$iTreg and Nrp-1$^-$iTreg were restimulated with mitomycin-C treated APC (Rag-/-) in the presence of 1 ug/ml anti-CD3/28. Cells were analyzed ~50 hours post restimulation. Control: no supplemental cytokines; +IL-2: + 40U/ml rh-IL-2; +IL-27: + 20ng/ml rm-IL-27. Cells were analyzed ~ 50 hours post restimulation. IL-10 and IL-10R were measured in Nrp-1$^+$iTreg and Nrp-1$^-$iTreg. **(C–E)** CD4^{cre}/Nrp1$^{flox/flox}$ mice were generated to conditional knockout Nrp-1 (Nrp-1 CKO) in CD4$^+$T cells. CD4^{cre} mice were set as wild-type (WT) control. Nrp-1 CKO iTreg and WT iTreg were generated from naïve CD4$^+$T cells in Nrp-1 CKO and Nrp-1 WT mice respectively. Foxp3 expression was equivalent in Nrp-1 CKO iTreg and WT iTreg. IL-10 mRNA expression was much higher in WT iTreg than that in Nrp-1 CKO iTreg (n=5). There was an inferior functional activity of Nrp-1 CKO iTreg in suppressing CD8$^+$T cells (Ratio 1/1) *in vitro* (n=5) by an analysis of flow-based suppression assay. Representative plots and the summarized data of different experiments were shown. The data indicate the Mean ± SEM of 3 separated experiments (*p<0.05, **p<0.01, ***p<0.001). **(F)** The methylation levels were detected by primers designed for the TSDR region in Nrp-1$^+$iTreg (n=4) and Nrp-1$^-$iTreg (n=4). Nrp-1$^+$iTreg and Nrp-1$^-$iTreg had similar DNA methylation. **(G)** Anti-IL-10 antibody or IgG were added in the suppression system of Nrp-1$^+$iTreg against CD8$^+$T cells (Ratio 1/1) *in vitro* (n=4) by an analysis of flow-based suppression assay (*p<0.05).

of Nrp-1, acted directly on CD4$^+$Nrp-1$^+$T cells, promoted IL-10 production, and affected their function (39). Using Nrp-1 CKO mice, we found that IL-10 mRNA expression was much higher in WT iTreg than that in Nrp-1 CKO iTreg. There was an inferior functional activity of Nrp-1 CKO iTreg *in vitro*, and the suppression of Nrp-1$^+$iTreg against the proliferation of CD8$^+$T cells was dependent on IL-10 *in vitro*. These results suggested that IL-10 signaling contributed to a superior functional activity of Nrp-1$^+$ iTreg. Another group found that Nrp-1 KO nTreg had a deficient suppressive function and was defective in IL-10 production (40). We further found that IRF4 was highly expressed in Nrp-1$^+$iTreg by RNAseq analysis. The transcription factor IRF4, cooperating with Foxp3, plays an important role in natural Treg differentiation and function (41, 42). IRF4 regulated IL-10 expression in Treg through the remodeling of chromatin at the *IL10* locus (41). IRF4 and Nrp-1 were both functionally involved in CD8$^+$T cells (25). We assume that IRF4 will enable Nrp-1$^+$iTreg with a higher amount of IL-10 and stronger regulatory function.

Importantly, Nrp-1 is required for Treg to limit anti-tumor immune responses and to cure established inflammatory colitis (34, 43, 44), but is dispensable for the suppression of autoimmunity and maintenance of immune homeostasis (34). The number of nTreg is relatively small in peripheral blood, and it takes a long time to expand *in vitro* before infusion of these cells into recipients. As polyclonal cells, iTreg can be massively expanded from naïve CD4$^+$T cells *in vitro*, and it is easy to obtain the number of cells in therapeutic doses, suggesting that iTreg based therapy is a good choice for the treatment of autoimmune diseases.

There are some limitations of this study. We do not complete a protein staining of TGF-β, IRF4, IL17RA, and STAT3. How does IRF4 regulate the IL-10 expression and the function of Nrp-1$^+$iTreg are unknown. The role of Nrp-1 in the function of human iTreg has not been mentioned.

In all, our data confirmed that Nrp-1 is a good marker to identify functional iTreg, as Nrp-1$^+$iTreg showed a greater suppressive activity and powerful function to maintain the immune homeostasis. These findings may provide a novel strategy for treating autoimmune diseases.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The

RNA seq data presented in the study are deposited in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/), accession number GSE201416.

ETHICS STATEMENT

The animal study was reviewed and approved by Penn State University and Cornell University for the Use and Care of Animals. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SZ and JL conceived the study. WC, WH, YX, YC, WQ, JM, AA, and JW performed the experiments. WC, WH, SZ, and JL analyzed the data. WC, WH, and SZ wrote the manuscript. 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/fimmu.2022.900139/full#supplementary-material

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Distinct Injury Responsive Regulatory T Cells Identified by Multi-Dimensional Phenotyping

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$CD4^+$ regulatory T cells (Tregs) activate and expand in response to different types of injuries, suggesting that they play a critical role in controlling the immune response to tissue and cell damage. This project used multi-dimensional profiling techniques to comprehensively characterize injury responsive Tregs in mice. We show that $CD44^{high}$ Tregs expand in response to injury and were highly suppressive when compared to $CD44^{low}$ Tregs. T cell receptor (TCR) repertoire analysis revealed that the $CD44^{high}$ Treg population undergo TCRαβ clonal expansion as well as increased TCR CDR3 diversity. Bulk RNA sequencing and single-cell RNA sequencing with paired TCR clonotype analysis identified unique differences between $CD44^{high}$ and $CD44^{low}$ Tregs and specific upregulation of genes in Tregs with expanded TCR clonotypes. Gene ontology analysis for molecular function of RNA sequencing data identified chemokine receptors and cell division as the most enriched functional terms in $CD44^{high}$ Tregs versus $CD44^{low}$ Tregs. Mass cytometry (CyTOF) analysis of Tregs from injured and uninjured mice verified protein expression of these genes on $CD44^{high}$ Tregs, with injury-induced increases in Helios, Galectin-3 and PYCARD expression. Taken together, these data indicate that injury triggers the expansion of a highly suppressive $CD44^{high}$ Treg population that is transcriptionally and phenotypically distinct from $CD44^{low}$ Tregs suggesting that they actively participate in controlling immune responses to injury and tissue damage.

Keywords: Tregs, trauma immunology, CyTOF, T cell receptor diversity, single-cell RNA sequencing

INTRODUCTION

Traumatic injury causes sudden, non-infectious tissue damage that initiates a complex immune response aimed at controlling excessive inflammation and maintaining immunological tolerance against exposure to sequestered self-antigens (1). When unchecked, the pro-inflammatory response to trauma, manifesting clinically as the systemic inflammatory response syndrome (SIRS), can lead

to significant morbidity and mortality from shock and organ failure (2, 3). Key immune features of SIRS include augmented Toll-like receptor (TLR) responsiveness, enhanced granulopoiesis, and inflammasome activation in innate immune cell types (4, 5). In contrast, the adaptive immune response to injury is skewed towards the compensatory anti-inflammatory response syndrome (CARS) phenotype that is characterized as enhanced regulatory T cell (Treg) activity, increased Th2-type cytokine production by T cells, and reduced antigen-specific Th1 responses (6–11). Imbalances in these innate and adaptive immune response phenotypes are central to the loss of immune homeostasis that can predispose people to secondary infections or inflammation-mediated complications following severe injury (12). Thus, there is a critical need to understand the complex immune regulatory responses that occur in response to traumatic injuries to forward the development of beneficial immunotherapies.

Tregs are vital to the maintenance of peripheral immune tolerance (13–15). In mice, Tregs were identified as being acutely activated by injury, and are the primary adaptive immune cell subset that controls the pro-inflammatory SIRS phenotype (12, 16). Furthermore, injury enhances the immune suppressive potency of Tregs from injury-site draining lymph nodes, but not Tregs from the spleens in a mouse burn injury model (10). This compartmentalized response by Tregs, as well as their rapid activation by injury, supports the possibility that Tregs can react to danger associated molecular patterns (DAMPs), inflammatory cytokines, stress, or protein antigens that are exposed by tissue damage.

Our group has recently demonstrated that $CD44^{high}$ Tregs are highly reactive to burn trauma in mice, and show expansion and upregulation of Treg effector molecules such as CTLA4, ICOS, and GITR (17). Moreover, this $CD44^{high}$ Treg subpopulation is similar to those found on expanded memory Tregs (mTregs) in inflammatory and autoimmune diseases like multiple sclerosis (18), coronary artery disease (19), nephritis (20), type I diabetes (21), allergy (22) and obesity (22). Little is known about how mTregs are activated or their biological functions, although it is thought that mTregs may react to self-antigens and function to control excessive inflammation at sites of tissue damage (23). Given that trauma induces rapid activation of mTregs, we hypothesize that a pool of mTregs is specifically reacting to DAMPs or other antigens that are released or exposed to immune cells following tissue trauma. To address this hypothesis, unsupervised transcriptomic and single-cell technologies were used to comprehensively characterize injury-reactive Tregs with the objective, to contribute novel insights into Treg biology and their phenotypic response to cell and tissue damage.

MATERIALS AND METHODS

Study Design

This project used unbiased systems immunology approaches including RNA sequencing, mass cytometry (CyTOF), and TCR repertoire analytical methods to test the hypothesis that the Tregs that respond to injury represent a subset of CD4 +FoxP3+ T cells in mice. All experiments were performed using a well-defined mouse burn trauma model following ARRIVE guidelines. Age- and sex-matched mice were randomized into control and experimental groups, with sample sizes chosen based on statistical power analysis and previous experience. Experimental replication for each experiment is indicated in the figure legends. The investigators were not blinded, and no data were excluded from analysis.

Mice

C57BL/6 wild-type (stock #000664), $Foxp3^{DTR}$ (stock #016958) and BALB/c (stock #000651) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Eight to16-weeks old mice were acclimated for at least 1 week before being used for experiments. All procedures performed in this study were reviewed and approved by the Brigham and Women's Hospital IACUC (2020N000458) and were found to be in accordance with guidelines set by the U.S. Department of Agriculture (Washington, DC) and the National Institutes of Health (Bethesda, MD).

Mouse Injury Model

A mouse burn injury model was used to model traumatic injury in mice as previously described (24). Briefly, mice were anesthetized by intraperitoneal (IP) injection with 125 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA). Buprenorphine at 0.6mg/kg was injected subcutaneously at the time of injury. The mice had their dorsal fur shaved and were placed in a plastic mold exposing 20% of their total body surface area. Injury was induced by immersing the exposed part of the dorsum for 9 seconds in a 90° C water bath. This approach causes a full-thickness and well-demarcated anesthetic injury due to complete loss innervation. Uninjured mice underwent the same procedure but were exposed to room temperature water for 9 seconds. All animals were resuscitated with an IP injection of 1 mL of 0.9% pyrogen-free normal saline containing ANTISEDAN (atipamezole, Zoetis, US) at 1mg/kg. The mortality from this burn trauma model is less than 2%.

Lymph Node Cell Preparations

Mice were euthanized by CO_2 asphyxiation. Injury-site draining lymph nodes (axillary, brachial, and inguinal) were harvested and immediately placed in ice-cold culture medium (RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1 mM glutamine, 10 mM HEPES, 100 μM nonessential amino acids, penicillin/streptomycin/fungizone, and 50 μM 2-mercaptoethanol, all purchased from Gibco-Invitrogen, Grand Island, NY). To prepare single cell suspensions, the lymph nodes were mechanically minced through sterile 70- μm cell strainers. Cell preparations were washed twice in culture medium and then strained to remove debris.

Flow Cytometry and Cell Sorting

Fluorescent conjugated antibodies were purchased from BioLegend (San Diego, CA) or eBioscience (Waltham, MA):

APC/Cy7-labeled anti-CD3 (145-2C11), APC-labeled anti-CD44 (IM7), eFluor450-labeled anti-FoxP3 (FJK-16s), and PE/Cy7-labeled anti-CD4 (GK1.5), FITC-labeled anti-CD25(PC61). FITC-labeled Anti-Mouse TCR Vβ Screening Panel was purchased from BD bioscience. Zombie NIR™ Fixable Viability Kit (BioLegend) was used for cellular viability staining. Flow cytometry stains were performed in PBS with 1% BSA and 0.1% sodium azide at room temperature. Cells were plated and Fc-block reagent (TruStain FcX Fc receptor blocking reagent, BioLegend, San Diego, CA) was added for 10 min to minimize nonspecific antibody staining before adding cell-surface fluorochrome-labeled antibodies. For intracellular stains, cells were permeabilized using the FoxP3/transcription factor staining buffer set from eBioscience (eBioscience/ThermoFisher Scientific, Waltham, MA). Stained samples were fixed in 4% PFA in PBS, washed by centrifugation, then reconstituted in PBS for flow cytometry analysis on a MACSQuant Analyzer (Miltenyi Biotech, San Diego, CA). Data analysis was performed using the FlowJo software program (Tree Star, Ashland, OR). The flow cytometry gating schemes for T cells, $CD4^+$ and $CD4^-$ T cells, $CD44^{high}$ and $CD44^{low}$ Tregs as well as TCRVβ are presented in **Supplemental Figures 6**, **7**. For sorting Tregs, the stains were performed in sorting buffer (RPMI 1640 without phenol red supplemented with 0.5% BSA, 1 mM glutamine, 10 mM HEPES, 100 μM nonessential amino acids, penicillin/streptomycin/fungizone, and 50 μM 2-mercaptoethanol). CD4+ T cells were purified by negative selection using Miltenyi MACS $CD4^+GFP^+$ Tregs or $CD4^+GFP^+CD44^{high}$ and $CD4^+GFP^+CD44^{low}$ cells were sorted respectively according to experimental requirements.

Treg Suppression Assay

$CD4^+CD25^-$ T cells (Tconv) for Treg functional assays were negatively selected using LS Columns (Miltenyi Biotec, 130-042-401) with MidiMACS™ Separator (Miltenyi Biotec,130-042-302) by staining the cells with Biotin-labeled anti-mouse CD8a (BioLegend 100704), CD25 (BioLegend 102004), TER119 (BioLegend 116204), CD45R/B220 (BioLegend 103204), CD49b (BioLegend 108904), and CD11b (BioLegend 101204). The negatively selected cells were collected and stained with Zombie NIR™ Fixable Viability kit and then CellTrace™ Violet Cell Proliferation Kit (ThermoFisher, C34571) for the Treg functional assay. $CD44^{high}$ and $CD44^{low}$ Tregs were sorted respectively from sham or day 7 after injury $Foxp3^{DTR}$ mice, co-cultured with CellTrace Violet labeled Tconv, and stimulated with mouse T cell activator anti-CD3/CD28 beads (Gibco, 11452D). Purified Tconv (8×10^4) were co-cultured with $CD44^{high}$ or $CD44^{low}$ Tregs at 1:1, 1:2, 1:4, 1:8 Treg : Tconv ratios or control Tconv alone. Proliferation of CD3/CD28 bead stimulated Tconv without added Tregs was set as control proliferation with 0% suppression. Proliferation was measured using the CellTrace Violet dilution assay by flow cytometry after 4 days co-culture that detects proliferated cells by reduced fluorescence intensity in cells that undergo one or more cell divisions. Suppression% was calculated as: $100 - (\frac{\text{Percent proliferated cells in Treg : Tconv}}{\text{Percent proliferated cells Tconv only}} \times 100)$

Depletion of Tregs in $FoxP3^{DTR}$ Mice, Adoptive Transfer of Tregs, and Secondary *Pseudomonas aeruginosa* Lung Infection Model

$Foxp3^{DTR}$ mice were treated with 40ng/kg diphtheria toxin (DT) at 2h and 24h after burn injury to deplete Tregs. Male C57BL/6 mice underwent burn trauma injury. At 7 days after injury, CD4 T cells were purified from injury-site draining lymph nodes (axillary, brachial, and inguinal) into $CD44^{high}$ or $CD44^{low}$ Tregs using anti-CD25 and anti-CD44 antibodies. Tregs from injured mice were transfused into the anesthetized injured $Foxp3^{DTR}$ mice depleted of Tregs at 50,000 cells/mouse by intracardiac injection in 0.2 ml of PBS. Mice were subsequently challenged with intranasal *Pseudomonas aeruginosa* 1 day after Treg-transfusion. Survival was monitored over a week period. For lung infections, *Pseudomonas aeruginosa* (ATCC 27853) were grown for 16 hours with gentle agitation in brain-heart infusion (BHI) broth medium at 37°C and were harvested by centrifugation at 450 x *g* for 10 minutes, then washed once with PBS by centrifugation. Based on absorption spectroscopy measurements (ABS_{600}), bacteria were diluted to contain 3-5 x 10^7 CFUs/ml in PBS. Mice were anesthetized by IP injection using Ketamine/Xylazine at 125/10 mg/kg. Mice were then held with their nares upright, and 40 μl of bacteria suspension was administered by intranasal route (1.2-2 x 10^6 CFUs). This inoculum dose was found to cause 50% mortality in male C57BL/6 mice over a 7 day period, with deaths first occurring at days 2-3 after infection. Bacteria CFUs were quantified by drop-plating of serial dilutions on Luria broth (LB) agar plates, and colonies were counted the following day after incubating the plates at 37°C.

RNA Sequencing of Sorted $FoxP3^+$ T Cell Populations

Lymph node $CD4^+CD44^{high}$FoxP3-GFP^+ and $CD4^+CD44^{low}$FoxP3-GFP^+ T cells or FoxP3-GFP^+ T cells were purified by FACS sorting at 7 days after injury from injured or uninjured $Foxp3^{DTR}$ mice (strategy shown in **Supplementary Figure S7**). RNA was purified from 50,000 cells from each sample using the RNeasy Micro Kit (Qiagen, CA) according to manufacturer's protocol. RNA sequencing (RNAseq) was performed by the Molecular Biology Core Facilities (MBCF) at Dana-Farber Cancer Institute (DFCI). cDNA was synthesized using Takara SMART-Seq v4 PLUS kit and was fragmented to ~150bp by Covaris Adaptive Focused Acoustics®-AFA® technology. The cDNA library prepared with Swift 2S™ Turbo DNA library kit from 2ng of cDNA was submitted for next-generation sequencing (NGS).

RNAseq Data Processing and Analysis

Analysis of RNA sequencing data was performed using Visualization Pipeline for RNA-seq (VIPER) workflow (25). The VIPER workflow provides sample to sample correlation and sample to feature heatmap plots to judge the correlation and clustering patterns of all samples (**Figures S2A, B**). Gene count stabilized variance of RNAseq data was determined using the *vst* function of DESeq2 (26). Principal component analysis (PCA)

plots were generated based on the variance stabilized counts. The top 2000 genes that were most differentially expressed in $CD44^{high}$ versus $CD44^{low}$ Tregs were identified using DESeq2 and input into the gene ontology (GO) molecular function term enrichment analysis webtool (27–29). Heat maps were generated using gene count Z-scores of highly variable cytokines, cell surface markers, and transcription factor genes. Hierarchical clustering of samples was done using the *hclust* function in R. Differentially expressed genes between Treg subsets and the effects of injury on gene expression were identified by DESeq2 and volcano plots showing differential expression of genes were generated using the Enhanced Volcano R package (30).

RNA-Based TCR Repertoire Sequencing

Viable $CD4^+CD44^{high}GFP^+$ and $CD4^+CD44^{low}GFP^+$ cells were sorted from lymph nodes harvested at 7-days after injury from burned or uninjured $Foxp3^{DTR}$ mice (cell numbers are detailed in **Supplementary Table 1**). All RNA extraction, cDNA synthesis, amplification, NGS library preparation and sequencing were performed by iRepertoire, Inc. (Huntsville, USA), RNA was extracted from FACS sorted cells using Qiagen's RNeasy Micro kit with DNase Treatment. One-third of the total RNA from each sample were used for the construction of mouse TCRα and β chain libraries by reverse transcription polymerase chain reaction (RT PCR) using Qiagen's OneStep RT PCR mix (Qiagen). First strand cDNA was selected, and unused primer was removed by SPRIselect beads (Beckman Coulter) followed by a second round of binding and extension with the V-gene primer mix. After binding and extension, SPRIselect beads were used to purify the first and second strand synthesis products. Library amplification was performed with a pair of primers that are specific for communal sites engineered onto the 5' end of the C- and V-primers used in first and second strand synthesis. The final constructed library includes Illumina dual index sequencing adapters, a 10-nucleotide unique molecular identifier region, and an 8-nucleotide internal barcode associated with the C-gene primer. The amplified libraries were multiplexed and pooled for sequencing on the Illumina MiSeq platform using a 600-cycle kit and sequenced as 250 pair-end read. The portion of TCRA and TCRB receptor genes that were sequenced included the second framework region stretching to the beginning of the TCR constant region, including the CDR2 and CDR3 regions.

TCR Sequence Analysis

Sequencing of raw data were analyzed using the previously described iRmap program (11, 31). Sequence reads were de-multiplexed according to both Illumina dual indices incorporated during the amplification process and barcode sequences at the 5' end of reads from the constant region. Reads were then trimmed according to their base qualities with a 2-baseslіding window. If either quality value in this window was lower than 20, this sequence stretching from the window to the 3' end, was trimmed out from the original read. Trimmed pair-end reads were joined together through overlapping alignment with a modified Needleman-Wunsch algorithm (32). If paired forward and reverse reads in the overlapping region were not perfectly matched, both forward and reverse reads were thrown out without further consideration. The merged reads were mapped using a Smith-Waterman algorithm to germline V, D, J and C reference sequences using an IMGT reference library. To define the CDR3 region, the position of CDR3 boundaries of reference sequences from the IMGT database was migrated onto reads through mapping results, and the resulting CDR3 regions were extracted and translated into amino acids.

TCR Repertoire Data Analysis

Treemap plots were generated by iRweb tools (iRepertoire, Inc. Huntsville, USA). V, D and J gene usage and CDR3 sequences were identified and assigned. In treemap plots, each unique CDR3 is shown as a colored rectangle. The size of each rectangle corresponds to the abundance of each CDR3 within the repertoire and the positioning is determined by the V region usage. To compare the relative diversity of TCR libraries, we used iRweb tools to generate diversity-50 (D50) values, diversity plots, and diversity index (Di) values. D50 is the percent of unique TCR CDR3 sequences that account for the cumulative 50% of the total CDR3s counted in the sample. $D50 = \frac{number\ of\ uCDR3\ sequences\ in\ top\ 50\ \%}{number\ of\ uCDR3\ in\ top\ 10{,}000\ CDR3\ sequences} \times 100$. Low D50 values indicate higher clonality. The diversity index (Di) was calculated as: $Di = \left[1 - \frac{\sum n(n-1)}{N(N-1)}\right] \times 100$. TCR diversity index values are proportional to the values where high values indicate high diversity, while low values indicate low diversity. Diversity index plots were generated by plotting the percentage of total TCR reads versus the percentage of unique CDR3 sequences. The curve illustrates the overall diversity of the population with full diversity represented by the dashed line in the middle. The higher the TCR diversity in the cell population, the closer the curve will be to the dashed line in the middle. The D50 values are indicated on these diversity index plots to illustrate the differences in TCRA and TCRB clonality in Treg populations from uninjured and injured mice.

Preparation of scRNAseq and scTCRseq Libraries

For single cell RNAseq (scRNAseq) and single cell TCRseq (scTCRseq), viable lymph node FoxP3 GFP^+ cells were sorted from injured or uninjured $Foxp3^{DTR}$ mice at 7 days after injury. The sorted FoxP3 GFP^+ cells were washed and resuspended in sorting buffer at a cell concentration of 1000 cells/μL. About 17,000 mouse cells were loaded onto a 10x Genomics Chromium™ instrument (10x Genomics) according to the manufacturer's recommendations. The scRNAseq libraries were processed using Chromium™ single cell 5' library & gel bead kit (10x Genomics). Matched scTCRseq libraries were prepared using 2 μL of post cDNA amplification material and Chromium Single Cell V(D)J Enrichment Kit, Mouse T Cell. Quality controls for amplified cDNA libraries and final sequencing libraries were performed using Bioanalyzer High Sensitivity DNA Kit (Agilent). The sequencing libraries for scRNAseq and scTCRseq were normalized to 4nM concentration and pooled using a volume ratio of 4:1. The pooled sequencing libraries were sequenced on Illumina NovaSeq S4 300 cycle platform. The sequencing parameters were: Read 1 of 150bp, Read 2 of 150bp and Index 1 of 8bp. The sequencing data were demultiplexed and aligned to GRCm38 using cell ranger version 3.1.0 pipeline (10x Genomics).

Single-Cell RNAseq Data Analysis

scRNAseq reads were aligned to the mouse reference genome (GRCm38) with the Ensembl GRCM28.91 GTF file using Cell Ranger (10x Genomics; sample statistics in **Supplementary Table 3**). The uninjured and 7D after injury samples were aggregated using the *aggr* function in Cell Ranger. Cell Ranger gene expression matrices were further analyzed with Seurat (v3.2.2). Matrices were filtered to exclude low-quality cells; cells with < 500 or > 4000 unique features. Cells with > 7% reads mapping to the mitochondrial genome were filtered out (thresholds chosen using analysis shown in **Supplementary Figures S4 C, D**). Feature counts were normalized using the *NormalizeData* function of Seurat. Per standard methods, we identified the 2,000 most variable genes and did PCA on these genes. Gene counts were scaled using the *ScaleData* function of Seurat. Clustering was done using the *FindNeighbors* (dims = 1:20) and *FindClusters* (resolution = 0.5) functions in Seurat. Cluster 6 was then removed as these cells expressed B cells markers. TCR clonotype metadata from the output of Cell Ranger VDJ was added to each cell using custom scripts. Cells with a CDR3 clonotype found in 2 or more cells were labeled as "Expanded". Clustering was redone with parameters to generate many clusters, *FindNeighbors* (dims = 1:25) and *FindClusters* (resolution = 2.0) functions in Seurat. Clusters composed of 15% or more expanded cells were labeled as "Expanded" phenotype. Normalized expression was visualized with t-SNE plot projections and violin plots using Seurat.

Data Processing of scTCRseq Libraries

For scRNAseq, the raw sequencing data generated by Cell Ranger 3.1.0 were loaded into the third-party tools for secondary analysis: Loupe Cell Browser 4.1.0 and Loupe VDJ Browser 3.0.0 (https://www.10xgenomics.com/). TCR sequencing data were aligned to the mm10 reference genome and RefSeq gene models using Cell Ranger VDJ TCA and TCB sequences from individual cell were used to infer clonotypes. The clonotype comparison feature in Loupe Cell Browser was then applied to pool TCR clonotypes across groups by matching CDR3 amino acids of both TCRA and TCRB. TCR clonotypes were designated as "expanded" if the paired CDR3 sequences were found in 2 or more cells.

Mass Cytometry (CyTOF)

CyTOF staining was performed at room temperature as previously described (33). Cells harvested from lymph nodes were incubated with cisplatin live/dead stain for 2 min (Fluidigm) and blocked with TruStain FcX Fc receptor blocking reagent for 10 min before antibody staining. For surface staining, cells were stained with the CyTOF antibody cocktail for 30 min. After washing twice with staining buffer (calcium/magnesium-free PBS, 0.2% BSA, 0.05% sodium azide), cells were fixed and permeabilized using eBioscience™ Fixation/Permeabilization kit (Invitrogen). Subsequently, cells were barcoded using a palladium-based barcode reagent (33). After washing out excess barcode reagent, samples were pooled together and stained with intracellular CyTOF antibodies. Details of all antibodies used for CyTOF staining are listed in **Supplementary Table 4**. Single-cell CyTOF data was collected using a Helios mass cytometer (Fluidigm). Data normalization and deconvolution of barcoded staining data was conducted using the normalizer and the single-cell-debarcoder software developed in the Nolan Lab (Stanford) (33, 34). Data analysis was conducted using Cytobank (35). A nonlinear dimensionality reduction algorithm, viSNE, was run on all markers for dimensionality reduction (36). A hierarchical clustering algorithm, SPADE was run on viSNE parameters to cluster cells for phenotypic identification (37).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software Inc. San Diego, CA, USA) or custom R scripts for RNAseq data. Data comparing multiple groups were analyzed by one-way ANOVA with Dunnett statistical hypothesis testing to correct for multiple comparisons and assuming Gaussian distribution of residuals. Data consisting of one variable and two factors were analyzed by two-way ANOVA and the Sidak or Tukey tests were applied for multiple comparison correction as appropriate. For protein expression validation studies by CyTOF, two-tailed unpaired t-tests were applied, and Welch's correction was used if two populations had different standard deviations. Analyzed data with $P < 0.05$ were considered as significantly different.

RESULTS

Trauma Induces Expansion of Highly Suppressive CD44^{high} Tregs

We first assessed the function of CD44^{high} Tregs in a mouse model of burn injury. Injury-site draining lymph nodes were harvested from mice to measure CD44^{high} and CD44^{low} CD4^{+}FoxP3^{+} T cell (Treg) abundances at 1 and 7 days after burn trauma injury using the scheme shown in **Figure 1A**. By day 7, there was a significant increase in CD44^{high} Tregs in injury-site draining lymph nodes compared to uninjured controls (**Figures 1B, C**). In contrast, there was no difference in the number of CD44^{low} Tregs between injured and control mice (**Figure 1D**). Next, we utilized a fluorescence-activated cell sorting (FACS) approach to purify CD44^{high} and CD44^{low} Treg subsets to assess Treg function and for subsequent RNA sequencing studies (**Figure 1E**). In Treg-mediated suppression assays, both CD44^{high} and CD44^{low} Tregs significantly suppressed the proliferation of CD4^{+}CD25^{-} T cells (Tconv). The CD44^{high} Tregs were identified as being much more potent than CD44^{low} Tregs at suppressing T cell proliferation and injury did not further enhance the suppressive function of CD44^{high} Tregs (**Figures 1F, G**). However, we did observe that CD44^{low} Tregs from injured mice were significantly more suppressive at a 1:1 Treg to Tconv cell ratio. Taken together, both Treg subsets are affected by injury showing either expansion (CD44^{high}) or enhanced Treg suppressive function (CD44^{low}).

Because infectious complications are a leading cause of death in trauma patients (38, 39), we next tested whether CD44^{high} or

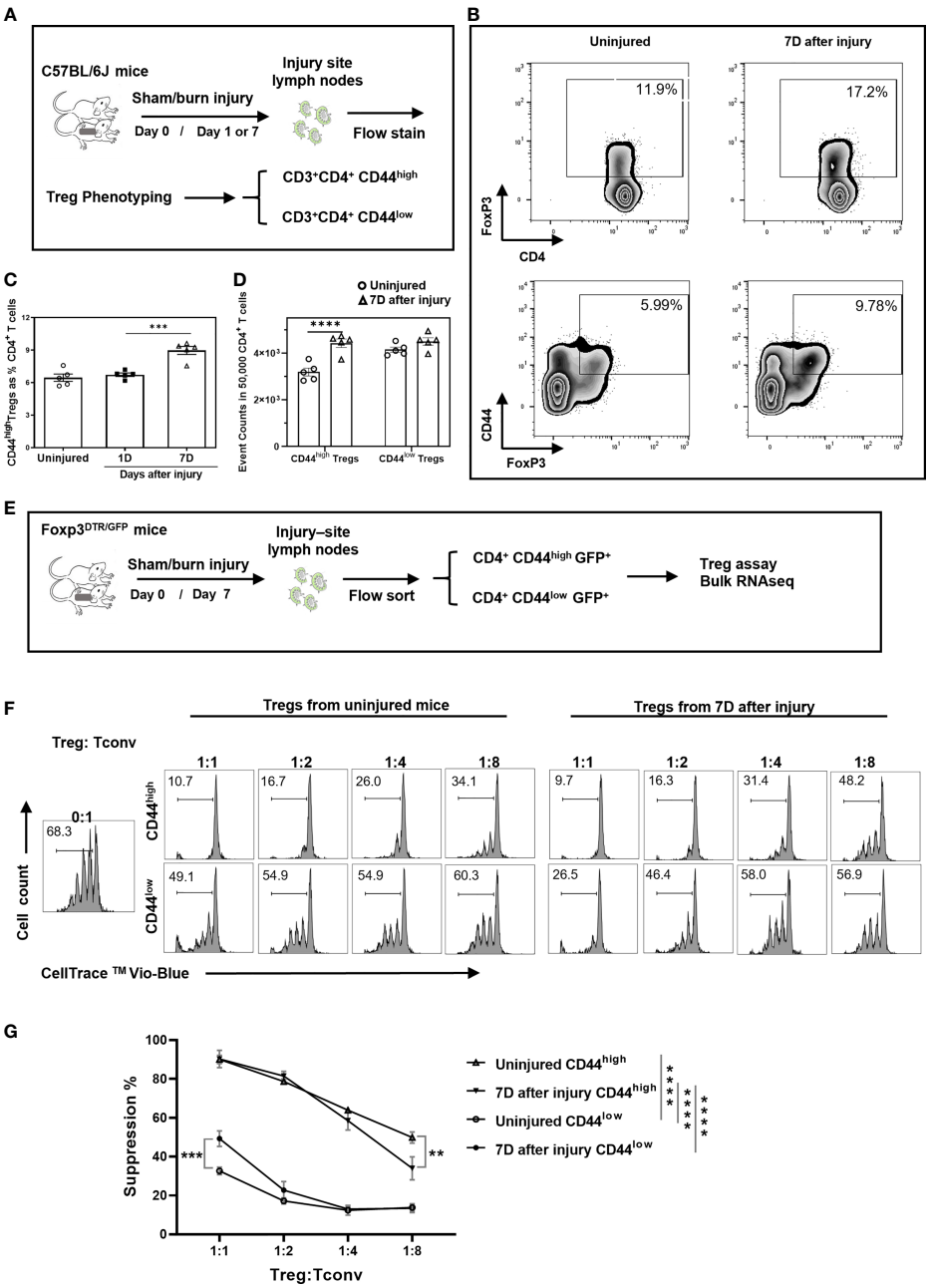

FIGURE 1 | Burn trauma induces the expansion of CD44^{high} Tregs in injury-site draining lymph nodes that more potently suppress T cell proliferation than CD44^{low} Tregs. **(A)** Experimental scheme for mouse Treg phenotyping by flow cytometry; **(B)** Representative flow cytometry contour plots illustrating Tregs and CD44^{high} Tregs in CD4^{+} T cells in injured and uninjured mice. **(C)** CD44^{high} Tregs are significantly increased in injury-site draining lymph nodes at 7 days (Δ), but not at 1 day (■) after injury compared to uninjured controls (○). Data are presented as mean ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA), P=0.0001. Significant comparisons by Dunnett's multiple comparisons test are denoted by *** (uninjured versus 7 days after burn), P=0.0002. **(D)** CD44^{high} and CD44^{low} Treg cell events in equal-sampled CD4^{+} T cells. Flow cytometry data represent two independent experiments (n=5 per group). Data were analyzed by two-way ANOVA, interaction P=0.0117. Significant comparisons by Sidak's multiple comparisons test are denoted by **** (uninjured control versus 7 days after injury in CD44^{high} Tregs), P<0.0001. **(E)** Experimental scheme for Treg functional assay and bulk RNA sequencing. **(F)** CD4^{+}CD25^{-} T cells (Tconv) FACS sorted from lymph nodes were labeled with CellTrace Violet and co-cultured with FACS sorted CD44^{high} and CD44^{low} Tregs from uninjured or injured Foxp3^{DTR} mice at the indicated cell concentration ratios. CellTrace Violet dilution was used to calculate percentage of proliferating Tconv cells after 4 days of co-culture with anti-CD3/CD28 antibody-coated T cell activation beads. Representative Treg suppression plots are shown. **(G)** Plots summarizing Treg suppressive activity. Error bars represent the mean ± SEM of n = 3 replicate wells. Data were analyzed by two-way ANOVA, interaction P<0.0001. Significant comparisons by Dunnett's multiple comparisons test are indicated by **P<0.01, ***P<0.001 and ****P<0.0001.

$CD44^{low}$ Tregs were beneficial or harmful in a secondary infection after burn injury (**Figure S1A**). In brief, $Foxp3^{DTR}$ mice were Treg depleted by administration of diphtheriae toxin after burn trauma as previously described (40). Treg depletion was validated by flow cytometry (**Figure S1B**). $CD44^{high}$ and $CD44^{low}$ Tregs sorted from injury-site draining lymph nodes of burn trauma mice were then adoptively transferred into Treg-depleted mice 2 days after injury. Mice were challenged intranasally with *Pseudomonas aeruginosa* (1.2-2 x 10^6 CFU per mouse) the next day and were observed over a week for survival (9, 10, 12). Injured mice given $CD44^{high}$ Tregs demonstrated a trend towards lower survival (43%) compared to mice given $CD44^{low}$ Tregs (75%, probability of survival, P=0.0617) (**Figure S1C**). Thus, $CD44^{high}$ Tregs suppress anti-microbial immune function more so than $CD44^{low}$ Tregs and their expansion may increase susceptibility to secondary infections following traumatic injury.

RNA Sequencing Analysis of $CD44^{high}$ and $CD44^{low}$ Treg Subsets Reveals Distinct Molecular Signatures

Next-generation RNA-sequencing (RNAseq) was used as an unbiased approach to compare gene expression variation and profiles between $CD44^{high}$ and $CD44^{low}$ Tregs from injured and uninjured mice. RNA from flow-sorted $CD44^{high}$ and $CD44^{low}$ Tregs from injured and uninjured FoxP3-GFP mice were prepared for RNA sequencing (**Figure 1E**). The resulting RNAseq data underwent quality control analysis to generate intra- and inter-group sample variability plots, sample-sample correlation plots, and sample-feature hierarchical clustering plots (**Figures S2A, B**). Principal component analysis (PCA) of sequencing data demonstrates that $CD44^{high}$ versus $CD44^{low}$ Tregs separated along principal component 1 (PC1) and accounted for the majority (89%) of gene expression variation (**Figure 2A**). Injury accounted for 5% of the observed variance in PC2. Gene Ontology (GO) analysis by enrichment of functional terms of DE genes indicated that genes associated with cell division and chemokine receptors were enriched in $CD44^{high}$ as compared to $CD44^{low}$ Tregs (**Figure 2B**). Given these findings, we specifically compared gene counts of the top differentially expressed chemokine receptors between $CD44^{high}$ and $CD44^{low}$ Tregs, which showed distinct differences in gene expression profiles (**Figure 2C**). Heatmaps showing expression levels of the most variably expressed cytokine, cell surface markers, and transcription factor were generated from RNAseq data to illustrate some of the key markers that distinguished these Treg subsets (**Figure 2D**). $CD44^{high}$ Tregs expressed higher levels of known Treg-related genes such as *Icos*, *Ctla4*, *Il10ra*, *Il10*, *Itgae*, *Tigit*, *Fgl2*, *Havcr2*, *Cd83*, *Nt5e*, and *Klrg1* than $CD44^{low}$ Tregs. Moreover, some notable cytokine and chemokine receptor genes such as *Il22ra2*, *Tgfbr1*, *Ildr1*, *Il18r1*, *Il10ra*, *Il3ra*, *Il17rb*, *Il23r*, *Il1r2*, *Il12rb*, *Ccl5*, *Cxcl10 Cxcl2*, *Cxcr3* were highly expressed in $CD44^{high}$ Tregs, while others like *Il2ra, Il6ra, Tgfbr3, Il4ra and Trnfsf8* were highly expressed in $CD44^{low}$ Tregs. $CD44^{high}$ Tregs from injured mice had higher expression levels of *Cd22, Cxcr5* and *Il7r*, as well as genes encoding components of TNF receptor signaling pathways, such as *Tnfrsf18*, *Tnfrsf9*, and *Tnfrsf4*, when compared to uninjured mice. Tregs from injured mice showed higher counts of *Cd69* and *Ifnr1* in both $CD44^{high}$ and $CD44^{low}$ groups. Notable transcription factor genes highly expressed in $CD44^{high}$ subsets were *Arnt2* (involved in responses to environmental stimuli), *Prdm1* (tissue-resident memory T cell signaling), *Atf6* (endoplasmic reticulum [ER]stress sensor), *Mybl2* (cell survival, proliferation, and differentiation); *Crem* (cAMP responsive element), T cell related genes (*Tbx21*, *Gata3*, *Rora*, *Irf4*, *Maf*, *Id2*, *Nfil3*), as well as cell cycle-related genes and some zinc finger proteins (**Figure 2C**). There were 24 differentially expressed (DE) genes increased and 25 DE genes decreased due to injury in the $CD44^{high}$ subset, and 35 increased and 15 decreased in the $CD44^{low}$ subset (**Figure 2D**). DE genes in $CD44^{high}$ versus $CD44^{low}$ Tregs in both the uninjured and injured groups are shown in **Figures S2C, D**. Among genes with a P value < 10^{-6}, there were 1006 genes with an increase of 2-fold or more and 486 genes with a decrease of 2-fold or more in the uninjured group $CD44^{high}$ Tregs. In the injured group, gene counts were 743 and 313, respectively. Upon burn injury, 24 genes were upregulated and 25 downregulated in the $CD44^{high}$ Tregs, for the $CD44^{low}$ Tregs, 35 upregulated and 15 downregulated (**Figure 2E**). Taken together, the bulk RNA sequencing results indicate that $CD44^{low}$ and $CD44^{high}$ Tregs are transcriptionally distinct populations.

Injury Induces Greater Changes in T Cell Receptor (TCR)α and TCRβ Clonality and Diversity in $CD44^{high}$ Treg Subsets

The experimental scheme for TCRα and TCRβ repertoire analysis is shown in **Figure 3A**. Treg TCR clonality and diversity were identified by RNA sequencing using the iRepertoire analytical platform. A summary of the TCR sequencing dataset is provided in **Supplementary Table 2**. Without injury, $CD44^{high}$ Tregs were found to have higher TCRα/TCRβ clonality than $CD44^{low}$ Tregs as determined lower D50 and diversity index (Di) values (**Figure 3B**). The D50 value indicates the percentage of TCR CDR3 sequences that account for 50% of the total unique CDR3 sequence reads, so low D50 values would indicate high clonality (41, 42). Interestingly, the D50 value decreased for TCRα in $CD44^{high}$ Tregs (uninjured = 9.7, injured = 5.5), but D50 for TCRβ did not change following injury suggesting that TCRα clonality is influence by injury. The Di values and curves support that $CD44^{high}$ Tregs have lower TCR diversity than $CD44^{low}$ Tregs (**Figure 3B**). The Di values were much lower for $CD44^{high}$ Tregs (TCRα/13.3, TCRβ/10.8) as compared to $CD44^{low}$ Tregs (TCRα/31.3, TCRβ/35.2). The shape of the diversity curves shown in **Figure 3B** illustrates that injury increased TCR diversity in $CD44^{high}$ Tregs by altering the curve to be closer to the diagonal dashed line, which identifies full TCR diversity. Accordingly, $CD44^{low}$ Tregs showed higher diversity than $CD44^{high}$ Tregs since the diversity curves are much closer to the diagonal line. Treemap plots illustrate that injury increased the diversity of $CD44^{high}$ Tregs, which is likely due to emergence of new $CD44^{high}$ Tregs or movement of $CD44^{high}$ Tregs from other tissues into lymph nodes following injury (**Figures 3C, D**). Plots comparing the top 10 TCRA and TCRB sequence percentages between $CD44^{high}$ and $CD44^{low}$ Tregs further

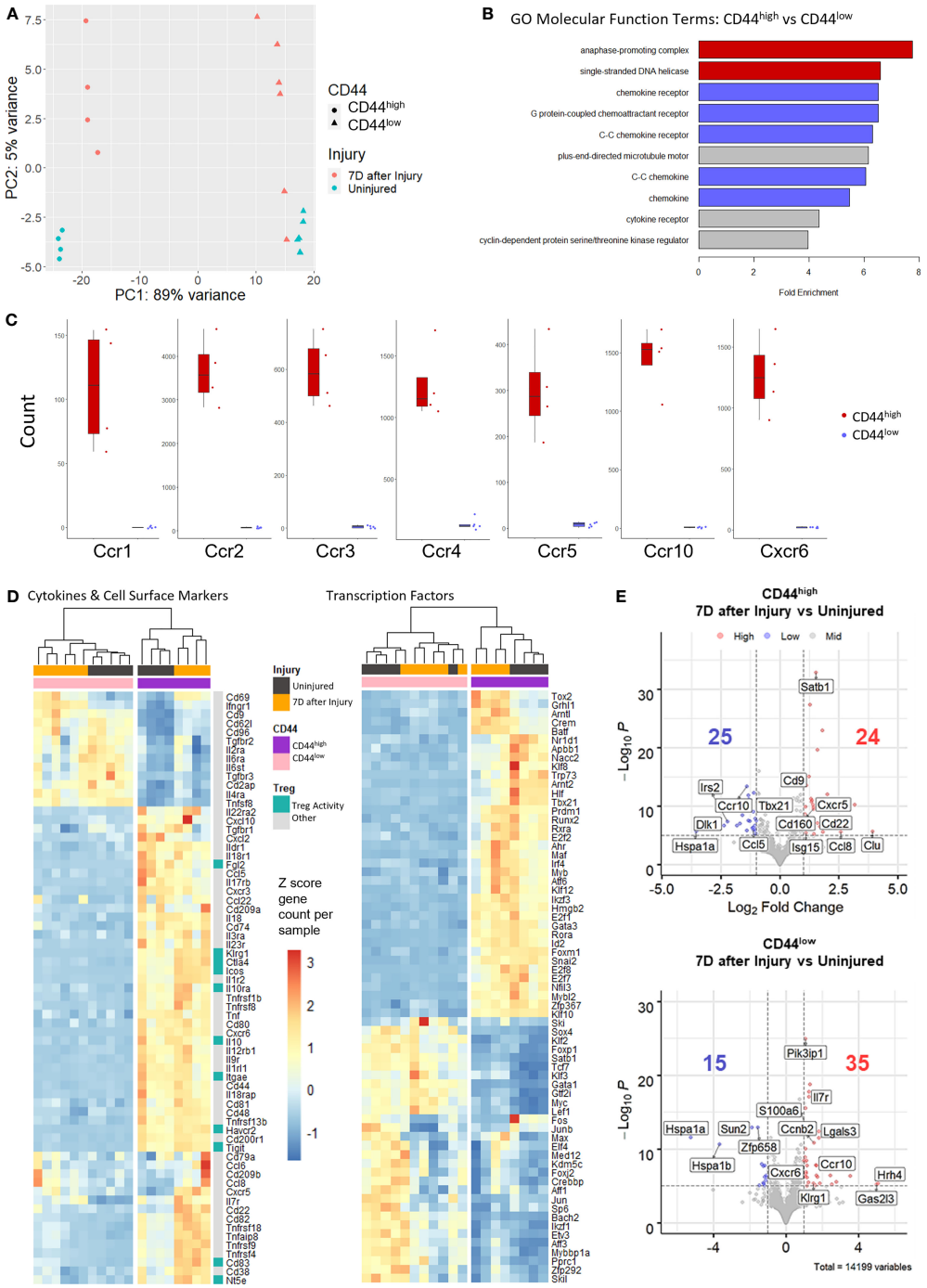

FIGURE 2 | Bulk RNA sequencing of sorted CD44^{high} and CD44^{low} Tregs from the lymph nodes of uninjured and injured mice. **(A)** Principal component analysis (PCA) plots demonstrating Treg subset distribution based on gene expression from uninjured and injured mice. **(B)** Bar plots of Gene Ontology (GO) molecular function analysis of the top 2000 genes upregulated in CD44^{high} versus CD44^{low} Tregs shows enrichment of cell division (red), chemokine receptor and chemokines (blue), and other (gray) terms. **(C)** Box plots illustrating the difference in chemokine receptor gene counts between CD44^{high} and CD44^{low} Tregs. **(D)** Gene expression levels of cytokines and cell surface markers and transcription factors (rows) in injured (black bars) and uninjured (orange bars) CD44^{high} (purple bars) and CD44^{low} (pink bars) Tregs (columns) from the top 2000 genes with the highest stabilized variance. **(E)** Volcano plots of injured vs. uninjured groups plotted as Log_2-fold change of differentially expressed (DE) genes in CD44^{high} and CD44^{low} Treg subsets. Genes with a p value less than 10^{-6} are colored blue if the gene shows a decrease of more than 2-fold and red if the gene shows an increase of more than 2-fold. Key DE genes are labeled. Counts of significantly up or down regulated genes are given with respective colors.

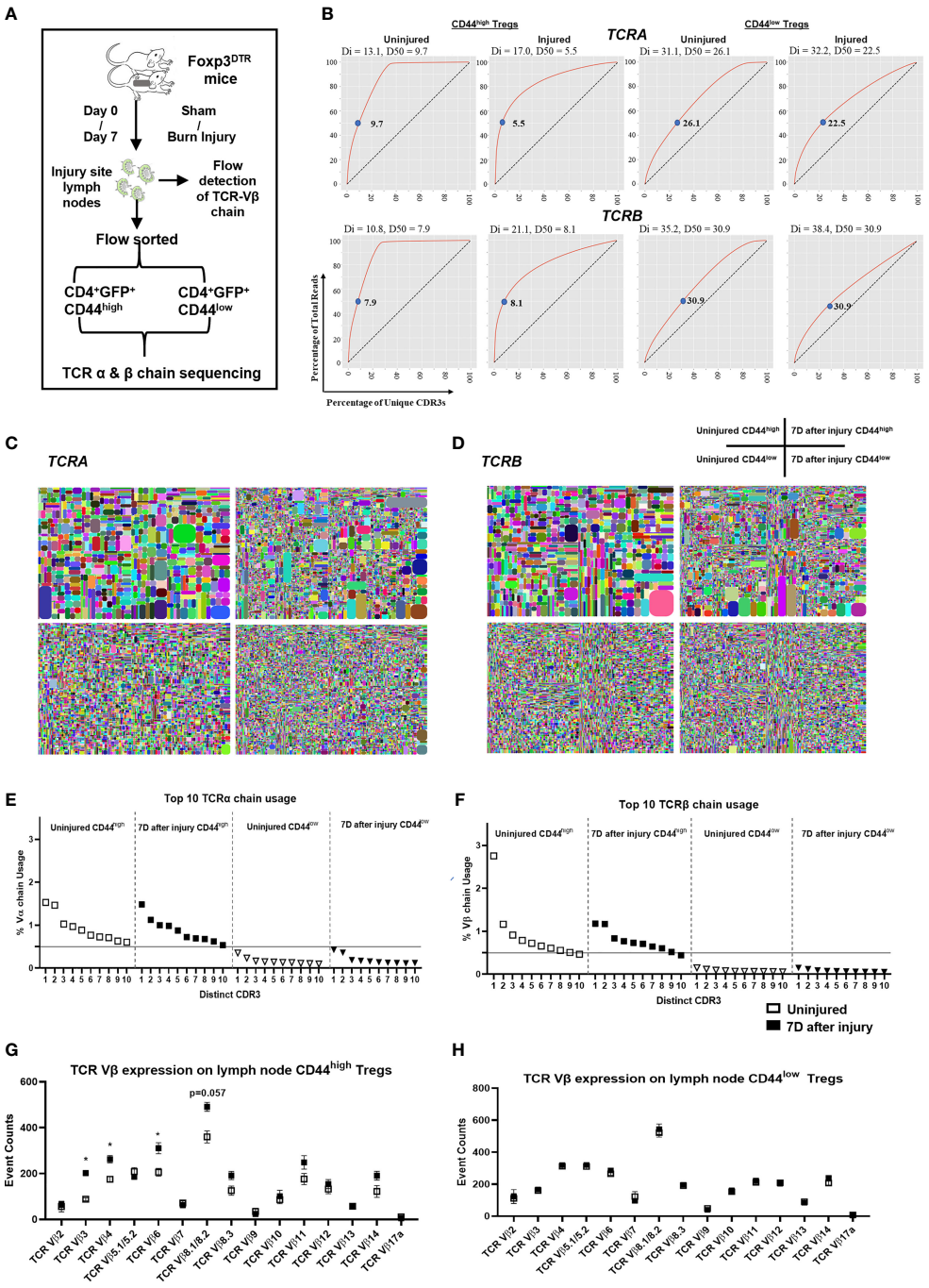

FIGURE 3 | Injury induces greater changes in αβ TCR oligoclonality as well as diversity in CD44^{high} Tregs than in CD44^{low} Tregs. **(A)** Experimental scheme of TCR repertoire analysis in injured and uninjured FoxP3^{DTR} mice. **(B)** Diversity curves illustrating the diversity of TCRA and TCRB sequences in CD44^{high} and CD44^{low} Treg populations from uninjured and injured mice. Curves show the cumulative percentage of total CDR3 sequence reads as a function of the percentage of unique CDR3 sequences. Curves further from the dashed line diagonal have higher clonality. D50 values (blue dots) indicate the percentage of unique CDR3 sequences that account for 50% of the total CDR3 sequence reads. Di values are shown above each graph. Treemap plots of TCRα **(C)** and TCRβ **(D)** sequencing analysis to illustrate TCR clonotype events from TCR RNA sequencing analysis. Each TCR clonotype is represented by a colored shape and the size of the shape reflects the frequency of each CDR3 clonotype variant. Smaller shapes and more varied colors equate to greater diversity in TCR clonality. Top 10 TCR-Vα usage **(E)** and TCR-Vβ usage **(F)** among CD44^{high} and CD44^{low} Tregs. Flow cytometry analysis of TCR-Vβ chain expression on **(G)** CD44^{high} and **(H)** CD44^{low} Tregs using 14 TCR-Vβ specific antibodies. Bars represent the mean ± SEM. Data were analyzed non-parametric by multiple Mann-Whitney test and are denoted by *P<0.05 or P value compared to injured control. Data represents 3 independent experiments (n=4 mice per group).

support differences in TCRα and TCRβ clonality between these Treg populations (**Figures 3E, F**).

Next, specific TCR-Vβ chain expression on Tregs prepared from 2 different inbred lines of injured mice were compared by flow cytometry. Among the 14 different TCR-Vβ chains tested, Tregs expressing TCR-Vβ 3, 4, and 6 were expanded in the CD44^{high} Treg population from injured C57BL/6 mice and TCR-Vβ 4, 6, 8.1/8.2, 8.3, and 14 were expanded CD44^{high} Treg from injured BALB/c mice. No TCR-Vβ expansion was observed on CD44^{low} Tregs or conventional CD4^{+} T cells in either inbred mouse lines (**Figures 3G, H**; **Figures S3A, B**). Thus, the oligoclonal expansion of CD44^{high} Tregs is not restricted to a single inbred mouse strain with different TCR repertoires and only CD44^{high} Tregs expanded in response to injury.

Single-Cell TCR and RNA Sequencing Identifies Injury-Induced Expanded TCR Clonotypes With Matched Transcriptome Profiles

Paired single-cell TCR and RNA sequencing on FACS sorted FoxP3-GFP^{+} cells was performed to identify αβ TCR clonotypes and matched transcriptome expression profiles in Tregs from injured and uninjured mice (**Figure 4A**). Quality control was performed on single-cell RNA sequencing data to exclude doublets and dying cells (**Figure S4**). Consistent with above TCR repertoire analysis done by the iRepertoire sequencing approach, we observed 182 expanded clonotypes (2 or more paired TCRαβ clones) in Tregs from burn injured mice and 83 clonotypes in uninjured mice (**Figure 4B**). There were no overlapping paired TCR clonotypes among the top 10 clonotypes identified in CD44^{high} or CD44^{low} Tregs from injured or uninjured mice. However, the frequency of Tregs with expanded TCR clonotypes was markedly higher in injured mice as compared to uninjured mice (**Table 1**). We next classified Treg scRNAseq clusters that have 15% or more of their cells with 2 or more paired TCR clonotypes as having an "expanded phenotype" (**Figure S4E, F**). Treg-related genes such as *Klrg1*, *S100a4*, *Tigit*, *S100a6*, *Icos* and *Itgae* were found to be significantly differentially expressed in expanded as compared to non-expanded Tregs (**Figure 4C**). Moreover, t-SNE plots showed that *Klrg1*, *S100a4*, *Tigit*, *S100a6*, *Icos* and *Itgae* expression overlapped in the areas of the t-SNE cell atlas where cells with expanded TCR clonotypes were found (**Figure 4D**). Expression *Cd44* was also found to be correlative with the expanded populations. Gene ontology (GO) analysis of the top differentially expressed genes between the expanded and non-expanded populations found the most significant GO term hits to be SRP-dependent translational protein targeting to membrane, T cell activation, negative regulation of immune system process, and T cell selection (**Figure 4E**). These results suggest that injury expanded Tregs acquire a highly activated phenotype.

CyTOF Analysis Identifies CD44^{high} and CD44^{low} Treg Clusters and Validates Differentially Expressed Genes at the Protein Level

Density contour plots of CyTOF staining data analyzed by tSNE for dimensionality reduction shows injury-induced increases in cell density in regions identified as FoxP3^{+} and Helios^{+} CD4^{+} T cells (**Figures 5A, C**). CyTOF data was clustered using the SPADE algorithm and cluster 5 was identified as CD44^{high} Treg and cluster 13 as CD44^{low} Treg clusters based on CD44, ICOS, CD25, Foxp3, CLTA-4 and OX40 expression patterns (**Figure 5B** and **Figures S5A, B**). The abundance of cells within the CD44^{high} Treg cluster increased at 7 days after injury but there was no significant increase in the CD44^{low} Treg cluster (**Figure 5D**). Expression of KLRG1, ICOS, CTLA-4, ITGAE (CD103), Helios, Galectin-3, PYCARD, ICAM-1, CXCR6, and CRLF2 proteins in the CD44^{high} Treg cluster was significantly higher than that in the CD44^{low} Treg cluster (**Figure 5E**). Deeper analysis of marker expression levels on Tregs showed that injury further upregulated Helios, Galectin-3 and PYCARD expression in the CD44^{high} Treg cluster (**Figure 5F**).

DISCUSSION

Regulatory T cells control immune tolerance and have potent counter-inflammatory functions in infectious and non-infectious diseases (9, 10, 12, 15). A better understanding of the specific functions of Tregs in reacting to and resolving tissue injuries caused by trauma, infections, tumors, and autoimmune responses will help towards designing ways to modulate Tregs as a therapeutic to resolve or enhance immune functions in these diseases. Immune reactions against non-infectious injuries are unique because they are initiated in part by the release of alarmins and DAMPs from sterile tissue and cell damage (1, 7, 9, 12). Recent findings indicate that a subpopulation of CD44^{high} Tregs react to burn trauma in mice by expanding and upregulating CTLA-4, ICOS, and GITR (17). Furthermore, this CD44^{high} Treg population demonstrated rapid activation in the lymph nodes and displayed memory-like behavior in response to injury in adoptive transfer experiments. Given the memory-like nature of CD44^{high} Tregs, we hypothesized that these Tregs might specifically react to DAMPs or other antigens that are released or exposed to the cellular immune surveillance system following tissue injury. In this report, we used advances in systems immunology technologies to perform unbiased and precise transcriptional profiling of injury reactive Tregs.

We took advantage of the FoxP3^{DTR} mouse model because all Tregs in these mice express GFP, making it possible to purify CD44^{high} and CD44^{low} Treg subpopulations for the cellular and molecular analyses performed in this project (44). In FoxP3^{DTR} mice, we validated that CD44^{high} Tregs expanded in response to injury. The CD44^{low} Tregs showed no significant increase in percentages or numbers but did show limited injury enhanced Treg mediated suppressive activity. By directly comparing the suppressive activity between CD44^{high} and CD44^{low} Tregs from injured and uninjured mice, we discovered that the CD44^{high} Treg population was much more potent at suppressing T cell proliferation than CD44^{low} Tregs; however, injury did not further enhance their suppressor potency. We suspect that this is because CD44^{high} Tregs have naturally high suppressive activity that cannot be further increased. Their specific

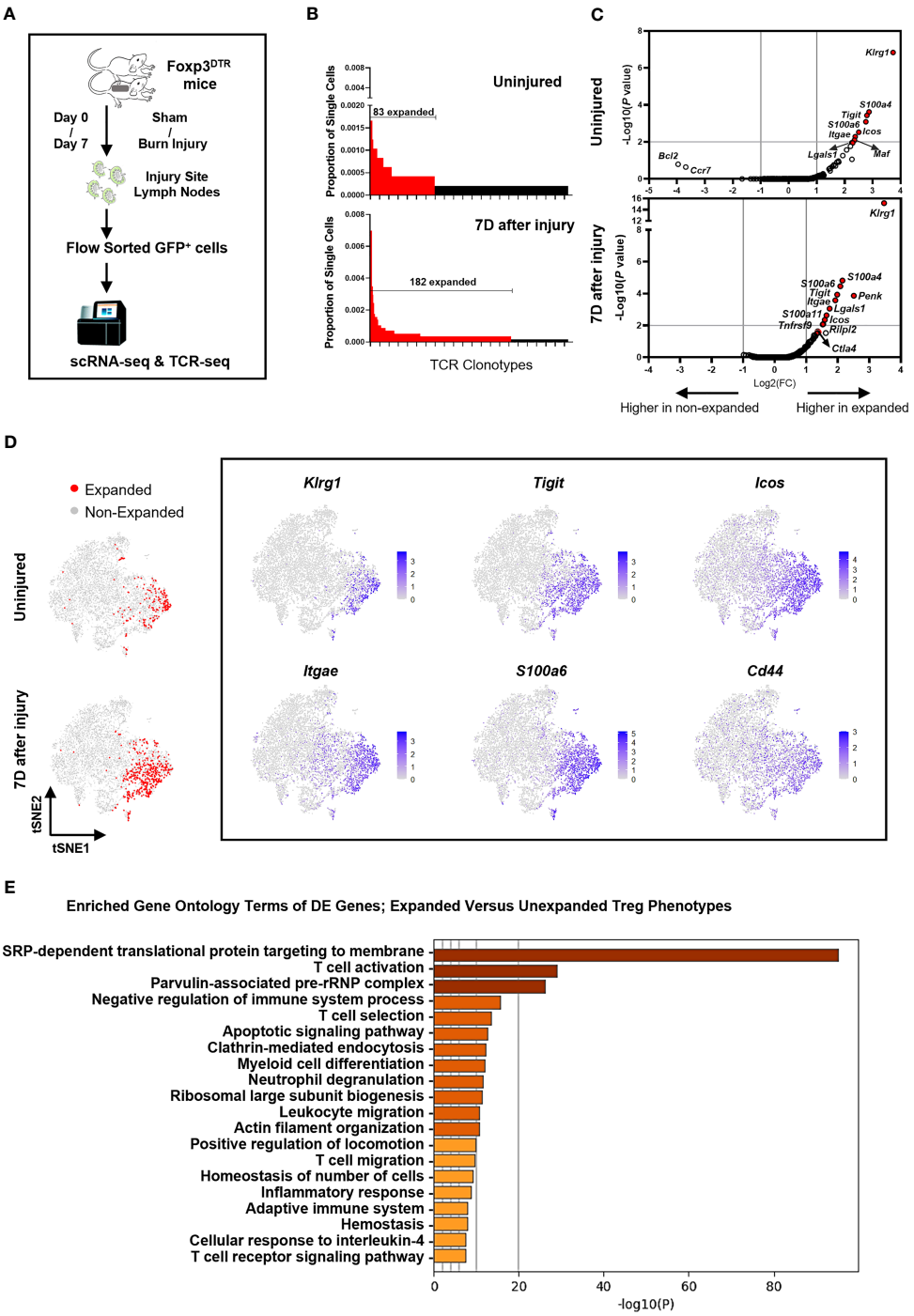

FIGURE 4 | Injury induces a KLRG1^{+} Treg subset with expanded TCR clonotypes. **(A)** Experimental scheme of scRNAseq and scTCRseq analysis of FACS sorted FoxP3-GFP^{+} Tregs from the lymph nodes of mice at 7 days after sham or burn trauma injury. **(B)** Comparison of the frequencies of the top 255 TCR clonotypes in Tregs from uninjured and injured mice. Red bars depict expanded (>2) TCR clonotypes. **(C)** Volcano plots showing genes that are differentially expressed between expanded and non-expanded Tregs from uninjured and injured mice. **(D)** tSNE plots showing the location of expanded Tregs identified by TCR sequencing, as well as the expression of 6 highly differentially expressed genes in injury expanded Tregs. **(E)** Bar plots showing enriched gene ontology (GO) terms that are significantly different between clusters of expanded versus unexpanded Tregs. These GO term plots were generated using the Metascape gene annotation and analysis resource (43).

TABLE 1 | Top 10 TCRα/β paired clonotypes in Tregs from injured and uninjured mice.

Injured	Type	V genes	J genes	C genes	CDR3s	Frequency	Proportion
1	TRA	TRAV13-2	TRAJ39	TRAC	CAIDRGNAGAKLTF	40	0.698%
	TRB	TRBV5	TRBJ2-7		CASSLHWGGSYEQYF		
2	TRA	TRAV13-1	TRAJ17	TRAC	CALAFAGNKLTF	20	0.349%
	TRB	TRBV1	TRBJ1-5	TRBC1	CTCSAPGQGNQAPLF		
3	TRA	TRAV9D-1	TRAJ43	TRAC	CAVSFYNNNAPRF	17	0.297%
	TRB	TRBV19	TRBJ1-6	TRBC1	CASSIGNSPLYF		
4	TRA	TRAV14-3	TRAJ57	TRAC	CAAGGSAKLIF	14	0.244%
	TRB	TRBV31	TRBJ2-1		CAWNWGNYAEQFF		
	TRB	TRBV4	TRBJ1-5	TRBC1	CASSPPRDRGTAPLF		
5	TRA	TRAV7D-4	TRAJ6	TRAC	CAASGGNYKPTF	10	0.174%
	TRB	TRBV5	TRBJ2-5		CASSPTGGEDTQYF		
6	TRA	TRAV4-4-DV10	TRAJ50	TRAC	CAAEASSSFSKLVF	9	0.157%
	TRB	TRBV5	TRBJ1-1	TRBC1	CASSQDTEVFF		
7	TRA	TRAV6-5	TRAJ7	TRAC	CALPDYSNNRLTL	9	0.157%
	TRB	TRBV19	TRBJ2-7		CASSRDWGGYEQYF		
8	TRA	TRAV6-6	TRAJ34	TRAC	CALGGSSNTNKVVF	9	0.157%
	TRB	TRBV12-2	TRBJ2-7		CASGDIYEQYF		
9	TRA	TRAV5N-4	TRAJ17	TRAC	CAAKTNSAGNKLTF	8	0.140%
	TRB	TRBV16	TRBJ2-4		CASSLDSQNTLYF		
10	TRA	TRAV12D-2	TRAJ57	TRAC	CALRNQGGSAKLIF	6	0.105%
	TRB	TRBV16	TRBJ2-5		CASSFKDTQYF		
Uninjured	**Type**	**V genes**	**J genes**	**C genes**	**CDR3s**	**Frequency**	**Proportion**
1	TRA	TRAV21-DV12	TRAJ40	TRAC	CILRVADTGNYKYVF	8	0.166%
	TRB	TRBV23	TRBJ1-1	TRBC1	CSSSQPGHANTEVFF		
2	TRA	TRAV6N-6	TRAJ6	TRAC	CALSVSGGNYKPTF	8	0.166%
	TRB	TRBV1	TRBJ2-5		CTCSAAWGQDTQYF		
3	TRA	TRAV4D-3	TRAJ21	TRAC	CAAEMSNYNVLYF	6	0.124%
	TRB	TRBV26	TRBJ2-7		CASSPLGGGYEQYF		
4	TRA	TRAV10	TRAJ50	TRAC	CAASRGASSSFSKLVF	5	0.104%
	TRB	TRBV31	TRBJ2-4		CAWSLDWVSQNTLYF		
5	TRA	TRAV21-DV12	TRAJ39	TRAC	CILRNNNAGAKLTF	5	0.104%
	TRB	TRBV13-3	TRBJ2-7		CASSDDSSYEQYF		
6	TRA	TRAV6-5	TRAJ34	TRAC	CALSSSNTNKVVF	5	0.104%
	TRB	TRBV5	TRBJ2-5		CASSQEHWGDTQYF		
7	TRA	TRAV7-4	TRAJ52	TRAC	CAARSNTGANTGKLTF	5	0.104%
	TRB	TRBV1	TRBJ2-3		CTCSAVWGGIETLYF		
8	TRA	TRAV13-4-DV7	TRAJ37		CAASGNTGKLIF	4	0.083%
	TRB	TRBV12-2	TRBJ2-2	TRAC	CASGNWGNTGQLYF		
9	TRA	TRAV14-1	TRAJ12		CAASAWGGYKVVF	4	0.083%
	TRB	TRBV2	TRBJ2-7	TRAC	CASSPRDRGFEQYF		
10	TRA	TRAV15-2-DV6-2	TRAJ34	TRAC	CALSELNTNKVVF	4	0.083%
	TRA	TRAV9-1	TRAJ34	TRAC	CAVSGPNTNKVVF		
	TRB	TRBV19	TRBJ2-5		CASSIFGGNQDTQYF		

expansion in response to injury combined with their high suppressive activity suggests that they may control autoimmune reactivity following tissue injuries.

Originally, we predicted that CD44^{high} Tregs could be protective in trauma by restraining excessive inflammation from both tissue injury and from secondary infections and sepsis (17). We decided to test this hypothesis using a clinically relevant two-hit trauma-bacterial infection mouse model (45, 46). In opposition to this hypothesis, we observed that injured Treg-depleted mice that were given CD44^{high} Tregs by adoptive transfer had higher mortality than mice that were given CD44^{low} Tregs. This outcome suggests that the persistence of highly suppressive CD44^{high} Tregs may increase susceptibility to opportunistic infections. Consistent with this, it has been demonstrated that pediatric burn patients have increased levels of circulating memory Tregs that resemble these injury responsive CD44^{high} Tregs in mice. These burn victims also showed long-term lower vaccine responses than uninjured controls, supporting our findings that injury-responsive memory Tregs are highly immunosuppressive (47). Consistent with our findings in mice, trauma patients have increased circulating levels of Tregs that can more potently suppressive T cell proliferation and patients that develop infections or sepsis showed higher circulating levels of Tregs (9, 48).

RNA sequencing of sorted CD44^{high} and CD44^{low} Tregs from injured and uninjured mice was used to discover differentially expressed (DE) genes in Treg populations, as well as those that are significantly altered by injury. We found that the 89% of the variance in gene expression was attributed to the CD44^{high} versus CD44^{low} Treg phenotype. This finding supports that CD44^{high} and CD44^{low} Tregs are transcriptionally distinct Treg subpopulations. Unsupervised hierarchical clustering of a

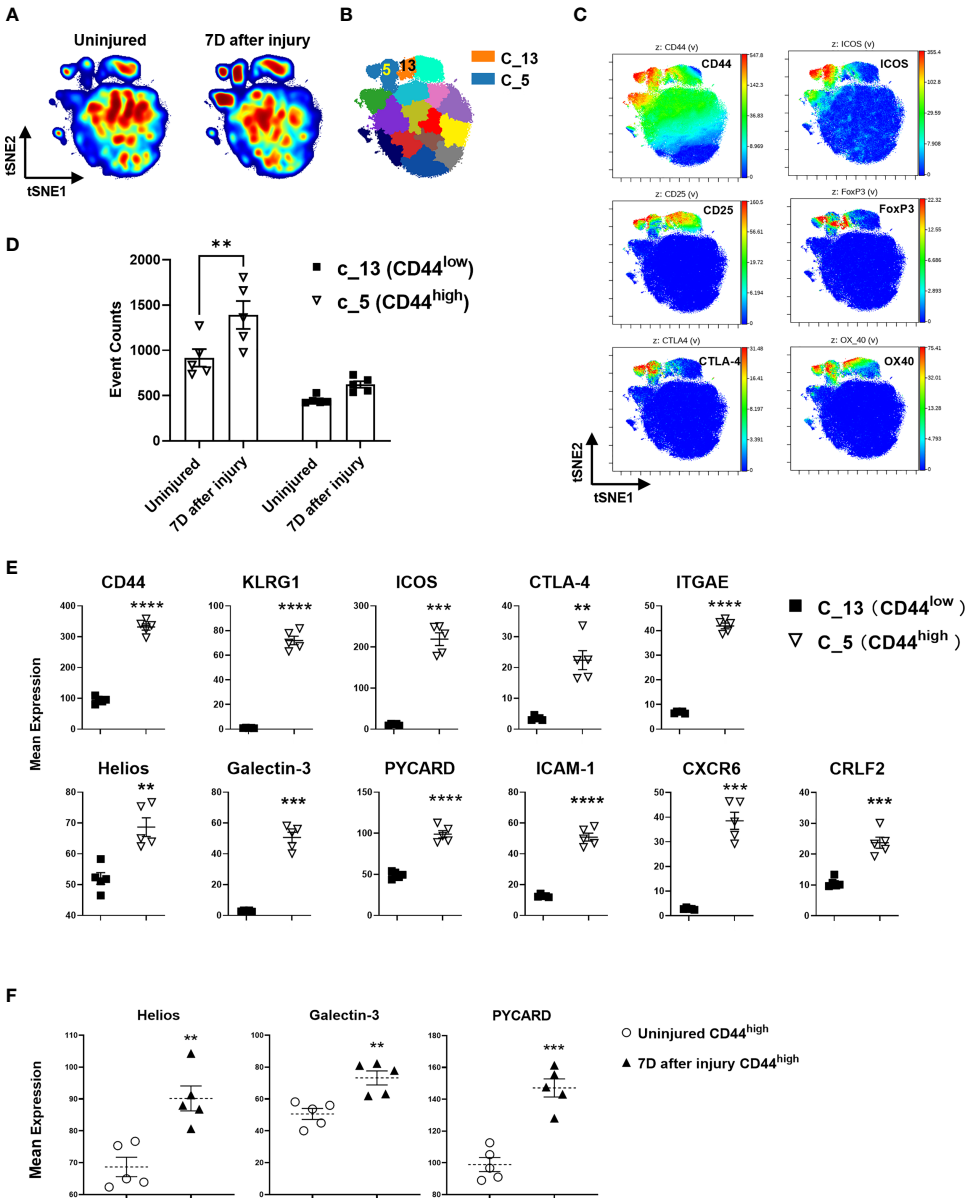

FIGURE 5 | CyTOF validation of protein expression of DE genes on Treg subsets identified by RNA sequencing. **(A)** Representative density contour tSNE plots generated by 39-marker CyTOF staining of equal-sampled gated $CD3^+/CD4^+$ T cells from the lymph nodes of uninjured and injured mice, and **(B)** unsupervised computational clustering by SPADE showing phenotypic clusters as patchwork colors. Clusters 5 and 13 (C_5, C_13) are Treg subset clusters. **(C)** tSNE plots colored by the indicated antibody staining channel confirming the identification of $CD44^{high}$ and $CD44^{low}$ Treg subsets as well as other canonical Treg identifying markers. **(D)** Event counts from cluster 5 and 13 comparing changes in uninjured and injured mice at day 7. Bars represent the means ± SEM. Data were analyzed by two-way ANOVA, interaction P=not significant. Significant comparison by Sidak's multiple comparisons test is denoted by ** P=0.005 ($CD44^{high}$ cluster 5, injured versus uninjured) **(E)** Mean expression levels of select protein markers detected by CyTOF corresponding to DE genes in $CD44^{high}$ and $CD44^{low}$ Tregs that were identified by RNA sequencing. **(F)** The impact of injury on the expression of Helios, Galectin-3, and PYCARD in $CD44^{high}$ Tregs 7 days after injury as measured by CyTOF staining. Bars represent the mean ± SEM of mean expression intensity levels. Data were analyzed by two-tailed unpaired t test and significance compared with uninjured control denoted by $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Data represent 2 independent experiments (n=5 mice per group).

subset of cytokine, cell surface marker, and transcription factor gene expression separated $CD44^{high}$ and $CD44^{low}$ Tregs into fully delineated clusters. The high expression of chemokines and chemokine receptors on $CD44^{high}$ Tregs indicates that they are more mobile Treg population than $CD44^{low}$ Tregs. In addition, the high expression of IL-1, IL-18, IL-12, IL-17 and TGFβ receptors strongly support that $CD44^{high}$ Tregs are reacting to the pro-inflammatory tissue environment caused by injury. The transcription factor genes that are more highly expressed in $CD44^{high}$ Tregs are known to control responses to environmental

stimuli, ER stress sensor, cell cycle, cAMP responsive element, immune related signaling, and tissue-resident memory T cell signaling. This transcription factor expression profile is consistent with the physiological changes caused by injuries and the enriched expression of IRF4 and Blimp1 transcription factors by $CD44^{high}$ Tregs is consistent with a prior study that elegantly showed that these transcription factors are required for Treg differentiation and suppressive function (49). Finally, $CD44^{high}$ Tregs express higher levels of known Treg suppressive genes than $CD44^{low}$ Tregs, consistent with the more potent suppressive activity of $CD44^{high}$ compared to $CD44^{low}$ Tregs. The injury modulated genes as well as those that are differentially expressed by $CD44^{high}$ and $CD44^{low}$ Tregs provide unique gene expression data sets for future data mining by us, and others interested in Treg biology or in modulating Tregs for immunotherapy.

Several possible mechanisms for injury-induced Treg expansion include antigen-specific T cell proliferation, pattern recognition receptor signaling by DAMPs, or response to cytokines and alarmins (50–52). In this study, we performed bulk and single-cell TCR repertoire analysis of Tregs using the iRepertoire PCR approach and the 10X Genomics platform with paired TCRα and TCRβ sequencing, respectively. Both approaches provided data to support $CD44^{high}$ Treg activation by injury and clonal expansion. The iRepertoire TCR sequencing data indicate that TCRα and TCRβ chains are less diverse on $CD44^{high}$ Tregs than $CD44^{low}$ Tregs. However, 7 days after injury, the $CD44^{high}$ Treg population showed an increase in TCRα/β chain diversity that was visualized in the Treemap plots. This finding supports that $CD44^{high}$ Tregs react to and expand after burn trauma or traffic from tissues to immune compartments. There are two major subsets of Tregs in mice – thymus educated natural Tregs (nTreg) and induced Tregs (iTreg) that develop in peripheral tissues. We suspect that $CD44^{high}$ Tregs are a population of nTreg with TCR bias towards recognizing self-antigens because they display low TCR diversity. Future studies will focus on delineating whether injury reactive Tregs are natural or induced Treg populations by lineage tracing or deeper molecular profiling approaches.

The exclusive expression of multiple chemokine receptors on $CD44^{high}$ versus $CD44^{low}$ Tregs supports the idea that $CD44^{high}$ Tregs are trafficking Tregs with the capacity to respond to chemokines that are produced at sites of injury or inflammation. Chemokines are produced by Tregs and CCL2, CCL3, and CCL4 have been shown to be central to the trafficking of Tregs to sites of inflammation and tissues (53–55). Moreover, surgical trauma has been shown to induce CCL18 levels, which in turn increases tumor-site Tregs to further suppress tumor immunity (56). Plots comparing chemokine receptor gene expression on $CD44^{high}$ versus $CD44^{low}$ Tregs illustrate the exclusive expression of 7 different chemokine receptors on $CD44^{high}$ Tregs and CyTOF staining showed that CXCR6 expression was restricted to $CD44^{high}$ Tregs. We believe that the differential expression of chemokines and chemokine receptors by $CD44^{high}$ Tregs plays a central role in their response to injury and strong suppressive activity.

Using bulk RNA sequencing, we were able to detect gene expression signatures in injury expanded versus unexpanded Tregs. Importantly, the genes that were significantly induced in expanded Tregs overlap with those found by bulk RNA sequencing of flow cytometry sorted $CD44^{high}$ Tregs from injured mice. Similar sets of genes have been identified to be upregulated in Tregs in other types of injuries and diseases that induce sterile tissue injuries (9, 10, 12). In a muscular dystrophy model, clonally expanded $CD44^{high}$ Tregs within injured skeletal muscle express higher levels of *Klrg1, Il1rl1, Itgae, Ccr2 and Ccr4* (57). Similarly, in an acute muscle injury model, *Klrg1* expressing Tregs showed TCR clonal expansion, which resulted in enhanced healing of injured muscle (58). The Tregs identified in these studies are likely similar to those identified in our mouse burn trauma model and appear to be beneficial in injury resolution. We suspect that $CD44^{high}$ Tregs in our burn trauma model play a similar role in resolving injury-induced tissue damage to promote healing.

We identified *Klrg1* gene expression in injury expanded Treg clonotypes and validated KLRG1 protein expression on $CD44^{high}$, but not $CD44^{low}$ Tregs. $KLRG1^+$ Tregs have been identified in the tumor microenvironment of murine models and human samples of non-small cell lung cancer (59–61). Consistent with our findings, these $KLRG1^+$ Tregs were strongly immunosuppressive (60). Severe COVID-19 patients that develop lung injury showed expanded $KLRG1^+$ Tregs that express *Cxcr3*, *Il10*, *Il12b1*, *Ilrb1*, *Tbx21*, *Gata3* gene signatures similar to what was found in this study (62). Though $KLRG1^+$ Tregs are normally potent immune suppressive cells, they have also been identified as being dysfunctional in autoimmune mouse models. For example, in the non-obese diabetic (NOD) mouse model, $KLRG1^+$ Tregs were not suppressive and tended to differentiate into Th1-or Th17-like effector cells in the pancreas causing heightened disease (63). Similarly, in a TCR transgenic NOD mouse model, $KLRG1^+$ Tregs were unable to suppress the development of type 1 diabetes (64). Thus, $KLRG1^+$ Tregs represent a Treg population with plasticity that contributes to the pathogenesis of infections, cancer, and autoimmune diseases. We speculate that tissue damage might be a common stimulus for Treg activation and differentiation in these sites of infection or injury.

In summary, we identify $CD44^{high}$ Tregs as a potently immunosuppressive population that expand in an oligoclonal manner in response to injury. Molecular profiling of $CD44^{high}$ versus $CD44^{low}$ Tregs indicates that they are distinct subsets with different transcriptional and functional profiles. We have not yet identified antigens or factors that contribute to their expansion. However, future studies will use the paired TCRαβ sequence information gained from injury expanded Treg clonotypes to identify antigens and antigen presenting cells that activate $CD44^{high}$ Tregs. We propose that the activation and expansion of injury responsive Tregs is a fundamental feature of many pathological immune responses that cause tissue damage. Thus, we anticipate that these data will contribute valuable information that will help with designing novel Treg targeted therapies to modulate their behavior in trauma, infections, cancer, and autoimmunity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, accession ID: GSE174790.

ETHICS STATEMENT

The animal study was reviewed and approved by Brigham and Women's Hospital IACUC.

AUTHOR CONTRIBUTIONS

Conceptualization: JL and FG; Methodology: FG, HL, KH, JK, AC, and FZ; Investigation: FG and HL; Visualization: FG, BH, and AG; Funding acquisition: JL; Project administration: JL and JK; Supervision: JL; Writing – original draft: FG; Writing, review, editing: AG, LC, JN, and JL. 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/fimmu.2022.833100/full#supplementary-material

Supplementary Figure 1 | $CD44^{high}$ Tregs decrease survival in a Treg-depleted mouse model of trauma injury with secondary lung infection. **(A)** Experimental scheme. C57BL/6 mice were subjected to 20% total body surface area burn injury. $Foxp3^{DTR}$ mice were treated with 40ng/kg diphtheria toxin (DT) at 2h and 24h after burn injury to deplete Tregs. 7 days after injury, $CD44^{high}$ or $CD44^{low}$ $CD4^{+}CD25^{+}$ cells (Tregs) were FACS sorted from injury-site draining lymph nodes (axillary, brachial, and inguinal). Tregs were transfused into the injured $Foxp3^{DTR}$ mice at 50,000 cells/mouse by intracardiac injection. The mice were subsequently challenged with intranasal *Pseudomonas aeruginosa* 1 day after Treg-transfusion. Survival was monitored over a week period. **(B)** Injury-site draining lymph nodes, spleen, and blood were collected from Treg depleted $Foxp3^{DTR}$ mice 24 hours after last injection to determine Treg depletion efficiency by flow cytometry. **(C)** Survival of mice [n=14 ($CD44^{high}$), n=24 ($CD44^{low}$)] subjected to burn injury with secondary *P. aeruginosa* pulmonary infection. Data are presented as a Kaplan-Meier survival curve and analyzed by Log-rank (Matel-Cox) test

Supplementary Figure 2 | Bulk RNA sequencing of FACS sorted $CD44^{high}$ and $CD44^{low}$ Tregs in injury-site lymph nodes of mice subjected to burn/sham injury. **(A)** Sample-Sample Clustering Map demonstrating the correlation values between samples. Groups are annotated at the top. **(B)** Sample-Feature (Gene) Hierarchical Clustering Map with samples along the x-axis and genes along the y-axis. Metadata columns are annotated along the top. Volcano plots showing the log_2 fold change of differentially expressed genes in $CD44^{high}$ and $CD44^{low}$ Treg subsets in **(C)** uninjured and **(D)** injured mice. Blue dots represent the genes in $CD44^{low}$ Tregs with a more than 2-fold increase compared to $CD44^{high}$ Tregs. Red dots indicate genes with a more than 2-fold increase in $CD44^{high}$ Tregs compared to $CD44^{low}$ Tregs [n=4 (uninjured $CD44^{high}$ Tregs, 7D after injury $CD44^{high}$ Tregs and uninjured $CD44^{low}$ Tregs), n=6 (7D after injury $CD44^{low}$ Tregs)].

Supplementary Figure 3 | TCRvβ clonotype analysis demonstrating expansion only in $CD44^{high}$ Tregs. TCRvβ staining shows that the clonotype expansion occurs on $CD44^{high}$ Tregs but not on other cell types, in both **(A)** C57BL/6 (n=4 injured or uninjured mice) and **(B)** BALB/c mice (n=5 injured or uninjured mice). Bars represent the means ± SEM. Data were analyzed non-parametric by multiple Mann-Whitney test and were denoted by * $P<0.05$ or ** $P<0.01$ compared to injured control. Data represents 3 independent experiments.

Supplementary Figure 4 | Single Cell Quality Metrics. **(A)** Sequencing saturation plot showing the Sequencing Saturation metric as a function of down-sampled sequencing depth (measured in mean reads per cell), up to the observed sequencing depth. Sequencing Saturation is a measure of the observed library complexity and approaches 1.0 (100%) when all converted mRNA transcripts have been sequenced. The slope of the curve near the endpoint can be interpreted as an upper bound to the benefit to be gained from increasing the sequencing depth beyond this point. The dotted line is drawn at a value reasonably approximating the saturation point. **(B)** Median genes per cell shows the median genes per cell as a function of down-sampled sequencing depth in mean reads per cell, up to the observed sequencing depth. The slope of the curve near the endpoint can be interpreted as an upper bound to the benefit to be gained from increasing the sequencing depth beyond this point. Violin plots demonstrating **(C)** number of unique features (genes) and **(D)** percentage of reads mapped to the mitochondrial genome. Dotted red lines show the cutoffs used for final data analysis. Cells with greater than 3000 or less than 200 genes, or cells with greater than 6% mitochondrial reads were filtered out. **(E)** tSNE plot showing the Seurat clusters used to group the expanded and unexpanded phenotypes. **(F)** tSNE showing the cells grouped into expanded and unexpanded phenotypes as defined by clusters containing at least 15% of single cells with >2 identical paired CDR3 sequences.

Supplementary Figure 5 | Identification of clusters identified by CyTOF stains from equal-sampled $CD3^{+}CD4^{+}$ T cells. **(A)** A heatmap of calculated ArcSinh ratio of mean expression levels of markers in each cluster controlled by row's minimum. **(B)** tSNE plots colored by channel showing the expression of the markers. Data are from equal-sampled $CD3^{+}CD4^{+}$ T cells of concatenated files. **(C)** Volcano plot showing translated proteins of differentially expressed genes between $CD44^{high}$ and $CD44^{low}$ Tregs.

Supplementary Figure 6 | Flow cytometry gating schemes. Representative flow cytometry plots demonstrating gating for $CD4^{+}$ and $CD4^{-}$ T cells, $CD44^{high}$ and $CD44^{low}$ Tregs, and $TCRV\beta^{+}$ populations.

Supplementary Figure 7 | FACS sort plots. Representative FACS sort plots of **(A)** $CD4^{+}CD44^{high}GFP^{+}$ ($CD44^{high}$ Tregs) and $CD4^{+}CD44^{low}GFP^{+}$ ($CD44^{low}$Tregs) for bulk RNAseq and iRepertoire analysis of TCRα and TCRβ, and **(B)** Sorting scheme used to prepare GFP^{+}(Tregs) for single cell mRNA sequencing analysis.

Supplementary Table 1 | Details of RNA-based TCR repertoire sequencing samples used for iRepertoire analysis.

Supplementary Table 2 | Sample information of RNA-based TCR repertoire sequencing.

Supplementary Table 3 | Sample statistics of scRNAseq analysis.

Supplementary Table 4 | CyTOF antibodies panel.

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A Novel GMP Protocol to Produce High-Quality Treg Cells From the Pediatric Thymic Tissue to Be Employed as Cellular Therapy

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Due to their suppressive capacity, the adoptive transfer of regulatory T cells (Treg) has acquired a growing interest in controlling exacerbated inflammatory responses. Limited Treg recovery and reduced quality remain the main obstacles in most current protocols where differentiated Treg are obtained from adult peripheral blood. An alternate Treg source is umbilical cord blood, a promising source of Treg cells due to the higher frequency of naïve Treg and lower frequency of memory T cells present in the fetus' blood. However, the Treg number isolated from cord blood remains limiting. Human thymuses routinely discarded during pediatric cardiac surgeries to access the retrosternal operative field has been recently proposed as a novel source of Treg for cellular therapy. This strategy overcomes the main limitations of current Treg sources, allowing the obtention of very high numbers of undifferentiated Treg. We have developed a novel good manufacturing practice (GMP) protocol to obtain large Treg amounts, with very high purity and suppressive capacity, from the pediatric thymus (named hereafter thyTreg). The total amount of thyTreg obtained at the end of the procedure, after a short-term culture of 7 days, reach an average of 1,757 x10^6 (range 50 x 10^6 – 13,649 x 10^6) cells from a single thymus. The thyTreg product obtained with our protocol shows very high viability (mean 93.25%; range 83.35% – 97.97%), very high purity (mean 92.89%; range 70.10% – 98.41% of $CD25^+FOXP3^+$ cells), stability under proinflammatory conditions and a very high suppressive capacity (inhibiting in more than 75% the proliferation of activated $CD4^+$ and $CD8^+$ T cells *in vitro* at a thyTreg:responder cells ratio of 1:1). Our thyTreg product has been approved by the Spanish Drug Agency (AEMPS) to be administered as cell therapy. We are recruiting patients in the first-in-human phase I/II clinical trial worldwide that evaluates the safety, feasibility, and efficacy of autologous thyTreg administration in children undergoing heart transplantation (NCT04924491). The high quality and amount of thyTreg and the differential features of the final product obtained with our protocol allow

preparing hundreds of doses from a single thymus with improved therapeutic properties, which can be cryopreserved and could open the possibility of an "off-the-shelf" allogeneic use in another individual.

Keywords: Treg, thymus, thyTreg, immunotherapy, GMP manufacturing, tolerance induction

INTRODUCTION

The immune system is the body's defense mechanism against pathogens and other harmful agents. However, it is also responsible for transplant rejection or autoimmune diseases, in which an exacerbated response to the graft or one's cells develops. Autoimmune diseases have a very high incidence affecting 4-8% of the population of developed countries. The graft-versus-host disease (GVHD), which is the leading cause of mortality and morbidity after hematopoietic cell transplantation, is also associated with excessive or undesired immune responses. Another scenario of disproportionate immune response is the cytokine release syndrome (CRS), a systemic inflammatory response characterized by a sharp increase of proinflammatory cytokines triggered by factors such as infections, drugs, chimeric antigen receptor T cell (CAR-T) therapy in oncologic patients, or GVHD (1). The ongoing COVID-19 pandemic has brought out CRS's fatal consequences caused by SARS-CoV-2 infection, being one of the leading causes of death in severe patients (2).

In most cases, the standard treatment to prevent these immune responses is the use of immunosuppressive drugs, but they still do not provide a definitive solution and produce side effects that are decisive in the patient's clinical course. Because immunosuppressants have a pleiotropic action, the entire immune system is suppressed, affecting the ability to defend the host against infections and the development of tumors or promoting autoimmune disorders. Besides, this strategy will always have the immune system's degradation and generalized chronic damage as a counterpart. Therefore, a widespread feeling among the scientific community is that only re-educating the immune system to promote immune tolerance will reduce the harmful immune responses without damaging the immune system's functional integrity. In this sense, cellular therapies based on the infusion of cells capable of inducing tolerance is generating great enthusiasm in the clinical practice, being regulatory T cells (Treg), a subtype of $CD4^+$ T cells with suppressive function, the most studied and promising alternative (3). The immunoregulatory capacity of Treg cells is not due to one particular suppression mechanism, but rather it is the set of several coordinated mechanisms capable of promoting immune regulation. Indeed, Treg can suppress the effector function of a wide range of cells, including $CD4^+$ and $CD8^+$ T cells, B cells, dendritic cells, macrophages, granulocytes, natural killer cells, and osteoclasts (4). The mechanisms they use to suppress the different immune cells can be considered direct when the Treg themselves are the ones that provoke a response directly on the target cell, or indirect, in which another cell or molecule is affected, leading to the suppression of the target cell (5). The crucial role of Treg in preventing the hyperactivation of the immune system has been confirmed in transplanted adults (6) and children (7–9), GVHD (10), Crohn's disease (11) and other autoimmune disorders (12–14). Therefore, a therapeutic strategy based on Treg cells could offer excellent results in the prevention or treatment of these diseases to significantly increase their number in circulation and enhance the intrinsic mechanisms of tolerance in these patients.

Except for the employment of Treg derived from cord blood in GVHD, all clinical trials and therapeutic approaches administering Treg cells follow the same design: Treg are purified from peripheral blood from the patient or donor, expanded *ex vivo* and reinfused as an autologous or allogeneic therapy respectively (15). The safety and potential efficacy of Treg therapy in humans is reflected in clinical trials already conducted, that show that peripheral or cord blood Treg infusion reduces or prevents various immune disorders such as GVHD or type 1 diabetes in the short term (16–19). The greatest risk of GVHD occurs during the first three months, and immune suppression by Treg therapy during this short critical period has been shown to be sufficient to provide long-term tolerance. However, in the case of solid organ transplants, the risk of rejection persists throughout the patient's life, which makes necessary a protective effect of Treg that would last over time to ensure the prevention of graft rejection. There are currently numerous clinical trials in progress in phase I or phase I/II that use Treg cell therapy to prevent rejection of the transplanted organ in adult patients, most of them in the context of kidney and liver transplantation (20, 21). However, to date, the efficacy results are not entirely conclusive, mainly due to the low or short therapeutic effect of the infused Treg (22, 23). This could be due to the low number of Treg that can be purified from peripheral blood and the relative quality of infused Treg inherent to their higher state of cellular differentiation, which could be worsened after extensive *ex vivo* expansion (24). Although umbilical cord blood is a promising source of Treg cells, compared to adult peripheral blood due to the relatively high frequency of naïve Treg cells and the scarcity of memory T cells (25), the number of Treg that can be recovered of a single cord blood unit is very low. This fact implies that these naïve Treg have to undergo many rounds of *ex vivo* expansion to generate enough cells for a clinical dose which could also have an impact on their quality (24, 26).

Therefore, the search for an alternative source of Treg with a predominantly naïve state that allows obtaining enough quantity of cells is crucial to overcome the limitations encountered in peripheral blood and umbilical cord blood. In this sense, the thymus, a primary lymphoid organ responsible for the maturation and differentiation of T and Treg cells, which is

located above the heart and discarded to gain access to the heart during pediatric cardiac surgeries, could be employed as a new source of highly undifferentiated Treg (27, 28).

MATERIALS AND METHODS

Thymic Tissue Obtention

Human thymuses used for this research were excised and discarded in pediatric cardiac surgeries at the Pediatric Cardiac Surgery Unit of Gregorio Marañón Hospital (HGUGM). Thymic tissue was collected in sterile containers with TexMACS GMP medium (Miltenyi Biotec) supplemented with 1% antifungal antibiotic (Penicillin-streptomycin-amphotericin B; Sigma-Aldrich) and kept at 4°C until processing. The study was conducted after the HGUGM ethics committee's approval and according to the principles expressed in the Declaration of Helsinki. Informed written consent from the legal guardians was obtained before the patient's enrolment.

ThyTreg Production in the Research Laboratory

Thymic tissue fragments were mechanically disaggregated in TexMACS GMP medium (Miltenyi Biotec) with the gentleMACS Dissociator (Miltenyi Biotec). Total thymocytes obtained were filtered through a 40 µm pore, and $CD25^+$ cells were immunomagnetically selected using human CD25 Microbeads II and LS columns (Miltenyi Biotec). After isolation, $CD25^+$ (thyTreg day 0) and CD25- (thyTconv day 0) were cultured in TexMACS GMP medium supplemented with 600 U/ml IL-2 (Miltenyi Biotec) at 10^6 cells/ml at 37°C and 5% CO_2. Cells were stimulated with T Cell TransAct (Miltenyi Biotec), a polymeric nanomatrix, to activate and expand human T cells *via* CD3 and CD28 following the manufacturer's instructions. On day 3, half of the medium was removed and replaced with fresh TexMACS GMP medium supplemented with 600 U/ml IL-2. Cells were monitored on days 4, 5 and 6 and passage was performed when required. On day 7, cells were harvested, and their phenotype, functionality and stability were analyzed (**Figure 1A**). Additionally, dried cell pellets and culture supernatants were stored at −80°C for further DNA methylation studies and cytokine quantification respectively.

GMP thyTreg Manufacturing Protocol in the Cell Production Unit

To implement the thyTreg isolation and culture process in a good manufacturing practice (GMP) compliant protocol, we had to adapt some procedures and reagents as described in **Supplementary Table 1**. The manufacturing process was carried out in the Cell Production Unit (CPU) of Gregorio Marañón Health Research Institute (IISGM), which is accredited by the Spanish Agency of Medicines and Medical Devices (AEMPS). Briefly, thymic fragments were dissociated with the gentleMACS Octo Dissociator equipment (Miltenyi Biotec). Total thymocytes were suspended in PBS/EDTA supplemented with 0.5% human serum albumin (Albutein 20%, Grifols) and poured into a transfer bag (Grifols). $CD25^+$ thyTreg were isolated using the CliniMACS CD25 GMP MicroBeads, CliniMACS Tubing Set and CliniMACS plus equipment (Miltenyi Biotec). The positive fraction containing the thyTreg was counted and centrifuged to remove the selection buffer and resuspended in TexMACS GMP medium at $1x10^6$ cells/ml. ThyTreg were cultured in flasks maintaining a concentration of 500,000 cells/cm^2. After 7 days, the necessary tests were carried out to demonstrate the quality of the thyTreg obtained in the CPU. Both dry cell pellets and cell culture supernatants were stored at −80°C.

Additional sterility analyses were performed during the entire manufacturing process of thyTreg cells at the CPU to confirm the absence of microorganisms. A series of blood cultures were carried out to assess the detection of aerobic and anaerobic microorganisms using the automatic system BACTEC (Beckton Dickinson), in addition to the detection of mycoplasma by bioluminescence in the cell culture supernatant. The detection of genetic abnormalities was performed through an array-CGH (KaryoNIM Stem) by NIMGenetics (Madrid, Spain).

Flow Cytometry and Cell Sorting

We evaluated the cell viability and phenotype in the different stages of the procedure by flow cytometry. Briefly, cell surface markers staining was followed by staining with Fixable Viability Dye-eFluor450 (eBioscience). Then, the cells were fixed and permeabilized using the FOXP3 transcription factor staining kit (eBioscience) for intracellular staining. All the antibodies are listed in **Supplementary Table 2**. Flow cytometry analysis of labeled cells was performed with a MACSQuant16 cytometer (Miltenyi Biotec), acquiring at least 100,000 events, and the data were analyzed using Kaluza software (Beckman Coulter).

To isolate the $CD4^+$ single-positive (SP) and $CD4^+CD8^+$ double-positive (DP) thyTreg cells, $50x10^6$ of total thyTreg cells were labeled with anti-CD4-VioBlue (Miltenyi Biotec) and anti-CD8-FITC (Beckman Coulter). Cells were washed and resuspended at $5x10^6$ cells/ml in MACSQuant Tyto Running Buffer (Miltenyi Biotec) and were subjected to two consecutive rounds of sorting with High-Speed MACSQuant Tyto Cartridges (MACSQuant Tyto cell sorter, Miltenyi Biotec). After the first round, $CD4^+$SP cells were collected from the positive fraction. The negative fraction was loaded into a second cartridge, and $CD4^+CD8^+$ DP cells were collected from the positive fraction.

In Vitro Suppression Assay

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors from the Madrid Transfusion Center and cryopreserved until further use. Thawed PBMC were cultured overnight in RPMI 1640 (Biochrome) supplemented with 5% serum fetal bovine serum (FBS, Biowest) and 60 U/ml of IL-2 (ImmunoTools). The following day, the PBMC were stained with 1 µM of CellTrace Violet (CTVio, Life Technologies). $1x10^5$ CTVio-labeled allogeneic PBMC were co-cultured with thyTreg at different thyTreg :

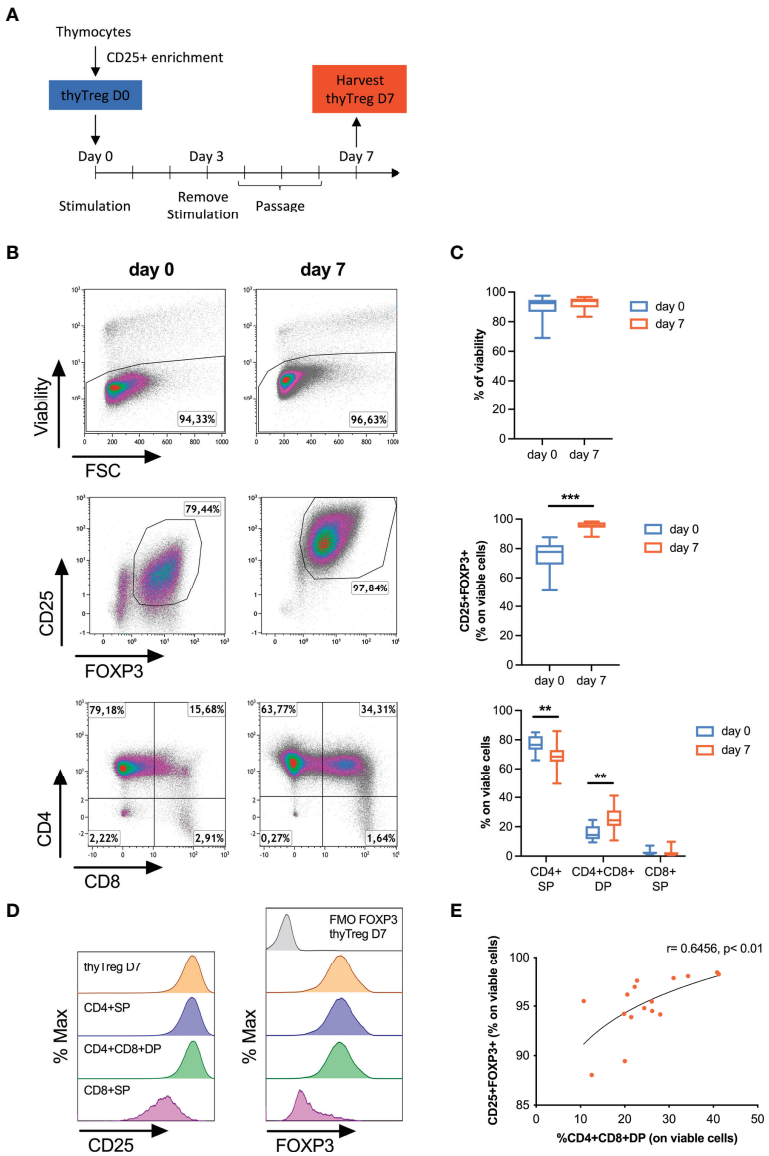

FIGURE 1 | Characteristics of manufactured thyTreg. **(A)** Isolation and culture protocol for thyTreg obtention. **(B)** Representative flow cytometry dot plots showing the viability, purity and CD4/CD8 phenotype of thyTreg right after isolation (day 0) or after culture (day 7). **(C)** Summary of the cell viability, purity and CD4/CD8 phenotype of n=16 thyTreg at days 0 (blue) and 7 (orange). The graph shows min-median-max. **,P < 0.01 and ***,P < 0.001 (paired Wilcoxon test). **(D)** Representative flow cytometry histograms showing CD25 (left) and FOXP3 (right) expression in thyTreg CD4/CD8 subsets. To determine the background signal, the fluorescence minus one (FMO) of FOXP3 is shown. **(E)** Correlation between the frequency of $CD4^+CD8^+$DP and the purity of thyTreg product (Pearson correlation analysis).

PBMC ratios (1:1, 1:2, 1:4 and 1: 8) in the presence of anti-CD3/anti-CD28 coated-beads (Dynabeads; Gibco) at a bead:PBMC ratio of 0.5:1 in X-VIVO 15 (Lonza) supplemented with 5% serum human AB (Sigma-Aldrich) and 600 U/ml of IL-2 (ImmunoTools) in round bottom 96 well culture plate. PBMC cultured alone in the presence or absence of Dynabeads were used as positive (C+) and negative (C-) control of proliferation, respectively. After 3 days in culture, the cells were labeled with anti-CD4-PC7 (Beckman Coulter), anti-CD8-FITC (Beckman Coulter) and 0.5 µg/mL of 7AAD (Sigma-Aldrich) to differentiate living and dead cells. Cells were acquired in a MACSQuant16 cytometer (Miltenyi Biotec), and data analysis was performed using Kaluza software (Beckman Coulter). The percentage of suppression of proliferation was calculated according to the "Division index method" (29) within $CD4^+$ and $CD8^+$ T cells.

Cytokine Production Analysis

The levels of different secreted cytokines or soluble proteins were measured using ELLA Protein-Simple (Biotechne) immunoassay

technology in the culture supernatant of the thyTreg product (day 7). The supernatants were thawed at room temperature and centrifuged to remove cell debris. Samples were pre-treated (in case of TGF- β detection) and diluted according to the manufacturer (Simple Plex, Protein Simple). Samples were then loaded along with the necessary controls into SimplePlex cartridges, following the kit instructions for their quantification by triplicate. Graphs show the concentration of each molecule in pg/ml; each point represents the mean of the replicate measurements. The limit of detection (LD) and the quantification range for each of the evaluated molecules are: IL-10, 0.14 (0.46-5530 pg/ml); TGF-β, 5.29 (20.8-12684 pg/ml); Granzyme-B, 0.385 (1.31-5000 pg/ml); LAG-3, 15 (39.6-151050 pg/ml); TIM-3, 0.33 (2.04-7780 pg/ml); IFN-γ, 0.05 (0.17-4000 pg/ml); IL-17A, 0.38 (0.82-8490 pg/ml); IL-4, 0.05 (0.319-1290 pg/ml); and PD-L1, 0.741 (3.45-13172 pg/ml). Values above the limit of quantification are shown as the maximum limit of quantification. Values below the limit LD are shown as 0.

Stability Assay Under Proinflammatory Conditions

The thyTreg cell product (day 7) was cultured at $1x10^6$ cells/ml in TexMACS GMP medium supplemented with 600 U/ml IL-2 and restimulated with TransAct alone or together with the following cytokines: 10 ng/ml of IL-12 (polarizing condition to Th1); and 10 ng/ml IL-1β, 10 ng/ml IL-6, 10 ng/ml of IL-23 and 20 ng/ml of TNF-α (polarizing condition to Th17). All cytokines were purchased from ImmunoTools. PBMC were also cultured in parallel under the same conditions. Cells were cultured for 3 days, removing excess TransAct matrix on day 2. On day 3, thyTreg and PBMC culture supernatants were frozen at −80°C for cytokine analysis, and thyTreg were assessed for cell viability, phenotype, and suppressive capacity as described above. The remaining cells were saved as dry pellets at −80°C for TSDR methylation studies.

Methylation Analysis

DNA was isolated from cell pellets using DNeasy Blood & Tissue Kit (Qiagen). The methylation status of 141 CpG sites located in 29 different genome regions comprising 20 different genes, including the Treg-specific demethylated region (TSDR), was analyzed by targeted Next-Gen bisulfite sequencing (NGS070V3 assay) performed by EpigenDx Inc (Hopkinton, MA, USA).

Statistical Analysis

The results are expressed as the mean ± SEM (Standard Error of the Mean) or min-median-max. Continuous data were tested for normality using the Shapiro-Wilk test. Comparisons were based on the unpaired Mann-Whitney U test and the paired Wilcoxon test for nonparametric data. The statistical test used to evaluate each experiment is specified within the respective figure legend. The statistical associations between variables were calculated by linear regression and Pearson correlation analysis. *p*-values < 0.05 were considered to be statistically significant. The following criteria to distinguish significance levels was used: * = < 0.05, ** = < 0.01 and *** = < 0.001.

RESULTS

ThyTreg Isolation and Phenotype

Thymocytes obtained by mechanical disaggregation from freshly removed pediatric thymuses (n=20; age range 0-48 months; **Table 1**) presented high viability (96.33% ± 0.99%) (**Supplementary Figure 1A**). Most of them (76.68% ± 2.03%) exhibited a $CD4^+CD8^+$ double-positive (DP) phenotype, while 12.57% ± 1.23% were $CD4^+$ single-positive (SP) cells, and 6.98% ± 1.26% were $CD8^+$SP cells (**Supplementary Figure 1B**). Because it has been shown that $CD4^+CD8^+$DP thymic Treg cells significantly contribute to the Treg pool in the human thymus (30–32), we decided to directly isolate $CD25^+$ thymocytes (2.36% ± 0.34%) without previous depletion of $CD8^+$ cells. The average frequency of $FOXP3^+$ cells on isolated $CD25^+$ thymocytes was 67.08% ± 2.22% (representative plot in **Supplementary Figure 1C**). The benefits of preserving DP thyTreg were supported by comparing the thyTreg cells obtained with or without $CD8^+$ depletion. The thyTreg yield was significantly higher without $CD8^+$ depletion (p=0.04; **Supplementary Figure 2A**), as well as the proportion of DP cells (p=0.003; **Supplementary Figure 2B**) while maintaining cell viability and percentages of $CD8^+$SP and $FOXP3^+$ cells (**Supplementary Figures 2B-D**). Following this strategy, we obtained 6.54 x 10^6 thyTreg per 10^9 thymocytes (range 2.44 x 10^6 – 11.65 x 10^6) after $CD25^+$ immunomagnetic selection (**Table 1**), with cell viability over 85%. Therefore, the estimated thyTreg number per gram of thymus was around 9.96 x 10^6 (range 1.32 x 10^6 – 21.59 x 10^6), which corresponds to 200.3 x 10^6 highly pure thyTreg for an average thymus weight of 20.10 grams.

ThyTreg Culture and Product Characterization

Following thyTreg isolation, cells were activated for 3 days and cultured for an additional 4 days, as depicted in **Figure 1A**. It is to note that the culture conditions were kept as simple as possible with the idea of maintaining the immature nature of the thyTreg, avoiding extra compounds usually employed during Treg expansion such as rapamycin and human AB serum, which showed no advantage in terms of thyTreg purity, phenotype or fold expansion (**Supplementary Figures 3A–F**). These cell characteristics were also maintained using TransAct instead of Dynabeads for cell activation to avoid the cell loss associated with the Dynabeads removal (**Supplementary Figures 3G–J**). Cell phenotype on day 0 and day 7 is shown in **Figures 1B, C** (n=16). The thyTreg cells harvested at day 7 presented very high viability (92.41% ± 1.02%) and purity in terms of $CD25^+FOXP3^+$ (95.2% ± 0.74%); being both parameters higher compared to day 0. During this short-time culture period, thyTreg proliferated 6.9 ± 1.42-fold (**Supplementary Figure 4A**). Considering 200.3 x 10^6 thyTreg isolated at day 0 and the average fold expansion, the theoretical number of thyTreg that could be obtained from a single thymus is around 1,500 x 10^6, reaching a yield of 13,649 $x10^6$ thyTreg cells from a single thymus in the best case.

We observed that the proportion of $CD4^+$SP thyTreg decreased during the cell culture, being offset by the increased proportion of $CD4^+CD8^+$DP thyTreg (**Figures 1B, C**; bottom

TABLE 1 | Characteristics of processed thymuses and thyTreg obtention.

Donor ID	Age (mo)	Thymus weight (g)	Thymocytes/g (x 10^9)	ThyTreg D0 x 10^6 (per 10^9 thymocytes)	ThyTreg D7 x 10^6 (per 10^9 thymocytes)
1	6	23.51	1.91	7.55	57.08
2	3	36.15	3.37	6.13	18.77
3	8	37.40	1.82	4.29	36.72
4	14	26.17	2.49	8.67	209.47
5	48	15.52	0.76	7.20	40.75
6	0.3	15.20	1.14	11.65	85.65
7	0.1	13.66	1.60	4.90	22.20
8	1	13.24	1.31	8.82	104.96
9	5	34.67	2.11	5.04	14.36
10	0.2	12.00	1.22	7.00	49.18
11	3	12.33	0.88	3.38	4.64
12	0	7.80	1.14	4.75	15.58
13	0.4	5.90	1.21	6.80	23.97
14	30	29.00	1.43	5.33	11.73
15	0.2	3.30	0.95	7.60	30.40
16	0.8	12.16	1.79	8.00	103.20
17*	4	35.20	1.80	9.20	39.65
18*	0.1	12.60	1.11	5.15	56.60
19*	28	47.00	0.54	2.44	20.95
20*	0.8	9.20	1.18	6.85	99.75
Mean	7.65	20.10	1.49	6.54	52.28
Range	0-48	3.3-47	0.54-3.37	2.44-11.65	4.64-209.47

*Individual data, mean and range are shown. *GMP-thyTreg.*

panels). Remarkably, at day 7, these $CD4^+CD8^+$DP thyTreg presented a similar phenotype to the $CD4^+$SP thyTreg, characterized by a high expression of CD25 and FOXP3 (**Figure 1D**). Indeed, there was a positive correlation between the proportion of $CD4^+CD8^+$DP thyTreg and the frequency of $CD25^+FOXP3^+$ thyTreg (**Figure 1E**).

To further characterize the thyTreg product, we analyzed a series of cellular markers related to Treg phenotype and functionality (**Figure 2A**). In summary, thyTreg product was characterized by high expression of the cytotoxic T-lymphocyte associated protein (CTLA-4), inducible T-cell co-stimulator (ICOS), thymic origin marker HELIOS, and CD27; intermediate expression of T cell immunoreceptor with Ig and ITIM domains (TIGIT), glucocorticoid-induced tumor necrosis factor receptor (GITR), latency-associated peptide (LAP), HLA-DR, and CD45RA; and low expression of CD39, CD73, and lymphocyte activation gene 3 (LAG-3). In addition, to evaluate the homing capacity of thyTreg cells, we determined the expression of the chemokine receptors CCR4, CXCR3, and the CD62L selectin (**Figure 2B**). ThyTreg cells showed high expression of CCR4, indicating their putative ability to migrate to organs with large epithelial surfaces (such as skin, gut or lungs) (33), and CD62L, favoring their location in lymph nodes (34). As previously shown (27), the expression of several functionality markers, including CTLA-4, CD73, ICOS, GITR and LAP, significantly increased during the cell culture; whereas CD39 expression decreased (**Supplementary Figure 4B**). Moreover, CD45RA expression increased, which could reflect the last switch from CD45RO to CD45RA occurring as a final step of maturation in the thymus (35) (**Supplementary Figure 4B**). Regarding the expression of chemokine receptors, CCR4 and CD62L significantly increased after 7 days of culture; whereas CXCR3 expression decreased (**Supplementary Figure 4C**), indicative of an undifferentiated phenotype (36).

We then analyzed the profile of secreted molecules by thyTreg in culture supernatants (**Figure 2C**). We detected high levels of the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β) (188.03 ± 36.04 and 237.73 ± 50.83 pg/ml, respectively); as well as high levels of other inhibitory molecules associated with Treg functionality, such as Granzyme B, soluble LAG-3 and soluble T-cell immunoglobulin mucin 3 (TIM3). On the contrary, we detected very low expression of proinflammatory cytokines such as IFN-γ, IL-4, IL-17A; and PD-L1. Finally, we evaluated *in vitro* the capacity of thyTreg cells to suppress the proliferation of $CD4^+$, and $CD8^+$ stimulated T cells (**Figures 2D, E**). We found that thyTreg exhibited a very high suppressive capacity, with more than 80% mean inhibition at a thyTreg: responder cells ratio of 1:1 and more than 40% at 1:4 ratio.

To determine the stability of the thyTreg product, we restimulated thyTreg cells exposed to a cocktail of cytokines polarizing to Th1 (IL-2, IL-12) or polarizing to Th17 (IL-2, IL-1β, IL-6, IL-23, TNF-α). We observed that thyTreg cell phenotype in terms of FOXP3, CTLA-4, CD39, and HLA-DR expression remains imperturbable (**Figures 3A, B**). Furthermore, thyTreg cells were not prompted to produce IFN-γ or IL-17A under polarizing conditions (**Figure 3C**) and conserved their suppressive function (**Figure 3D**). To support these findings, we determined the stability of *FOXP3* expression by analyzing the methylation profile of the TSDR (**Figures 3E, F**). We observed an intermediate level of TSDR demethylation in thyTreg cells at day 0 (62.93% ± 4.3% for males and 22.74% ± 8.03% for females), which increased after culture to 89.03% ± 2.57% for males and 51.26% ± 0.36% for females. Differences in demethylation levels between gender is due to the methylation-mediated inactivation of one

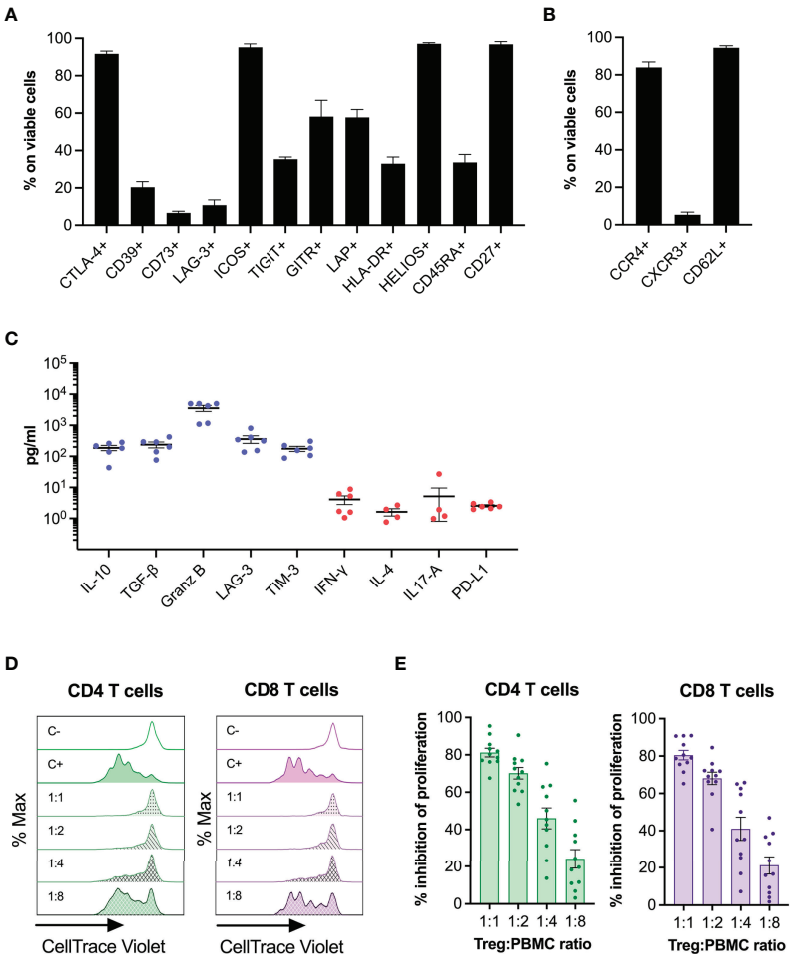

FIGURE 2 | Phenotype and functionality of thyTreg cell product. **(A)** Frequency of phenotypic and functionality markers within thyTreg cells (day 7). **(B)** Frequency of homing markers within thyTreg cells (day 7). **(C)** Quantitation of molecules secreted in day 7 thyTreg culture supernatants. Anti-inflammatory molecules in blue; proinflammatory molecules in red. **(D)** Representative flow cytometry histograms showing CD4 (green) and CD8 T (purple) cell proliferation as CellTrace Violet lost. C-, negative control of proliferation, PBMC cultured alone without stimulation; C+, positive control of proliferation, PBMC cultured alone with anti-CD3/anti-CD28 stimulation; 1:1 to 1:8, stimulated PBMC cultured with thyTreg cells at different thyTreg : PBMC ratios. **(E)** Summary of the suppressive capacity of thyTreg cells defined as % inhibition of CD4 (green) and CD8 T (purple) cell proliferation at the indicated ratios. Graphs show mean ± SEM.

X-chromosome in females. In contrast, the TSDR demethylation of freshly isolated (day 0) or cultured (day 7) thymic CD25- (thyTconv) was around 5%. The differential methylation pattern between thyTreg and thyTconv was observed not only in the *FOXP3* gene but in other 8 out of 19 genes related to Treg, including *CTLA-4*, *IKZF2* or *ILR2A* (**Supplementary Figure 4D**). Notably, the TSDR demethylation status in thyTreg cells was maintained under proinflammatory conditions (**Figure 3G**).

Since one of the hallmarks of our thyTreg product is the existence of a $CD25^+FOXP3^+CD4^+CD8^+$ DP population, we decided to evaluate their commitment to a Treg phenotype by analyzing the methylation status of the TSDR. For that, after 7 days of thyTreg culture, we sorted the $CD4^+$SP and the $CD4^+CD8^+$DP populations and analyzed their TSDR demethylation status compared with the whole thyTreg cell product (**Supplementary Figure 5**). We indeed confirmed that the proportion of $CD4^+CD8^+$DP cells with a demethylated TSDR (94.1%) was similar to that observed in the $CD4^+$SP or the total thyTreg population (92.8% and 91.6%, respectively), confirming the stability of *FOXP3* expression in this cell subset.

GMP thyTreg

With a focus on the possible therapeutic use of thyTreg cells, all the manufacturing protocol carried out at the research level was made considering that the reagents and equipment used were GMP-compliant or that GMP-certified equivalents were available in the market (**Supplementary Table 1** and **Figure 4A**). To confirm that thyTreg cells presented the same quality and functionality under GMP conditions, four thyTreg cell products (mean age of thymus donor = 8.23 months, **Table 1**) were manufactured at the Cell Production Unit (CPU) of Gregorio Marañón Health Research Institute.

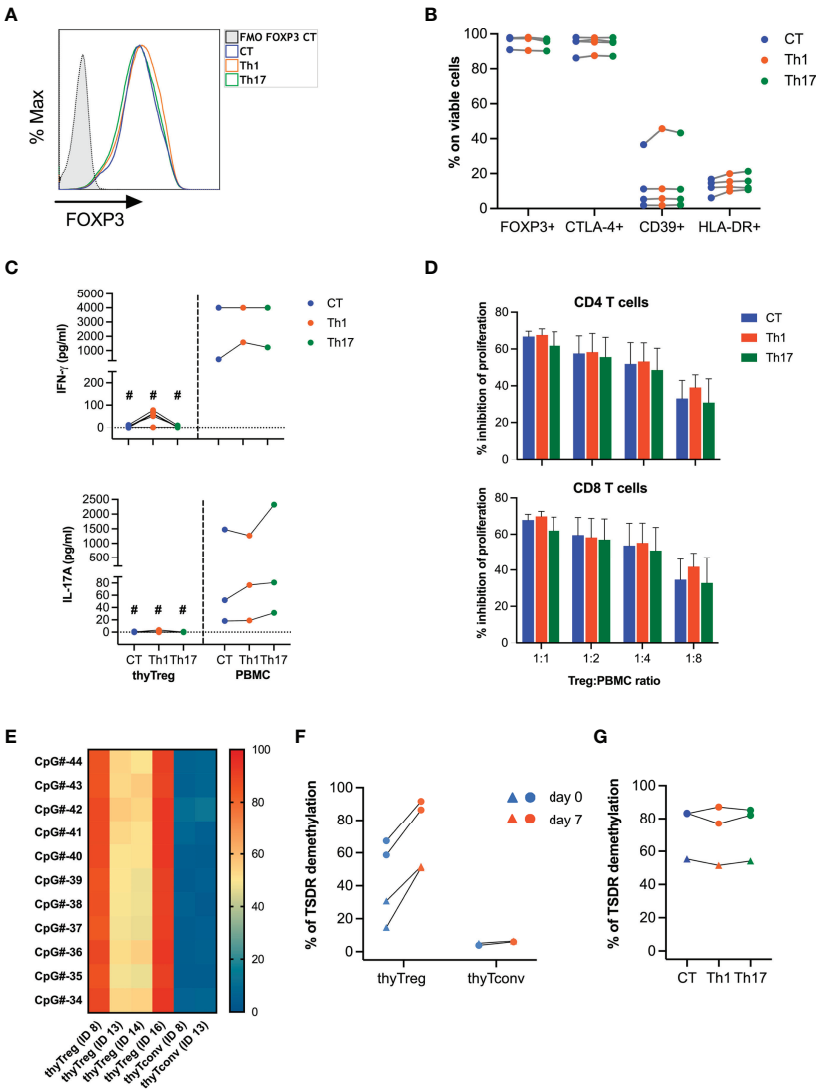

FIGURE 3 | Stability of thyTreg cell product. **(A–D, G)** thyTreg cell product was restimulated under control conditions (CT, blue), or under Th1 (orange) or Th17 (green) polarizing conditions and evaluated after 3 days of culture. PBMC were cultured in parallel under the same conditions. **(A)** Representative flow cytometry histogram showing FOXP3 expression. To determine the background signal, the fluorescence minus one (FMO) of FOXP3 is shown. **(B)** Frequency of FOXP3, CTLA-4, CD39 and HLA-DR within thyTreg under different culture conditions. Paired Wilcoxon test showed no significant differences between conditions. **(C)** Quantitation of secreted IFN-γ and IL-17A by thyTreg or PBMC under different culture conditions. Comparison between culture conditions within the same cell type was performed using paired Wilcoxon test, and comparison within the same condition between thyTreg and PBMC were performed using unpaired Mann-Whitney test ($^{\#}P < 0.05$). **(D)** Summary (n=4) of the suppressive capacity of thyTreg cells cultured under different polarizing conditions defined as % inhibition of CD4 (upper panel) and CD8 T (lower panel) cell proliferation at the indicated ratios. Graphs show mean ± SEM. Paired Wilcoxon test showed no significant differences between conditions. **(E)** Demethylation level of 11 conserved CpGs at the TSDR region of *FOXP3* in n=4 thyTreg cell products and n=2 thyTconv cultured in parallel for 7 days. ID13 and ID14 are female donors. **(F)** Global TSDR demethylation level (calculated as the mean of demethylation of the 11 CpGs) of thyTreg and ThyTconv right after cell isolation (day 0, blue) or after 7 days of culture (day 7, orange). Triangles represent female donors, and circles represent male donors. **(G)** Global TSDR demethylation level of thyTreg cultured under different polarizing conditions.

Following thymic dissociation, the recovery of thyTreg cells after the $CD25^{+}$ selection with the CliniMACS was around 5.91 x 10^{6} per 10^{9} labeled thymocytes, a value that was similar to that obtained using the column selection in the laboratory (6.69 x 10^{6} per 10^{9} labeled thymocytes). GMP-thyTreg cell products obtained in the CPU after 7 days of culture showed a very similar quality to the thyTreg obtained in the laboratory (**Table 2**). The results in **Table 2** show that the GMP-thyTreg products have high viability and purity (**Figures 4B, C**), comparable to laboratory thyTreg. The GMP-thyTreg proliferation in culture was similar to that obtained in the laboratory (**Supplementary Figure 6A**). The GMP-thyTreg cell

TABLE 2 | Comparison of the main thyTreg characteristics between Research and GMP manufacturing protocol.

Characteristic	GMP thyTreg (n=4)	ThyTreg (n=16)
Donor age (mo)	8.23 ± 6,65	7.50 ± 3.32
Thymocytes/g (x 10^9)	1.16 ± 0.26	1.57 ± 0.17
thyTreg D0 (per 10^9 thymocytes)	5.91 ± 1.42	6.69 ± 0.52
thyTreg D7 (per 10^9 thymocytes)	54.24 ± 16.83	51.79 ± 13.17
thyTreg D7 phenotype		
% of Viability	96.58 ± 0.71	92.41 ± 1.02
% of Purity ($CD25^+FoxP3^+$)	83.65 ± 4.87	95.20 ± 0.74
% of $CD4^+$SP	62.81 ± 2.70	67.73 ± 2.30
% of $CD4^+CD8^+$DP	28.43 ± 2.59	25.49 ± 2.26
% of CTLA-4^+	73.71 ± 1.55	91.68 ± 1.53
% of $CD39^+$	9.64 ± 2.68	20.39 ± 3.00
% of HLA-DR^+	22.76 ± 4.00	32.92 ± 3.58
IL10 secretion (pg/ml)	560.33 ± 137.56	188.03 ± 36.04
% of Inhibition of T CD4 proliferation (1:1)	71.48 ± 2.52	81.24 ± 2.31
% of Inhibition of T CD8 proliferation (1:1)	66.96 ± 7.16	80.52 ± 2.54
TSDR demethylation (in males)*	68.08 ± 9.15	89.03 ± 2.57

**Demethylation data corresponds to n=4 for GMP thyTreg and n=2 for research thyTreg. Data are mean ± SEM.*

product presented a similar phenotype, in terms of $CD4^+$SP and $CD4^+CD8^+$ DP abundance and other maturation and functionality markers (**Figures 4D, E**). It is to note that the expression of CTLA-4 and CD39 in the GMP-thyTreg cell products was slightly lower than the results obtained in the laboratory thyTreg. Still, their values fall within the ranges observed in laboratory thyTreg cells and are comparable to those of other studies (27). They also secreted a similar pattern of modulatory molecules: high levels of IL-10 (560.33 ± 137.56 pg/ml), TGF-β (129 ± 12.1 pg/ml), Granzyme B, soluble LAG-3 and soluble TIM3; and very low amounts of the proinflammatory cytokines IFN-γ, IL-4, IL-17A; and PD-L1 (**Figure 4F**). In accordance, GMP-thyTreg cells maintained a high suppressive capacity, inhibiting $CD4^+$ and $CD8^+$ T cell proliferation (**Figure 4G**). Furthermore, the GMP-thyTreg product was stable under proinflammatory Th1 and Th17 conditions, maintaining its phenotype (**Figures 4H, I**), without overexpressing IFN-γ (Th1) or IL-17A (Th17) (**Supplementary Figure 6B**), and its functionality remains unchanged (**Supplementary Figure 6C**). They also presented a high percentage of TSDR demethylation, indicative of the stability of the *FOXP3* expression (**Figure 4J**). Additionally, thyTreg cells met all the safety criteria for cell therapy liberation in terms of absence of contaminants and lack of genomic abnormalities.

In summary, the adaptation and scaling of the protocol to GMP conditions with the validations performed, provided evidence that we are manufacturing a product of thyTreg cells complying with the specifications required for their use in humans. In fact, our therapeutical product defined as "Treg lymphocytic cells, autologous, obtained from thymic tissue, expanded and stimulated with IL-2 (thyTreg)" received the approval of the Spanish Medical Agency (AEMPS) to be employed as cell therapy in humans.

DISCUSSION

The existing scientific evidence points to the fact that only through the induction of immunological tolerance we will be able to overcome harmful immune responses, eliminating the use of pharmacological immunosuppression, thus avoiding the toxic effects of these therapies and maintaining a competent immune system (15, 37). Among the different cell-based therapy strategies aiming at this purpose, Treg cells have been shown to play a crucial role in restoring immunological balance (15, 22, 38). In the context of solid organ transplantation, numerous clinical trials have been conducted in adults using autologous therapy of Treg cells obtained from peripheral blood to prevent solid organ rejection (21). However, very few have published efficacy results in its use as a therapy to prevent transplant rejection. One of the pioneers employing therapeutic Treg is the international consortium "The ONE Study", which administered peripheral blood autologous Treg in adult kidney transplant recipients. Published results proved the feasibility and safety of autologous Treg administration and showed that, while rejection rates were not modified in the first year, Treg infusion was associated with a lower incidence of infections, compared with the reference group (23).

The therapeutic use of *ex vivo* expanded peripheral blood Treg has presented a series of limitations that have compromised its effectiveness. The Treg frequency in peripheral blood is only 5-10% of CD4 T cells (39), therefore the maximum amount of Treg that could be obtained from an adult is around 30 million. Moreover, most peripheral blood adult Treg cells present a memory phenotype (CD45RA−), which indicates a higher phenotypic instability due to a more methylated status of the *FOXP3* and limited suppressive capacity (40). Also, shortened telomers of adult blood-derived Treg affect their replicative potential and *in vivo* survival, limiting the duration of the therapeutic effect (41, 42). This limited quality of adult Treg is worsened due to the long expansion rounds required to reach a sufficient number of Treg for therapeutic use, causing a more senescent phenotype, a marked loss of suppressive capacity and even the conversion of Treg into effector cells that could pose an added risk of rejection (24, 43). In pediatric subjects, Treg cells exhibit a predominantly naïve phenotype with still immature cells that have not been exposed to marked activation and differentiation processes. Several authors have confirmed the

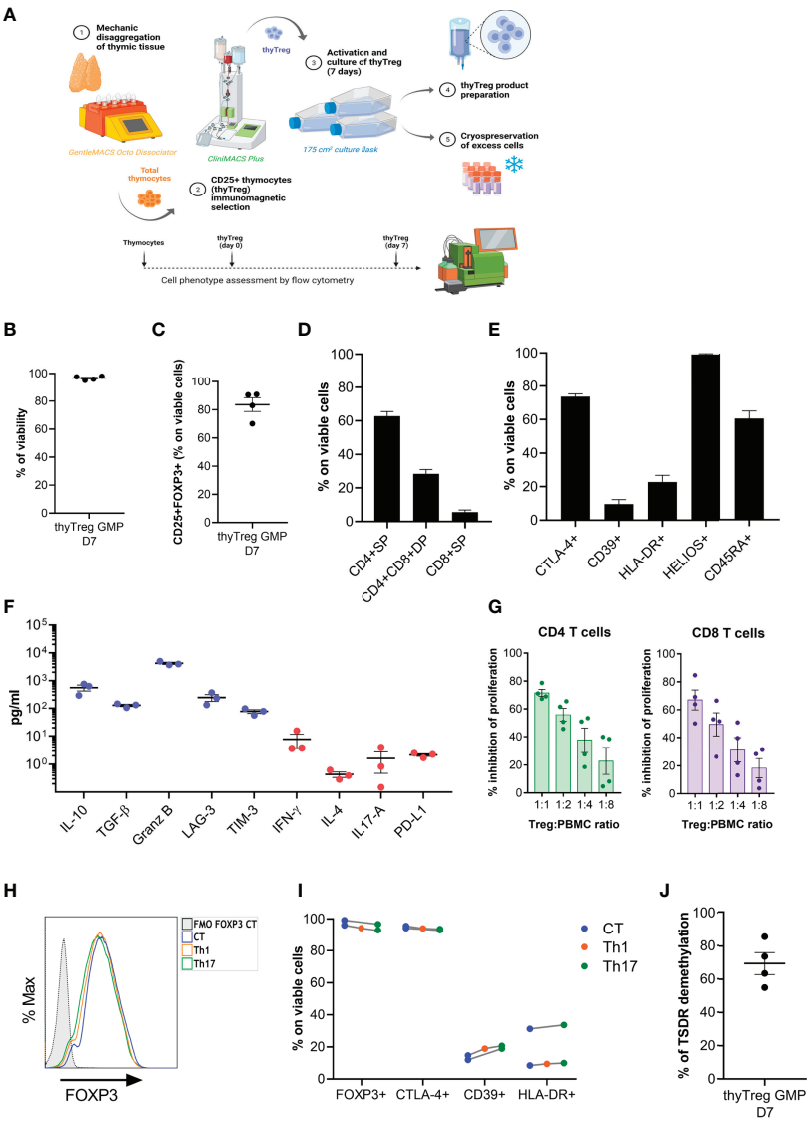

FIGURE 4 | GMP manufacturing of thyTreg. **(A)** Schematic representation of the procedure and equipment used in the Cell Production Unit for the thyTreg GMP manufacturing. Additionally, the dotted line represents the scheme of the quality evaluation process performed at different stages. **(B–E)** Summary of the cell viability **(B)**, purity **(C)** and phenotype **(D, E)** of n=4 GMP thyTreg. Graphs show mean ± SEM. **(F)** Quantitation of molecules secreted in day 7 GMP thyTreg culture supernatants. Anti-inflammatory molecules in blue; proinflammatory molecules in red. **(G)** Summary of the suppressive capacity of GMP thyTreg cells defined as % inhibition of CD4 (green) and CD8 T (purple) cell proliferation at the indicated ratios. Graphs show mean ± SEM. **(H)** Representative flow cytometry histogram showing the stability of FOXP3 expression of GMP thyTreg cell product under control conditions (CT, blue) or under Th1 (orange) or Th17 (green) polarizing conditions evaluated after 3 days after re-stimulation. To determine the background signal, the fluorescence minus one (FMO) of FOXP3 is shown. **(I)** Frequency of FOXP3, CTLA-4, CD39 and HLA-DR within GMP thyTreg under different polarizing conditions. **(J)** Global TSDR demethylation level (calculated as the mean of demethylation of the 11 CpGs) of GMP thyTreg cell product (n=4 males).

higher quality of the naïve Treg (40), indicating that the population of CD45RA$^+$ Treg cells, more abundant in children, would be the most appropriate to expand for therapeutic purposes (44, 45). Indeed, therapeutic Treg isolation strategies, including CD45RA+ enrichment, allow to obtain a cell population that maintains its suppressive properties and effectiveness longer (46). Despite the high quality of pediatric Treg, the usual strategy of purifying them from peripheral blood would be unapproachable due to the low blood volume that could be drawn from pediatric subjects, being the maximum amount of recovered Treg around 5 million cells. This could be solved by *ex vivo* expansion cycles, but it would lead to the loss of their undifferentiated phenotype. Another successful strategy is the obtention of Treg from umbilical cord blood (47). These cells share the advantage of a mostly naïve phenotype but also has limitation in the number of cells that can be recovered. As

described in Riley et al., 5-7 million Treg (26) can be obtained from a cord blood unit, which would still be a deficient number for a therapeutic dose, requiring therefore numerous rounds of expansion. Indeed, in some cases, it was necessary to expand up to 27,000 times to get a single therapeutic dose (18, 48). Despite the limitation in the number of cells available, the potential efficacy of cord blood Treg has been demonstrated by the excellent results obtained when using them as allogeneic therapy in the prevention of GVHD in adults, reducing the incidence of grade II-IV acute GVHD and eliminating the incidence of chronic GVHD (17, 18).

In a further attempt to improve the therapeutic Treg quality and overcome the current limitations regarding cell number and phenotype, the thymus, a primary lymphoid organ where the T cells mature, has been proposed as a new source of Treg. Indeed, Dijke and collaborators (27) showed that a large amount of stable, long-lived and potent FOXP3$^+$ Treg could be isolated and expanded from a single thymus. Furthermore, Romano and colleagues (28) have recently reported a good manufacturing practice (GMP) compliant protocol to isolate and expand thymus-derived Treg cells, confirming the feasibility of the strategy. This is a revolutionary approach since children with heart diseases requiring cardiac surgery often undergo thymectomy to clear the surgical field. Therefore, the thymus is routinely discarded and could provide an excellent source for therapeutic Treg (as an example, around 100 thymuses are discarded per year at our institution, the Pediatric Hospital Gregorio Marañón).

Our thyTreg manufacturing protocol also employs the thymus as a Treg source, but differentiates from the others in several aspects. First, the Treg purification procedure is performed in a single step (immunomagnetic selection of CD25$^+$ cells), without previous depletion of CD8$^+$ cells. In addition to increasing cell yield, this alternative preserves a CD25$^+$ population which is CD4$^+$CD8$^+$ double-positive (thyTreg DP), for which epigenetic and transcriptional analysis have demonstrated their Treg commitment (32). Moreover, their immature phenotype and high expression of FOXP3 that we observed in thyTreg DP could contribute to the greater purity and suppressive capacity in the final product. Second, we replaced the mechanism of activation used in the other strategies, which are magnetic spheres coupled to anti-CD3/anti-CD28, with a new soluble nanomatrix system that can be easily removed by centrifugation, preventing the loss of a large part of cells in the process of elimination of the spheres prior to administration. Third, the culture medium has been kept to minimum components (TexMACS + human IL-2), avoiding the use of chemical compounds (such as rapamycin) or human sera. Finally, the culture duration is very short (7 days), allowing the activation and proliferation of thyTreg, but avoiding extensive expansion rounds that could potentially decrease the quality of Treg. The thyTreg product obtained exhibits high purity and suppressive capacity, with a stable FOXP3 expression, whose characteristics are maintained under inflammatory conditions. Regarding the number of therapeutic Treg cells obtained, there is a wide variety of product yields, functionality and fold changes depending on the cell source and the protocol employed to isolate and culture Treg (3, 49, 50). Considering the T-cell receptor (TCR) repertoire, both thymic and peripheral Treg have been shown to present very diverse TCR repertoires (51, 52). We acknowledge that Treg isolated from the thymus could potentially present different maturation statuses and indeed be subjected to a partial thymic selection. Nevertheless, most FOXP3$^+$ thymocytes are found in the thymic medulla (53), where cells have already been selected. Furthermore, the thyTreg product has been cultured for 7 days and underwent a final step maturation as suggested by the phenotypic marker's evolution CD45RA, CD62L and CD39. Nonetheless, we are planning to evaluate the TCR diversity of the thyTreg product. Although numerous studies reflect the difficulty of freezing Treg cells while preserving their phenotype and suppressive capacity (54), preliminary data indicates that our thyTreg product can be cryopreserved under a GMP compliant protocol capable of maintaining the viability, phenotype and functionality of the thyTreg, which would make the use of frozen cells feasible. Nevertheless, further investigation in this line is being performed in order to have conclusive results.

The loss of immunological homeostasis and the appearance of excessive or unwanted immune responses in the form of inflammatory phenomena can trigger various serious pathologies. The improved quality and amount of thyTreg obtained with our protocol allow us to prepare hundreds of therapeutic doses from a single thymus, which can be cryopreserved and could be employed for sequential autologous doses or as an "off-the-shelf" allogeneic therapy in another individual. Although the autologous application of thyTreg could be the most straightforward, we postulate that their allogeneic use would be a realistic approach, opening the possibility to treat other diseases, both in children and adults, such as the rejection of different types of organs, GVHD, autoimmune processes, or even in the most severe COVID-19 patients. For all these reasons, we are currently exploring whether the administration of allogeneic thyTreg will maintain its therapeutic suppressor effect without being recognized as foreign and rejected by the recipient´s immune system. This hypothesis is based on preliminary results of our group and others (55) that indicate that the immature or undifferentiated character of the thyTreg is associated with a very low frequency of immunogenicity markers that allow them to be recognized as foreign cells, therefore, being unnoticed by the recipient's immune system. Indeed, there are already different successful studies that use allogeneic Treg in the context of GVHD employing donor peripheral blood (56) or third-party donor umbilical cord blood (18, 48). However, until the low immunogenicity of our thyTreg product is completely proved, we should consider the importance of HLA-concordance to prevent rejection when using them allogeneically. In addition to the allogenic use of thyTreg, we are also exploring their genetic modification to enhance their effectiveness and versatility. In particular, we are genetically modifying thyTreg to make them antigen-specific by inducing the expression of the Chimeric Antigen Receptor (CAR) and universal by eliminating the HLA from the surface of the CAR-thyTreg.

Importantly, our thyTreg product has been approved by the Spanish Drug Agency (AEMPS) to be administered as cell therapy, and we are recruiting patients in a phase I/II clinical trial that evaluates the safety and efficacy of autologous thyTreg administration to prevent rejection in heart transplant children (NCT04924491). Our ongoing clinical trial, with four patients already treated, is the first to employ a Treg therapy to prevent rejection in transplanted children, but above all, it is the first worldwide to use thyTreg in humans as an alternative to Treg obtained from blood. The confirmation in this trial of the feasibility and safety of our strategy paves the way for the development of new indications for this therapy, which could revolutionize the treatment of different pathologies with high incidence.

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 studies involving human participants were reviewed and approved by Ethical Committee (CEIM) from Gregorio Marañon University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EB-Q and MM-B designed and performed experiments, and analyzed data and wrote the manuscript. BC and RL performed experiments and analyzed data. JG-J, CP, AP, and RP-C provided samples of thymic tissue. MCa and NG participated in the enrolment of patients. MCl, MF-S, and SS provided scientific input and support in implementing the manufacturing protocol in the Cell Production Unit. MP and RC-R conceptualized the study, supervised the project and wrote the manuscript. 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/fimmu.2022.893576/full#supplementary-material

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HYPOTHESIS AND THEORY
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How Thymocyte Deletion in the Cortex May Curtail Antigen-Specific T-Regulatory Cell Development in the Medulla

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CD4$^+$ T cell responses to self-antigens are pivotal for immunological self-tolerance. Activation of Foxp3$^-$ T-conventional (T-conv) cells can precipitate autoimmune disease, whereas activation of Foxp3$^+$ T-regulatory (T-reg) cells is essential to prevent autoimmune disease. This distinction indicates the importance of the thymus in controlling the differentiation of self-reactive CD4$^+$ T cells. Thymocytes and thymic antigen-presenting cells (APC) depend on each other for normal maturation and differentiation. In this Hypothesis and Theory article, we propose this mutual dependence dictates which self-antigens induce T-reg cell development in the thymic medulla. We postulate self-reactive CD4$^+$ CD8$^-$ thymocytes deliver signals that stabilize and amplify the presentation of their cognate self-antigen by APC in the thymic medulla, thereby seeding a niche for the development of T-reg cells specific for the same self-antigen. By limiting the number of antigen-specific CD4$^+$ thymocytes in the medulla, thymocyte deletion in the cortex may impede the formation of medullary T-reg niches containing certain self-antigens. Susceptibility to autoimmune disease may arise from cortical deletion creating a "hole" in the self-antigen repertoire recognized by T-reg cells.

Keywords: thymus, T-cell selection, T-cell tolerance, T-cell deletion, T-regulatory cells, self-antigen recognition, autoimmune disease

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INTRODUCTION

Foxp3$^+$ T-regulatory (T-reg) cells are an immunosuppressive lineage of T cells essential for immune tolerance (1). The development and function of T-reg cells depend on interactions between the T cell receptor (TCR) and peptide-major histocompatibility complex class II (pMHCII) antigens on the surface of other cells (2, 3). Some pMHCII self-antigens induce thymic lymphocytes (thymocytes) to upregulate Foxp3 (4–6); we refer to these pMHCII self-antigens as $T_{regitopes}$ (T-reg epitopes). Some self-peptides fail to form a $T_{regitope}$ because they cannot bind stably to the peptide-binding grooves of any MHCII alleles expressed in a given organism (7). In addition, the expression pattern of a self-peptide affects its capacity to form a $T_{regitope}$. Self-peptides not expressed in the thymus do not affect the development of responding thymocytes, whereas highly expressed self-peptides induce thymocyte deletion (8, 9). Highly expressed self-peptides induce thymocyte

deletion because the high number of pMHCII complexes per APC, or high number of pMHCII$^+$ APC, triggers persistent TCR signaling in thymocytes. Alternatively, highly expressed self-peptides may induce deletion because they are presented in the cortex to immature thymocytes that are more sensitive to deletion than mature thymocytes in the medulla (10, 11). Thus, according to current concepts, self-peptides with low or sparse presentation in the thymic medulla should form $T_{regitopes}$.

A self-peptide derived from the α3 chain of type IV collagen (α3) forms a $T_{regitope}$ when presented by the MHCII molecule, human leucocyte antigen (HLA)-DR1, but not when presented by HLA-DR15 (4). HLA genotype would not be expected to affect α3 expression, which has been observed in the thymic medulla in a pattern suitable for $T_{regitope}$ formation (12). Although HLA-DR1 and HLA-DR15 both present the α3 self-peptide to T cells, the peptide anchor residues are offset by one position so that the TCR "sees" different amino acids of the peptide when it is presented by HLA-DR1 *versus* HLA-DR15 (4). The distinct fates of α3-specific CD4$^+$ T cells are of special interest because humans and mice expressing HLA-DR15 are susceptible to Goodpasture's disease, also known as anti-glomerular basement membrane disease, characterized by pro-inflammatory T cell responses towards α3/DR15 (13, 14). However, co-expression of HLA-DR1 induces development of α3/DR1-specific T-reg cells and prevents Goodpasture's disease in a manner that depends on T-reg cells (4).

To account for the distinct fates of CD4$^+$ T cells specific for α3/DR1 *versus* α3/DR15, and other findings, here we propose an extension to current concepts of thymic T-reg cell development. We postulate the potential of medullary pMHCII self-antigens to form a $T_{regitope}$ can be extinguished when a high percentage of cognate antigen-specific thymocytes are deleted by encountering the same or similar pMHCII self-antigens in the cortex. We suggest antigen-specific CD4$^+$ CD8$^-$ (CD4 single-positive, CD4SP) thymocytes deliver signals that induce medullary thymic epithelial cells (mTEC) to "lock in" expression of their cognate self-antigen. Self-reactive CD4SP thymocytes may thereby generate a medullary niche for subsequent development of T-reg cells specific for the same self-antigen. Thus, antigen-specific T-reg niche size may be inversely related to the extent of cortical deletion of antigen-specific thymocyte populations. Implications of this extended model for the pathogenesis of organ-specific autoimmune diseases are discussed.

IMPACT OF THYMOCYTE DELETION IN THE CORTEX ON T-REG SELECTION IN THE MEDULLA

Thymocyte deletion has been dissected based on the maturation stage and/or the intrathymic location of the thymocytes undergoing deletion (15). Most CD4$^+$ CD8$^+$ (double positive, DP) thymocytes are located in the cortex, whereas CD4SP thymocytes migrate between cortex and medulla, preferentially residing in the medulla (16, 17). In models in which deletion occurs at the DP stage, increased numbers of apoptotic cells are found in the cortex (18–20), whereas deletion at the CD4SP stage results in increased numbers of apoptotic cells in the medulla (18). Thus, it is plausible that deletion of DP thymocytes occurs in the cortex and deletion of CD4SP thymocytes occurs in the medulla. However, DP CD69$^+$ thymocytes can enter the medulla in a CCR4-dependent mechanism (21). In mixed chimeras, *Ccr4*$^{-/-}$ thymocytes are overrepresented in all TCR-signalled thymocyte subsets starting at the DP CD69$^+$ stage (21). Those findings indicate CCR4 is required for normal deletion and suggest this deletion may occur in DP thymocytes inside the medulla. Still, considering the high frequency of thymocytes that undergo deletion at the DP stage (22–24), the relatively mild effect of CCR4 deficiency on deletion (21, 25) suggests that a substantial amount of deletion at the DP stage is independent of CCR4. While the relative contributions of the cortex and medulla to thymocyte deletion at the DP stage remain unclear, for conceptual clarity, in this Hypothesis and Theory article we have assumed that deletion at the DP stage occurs predominantly in the cortex, and we refer to this process as cortical deletion. Cortical deletion is widely considered to be inconsequential to T-reg selection because a thymocyte deleted in the cortex cannot directly affect events taking place in the medulla. However, we postulate that cortical deletion can affect T-reg selection by creating variation in the number of antigen-specific CD4SP thymocytes in the medulla.

An antigen-specific CD4$^+$ T cell is typically identified by the binding of its TCR to a given pMHCII tetramer (26). In a naïve C57BL/6 (B6) mouse, the number of self-antigen-specific CD4SP thymocytes varies by 100-fold depending on the peptide embedded in the MHCII tetramer (27). Most of this effect arises from variation in the proportion of antigen-specific thymocytes that undergo deletion (28). Deletion of antigen-specific thymocytes need not be triggered by the antigen itself. For example, the IgM:I-A^b-specific CD4SP thymocyte population in B6 mice is small (28). This population is ~ 8 times larger in mice that lack B cells–the only source of the IgM self-peptide–indicating IgM:I-A^b itself is required for the deletion of some IgM:I-A^b-specific thymocytes. However, the IgM:I-A^b-specific CD4SP thymocyte population is 450 times larger in mice with truly defective deletion due to MHCII expression being confined to cortical thymic epithelial cells, demonstrating that most IgM:I-A^b-specific thymocytes can be deleted by self-antigens other than IgM:I-A^b (28). Enumerating CD4$^+$ T cells specific for a panel of foreign pMHCII antigens revealed the extent of such "deletion *via* TCR cross-reactivity" correlates with the number of self-peptides with the same or similar TCR-exposed amino acids (29). Deletion of thymocytes expressing a cross-reactive TCR (30) is indistinguishable from deletion of thymocytes specific for ubiquitous self-antigen (20). Both are initiated at the DP stage and the thymocytes never reach the CD4SP stage at which Foxp3 is upregulated in developing T-reg cells (31, 32). Cortical deletion prevents cross-reactive and ubiquitously self-reactive thymocytes from developing into T-reg cells.

The perinatal period is a critical time for immune tolerance (33). Perinatal T-reg cells are more effective than adult T-reg cells

at preventing autoimmune disease provoked by Aire deficiency (34). The perinatal T-reg TCR repertoire is distinct from, and more diverse than, the adult T-reg TCR repertoire (34). The age-dependent change in T-reg selection is partly attributable to mTEC directly presenting a higher number of self-antigens in perinates than in adults (34). However, several findings suggest the extent of cortical deletion is also different in perinates and adults. The percentage of strongly TCR-signalled (Helios$^+$) cells in the immature (CCR7$^-$ CD24$^+$) thymocyte population increases with age (**Figure 1**). This effect, which was reported previously (35), suggests cortical deletion is smaller in magnitude during the perinatal period than in adult life. This may be due to MHCII^{high} CD8α$^+$ DC being less frequent in the perinatal thymus than in the adult thymus (34). Although cortical thymic epithelial cells can induce strong TCR signaling in some thymocytes (20, 36, 37), BM-derived APC (BM-APC), including DC, are present in the cortex (38, 39) and are required for normal cortical deletion (20, 36, 37, 40). An age-dependent change in the fate of thymocytes specific for one natural self-antigen has been documented (6). In mice with transgenic expression of the TCRβ chain from the Yae62 TCR (Yae62β-tg) (41), peptidyl arginine deiminase type IV (Padi4):I-A^b is a $T_{regitope}$ at 1-3 weeks after birth; however, Padi4:I-A^b-specific thymocytes are deleted at the DP stage or at the DP-CD4SP transition from 4 weeks after birth onwards (6). Analysis of *Padi4*$^{-/-}$ Yae62β-tg mice confirmed Padi4:I-A^b-specific thymocytes undergo T-reg development in perinates and deletion in adults in response to Padi4 itself, with Padi4 expression in BM-APC sufficient to induce deletion in adult mice (6). Thymocytes with the potential to develop into T-reg cells can be deleted instead of developing into T-reg cells if they encounter a related self-peptide, or the cognate self-peptide itself, at the DP stage in the thymic cortex.

Events that occur within the DP stage in thymocytes that become T-reg cells have been puzzling to resolve. Commitment to the T-reg lineage within the DP stage was thought to be common, based on flow cytometry data indicating ~ 33% of Foxp3$^+$ thymocytes in adult wild-type mice were DP cells (42). However, another study reported <10% of Foxp3$^+$ thymocytes were DP cells including during the perinatal period (31). After rigorous exclusion of doublet events during flow cytometric analysis, <5% of Foxp3$^+$ thymocytes had a DP phenotype (32). Accordingly, analysis of thymocytes that had incorporated a DNA label at the DP stage showed that Foxp3 upregulation predominantly occurs 4-8 days after label uptake, by which time the labelled cells have acquired a CCR7$^+$ CD4SP phenotype (43) and moved to the medulla (44). Although we cannot exclude the possibility that commitment to the T-reg lineage can occur at the DP stage in the cortex, we favour the view that this usually occurs at the CCR7$^+$ CD4SP stage when the thymocytes are in the medulla.

CURRENT CONCEPTS OF DEVELOPMENTAL NICHES FOR T-REG CELLS IN THE THYMIC MEDULLA

T-reg cells that develop in the thymus are thought to encounter their cognate self-antigen for the first time in the medulla (45, 46). This is plausible because the intra-thymic expression of some self-antigens, including tissue-restricted antigens (TRA), is confined to mTEC (47). Two major mTEC subsets are distinguished by expression of CCL21 (48), a chemokine that attracts CCR7$^+$ thymocytes to the medulla (49), or the nuclear protein, Aire, which is required for normal expression of thousands of TRA by mTEC (50–52). Another nuclear protein, Fezf2, which is required for a distinct program of TRA expression independent of Aire (53), is expressed by CCL21$^+$ mTEC and Aire$^+$ mTEC (54, 55). Self-antigens expressed by

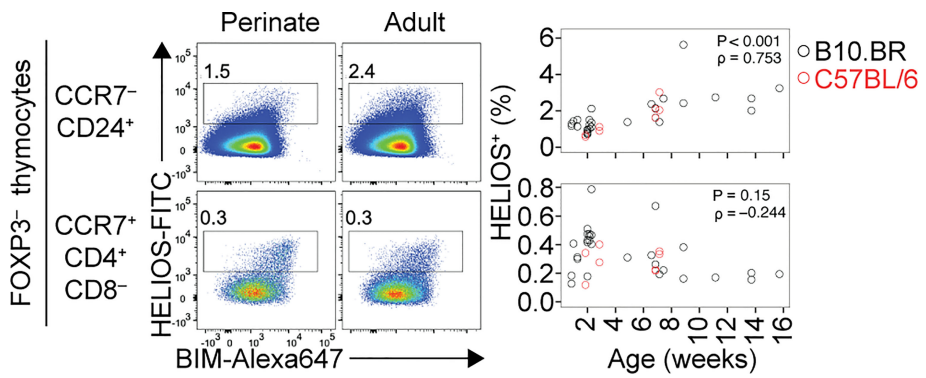

FIGURE 1 | Age-dependent shift in cortical and medullary tolerance induction in the thymus. Flow cytometry plots (left) show HELIOS/BIM phenotypes of Foxp3$^-$ thymocytes, divided into CCR7$^-$ CD24$^+$ (cortical) and CCR7$^+$ CD4$^+$ CD8$^-$ (medullary) populations. Note that HELIOS and BIM tend to be co-expressed. Within each population, numbers on the plots show the percentage of HELIOS$^+$ cells among all thymocytes, with graphs (right) showing results for multiple mice of the indicated ages and strains. Cortical tolerance appears less prominent during perinatal life, potentially enabling a higher frequency of strongly self-reactive thymocytes to develop into T-reg cells in the medulla in perinates compared to adults. P and rho values were determined using Spearman's test for correlation.

mTEC can be taken up and presented by BM-APC (56, 57) and the presentation of some self-antigens to thymocytes is completely dependent on this mechanism (58, 59). While the mTEC population collectively expresses almost all protein-coding genes, the expression of individual genes varies widely both at the level of transcript abundance and in the frequency of mTECs that express the transcript (52). Many self-antigens are thought to be presented to thymocytes in small and discrete foci, which form a "mosaic" of developmental niches for antigen-specific T-reg cells in the medulla (45, 60).

This "mosaic" of self-antigen expression is shaped by proliferation, differentiation, and maturation of mTEC. Proliferating mTEC, which express many chromatin-modifying factors and some TRA, give rise to cells that express Aire and a higher number of TRAs per mTEC (54, 61). Whether CCL21$^+$ mTEC are precursors or progeny of proliferating mTEC remains unclear (35, 54, 61). The current concept is that an individual mTEC expresses different sets of self-antigens over its lifetime (61–63). In support of this "colinear differentiation" model (62), an individual mTEC can switch off expression of one self-antigen and switch on expression of another (62, 64). In addition, single-cell RNA sequencing identified sets of self-antigens that were co-expressed in multiple mTEC (61, 63, 65). In this model, the presence of cells spanning all mTEC subsets and all maturation stages is necessary and sufficient for the expression of a full "mosaic" of self-antigens in the medulla.

The thymic medulla is smaller in mice lacking CD4SP thymocytes compared to wild-type mice or mice lacking CD8SP thymocytes (66). Development of the mature mTEC population, defined by high expression of MHCII and the costimulatory molecule CD80, and comprising an Aire$^+$ subset, requires cognate interactions between the TCR on CD4SP thymocytes and pMHCII on mTECs (67). CD4SP thymocytes express the ligands for RANK, CD40, and LTβR, which are cell-surface receptors necessary for mTEC maturation (66, 68, 69). Anti-RANK ligand (RANKL) antibody treatment and the absence of self-reactive CD4SP thymocytes both cause deficiency of Aire$^+$ mTEC, whereas CCL21$^+$ mTEC remain largely intact (54, 55). Notably, anti-RANKL antibodies diminish the frequency of proliferating cells in the mTEC population (54, 70), whereas the absence of self-reactive CD4SP thymocytes does not (55). It is possible that invariant NKT cells provide enough RANKL to support mTEC proliferation (71). However, normal transition from the proliferating stage to the Aire$^+$ stage in mTEC development requires signals uniquely provided during cognate interactions with self-reactive CD4SP thymocytes (66). Similarly, normal development of mature thymic DCs requires cognate TCR-pMHCII interactions with CD4SP thymocytes (72).

CD4SP thymocytes also contribute to thymic T-reg cell niches by producing IL-2 (73, 74). Consumption of this IL-2 prevents deletion of strongly TCR-signalled CD4SP thymocytes (75) and enables these T-reg precursors to upregulate Foxp3 expression (76, 77). CD4SP thymocytes are thus both inducers and "clients" of antigen-specific T-reg cell niches in the thymic medulla.

A ROLE FOR CD4SP THYMOCYTES IN GENERATING THE T-REG NICHE CONTAINING THEIR COGNATE SELF-ANTIGEN?

To this picture we wish to add the hypothesis that strongly self-reactive CD4SP thymocytes foment the niche containing their cognate self-antigen. Sustained, repetitive, cognate interactions between CD4SP thymocytes and mTEC may be necessary for the survival of post-cycling Aire$^+$ mTEC and may induce the mTEC to pause or arrest its "colinear differentiation" program. In other words, these interactions may "lock in" continued expression of those self-antigens that the mTEC is expressing at the time. Currently available data do not exclude this extended model. During the post-cycling Aire$^+$ stage, different studies found the mean number of TRAs expressed per Aire$^+$ mTEC remained constant (61) or increased by a factor of only two (54). Multiple mTEC that co-express sets of self-antigens may be "daughters of the same epithelial cell progenitor" (65).

Through this process, antigen-specific CD4SP thymocytes may seed a niche for the development of T-reg specific for the same self-antigen or another self-antigen in the same co-expression "module" (61). Generating a functional T-reg niche requires collaboration between CD4SP thymocytes because no single cell can fulfil all functions required of CD4SP thymocytes. These functions include: (i) to induce the post-cycling Aire$^+$ mTEC to survive and "lock in" its current self-antigen expression profile; (ii) to induce the mTEC and local DC to upregulate antigen-presenting and costimulatory molecules; (iii) to produce IL-2; and (iv) to develop from a naïve CD4SP thymocyte into a T-reg precursor and then into a T-reg cell. At another level, there is inter-niche competition because the medullary volume limits the number of niches present at a given time. Success in this inter-niche competition may be proportional to the extent of intra-niche collaboration, which is in turn dictated by the size of the antigen-specific CD4SP thymocyte population in the medulla (**Figure 2**).

This extended hypothesis can accommodate some unexplained findings. Two TCRs, called DO11 and N7, can facilitate T-reg development in mice expressing the neo-self-antigen, ovalbumin (OVA) (78). For these two TCRs, as had been observed in other models (2, 79), antigen-specific CD4SP thymocyte population size and Foxp3$^+$ cell frequency were inversely related, consistent with T-reg development being constrained by OVA:I-A^d availability (78). Surprisingly, and in contrast to the inverse relationship, T-reg development failed when the CD4SP thymocyte populations expressing the DO11 or N7 TCR were very small (78). We suggest the OVA:I-A^d-specific population size had a lower limit, below which intra-clonal collaboration between the TCR-transgenic CD4SP thymocytes was insufficient to generate a niche for effective OVA:I-A^d-specific T-reg development.

Our hypothesis also accommodates complementary findings based on CD4$^+$ T cell responses to natural self-antigens. Although T-reg cell populations specific for myelin

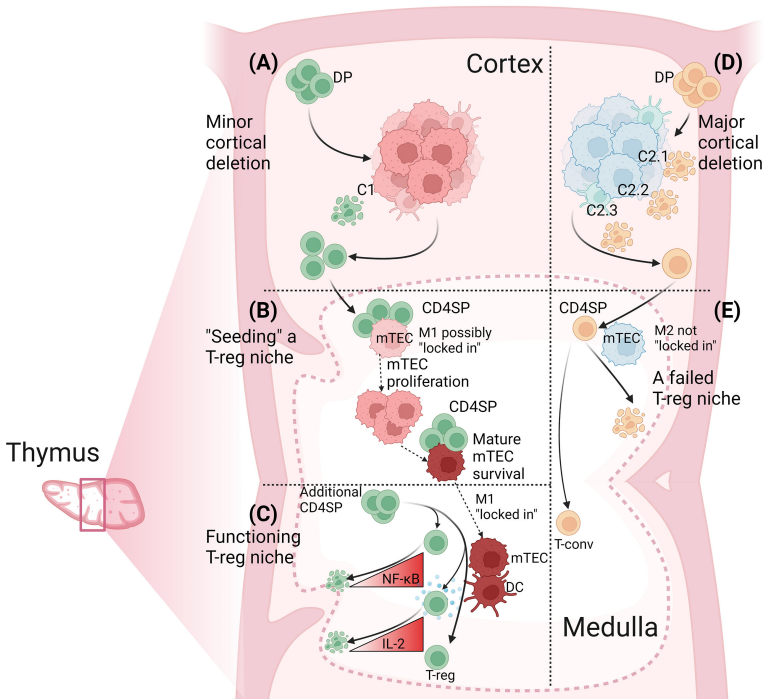

FIGURE 2 | Determinants of antigen-specific T-reg niche size in the thymus. **(A)** The extent of cortical deletion is minor when the relatedness between cortical and medullary peptides is low. Out of four DP thymocytes specific for a medullary peptide, M1, one is deleted by a related cortical peptide, C1, and the other three progress to the CD4SP stage and migrate into the medulla. **(B)** Cognate CD4SP-mTEC interactions "seed" the T-reg niche. CD4SP thymocytes could mediate this effect by interacting with immature mTEC prior to the onset of proliferation and/or by promoting mTEC survival at the post-cycling stage through sustained engagement of RANK, CD40 and LTβR expressed on mTEC. The key outcome is that M1-specific CD4SP thymocytes induce the M1-presenting mTEC to "lock in" M1 expression. **(C)** A functioning T-reg niche. By now, the mature M1-expressing mTEC has high expression of MHCII, CD80, Aire and the protein from which the M1 peptide is derived. M1-specific CD4SP thymocytes also induce local DC to increase MHCII and CD80 expression. M1-specific CD4SP thymocytes also produce IL-2. However, most M1-specific CD4SP thymocytes undergo deletion due to insufficient NF-κB activation or insufficient IL-2 consumption. Rare M1-specific CD4SP thymocytes activate sufficient NF-κB and consume sufficient IL-2 to survive, upregulate Foxp3 and progress to the next stage of T-reg development. **(D)** The extent of cortical deletion is major when the relatedness between cortical and medullary peptides is high. Out of four DP thymocytes specific for medullary peptide, M2, three are deleted in the cortex by related self-peptides, C2.1, C2.2 and C2.3, and only one progresses to the CD4SP stage and migrates into the medulla. **(E)** A failed T-reg niche. The number of M2-specific CD4SP thymocytes is too low to provide inductive signals to the M2-expressing mTEC. The mTEC may switch off expression of M2 and switch on expression of different self-antigens. Activation of M2-specific CD4SP thymocytes is insufficient to induce T-reg development but may be sufficient to induce deletion of some cells. No M2-specific T-reg niche forms and the small M2-specific CD4SP population develops into T-conv cells. Figure created with BioRender.com.

oligodendrocyte glycoprotein (MOG):I-A^b can be expanded in B6 mice immunized with MOG peptide (8, 80), MOG:I-A^b-specific T-reg cells are rare in naïve B6 mice (27, 81). A notably different phenotype is observed in B6.*Kaa* mice (81), which express a transgenic TCRβ chain repetitively found in MOG:I-A^b-specific T cells (82). Compared to naïve B6 mice, naïve B6.*Kaa* mice have 4 times more MOG:I-A^b-specific $CD4^+$ T-conv cells and 16 times more MOG:I-A^b-specific $CD4^+$ T-reg cells (81). Furthermore, proliferation and suppressive function of MOG:I-A^b-specific $CD4^+$ T-reg cells were demonstrable in T-reg populations from naïve B6.*Kaa* mice but not from naïve B6 mice (81). Similar findings were made in an analogous study of $CD4^+$ T cells specific for proteolipid protein (PLP):I-A^b (83). In the latter study, comparison of *Plp1*$^{+/+}$ and *Plp1*$^{-/-}$ mice on the TCRβ-transgenic background provided the additional insight that PLP expression induced the post-thymic expansion of PLP:I-A^b-specific $CD4^+$ T-reg and $Foxp3^-$ $FR4^+$ $CD73^+$ anergic (84) populations, but not the $Foxp3^-$ $FR4^-$ $CD73^-$ naïve T-conv cell population (83). In both studies (81, 83), enlarging the antigen-specific CD4SP thymocyte population *via* a TCRβ transgene expanded the antigen-specific T-reg cell niche.

A challenge to our hypothesis is that the MOG:I-A^b-specific $CD4^+$ population in naïve B6 mice is relatively large, close to the top of the spectrum of foreign pMHCII-specific population sizes (29). Our hypothesis would predict the large MOG:I-A^b-specific CD4SP thymocyte population in B6 mice ought to establish a MOG:I-A^b-specific T-reg cell niche. To compare the intra-thymic expression of MOG with natural self-antigens known to form $T_{regitopes}$ (4–6), we analyzed data from two studies that conducted RNA sequencing on mTEC samples (52, 63). In this panel of 20 self-antigens, both studies found MOG had the lowest expression in mTECs (**Figure 3**), suggesting low "basal" MOG expression in the thymic medulla might limit MOG:I-A^b-specific T-reg niche generation in B6 mice. An initial test of our hypothesis would be to compare the abundance of MOG transcripts in mTEC from B6 *versus* B6.*Kaa* mice. We predict

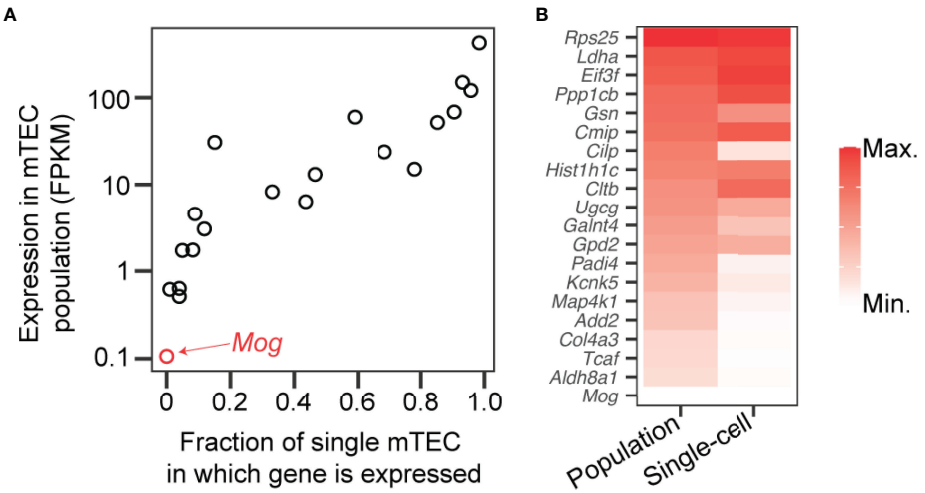

FIGURE 3 | Low expression of Mog compared to self-antigens known to induce T-reg cell development in the thymus. **(A)** Transcripts encoding myelin oligodendrocyte glycoprotein (*Mog*) and 19 self-antigens known to induce thymic T-reg development (4–6) were measured by RNA sequencing of mTEC at the population level (*y*-axis) (52) and in 305 single cells (*x*-axis) (63). FPKM, fragments per kilobase of exon per million mapped fragments. **(B)** Transcription levels of the 20 genes represented in **(A)** in the mTEC population (left) and in single mTEC (right) with red shading according to the scales shown on the axes in **(A)**.

the unusually large MOG:I-A^b-specific CD4SP thymocyte population in B6.*Kaa* mice would result in higher MOG transcription in the mTEC population. Our hypothesis would also predict the presence of B6.*Kaa* thymocytes should enhance T-reg development in co-resident wild-type thymocytes in mixed chimeras.

IMPACT OF THYMOCYTE DELETION IN THE MEDULLA ON T-REG SELECTION

Outside the thymus, in a self-tolerant and functional immune system, T-reg cells are thought to outcompete T-conv cells for APCs that are presenting self-antigens, whereas the reverse would apply for APCs presenting foreign antigens (85). Theoretically, self-tolerance should be most robust if the thymus selected the most self-reactive thymocytes into the T-reg lineage, in order to maximize the difference in self-reactivity between T-reg and T-conv cells. However, this is not observed experimentally. Antigen-specific T-reg cells bind fewer pMHCII tetramer molecules per cell than some antigen-specific T-conv cells in mice lacking the self-antigen (8, 80). This suggests the self-antigen deletes those thymocytes that express the most self-reactive TCRs. Consistent with this conclusion, the half-lives and functional avidities of 10 Padi4:I-A^b-specific TCRs indicated the high, intermediate and low ranges of TCR self-reactivity induced deletion, (perinatal) T-reg cell development, and T-conv cell development, respectively (6). However, there is also compelling evidence that CD4SP thymocytes can undergo deletion as a result of their TCR self-reactivity being too low for T-reg development (86). Accordingly, in a panel of 4 PLP:I-A^b-specific TCRs, the TCRs with the highest and lowest functional avidity induced deletion, whereas the 2 TCRs with intermediate functional avidity induced T-reg development (83). The TCR self-reactivity most conducive to T-reg development would appear to be "sandwiched" between two ranges of TCR self-reactivity that induce deletion in the thymic medulla.

After CD4SP thymocytes initiate strong TCR signaling in the medulla, the thymocyte-intrinsic pathways required to prevent deletion change as the thymocyte matures. Canonical NF-κB activation prevents deletion within the $Foxp3^-$ T-reg precursor stage (22, 87–89), whereas IL-2 signaling prevents deletion at a later stage, close to the time of Foxp3 upregulation (75). DOCK8 inhibits deletion at both of these stages (90). For CD4SP thymocytes inside a medullary T-reg cell niche, survival requires signaling that is not required for deletion. Evidence that most of these cells are deleted (22, 75) suggests those cells that complete T-reg development are rigorously selected.

CD4SP thymocytes can also develop into T-reg cells *via* a developmental pathway that includes a $Foxp3^+$ $CD25^-$ T-reg precursor stage (31, 91). Compared to $Foxp3^-$ $CD25^+$ T-reg precursors, $Foxp3^+$ $CD25^-$ T-reg precursors take longer to develop, tend to have lower TCR self-reactivity and are less susceptible to deletion (92). This alternative pathway may be used by TCRs such as the OVA:I-A^d-specific TCR called R4 (78) and another TCR called G113 (2). The R4 and G113 TCRs still induce T-reg development when they are expressed by very few CD4SP thymocytes (2, 78), implying intra-clonal collaboration is unnecessary for these TCRs to support T-reg development. Unlike the DO11 and N7 TCRs, the R4 and G113 TCRs do not induce measurable deletion (2, 78), suggesting only TCRs that trigger deletion require the antigen-specific CD4SP thymocyte population size to exceed a lower limit in order to induce T-reg development. CD4SP thymocytes with a TCR self-

reactivity too low to induce deletion would be expected to have a longer lifespan in the thymic medulla, which may increase their probability of finding a pre-existing, functional T-reg niche, and surviving long enough to upregulate Foxp3. Whether an antigen-specific CD4SP Foxp3$^-$ thymocyte is at risk of deletion or not, it can still contribute to the antigen-specific T-reg cell niche by providing inductive signals to APCs and by producing IL-2 (**Figure 2**).

The adult thymus also contains recirculating or thymus-resident T-reg cells (93), which may impact *de novo* thymic T-reg development. GK-transgenic mice, which have few peripheral CD4$^+$ T cells and few non-nascent T-reg cells in the thymus due to transgenic expression of an anti-CD4 antibody, have a slightly higher frequency of Foxp3$^+$ thymocytes than wild-type mice (94). Non-nascent T-reg cells may thus limit *de novo* thymic T-reg development by competing for limiting IL-2 (94, 95). However, *de novo* thymic T-reg development is not reduced in mice with enlarged non-nascent thymic T-reg cell populations (96, 97). As non-nascent T-reg cells express more *Tnfsf11* and *Cd40lg* transcripts (which encode RANKL and CD40L, respectively) than nascent thymic T-reg cells (94), they may also positively affect the thymic T-reg niche by providing inductive signals to APC.

DISCUSSION

Certain self-antigens reproducibly "select" CD4SP thymocytes to enter the T-reg lineage (4–6). We refer to these self-antigens as $T_{regitopes}$. Here, we postulate a mechanism that operates in the opposite direction, wherein CD4SP thymocytes "select" self-antigens to become $T_{regitopes}$. This hypothesis draws on evidence that the major T-reg-inducing APC subsets in the thymus, mTEC and DC, require cognate TCR-pMHCII-dependent interactions with CD4SP thymocytes in order to form mature populations (55, 66, 67, 72). We propose CD4SP thymocytes deliver signals that promote mature mTEC survival and "lock in" the set of self-antigens being expressed by the mTEC at the time. This endows a self-reactive CD4SP thymocyte with the ability to generate a medullary niche containing its cognate self-antigen, enabling subsequent development of T-reg cells specific for the same self-antigen. We propose the "mosaic" of antigen-specific T-reg niches in the thymic medulla (45, 60) is not predetermined but is shaped by the antigen specificities of CD4SP thymocytes in the medulla. Deletion creates variation in the number of CD4SP thymocytes specific for different self-antigens (27, 28). The size of the antigen-specific T-reg niche in the medulla may be inversely related to the extent of cortical deletion of antigen-specific thymocyte populations (**Figure 2**).

It is unclear why α3/DR1 is a $T_{regitope}$, whereas α3/DR15 is not (4). Although the thymus was not analyzed, peripheral CD4$^+$ T cell populations in mice expressing these human HLA molecules contained a higher frequency of α3/DR1-specific cells than α3/DR15-specific cells (4). This difference may be due to greater cortical deletion of α3/DR15-specific thymocytes compared to α3/DR1-specific cells. If so, then this cortical deletion is unlikely to be triggered by α3/DR15 itself, as the α3 protein is sparsely expressed in the thymic medulla (12). Furthermore, α3/DR1 is a $T_{regitope}$, suggesting the α3 self-peptide is not displayed to cortical thymocytes. We infer that cortical deletion of α3/DR15-specific thymocytes is mediated by related self-peptides with similar TCR-exposed residues (29). An initial test of this hypothesis may involve enumerating antigen-specific thymocytes at distinct maturation stages, as described (6). Our hypothesis would predict the presence of DR1 ought to "lock in" α3 expression and augment selection of α3/DR15-specific T-reg cells. However, DR1 expression did not affect the α3/DR15-specific T-reg or T-conv cell frequency in DR1$^+$ DR15$^+$ mice compared to DR15$^+$ mice (4). Differential affinity of the α3 peptide for DR1 *versus* DR15 may lead to differences in the quantity of the two pMHCII complexes. Alternatively, differences in the chemistry of the different TCR-exposed peptide residues may lead to differences in the TCR affinity distribution of CD4SP thymocytes specific for the two pMHCII complexes. These differences may bias T-reg development towards the α3/DR1 $T_{regitope}$ despite co-expression of the two MHC alleles.

Associations between human autoimmune diseases and particular MHC alleles (98) indicate a role for TCR-peptide-MHC interactions in pathogenesis. The current paradigm is that autoimmune diseases are mediated by pro-inflammatory T-conv cells specific for self-peptides presented by disease-associated MHC alleles (99). Interestingly, compared to TCR-peptide-MHC interactions elicited by infection or immunization, some autoimmune interactions have unusual features, including atypical positioning of the TCR or the self-peptide, post-translational self-peptide modifications and self-peptide fusions (99). These findings shed light on the nature of inappropriate self-antigen recognition by T cells. However, they do not explain why only some people with disease-associated MHC alleles develop autoimmune disease. This implies the action of an additional predisposing factor, such as the absence of an antigen-specific T-reg cell population that would otherwise prevent autoimmune disease. The association between autoimmune diseases and particular MHC alleles may reflect the lack of an organ-specific self-peptide that can form a $T_{regitope}$ when presented by the disease-associated MHCII allele. We refer to this as $T_{regitope}$ deficiency.

Other genetic factors may combine with a disease-associated MHCII allele to avert or contribute to $T_{regitope}$ deficiency. Co-expression of an MHCII allele that can form a $T_{regitope}$ can avert $T_{regitope}$ deficiency, as exemplified with HLA-DR1 in Goodpasture's disease (4, 13). $T_{regitope}$ sufficiency or deficiency may explain why pairs of HLA haplotypes are associated with a decreased or increased risk of autoimmune diseases beyond the additive contributions of each haplotype (100). In addition, $T_{regitope}$ deficiency may require a high relatedness between (at least) two self-peptides, one presented in the cortex and the other in the medulla, a situation that may extinguish the potential of the medullary self-peptide to serve as a $T_{regitope}$. Hence, $T_{regitope}$ deficiency would be expected in only a subset of individuals who inherit a disease-associated MHCII allele, providing an explanation for why most such individuals never develop autoimmune disease.

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 The Animal Experimentation Ethics Committee of the Australian National University.

AUTHOR CONTRIBUTIONS

CW and SD jointly prepared figures and wrote the paper. All authors contributed to the article and approved the submitted version.

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Human $CD4^+CD25^+CD226^-$ Tregs Demonstrate Increased Purity, Lineage Stability, and Suppressive Capacity Versus $CD4^+CD25^+CD127^{lo/-}$ Tregs for Adoptive Cell Therapy

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Regulatory T cell (Treg) adoptive cell therapy (ACT) represents an emerging strategy for restoring immune tolerance in autoimmune diseases. Tregs are commonly purified using a $CD4^+CD25^+CD127^{lo/-}$ gating strategy, which yields a mixed population: 1) cells expressing the transcription factors, FOXP3 and Helios, that canonically define lineage stable thymic Tregs and 2) unstable $FOXP3^+Helios^-$ Tregs. Our prior work identified the autoimmune disease risk-associated locus and costimulatory molecule, CD226, as being highly expressed not only on effector T cells but also, interferon-γ (IFN-γ) producing peripheral Tregs (pTreg). Thus, we sought to determine whether isolating Tregs with a $CD4^+CD25^+CD226^-$ strategy yields a population with increased purity and suppressive capacity relative to $CD4^+CD25^+CD127^{lo/-}$ cells. After 14d of culture, expanded $CD4^+CD25^+CD226^-$ cells displayed a decreased proportion of pTregs relative to $CD4^+CD25^+CD127^{lo/-}$ cells, as measured by $FOXP3^+Helios^-$ expression and the epigenetic signature at the *FOXP3* Treg-specific demethylated region (TSDR). Furthermore, $CD226^-$ Tregs exhibited decreased production of the effector cytokines, IFN-γ, TNF, and IL-17A, along with increased expression of the immunoregulatory cytokine, TGF-β1. Lastly, $CD226^-$ Tregs demonstrated increased *in vitro* suppressive capacity as compared to their $CD127^{lo/-}$ counterparts. These data suggest that the exclusion of CD226-expressing cells during Treg sorting yields a population with increased purity, lineage stability, and suppressive capabilities, which may benefit Treg ACT for the treatment of autoimmune diseases.

Keywords: CD226, Treg, lineage stability, suppressive function, autoimmune disease, adoptive cell therapy

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INTRODUCTION

Human regulatory T cells (Tregs) possess the unique capacity to suppress innate and adaptive immune subsets throughout the body using a variety of mechanisms, including consumption of growth factors, degradation of inflammatory substrates, expression of negative regulators of costimulation, secretion of immunoregulatory cytokines, and trogocytosis (1–3). Impairment of Treg suppression *in vivo* leads to the proliferation of autoreactive T cells, which has been associated with the development of autoimmune diseases, such as type 1 diabetes (T1D) and systemic lupus erythematosus (SLE) (4, 5). Therefore, Tregs represent a critical target or even deliverable component of immunotherapies seeking to inhibit the pathogenesis of autoimmune diseases (6, 7).

Early proof-of-principle studies in the non-obese diabetic (NOD) mouse provide evidence that adoptive transfer of Tregs can reverse autoimmune diabetes (8–10). Translating this concept to patients with or at risk for T1D requires the isolation and subsequent *ex vivo* expansion of Tregs for adoptive cell therapy (ACT), due to the rarity of Tregs in both peripheral and umbilical cord blood (6, 11–14). Polyclonal autologous Treg-ACT was shown to be safe yet ineffective at preserving insulin production in individuals with recent-onset T1D (6), potentially due to limited Treg persistence *in vivo*. In a recent phase I clinical trial, low dose IL-2 bolstered polyclonal Treg engraftment in patients with T1D but also, imparted undesirable off-target expansion of cytotoxic cell subsets, such as activated natural killer (NK), mucosal associated invariant T (MAIT), and $CD8^+$ T cells (15). Hence, there is a clear need to optimize Treg ACT, including through isolation of a Treg population that maintains lineage stability and suppressive functionality following *ex vivo* expansion.

Early Treg enrichment strategies relied on the observation that Tregs constitutively express the IL-2 receptor alpha chain (IL-2Rα/CD25), conferring a high affinity for the T cell growth factor, IL-2 (16). However, observations of activation-induced upregulation of CD25 on $CD4^+$ conventional T cells (Tconv) (17, 18) supported the need for additional markers for effective Treg isolation (19). Current Treg isolation methods involve Fluorescence-Activated Cell Sorting (FACS) of $CD4^+CD25^{hi}$ T cells with low to no expression of the IL-7 receptor, CD127 (20). However, CD127 can be downregulated by Tconv in response to signaling by IL-7 and other common γ-chain cytokines (20). Moreover, in instances of lymphopenia, increased serum levels of IL-7 are known to decrease CD127 expression on Tconv, significantly complicating efforts to isolate tolerogenic Tregs for ACT in patients with autoimmune diseases (21–23).

The $CD127^{lo/-}$ Treg isolation strategy yields a heterogeneous population containing both lineage stable $FOXP3^+Helios^+$ Tregs as well as $FOXP3^+Helios^-$ Tregs, which are susceptible to phenotypic instability upon activation (24, 25). While subject to debate (26), the $FOXP3^+Helios^+$ transcription factor combination is generally accepted as identifying the thymically-derived Treg subset (tTregs) while $FOXP3^+Helios^-$ designates the peripherally-induced Treg fraction (pTregs) (25, 27). Compared to tTregs, pTregs exhibit increased production of inflammatory cytokines, including IFNγ, as well as methylation at the conserved non-coding sequence 2 (CNS-2), referred to as the Treg-specific demethylation region (TSDR) (28). As a result, the $CD4^+CD25^{hi}CD127^{lo/-}$ population is predisposed to an outgrowth of less suppressive Tregs and increased expression of inflammatory/effector molecules during expansion, all of which may negatively impact ACT therapeutic efficacy (29). Furthermore, an increased proportion of IFNγ-secreting $FOXP3^+Helios^-$ Tregs in patients with T1D versus healthy controls suggests that detrimental Treg plasticity may be augmented in settings of inflammation or autoimmunity (28).

Previous work in our laboratory characterizing the phenotype of IFNγ-secreting $FOXP3^+Helios^-$ Tregs revealed high expression of the costimulatory molecule CD226 (30). CD226 is an activating costimulatory receptor associated with the initiation of Th1 and Th17 immune responses (31, 32). Following its ligation with CD112 or CD155 on antigen-presenting cells (APCs), CD226 becomes activated *via* phosphorylation of its immunoreceptor tyrosine-based activation motif (ITAM) (33), augmenting downstream Ras/MAPK signaling, which is known to result in increased secretion of the pro-inflammatory cytokines IFN-γ and IL-17A (31). In our studies, CD226 expression correlated positively with CD127 and negatively with FOXP3 expression; moreover, freshly isolated $CD226^{lo}$ Tregs exhibited increased demethylation at the TSDR as compared to $CD226^+$ Tregs, suggesting high CD226 expression might be associated with an effector phenotype (30).

In addition to contributing to decreased regulatory function, *CD226* has been identified to contain a potential gain-of-function risk variant contributing to a propensity for multiple autoimmune diseases including T1D, SLE, rheumatoid arthritis (RA), and multiple sclerosis (MS) (32, 34–36). We previously reported that knockout (KO) of *Cd226* in NOD mice resulted in reduced severity of insulitis and diabetes incidence (37), and Wang et al. similarly observed that *Cd226* KO reduced disease severity in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS, further highlighting the role of CD226 in autoimmune disease pathogenesis (38).

To identify an improved surrogate surface marker for lineage stable Tregs, we performed extensive *ex vivo* analyses to evaluate the therapeutic potential of $CD4^+CD25^+CD226^-$ sorted T cells as compared to the conventional $CD4^+CD25^+CD127^{lo/-}$ strategy. Specifically, we hypothesized that this marker profile would allow for isolation and expansion of increased proportions of $FOXP3^+Helios^+$ Tregs, minimizing contamination of IFNγ-producing $FOXP3^+Helios^-$ Tregs, to yield a more stable and functionally suppressive population.

MATERIALS AND METHODS

Human Subjects

Fresh peripheral blood mononuclear cell (PBMC) samples were isolated from human leukapheresis-enriched blood of healthy donors (median age: 22 years, range 18-39 years, *N*=20, 45% female) purchased from LifeSouth Community Blood Centers (Gainesville, FL).

CD4+ T Cell Enrichment From Human PBMC Samples

Before Treg isolation, CD4+ T cells were enriched by negative selection using a CD4+ T cell enrichment RosetteSep™ cocktail (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions while autologous PBMCs required for suppression assays were isolated from unenriched peripheral blood. CD4+ T cell-enriched and unenriched components were diluted 1:1 with PBS and overlayed onto Ficoll-Paque Plus medium (Thermo Fisher, Waltham, MA, USA) for density gradient centrifugation (1200 x g, 20 min). PBMCs were suspended in Ammonium-Chloride-Potassium (ACK) Lysis Buffer (Gibco, Waltham, MA, USA), washed, and resuspended in PBS, according to the manufacturer's instructions. Quantification of cell viability was accomplished by staining with Acridine Orange/Propidium Iodide (AO/PI) before reading on an Auto2000 Cellometer (Nexcelom Biosciences, Lawrence, MA, USA).

FACS Isolation of Treg Subsets

CD4+ T cell-enriched PBMCs were split and stained with: 1) CD4-BV510, CD25-APC, and CD127-PE or 2) CD4-BV510, CD25-APC, and CD226-PE-Cy7 (clone and manufacturer information provided in **Table 1**). Matched sets of $CD4^+CD25^+CD127^{lo/-}$ and $CD4^+CD25^+CD226^-$ Tregs were isolated (**Figure 1**) using a FACSAria™ III Cell Sorter (Beckton Dickinson, Franklin Lakes, NJ, USA; $CD4^+CD25^+CD127^{lo/-}$ median sort purity: 96.1%, range: 85.8-99.9%, *N*=6; $CD4^+CD25^+CD226^-$ median sort purity: 97.8%, range: 88.7-99.9%, *N*=6).

Treg Expansion

Following FACS isolation, cells were expanded for 14 days *ex vivo* (13). In brief, Tregs were cultured in complete RPMI media (cRPMI; RPMI 1640 media Phenol Red w/o L-Glutamine (Lonza, Basel, CH-BS, Switzerland), 5mM HEPES (Gibco, Waltham, MA, USA), 5 mM MEM Non-Essential Amino Acids (NEAAs; Gibco), 2mM Glutamax (Gibco), 50 µg/mL penicillin (Gibco), 50 µg/mL streptomycin (Gibco), 20 mM sodium pyruvate (Gibco), 50 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 20 mM sodium hydroxide (Sigma-Aldrich) and 10% FBS (Genesee Scientific, El Cajon, CA, USA)) with Teceleukin recombinant human IL-2 (rhIL-2; Roche, Basel, CH-BS, Switzerland) at 300 IU/mL, with media and rhIL-2 being replaced every 3-4 days. Tregs were stimulated using MACS® GMP ExpAct Treg Beads (Miltenyi Biotec, Bergisch Gladbach, NW, Germany) at a 4:1 bead:cell ratio. Beads were replaced at day seven, and cells were expanded through day 14.

Analysis of TSDR Epigenetic Signature

Demethylation of the FOXP3-TSDR, or conserved non-coding sequence (CNS2), represents a robust epigenetic indicator of tTreg purity (39). We quantified demethylation within the FOXP3-TSDR by real-time polymerase chain reaction (RT-PCR) as previously described (30), with the following modifications. DNA extraction was conducted using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, NW, Germany) as described by the manufacturer's protocol. Following extraction, DNA was quantified using the Qubit™ Double-Stranded DNA (dsDNA) High Sensitivity (HS) Assay Kit (Invitrogen, Waltham, MA, USA) on the Qubit™

TABLE 1 | Antibodies used for flow cytometry.

Target	Clone	Fluorochrome	Vendor	Concentration	RRID
CD4	SK3	BV510	BD Biosciences	0.05 µg/mL	AB_2744424
CD8	RPA-T8	PE-CF594	BD Biosciences	0.10 µg/mL	AB_11154052
CD25	BC96	APC	BioLegend	0.50 µg/mL	AB_314280
CD25	BC96	BV605	BioLegend	0.50 µg/mL	AB_11218989
CD39	eBioA1	APC	eBioscience	0.50 µg/mL	AB_1963578
CD40L	24-31	APC-Cy7	BioLegend	0.50 µg/mL	AB_2076096
CD45RA	HI100	BV605	BioLegend	0.10 µg/mL	AB_2563814
CD73	AD2	PE	BD Pharmingen	0.50 µg/mL	AB_393561
CD127	A019D5	PE	BioLegend	0.20 µg/mL	AB_1937251
CD197 (CCR7)	2-L1-A	APC-R700	BD Biosciences	0.10 µg/mL	AB_2869856
CD226	11A8	PE-Cy7	BioLegend	0.40 µg/mL	AB_2616645
CLTA-4	L3D10	PE-Cy7	BioLegend	0.50 µg/mL	AB_2563098
FOXP3	206D	Alexa Fluor 488	BioLegend	0.50 µg/mL	AB_430883
FOXP3	259D	Alexa Fluor 488	BioLegend	0.50 µg/mL	AB_430887
GITR	621	PE-Cy5	BioLegend	0.50 µg/mL	AB_2240646
Helios	22F6	Pacific Blue	BioLegend	0.25µg/mL	AB_10690535
IL-10	JES3-9D7	BV421	BioLegend	0.08 µg/mL	AB_2632952
IL-17A	BL168	BV605	BioLegend	0.12 µg/mL	AB_2563887
IFN-γ	4S.B3	BV570	BioLegend	0.10 µg/mL	AB_2563880
PD-1	EH12.2H7	Alexa Fluor 647	BioLegend	0.50 µg/mL	AB_940471
TGF-β1	TW4-2F8	Alexa Fluor 647	BioLegend	0.40 µg/mL	AB_2721298
TGF-β1	FNLAP	PerCP-eFluor 710	eBioscience	0.50 µg/mL	AB_2573900
TNF	Mab11	BV650	BioLegend	0.20 µg/mL	AB_2561355

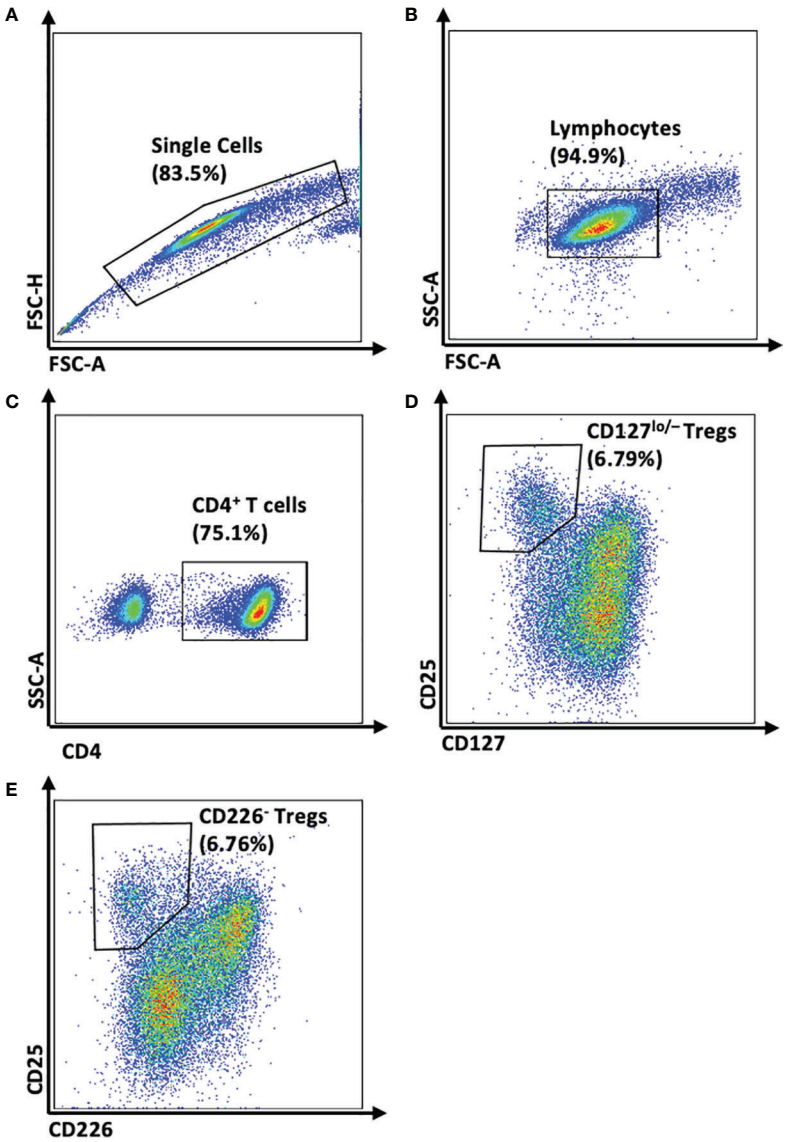

FIGURE 1 | Gating Strategy for FACS Isolation of Paired $CD4^+CD25^+CD127^{lo/-}$ and $CD4^+CD25^+CD226^-$ Tregs. Representative flow plots demonstrate the method by which $CD127^{lo/-}$ or $CD226^-$ Tregs were isolated from $CD4^+$ T-cell enriched PBMC using a BD FACSAriaIII Cell Sorter. **(A)** Singlet gating was performed using forward scatter area (FSC-A) versus forward scatter height (FSC-H). **(B)** Lymphocytes were gated on FSC-A and side scatter area (SSC-A). **(C)** From the $CD4^+$ T cell fraction, **(D)** $CD25^+CD127^{lo/-}$ Tregs and **(E)** $CD25^+CD226^-$ Tregs were isolated.

Fluorometer system (Invitrogen). Bisulfite conversion of DNA was conducted using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA). RT-PCR was performed using a StepOne™ system (Applied Biosystems, Waltham, MA, USA).

Flow Cytometric Analysis of Treg Phenotype

To assess the phenotype and purity of Tregs before and following 14 days of expansion, 1 x 10^5 $CD4^+CD25^+CD226^-$ and $CD4^+CD25^+CD127^{lo/-}$ Tregs were stained with Live/Dead™ Near-IR viability dye (Thermo Fisher) for 10 minutes at 4°C before washing with stain buffer (PBS + 2% FBS + 0.05% NaN_3 w/v). Cells were then stained with an extracellular antibody cocktail, consisting of CD4-BV510, CD25-APC, CD45RA-BV605, CD127-PE, CD197-APC-R700, and CD226-PE-Cy7 for 30 minutes at 4°C (antibody clone and concentration are provided in **Table 1**). Cells were fixed and permeabilized using the eBioScience™ FOXP3 Transcription Factor Staining Buffer Set (Invitrogen) according to the manufacturer's instructions, then stained with an intracellular transcription factor antibody cocktail, consisting of FOXP3-Alexa Fluor 488 and Helios-Pacific Blue (**Table 1**). Data were collected on an Aurora 3L (16V-14B-8R) spectral flow cytometer (Cytek, Fremont, CA, USA), and analysis

was conducted using FlowJo™ version 10.6.1 Software (BD Life Sciences, Ashland, OR, USA). Tregs were classified as $CD4^+CD25^+FOXP3^+$, $CD4^+CD25^+FOXP3^+Helios^+$, and $CD4^+CD25^+FOXP3^+Helios^-$ with phenotype established based on CD45RA and CD197 (CCR7) expression as follows: $CD45RA^+CCR7^+$ naïve, $CD45RA^-CCR7^+$ central memory (T_{CM}), $CD45RA^-CCR7^-$ effector memory (T_{EM}), and $CD45RA^+CCR7^-$ effector memory re-expressing CD45RA (T_{EMRA}) cells. The detailed gating strategy is shown in **Figure S1**. Protein expression levels were reported as stain indices [SI = geometric mean fluorescence intensity (gMFI) of the stained sample/gMFI of the applicable fluorescence minus one (FMO) control].

Flow Cytometric Analysis of Intracellular Cytokine Production

Following 14 days of *ex vivo* expansion as described above, MACS® GMP ExpAct Treg Beads were removed, then $CD4^+CD25^+CD127^{lo/-}$ and $CD4^+CD25^+CD226^-$ sorted Tregs were immediately assessed for intracellular cytokine expression. Cells were either stimulated with PMA (10 μg/mL; Thermo Fisher) and ionomycin (500 nM; Thermo Fisher) or unstimulated for four hours in the presence of GolgiStop (0.66 μL/mL; BioLegend, San Diego, CA, USA). Stimulated cells underwent staining for viability and extracellular markers, including CD4-BV510, CD25-APC, CD127-PE, CD226-PE-Cy7, and TGF-β1-PerCP-eFluor 710 (**Table 1**), and were subsequently permeabilized as described above. Following permeabilization, cells were stained with the FOXP3-AF488 and Helios-Pacific Blue cocktail, as well as an intracellular cytokine cocktail consisting of IL-10-BV421, IL-17A-BV605, IFN-γ-BV570, TGF-β1-Alexa Fluor 647, and TNF-BV650 (**Table 1**). Fold change of cytokine expression levels were assessed by dividing the gMFI of the stained, stimulated sample by the gMFI of the applicable stained, unstimulated control. Differences between fold change of cytokine expression are reported as Z-scores, [Z = (Mean fold change for Treg subset – Mean fold change for all Tregs assessed)/ standard deviation of the sample].

Flow Cytometric Analysis of Treg Activation Markers

Following 14 days of *ex vivo* expansion, $CD4^+CD25^+CD127^{lo/-}$ and $CD4^+CD25^+CD226^-$ sorted Tregs were labeled with CellTrace™ Violet (CTV; Thermo Fisher) as recommended by the manufacturer, then cultured with no PBMCs or stimulation (0 hour condition) or with autologous PBMCs at a 1:1 ratio in the presence of soluble anti-CD3 (8 μg/mL, Clone OKT3, BioLegend, RRID: AB_11150592) and soluble anti-CD28 (4 μg/mL, Clone CD28.2, Thermo Fisher, RRID: AB_468926) for 24 or 48 hours. Cells were stained for viability with Live/Dead™ Blue viability dye (Thermo Fisher) and underwent surface staining for CD4-BV510, CD25-BV605, PD-1-AF647, CD39-APC, CD73-PE, CTLA-4-PE-Cy7, GITR-PE-Cy5, and CD40L-APC-Cy7 (**Table 1**). The cells were subsequently permeabilized as described above and stained with FOXP3-AF488 and Helios-Pacific Blue before flow cytometric assessment on a Cytek Aurora 5L (16UV-16V-14B-10YG-8R) spectral flow cytometer and analyzed in FlowJo version 10.6.1 Software.

Treg Suppression Assays

Post-expansion $CD4^+CD25^+CD226^-$ Tregs and $CD4^+CD25^+CD127^{lo/-}$ Tregs were collected on day 14 and immediately labeled with cell proliferation dye (CPD-eFluor 670; Biolegend), while autologous PBMCs were labeled with CTV as recommended by the manufacturers' protocol. Tregs were co-cultured with PBMCs (Treg : PBMC ratios of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32) in the presence of soluble anti-CD3 (8 μg/mL, Clone OKT3) and soluble anti-CD28 (4 μg/mL, Clone CD28.2) in triplicate for 96 hours. Replicates were pooled, subjected to surface staining for CD4-BV510 and CD8-PE-CF594 (**Table 1**), assessed using a Cytek Aurora 5L (16UV-16V-14B-10YG-8R) spectral flow cytometer, and analyzed in FlowJo version 10.6.1 Software. Percent suppression of responder cells was established by the division index (DI) method using proliferation modeling (40).

Statistical Analysis

Generation of figures and statistical analysis were conducted using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, CA, USA). Data were analyzed by two-way ANOVA with Bonferroni's *post hoc* test for multiple testing correction unless otherwise stated. Area under the curve (AUC) values were compared using paired t-tests (41). The p-value ≤ 0.05 was considered significant.

RESULTS

CD25 Expression is Elevated on $CD4^+CD25^+CD226^-$ Tregs

To characterize the efficacy of the sorting strategies in isolating $CD4^+CD25^+CD226^-$ versus $CD4^+CD25^+CD127^{lo/-}$ Tregs (**Figure 1**), we examined surface expression levels of CD226, CD127, and CD25 on $CD4^+CD25^+FOXP3^+$ total Tregs, $FOXP3^+Helios^+$ Tregs, as well as $FOXP3^+Helios^-$ Tregs by flow cytometry (**Figure S1**), both prior to and following *ex vivo* expansion (42). As expected, CD226 expression was significantly lower on $CD4^+CD25^+CD226^-$ sorted cells, including total $FOXP3^+$ Tregs (0.49-fold, p<0.0001) as well as $FOXP3^+Helios^+$ (0.55-fold, p<0.0001) and $FOXP3^+Helios^-$ subsets (0.60-fold, p<0.0001; **Figures 2A, B**). Prior to expansion, $CD4^+CD25^+CD226^-$ and $CD4^+CD25^+CD127^{lo/-}$ sorted Tregs displayed comparably low CD127 expression (**Figures 2C, D**). Yet, the $CD4^+CD25^+CD226^-$ isolation strategy yielded a significantly higher CD25 gMFI on Tregs (1.13-fold, p<0.0001), including both the $FOXP3^+Helios^+$ (1.08-fold, p=0.0011) and $FOXP3^+Helios^-$ subsets (1.22-fold, p<0.0001; **Figures 2E, F**). As a result of expansion, $CD4^+CD25^+CD226^-$ sorted Tregs re-expressed CD226 at similar levels to $CD4^+CD25^+CD127^{lo/-}$ Tregs following *ex vivo* expansion, with the most dramatic upregulation of CD226 occurring in the $FOXP3^+Helios^-$ fraction (**Figures 2G, H**). However, CD127 levels remained comparably low across all Treg subsets (**Figures 2I, J**),

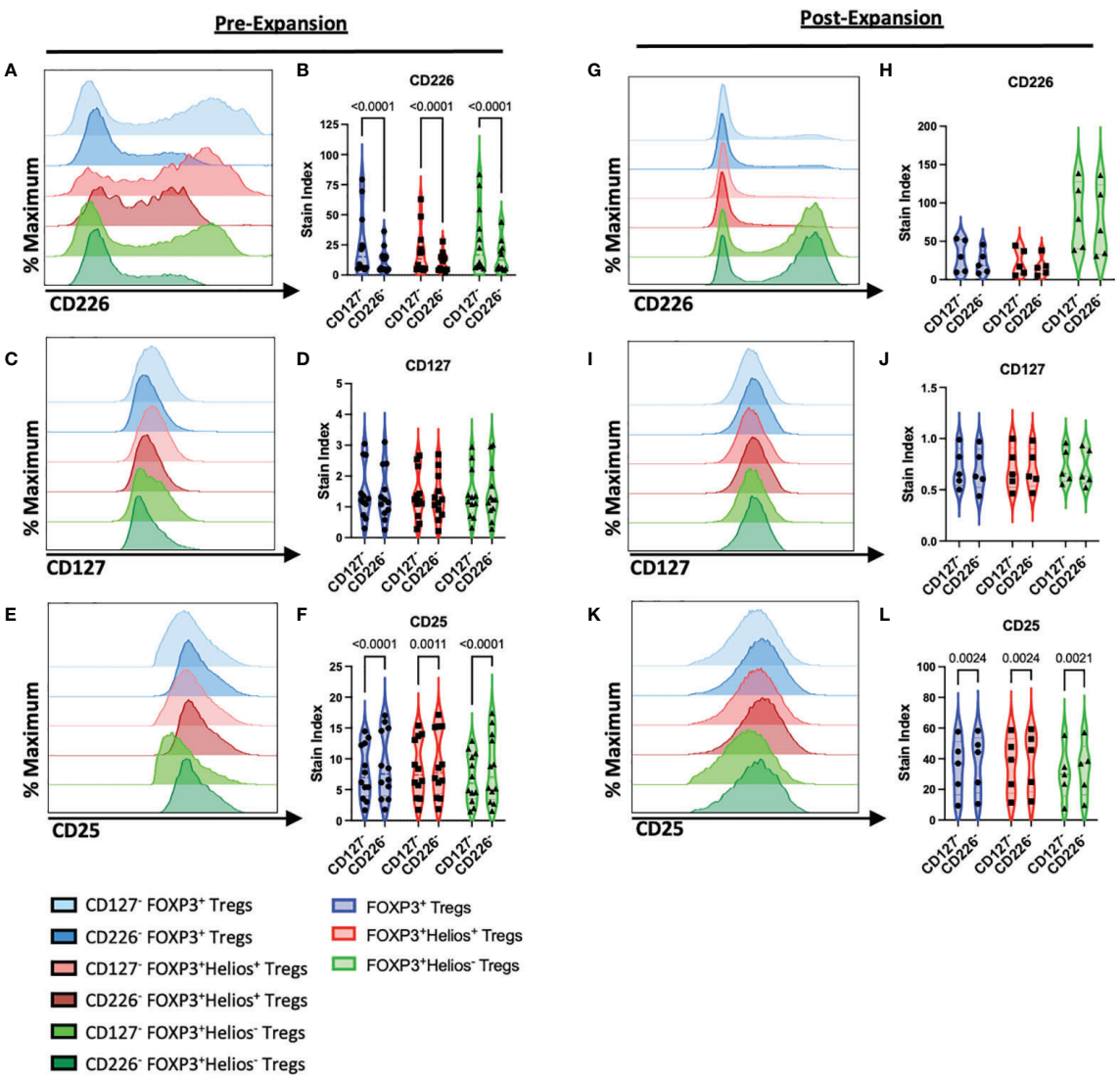

FIGURE 2 | CD4+CD25+CD226- Tregs Exhibit Increased CD25 Expression. Representative histograms show expression of cell surface markers on CD127lo/- (lighter blue) and CD226- CD4+CD25+FOXP3+ Tregs (darker blue), CD127lo/- (lighter red) and CD226- CD4+CD25+FOXP3+Helios+ Tregs (darker red), and CD127lo/- (lighter green) and CD226- CD4+CD25+FOXP3+Helios- Tregs (darker green) with violin plots showing stain index (SI) fold change from FMO controls, **(A–F)** prior to expansion (*n* =12 biological with *n* = 2 technical replicates) and **(G–L)** following 14 days of *ex vivo* expansion (*n* = 5 biological with *n* = 2 technical replicates). **(A, B, G, H)** CD226, **(C, D, I, J)** CD127, **(E, F, K, L)** CD25. Significant P-values are reported on the figure for two-way ANOVA with Bonferroni correction for multiple comparisons of Treg isolation conditions from matched subjects.

and CD25 levels remained augmented on CD4+CD25+CD226- versus CD4+CD25+CD127lo/- sorted cells, including total Tregs (1.08-fold, p=0.0024), FOXP3+Helios+ Tregs (1.08-fold, p=0.0024) and FOXP3+Helios- Tregs (1.10-fold, p=0.0021; **Figures 2K, L**).

CD4+CD25+CD226- Tregs Maintain Higher Purity and Lineage Stability

We previously identified CD4+CD25+CD127lo/-CD226+ Tregs as a subset with a higher proportion of IFNγ-producing pTregs, as compared to CD4+CD25+CD127lo/-CD226- Tregs (30). To evaluate the potential of using a CD4+CD25+CD226- sort for isolation of more lineage stable Tregs, as compared to the typical CD4+CD25+CD127lo/- strategy, we examined the expression of the canonical Treg lineage-defining transcription factors, FOXP3 and Helios, using flow cytometry (**Figure S1**). We identified significantly increased percentages of FOXP3+ Tregs prior to expansion (+3.60%, p=0.0026), including an increased proportion of FOXP3+Helios+ Tregs (+4.70%, p=0.0001) within the CD4+CD25+CD226- sorted population, compared to CD4+CD25+CD127lo/- (**Figures 3A, B**). Importantly, these differences were not related to variations in donor sex (**Figure**

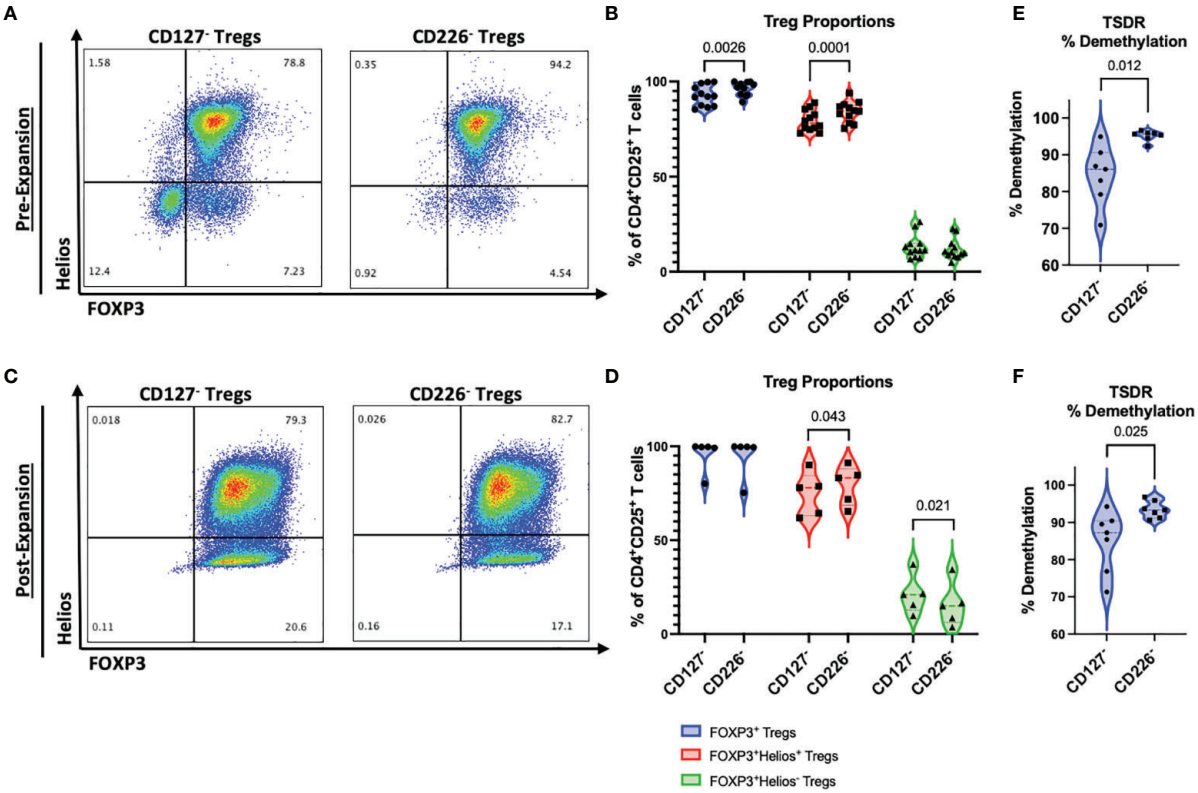

FIGURE 3 | CD226$^-$ Tregs maintain higher purity than conventionally sorted CD127$^{lo/-}$ Tregs. Tregs from each FACS method were examined for FOXP3 and Helios expression **(A, B)** at day 0 following isolation (n = 12 biological with n = 2 technical replicates) and **(C, D)** at day 14 following *ex vivo* expansion (n = 5 biological with n = 2 technical replicates). **(A, C)** Representative flow plots pre-gated on live CD4$^+$CD25$^+$ cells show percentages of Treg subsets for CD127$^{lo/-}$ sorted Tregs and CD226$^-$ sorted Tregs. **(B, D)** Percentages of CD4$^+$CD25$^+$FOXP3$^+$ Tregs (blue), CD4$^+$CD25$^+$FOXP3$^+$Helios$^+$ Tregs (red), and CD4$^+$CD25$^+$FOXP3$^+$Helios$^-$ Tregs (green) per FACS isolation method. P-values are reported on the figure for two-way ANOVA with Bonferroni *post-hoc* correction for multiple comparisons of Treg isolation conditions from matched subjects. **(E, F)** Percent demethylation at the TSDR of CD127$^{lo/-}$ and CD226$^-$ sorted Treg cultures, **(E)** pre-expansion and **(F)** post-expansion. Significant P-values are reported on the figure for paired T-tests, n = 7 biological with n = 2 technical replicates.

S2A) or age (**Figure S2B**); though, we did identify a significantly non-zero slope (p=0.023) suggesting that the isolation of CD127$^{lo/-}$ FOXP3$^+$Helios$^+$ Tregs increased with age for our data set (**Figure S2B**). Following a 14-day expansion period, significant increases in the FOXP3$^+$Helios$^+$ Treg (+3.57%, p=0.043) and decreases in the FOXP3$^+$Helios$^-$ Treg (-4.43%, p=0.021) subpopulations were observed from CD4$^+$CD25$^+$CD226$^-$ versus CD4$^+$CD25$^+$CD127$^{lo/-}$ sorted cells, despite comparable frequencies of total Tregs (**Figures 3C, D**). These data suggest that isolation of CD4$^+$CD25$^+$CD226$^-$ Tregs may yield a more stable Treg population after *ex vivo* expansion, without compromising post-expansion yield (**Figure S3**).

tTregs display a distinct epigenetic profile, including the selective demethylation of the FOXP3-TSDR region (39). We therefore evaluated levels of TSDR methylation by RT-PCR. This analysis showed increased levels of TSDR demethylation in the CD4$^+$CD25$^+$CD226$^-$ Treg population both before (+10.69%, p=0.012) and following expansion (+8.46%, p=0.025) compared to Tregs isolated by the CD4$^+$CD25$^+$CD127$^{lo/-}$ marker profile (**Figures 3E, F**). These data corroborate our flow cytometry results identifying a higher purity of lineage stable Tregs in CD4$^+$CD25$^+$CD226$^-$sorted cells (**Figures 3A–D**). Together, these results demonstrate high purity and lineage stability of CD4$^+$CD25$^+$CD226$^-$ Tregs throughout *ex vivo* expansion.

CD4$^+$CD25$^+$CD226$^-$ Tregs Display a More Naïve Phenotype

We next sought to assess the extent of differentiation in CD4$^+$CD25$^+$CD226$^-$ and CD4$^+$CD25$^+$CD127$^{lo/-}$ Tregs, pre- and post-expansion. Before expansion, CD4$^+$CD25$^+$CD226$^-$ Tregs were found to contain significantly more naïve total Tregs (+8.92%, p<0.0001), FOXP3$^+$Helios$^+$ Tregs (+8.58%, p<0.0001), and FOXP3$^+$Helios$^-$ Tregs (+2.95%, p=0.029), as well as fewer T_{CM} total Tregs (-1.78%, p=0.042), yet T_{CM} FOXP3$^+$Helios$^-$ Treg frequencies were increased versus CD4$^+$CD25$^+$CD127$^{lo/-}$ Tregs (+2.54%, p=0.0063, **Figures 4A–C**). Pre-expansion CD4$^+$CD25$^+$CD226$^-$ Tregs also comprised fewer T_{EM} total Tregs (-6.97%, p=0.0002), FOXP3$^+$Helios$^+$ Tregs (-6.75%, p=0.0002), FOXP3$^+$Helios$^-$

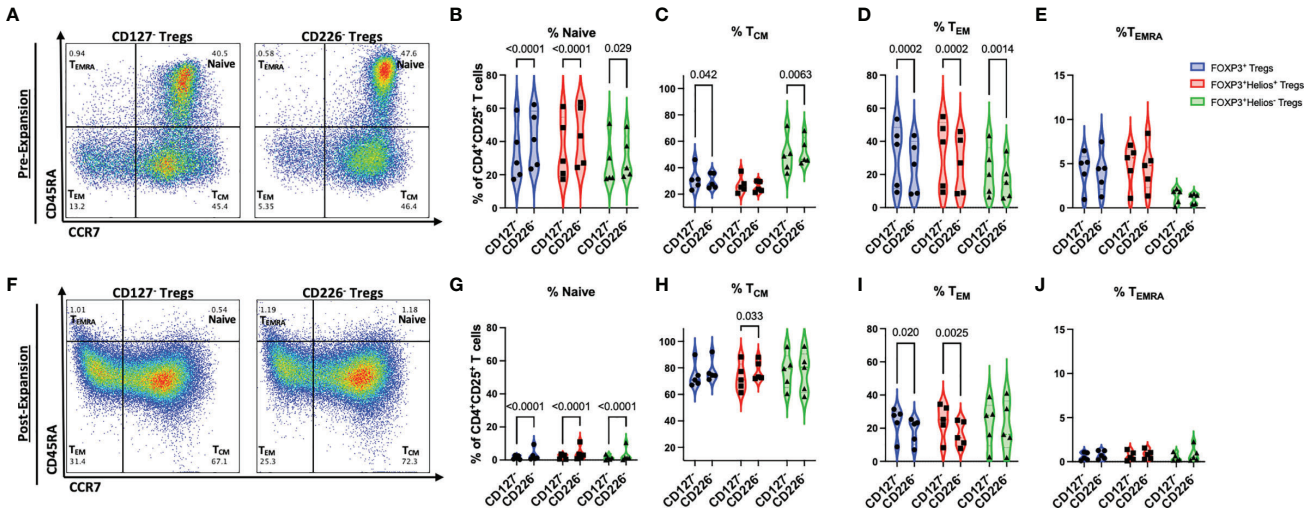

FIGURE 4 | CD226$^-$ Tregs display a more immunoregulatory phenotype than CD127$^{lo/-}$ Tregs. Memory differentiation of Tregs from each FACS method was assessed by CD45RA and CD197 (CCR7) expression **(A–E)** at day 0 following isolation and **(F–J)** at day 14 following *ex vivo* expansion (n = 5 biological with n = 2 technical replicates). **(A, F)** Representative flow plots pre-gated on live CD4$^+$CD25$^+$ cells show percentages for each T cell memory subset for CD127$^{lo/-}$ sorted Tregs and CD226$^-$ sorted Tregs. Relative proportions of **(B, G)** CD45RA$^+$CCR7$^+$ naïve, **(C, H)** CD45RA$^-$CCR7$^+$ T_{CM}, **(D, I)** CD45RA$^-$CCR7$^-$ T_{EM}, and **(E, J)** CD45RA$^+$CCR7$^-$ T_{EMRA} subsets from CD127$^{lo/-}$ sorted Treg and CD226$^-$ sorted Treg cultures after gating on CD4$^+$CD25$^+$ FOXP3$^+$ Tregs (blue), CD4$^+$CD25$^+$FOXP3$^+$Helios$^+$ Tregs (red), and CD4$^+$CD25$^+$FOXP3$^+$Helios$^-$ Tregs (green). P-values are reported on the figure for two-way ANOVA with Bonferroni *post-hoc* correction for multiple comparisons of Treg isolation conditions from matched subjects.

Tregs (-5.21%, p=0.0014), with no significant differences in T_{EMRA} compared to CD4$^+$CD25$^+$CD127$^{lo/-}$ Tregs (**Figures 4D, E**). Additionally, CD4$^+$CD25$^+$CD226$^-$ cells represented a significantly greater proportion (1.89-fold, p=0.019) and absolute cell count (p=0.037) within the CD4$^+$CD25$^+$CD127$^{lo/-}$ sorted Treg population, as compared to CD4$^+$CD25$^+$CD127$^{lo/-}$ CD45RA$^+$ cells (**Figure S4**), suggesting that CD226$^-$ enrichment for naïve and T_{CM} cells does not compromise post-sort yield as drastically as a four-marker CD4$^+$CD25$^+$CD127$^{lo/-}$CD45RA$^+$ naïve Treg isolation strategy (43, 44).

Post-expansion, higher percentages of remaining naïve Tregs in the total Treg (+1.47%, p<0.0001), FOXP3$^+$Helios$^+$ (+1.79%, p<0.0001), and FOXP3$^+$Helios$^-$ (+1.58%, p<0.0001) subpopulations, as well as higher percentages of T_{CM}-differentiated FOXP3$^+$Helios$^+$ Tregs (+4.96%, p=0.033) were observed in the CD4$^+$CD25$^+$CD226$^-$ sorted Tregs as compared to CD4$^+$CD25$^+$CD127$^{lo/-}$ sorted cells (**Figures 4F–H**). Additionally, CD4$^+$CD25$^+$CD226$^-$ Treg expansion yielded significantly lower percentages of T_{EM}-differentiated total Tregs (-5.63%, p=0.020) and FOXP3$^+$Helios$^+$ Tregs (-8.05%, p=0.0025), with no differences in T_{EMRA}-differentiated Tregs observed post-expansion (**Figures 4I, J**). Collectively, these data support the use of CD4$^+$CD25$^+$CD226$^-$ for the isolation of naïve Tregs which differentiate more readily into a T_{CM} as opposed to T_{EM} phenotype after expansion. This finding has potential implications for Treg ACT in settings of autoimmunity and transplantation: specifically, the preferential outgrowth of T_{CM} from CD226$^-$ Tregs may lead to better engraftment efficiency and localization to secondary lymphoid organs where autoimmune priming and graft versus host disease (GvHD) are initiated (45).

CD4$^+$CD25$^+$CD226$^-$ Tregs Display a More Immunoregulatory Cytokine Profile

The production of pro-inflammatory cytokines is a hallmark of Treg instability, which may contribute to a loss of immune tolerance in autoimmune disorders (30, 46). Therefore, we sought to determine whether CD4$^+$CD25$^+$CD226$^-$ Tregs possess a more immunoregulatory cytokine profile than CD4$^+$CD25$^+$CD127$^{lo/-}$ Tregs. Flow cytometric assessment of cytokine production in unstimulated cells revealed no differences in cytokine production at rest (**Figure S5**), but following PMA/Ionomycin stimulation, we observed decreased pro-inflammatory IL-17A expression by CD4$^+$CD25$^+$CD226$^-$ sorted Tregs within the total Treg (0.92-fold, p<0.0001), FOXP3$^+$Helios$^+$ (0.92-fold, p<0.0001), and FOXP3$^+$Helios$^-$ (0.94-fold, p=0.0010) populations (**Figures 5A, B**). Additionally, significant decreases were observed in IFN-γ expression by CD4$^+$CD25$^+$CD226$^-$ sorted total Tregs (0.86-fold, p<0.0001), FOXP3$^+$Helios$^+$ (0.85-fold, p<0.0001), and FOXP3$^+$Helios$^-$ (0.87-fold, p<0.0001) as well as TNF expression by CD4$^+$CD25$^+$CD226$^-$ sorted total Tregs (0.80-fold, p<0.0001), both of which are pro-inflammatory cytokines associated with a Th1 effector profile (**Figures 5A, C, D**) (47, 48). We found significantly increased expression of both extracellular and intracellular TGF-β1 by CD4$^+$CD25$^+$CD226$^-$ sorted total Tregs (1.87-fold, p<0.0001; 1.15-fold, p=0.0004, respectively), FOXP3$^+$Helios$^+$ Tregs (1.55-fold, p<0.0001; 1.16-fold, p<0.0001),

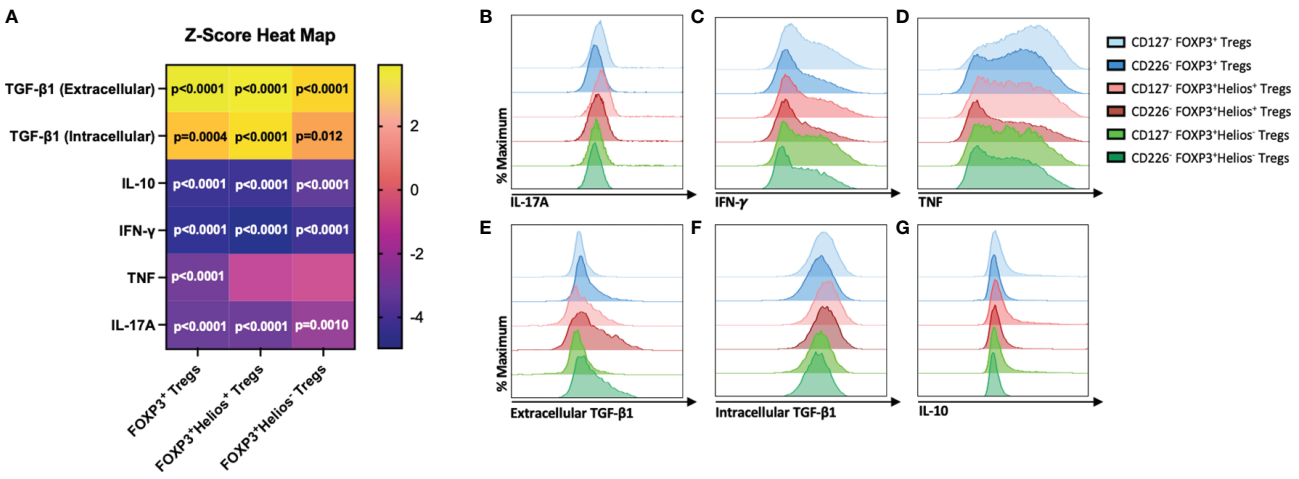

FIGURE 5 | CD226- Tregs exhibit a less inflammatory cytokine profile than CD127lo/- Tregs. Cytokine production by 14-day *ex vivo* expanded CD127lo/- sorted Treg and CD226- sorted Treg cultures was examined by flow cytometry following a four-hour PMA/Ionomycin stimulation in the presence of a protein transport inhibitor. **(A)** Heat map shows the mean fold change of protein expression (z-score) of cytokines (rows) by CD226- FOXP3+, FOXP3+Helios+, and FOXP3+Helios- Treg populations (columns) compared to CD127lo/- Treg populations. *n* = 9 biological with *n* = 2 technical replicates. Significant P-values are reported on the figure for two-way ANOVA with Bonferroni's multiple comparisons between Treg isolation conditions of matched subjects. Representative histograms show expression of **(B)** IL-17A, **(C)** IFN-γ, **(D)** TNF, **(E)** Extracellular TGF-β1, **(F)** Intracellular TGF-β1, and **(G)** IL-10 for CD127lo/- (lighter blue) and CD226- CD4+CD25+FOXP3+ Tregs (darker blue), CD127lo/- (lighter red) and CD226- CD4+CD25+FOXP3+Helios+ Tregs (darker red), and CD127lo/- (lighter green) and CD226- CD4+CD25+FOXP3+Helios- Tregs (darker green).

and FOXP3+Helios- Tregs (1.56-fold, p<0.0001; 1.10-fold, p=0.012) (**Figures 5A, E, F**). Given that TGF-β1 is critical for inhibiting Th1 differentiation (49, 50), these findings corroborate the observations of decreased pro-inflammatory cytokine expression by CD4+CD25+CD226- Tregs. Interestingly, expression of the anti-inflammatory cytokine IL-10 was significantly decreased in CD4+CD25+CD226- sorted total Tregs (0.83-fold, p<0.0001), FOXP3+Helios+ Tregs (0.83-fold, p<0.0001), and FOXP3+Helios- Tregs (0.85-fold, p<0.0001) (**Figures 5A, G**). IL-10, however, is commonly produced by Tr1-like T cells, which differentiate from conventional CD4+ T cells and may only transiently express FOXP3 (2), providing a potential mechanism for its decreased production. This observation is consistent with CD4+CD25+CD226- T cell sorting resulting in a decreased frequency of effector subsets. Overall, these data suggest that CD4+CD25+CD226- Tregs maintain a more immunoregulatory cytokine profile than the CD4+CD25+CD127lo/- counterpart.

CD4+CD25+CD226- Tregs Present Increased Surface Expression of PD-1 and CD39 Following Activation

To identify whether CD4+CD25+CD226- sorted Tregs demonstrate differential expression of proteins associated with Treg-mediated suppression, we evaluated PD-1, CD39, CD73, CD40L, GITR, and CTLA-4 by flow cytometry on Tregs expanded from CD226- and CD127lo/- preparations, prior to and following activation by autologous PBMCs. After 24 hours of stimulation, CD226- sorted Tregs displayed increased expression of PD-1 on both total FOXP3+ Tregs (1.89-fold, p=0.0067) as well as within the FOXP3+Helios+ Treg (1.82-fold, p=0.016) and FOXP3+Helios- Treg subsets (2.24-fold, p=0.0020) as compared to CD127lo/- sorted counterparts; however, there were no significant differences observed at baseline or after 48 hours of stimulation (**Figures 6A–C**). Furthermore, CD226- Tregs exhibited significantly increased expression of CD39 at 24 and 48 hours in both total Tregs (1.75-fold, p=0.037; 1.56-fold, p=0.027, respectively) and the FOXP3+Helios+ subset (1.73-fold, p=0.030; 1.47-fold, p=0.022, respectively) compared to CD127lo/- Tregs (**Figures 6D–F**). Compared to CD127lo/- sorted Tregs, CD226- sorted Tregs did not exhibit any significant differences in surface expression of CD73, CD40L, GITR, or CTLA-4, including after stimulation (**Figure S6**). Taken together, these data suggest that CD226- Tregs may exhibit enhanced suppression of responder T cells *via* the PD-1/PD-L1 and CD39/CD73 ectonucleotidase pathways (51, 52).

CD4+CD25+CD226- Tregs Demonstrate Increased *Ex Vivo* Suppressive Capabilities

During expansion, Treg cultures can be prone to lineage instability as well as outgrowth of Tconv contaminants, ultimately impacting therapeutic potential by reducing suppressive capabilities (53). Given that CD4+CD25+CD226- sorted Tregs exhibited a greater frequency of lineage stable FOXP3+Helios+ Tregs than CD4+CD25+CD127lo/- Tregs, corroborated by epigenetic, differentiation and cytokine profile data, we sought to understand how sorting method might impact suppressive capacity. To accomplish this, we conducted dual-color *ex vivo* suppression assays using serial dilutions of

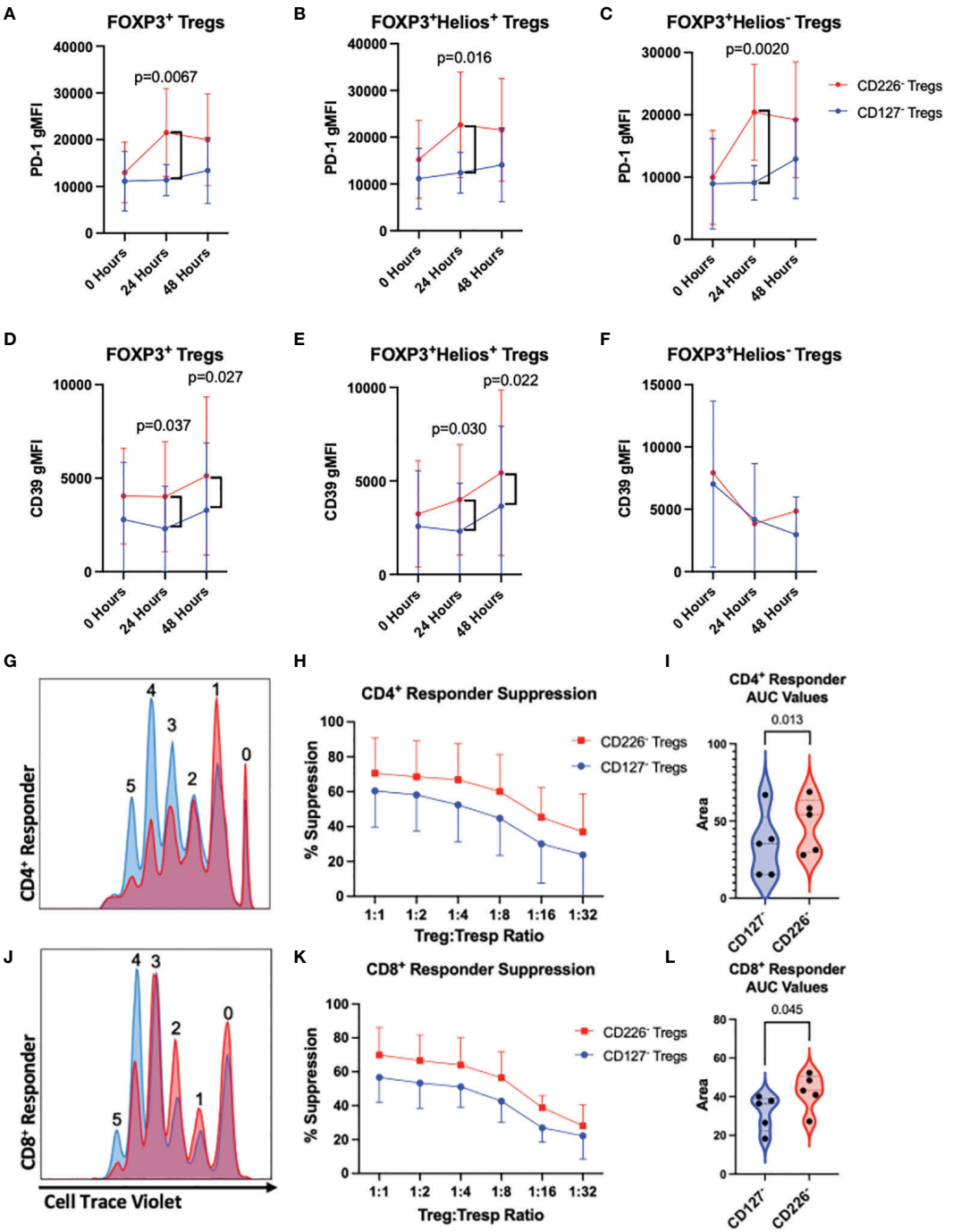

FIGURE 6 | CD226- Tregs demonstrate an increased suppressive phenotype and *ex vivo* suppressive capabilities as compared to CD127$^{lo/-}$ Tregs. **(A–C)** PD-1 and **(D–F)** CD39 expression was assessed by flow cytometry on total FOXP3+ Tregs **(A, D)**, FOXP3+Helios+ Tregs **(B, E)**, and FOXP3+Helios- Tregs **(C, F)** from 14-day *ex vivo* expanded CD127$^{lo/-}$ sorted Tregs versus CD226- Tregs following co-culture with autologous PBMCs in the presence of soluble α-CD3 and α-CD28 for 0, 24, or 48 hours. $n = 5$ biological replicates. Significant P-values reported on the figure for two-way ANOVA with Bonferroni's multiple comparison between Treg isolation conditions of matched subjects. CD127$^{lo/-}$ sorted Treg (blue) and CD226- sorted Treg cultures (red) were by expanded for 14-days *ex vivo*, then labeled with Cell Proliferation Dye eFluor670 and co-cultured in decreasing two-fold dilutions with CellTrace Violet-labeled autologous responder PBMCs in the presence of soluble α-CD3 and α-CD28 antibodies for four days before flow cytometric assessment of **(G–I)** CD4+ and **(J–L)** CD8+ T cell proliferation. **(G, J)** Representative dye dilution plots demonstrating suppression of responders. Percent suppression of **(H)** CD4+ and **(K)** CD8+ responders was quantified by the division index method, and comparisons between FACS conditions were made using **(I, L)** area under the curve (AUC) values for each percent suppression curve (40, 41). Data reflects $n = 5$ biological with $n = 3$ technical replicates. Significant P-values are reported on the figure for paired T-tests comparing Treg isolation conditions from matched subjects.

expanded Tregs (sorted *via* $CD4^+CD25^+CD127^{lo/-}$ or $CD4^+CD25^+CD226^-$) with autologous whole PBMC responder cells. $CD226^-$ sorted Tregs exhibited significantly increased suppression of both $CD4^+$ and $CD8^+$ effector T cell subsets, as demonstrated by decreased division indices for CTV labeled responder cells, as compared to those observed in $CD127^{lo/-}$ sorted Treg cocultures (**Figures 6G–L**). Importantly, we did not observe dilution of CPD in either Treg population, suggesting that differences in suppression were not due to further expansion of $CD226^-$ sorted Tregs. These data suggest that isolation of $CD4^+CD25^+CD226^-$ Tregs yields a more suppressive Treg population that may improve ACT efficacy compared to conventional $CD4^+CD25^+CD127^{lo/-}$ Tregs.

DISCUSSION

The requirement for long-term stability following engraftment in Treg-ACT necessitates the use of a combination of surface markers that function as a surrogate for the tTreg lineage-defining transcription factors. In this study, we compared the commonly employed Treg FACS isolation method using the $CD4^+CD25^+CD127^{lo/-}$ marker profile to an alternative $CD4^+CD25^+CD226^-$ approach. Importantly, sorted cells were assessed both pre- and post-expansion with the latter rested for 7 days prior to phenotypic, epigenetic, and functional characterization in order to mitigate transient upregulation of Helios in pTreg and contaminating Tconv (54) as a potential confounding factor. The resulting data demonstrate that isolation of $CD4^+CD25^+CD226^-$ Tregs yields a greater frequency of lineage stable $FOXP3^+Helios^+$ Tregs, a reduced percentage of $FOXP3^+Helios^-$ Tregs, and increased demethylation at the *FOXP3* TSDR compared to $CD4^+CD25^+CD127^{lo/-}$ Tregs. Among total Treg, $FOXP3^+Helios^+$ Treg and $FOXP3^+Helios^-$ Treg subsets, increased expression of CD25 was observed on $CD4^+CD25^+CD226^-$ sorted cells without compromising the low CD127 expression typically achieved using the $CD4^+CD25^+CD127^{lo/-}$ strategy. Together, this suggests that $CD4^+CD25^+CD226^-$ Tregs may have a higher avidity for IL-2, which is putatively reported to result in downstream pSTAT5-signaling to reinforce lineage stability, fitness, and function (55–57). While the increase in purity we observed is modest relative to $CD4^+CD25^+CD127^{lo/-}$ Tregs, we expect that these small improvements in initial purity may have a significant biological impact on the long-term survival and stability of a transferred population in ACT applications (58).

It is important to note that the data herein were derived from PBMC samples from healthy subjects (i.e., the general population). We speculate that the differences observed between $CD4^+CD25^+CD127^-$ and $CD4^+CD25^+CD226^-$ Tregs may be more prominent in autoimmune subjects, particularly during periods of acute inflammation where IL-2R signaling defects have been observed (59–61). This concept is critical when considering the transfer of islet antigen-specific Tregs created using genetically-modified T cell receptors (TCR) or chimeric antigen receptors (CAR) that could potentially become directly pathogenic toward islets and/or β-cells in situations of Treg instability (62–64).

To further characterize $CD4^+CD25^+CD226^-$ Tregs, we assessed T cell memory differentiation markers and found increased proportions of naïve Tregs both before and after expansion, along with reduced proportions of effector memory Tregs post-expansion as compared to the traditional $CD4^+CD25^+CD127^{lo/-}$ strategy. These results suggest that $CD4^+CD25^+CD226^-$ may not only serve as a better set of markers to identify lineage stable Tregs but also, to avoid effector contaminants. This notion is supported by prior work by Hoffmann and colleagues who initially demonstrated that $CD45RA^+$ naïve Tregs displayed increased stability upon expansion as compared to $CD45RA^-$ memory Tregs (65), a finding that we have consistently replicated from both umbilical cord and adult peripheral blood samples (13, 28, 66). Previous studies have identified the marker profile $CD4^+CD25^+CD127^{lo/-}CD45RA^+$ as selecting for predominately naïve tTregs; however, this isolation method yields a much smaller population than required for many ACT applications (43, 44). While our three-marker sorting strategy enriches for naïve Tregs, it also captures $CD4^+CD25^+CD226^-$ memory Tregs, resulting in a greater FACS yield compared to a $CD4^+CD25^+CD127^{lo/-}CD45RA^+$ strategy. Hence, the $CD4^+CD25^+CD226^-$ sorting strategy strikes a practical balance between the desire to enrich for naïve Tregs versus $CD4^+CD25^+CD127^{lo/-}$ isolation but also, maximize cell yield as compared to $CD4^+CD25^+CD127^{lo/-}CD45RA^+$ isolation. Similarly, while TIGIT has been identified as a marker of lineage stable tTregs, we previously reported that $TIGIT^+$ cells had a limited expansion capacity and therefore, would not produce enough Tregs for ACT (30, 31). Importantly, we observed a significantly lower proportion of T_{EM}-differentiated Tregs and a higher proportion of T_{CM}-differentiated Tregs expanded *ex vivo* from the $CD226^-$ Treg population. This observation suggests that $CD4^+CD25^+CD226^-$ Tregs may not only be longer-lived, but potentially, localize more readily in secondary lymphoid organs (17). This remains a critical issue for Treg-ACT applications in the context of T1D, as Tregs must be able to migrate to sites of inflammation and priming, specifically to the pancreatic draining lymph nodes where recent studies have revealed the presence of a stem-cell like $CD8^+$ T cell progenitor population that significantly contributes to pancreatic β-cell destruction in the NOD model of T1D (67).

During our assessment of the therapeutic potential of this $CD4^+CD25^+CD226^-$ Treg subset, we found decreased expression of the pro-inflammatory cytokines IL-17A, TNF, and IFN-γ, associated with Th17 and Th1 responses, in comparison to $CD4^+CD25^+CD127^{lo/-}$ Tregs following PMA/Ionomycin stimulation. This finding is especially important in the context of autoimmunity, as Th1 and Th17 effectors have been associated with several autoimmune diseases, including T1D and MS, suggesting that $CD4^+CD25^+CD226^-$ Treg isolation may potentially deplete these pathogenic Tregs and present reduced risk of pro-inflammatory ex-Treg outgrowth compared to $CD4^+CD25^+CD127^{lo/-}$ Treg isolation (68–70). Beyond this, PMA/Ionomycin stimulated $CD226^-$ sorted Tregs had increased intracellular and surface expression of the

immunoregulatory molecule, TGF-β1 (71). Following TCR activation, CD226- Tregs had increased expression of the immunoregulatory checkpoint molecules, PD-1 and CD39, which are associated with programmed cell death and ATP hydrolysis, respectively (52, 72). Inhibition or dysregulation of these checkpoint regulators has been associated with the development of autoreactivity (73, 74). Finally, given our observation that CD226- sorted Tregs had higher CD25 expression post-expansion, it is possible that the augmented suppressive capacity observed could, at least in part, be related to increased competition for IL-2. Altogether, our data support the notion that differences in IL-2 consumption, cytokine production, and contact-dependent mechanisms may all contribute toward the increased level of suppression observed with CD226- versus CD127lo/- sorted Tregs.

Tregs are emerging as a powerful therapeutic modality in a broad array of autoimmune settings (75, 76). While our study supports CD226- Tregs as a robust population to yield stable FOXP3+Helios+ Tregs, we note the limitation that our studies were conducted using general population control samples. Further research is needed to determine if CD226- Tregs will provide increased purity and stability in patients with active autoimmune disease. Indeed, there are a number of outstanding questions regarding the TCR repertoire, homing receptors, and *in vivo* trafficking of CD226- Tregs relative to CD127lo/- Tregs (77).

These findings also raise a number of considerations regarding ACT with CD226- Tregs and therapeutic targeting of the CD226 pathway in situations of autoimmunity. On one hand, our data related to CD226 being highly expressed on effector T cells supports additional studies targeting this pathway *in vivo* to block destructive autoimmunity. However, this approach should be taken with some caution, as we note that CD226 is also highly expressed by IL-10-secreting Tr1-like T cells (78). Thus, any immunotherapy seeking to inhibit CD226 on Tregs would likely need to be carefully dosed to increase Treg stability without compromising CD226-mediated Tr-1 like T cell function. Furthermore, these results support the continued investigation of the precise mechanisms by which reduced CD226 expression allows for increased Treg lineage stability and suppressive capacity. We note that additional studies are currently underway in our laboratory to assess the impact of CD226 on Tregs using both targeted biologics, along with global and conditional knockout approaches in animal models (37), as well as through gene targeting approaches in human Tregs. In summary, our findings present a novel method to generate a highly stable and suppressive Treg subset for use in ACT by initially eliminating Tregs expressing the costimulatory molecule, CD226.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MB: writing – original draft, writing – review and editing, formal analysis, visualization, validation, investigation, project administration, and supervision. LP: investigation, writing – review and editing, and funding acquisition. SH, JA, LS, KN, and EC: investigation and writing – review and editing. HS, CF, and AP: writing - review and editing. MS: writing - review and editing and funding acquisition. TB: conceptualization, writing – review and editing, funding acquisition, project administration, and supervision. TB is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. 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/fimmu.2022.873560/full#supplementary-material

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REVIEW
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Untangling the Knots of Regulatory T Cell Therapy in Solid Organ Transplantation

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Numerous preclinical studies have provided solid evidence supporting adoptive transfer of regulatory T cells (Tregs) to induce organ tolerance. As a result, there are 7 currently active Treg cell-based clinical trials in solid organ transplantation worldwide, all of which are early phase I or phase I/II trials. Although the results of these trials are optimistic and support both safety and feasibility, many experimental and clinical unanswered questions are slowing the progression of this new therapeutic alternative. In this review, we bring to the forefront the major challenges that Treg cell transplant investigators are currently facing, including the phenotypic and functional diversity of Treg cells, lineage stability, non-standardized *ex vivo* Treg cell manufacturing process, adequacy of administration route, inability of monitoring and tracking infused cells, and lack of biomarkers or validated surrogate endpoints of efficacy in clinical trials. With this plethora of interrogation marks, we are at a challenging and exciting crossroad where properly addressing these questions will determine the successful implementation of Treg cell-based immunotherapy in clinical transplantation.

Keywords: regulatory T-cells, tolerance induction, transplantation, cellular therapy, adoptive therapies

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INTRODUCTION

Since the inception of transplant programs, the discovery and use of immunosuppressive drugs have played a critical role in preserving allograft function. After several decades of implementation, these immunosuppressive regimens have efficiently decreased the incidence of acute graft loss. However, long-term and chronic allograft rejection rates remain pervasive and, together with the severity of side effects in the allograft recipient population, makes the pursuit of therapeutic alternatives a medical necessity. A better understanding of self-tolerance mechanisms has facilitated different approaches aiming at rebalancing alloantigen-reactive conventional T-cells (Tconv) and immunosuppressive regulatory T cells (Tregs). This is a clear conceptual shift from the current standard multidrug-based protocols focused on halting effector immune responses.

$CD25^{hi}FoxP3^{+}$ Treg cells represent 1-5% of circulating $CD4^{+}$ T lymphocytes and are essential in maintaining peripheral immune tolerance and homeostasis. After transplantation, the frequency of circulating Tregs in tolerant recipients is higher compared to patients with acute allograft rejection (1, 2). Increasing evidence also suggests that the balance between graft-reactive effector cells and

graft-protective suppressor Tregs plays a role in organ engraftment and long-term allograft survival (3, 4).

Despite a decade of major progress in Treg research, technical limitations and significant gaps in our knowledge of Treg cell biology continue to hinder our ability to harness the therapeutic potential of these cells to induce allograft tolerance. This review summarizes achievements, current status and future challenges in the clinical implementation of Treg cell-based immunotherapy in solid organ transplant (SOT) recipients.

ACHIEVEMENTS AND CURRENT STATUS OF CLINICAL TRIALS

The ability to isolate and expand Treg cells under good manufacturing practice (GMP)-compliant conditions paved the way for the clinical use of adoptive Treg cell transfer to induce allospecific tolerance in SOT patients. The first pilot study in SOT was reported by Todo et al. (5) in 10 liver transplant recipients using donor-specific Treg-enriched cell product in combination with standard immunosuppressive drugs that were gradually discontinued over a period of 18 months. All 10 recipients maintained stable graft function. Seven patients successfully achieved weaning of drugs between 16 and 33 months. All three patients who developed mild rejection during the immunosuppression weaning process underwent transplantation for autoimmune liver disease, which original autoimmune effector-regulatory unbalance may account for the difficult long-term control of effector responses. Since Todo's report, five more original manuscripts have been published to date in SOT, four of them in kidney transplant patients and another in liver recipients (summarized in **Table 1**). Across all studies with at least one-year follow-up, fifty-four SOT recipients who received a single infusion of autologous Tregs had 100% survival, no episodes of graft loss, no increased risk of infection, and no report of *de novo* cancer (6–10). Only two patients suffered mild adverse events: one experienced mild and transient cytokine release syndrome (9), and another developed donor-specific antibodies one-year-post transplant and primary disease recurrence after a two-year follow-up (7). Furthermore, among 28 kidney transplant recipients receiving autologous transfer therapy with Tregs, the ONE study reported a significant decrease in the incidence of viral infections after transplant (12). Like the Todo et al. study, the stability of transplant function in Harden et al. study (10) also permitted minimization of immunosuppression, revealing a significant reduction of inflammatory cell populations in the transplanted organ as a result of Treg transfer. Overall, the published results support feasibility and safety of Treg infusion procedures in SOT patients and disclosed promising early data on feasibility of drug immunosuppressive minimization/discontinuation (**Table 1**). They are also uncovering multiple challenges that may harness the progression of immunotherapies in the clinic, including phenotypic and functional diversity of Treg cells, lineage stability, optimization of *ex vivo* Treg cell manufacturing process, adequacy of administration route, inability of monitoring and tracking infused cells, absence of organ specificity/trafficking markers in Treg cells, and lack of biomarkers or validated surrogate endpoints of efficacy in clinical trials. Importantly, measurements and report outcomes are often not comparable among different trials or centers, which makes it difficult to standardize methodologies and verify and validate data for consistency.

PHENOTYPIC DIVERSITY

The efforts to characterize Tregs have revealed a broad spectrum of phenotypes in cells capable of engaging different suppressive mechanisms to control particular immune effector cell responses. The initial identification of these suppressor cells as $CD4^+$ $CD25^+$ T cells was substantiated by mouse experiments where their removal led to severe autoimmunity, which could be prevented after reconstituting these cells back to circulation (13, 14). In 2003, the forkhead box transcription factor FoxP3 was identified as an essential molecular marker of Treg cell development, differentiation and function. Since then, FoxP3 been considered as the defining Treg cell lineage "master-regulator" (15) and $CD4^+$ $CD25^+$ $FoxP3^+$ as the distinctive core Treg phenotype. Expression of the interleukin-7 receptor (IL-7R) α chain (CD127) on the surface of Treg cells inversely correlates with FoxP3 expression and is another convenient marker for Treg cells as it provides an additional distinction between $CD127^{high}$ ($FoxP3^{-/low}$) and $CD127^{-/low}$ ($FoxP3^{high}$) subpopulations. In combination with CD25 during flow cytometry analysis, CD127 can be used as biomarker for analysis and, because of the expression on the cell membrane, for isolation of Tregs (16, 17).

Treg cells can be also categorized by the expression of another membrane marker, CD45RA. Consequently, functionally suppressive Treg population can be distinguished between naïve resting Tregs, with high proliferative potential ($FoxP3^{low}$ $CD25^{low}$ $CD45RA^+$), and terminally differentiated, short-living Treg cells with low proliferative potential ($FoxP3^{high}$ $CD25^{high}$ $CD45RA^-$) (18, 19). Accordingly, as proposed by Arroyo-Hornero et al. (20) and supported by Canavan et al. in Crohn's disease patients (21), the segregation of the initial population of Treg cells based on the expression of CD45RA should be taken into consideration as $CD45RA^+$ Tregs, but not $CD45RA^-$, maintain a stable Treg signature after expansion. In a similar context, the expression of the Ikaros transcription factor family member, Helios, has been associated with lineage-committed, thymus-originated $FoxP3^+$ Treg cell and, therefore, regarded as potential biomarker for therapeutic competent Treg cells. In mice, $Helios^+$ and $Helios^-$ Treg subpopulations are phenotypically and functionally distinct and express different TCR repertoires (22, 23). However, similar studies in human Tregs have not generated consistent results (24–27). A recent report by Lam et al. (28) suggests that Helios expression in Treg cells may be an important marker of lineage stability, although it does not have a direct role in the maintenance of the lineage-committed state. Co-expression of the surface markers T cell

TABLE 1 | Published studies evaluating Treg transfer therapy after solid organ transplantation.

Study	Clinical setting	Manufacturing process	Phenotype and purity of infused Treg cells	Administration and Tracking	Outcomes and safety
Todo et al. (5)	• Post-liver transplant patients. • 10 patients, 3-5 year follow up. • Weaning from immunosuppression 6 months after transplant	**Isolation:** No isolation. **Expansion**: 2-weeks co-culture recipient lymphocytes with irradiated donor cells with anti-CD80/CD86. **Preservation**: no preservation	Infused lymphocytes. 58.6% $CD4^+$, 16.9% $CD8^+$. Tregs represented 24.8% of infused CD4+ Tcells.	• Peripheral IV infusion. • No tracking.[Monitored differential lymphocytes counts in peripheral blood, including Tregs.]	• Cell infusion well tolerated by all recipients. • Seven patients successfully achieved uneventful weaning and completed cessation of immunosuppressive therapy. • Three patients showed acute cellular rejection symptoms during weaning
Chandran et al. (6)	• Three kidney transplant recipients. • Follow up biopsies at 2 weeks and 6 months after infusion. • Follow-up for one year after infusion.	**Isolation**: FACS sorting of $CD4^+$ $CD127^{low/-}CD25^+$ from one unit of blood **Expansion**: 14-day culture with anti-CD3-CD28 paramagnetic beads, IL-2 and deuterated glucose (No Rapamycin). **Preservation**: none.	$1x10^9$ Tregs with an average of 95% purity for $FoxP3^+$ cells, >97% for CD4, and viability >99%. (Post-expansion)	• Peripheral IV infusion. • Tracking: Deuterated glucose.	• Cell infusion well tolerated by all recipients. • One patient developed (spontaneously resolved) leukopenia. • 100% patients and graft survival ater 1 year • Tregs circulating concentration peaked at one week. Deuterium signals detected up to 3 months after infusion ONLY in Treg cells
Mathew et al. (7)	• Nine kidney transplant recipients. • Three tiers of cell dosing (n = 3 per group): 0.5×10^9, 1×10^9, and 5×10^9 Tregs/recipient. • Control group: historical cohort with identical immunosuppression	**Isolation:** Immunomagnetic isolation of CD4+, CD25+ cells from cryopreserved leukapheresis product. **Expansion:** 21-day culture with anti-CD3-CD28 paramagnetic beads, IL-2 TGFβ and Rapamycin. **Preservation :** Leukapheresis product collected one month before transplant.	>98% purity for $CD4^+$ $CD25^+$ cells and >80% for $FoxP3^+$ $FoxP3^+$ cells (Post-expansion)	• Peripheral IV infusion on postoperative day 60. • No tracking. [Monitored differential lymphocytes counts in peripheral blood, including Tregs.] • 5–20 fold increase of Tregs percentages in all Treg infusion recipients. Increase stable in most patients until the one-year mark.	• Cell infusion well tolerated by all recipients. • 100% patients and graft survival after 2 years. • Biopsy 3 months after cell infusion: no signs of rejection. Biopsy 1 year after cell infusion: one episode of subclinical rejection associated with immunosuppression non-compliance. • One subject with lowest Treg dose infusion developed donor-specific antibodies 1-year post-transplant. In the two-year follow-up, the patient developed primary disease recurrence.
Roemhild et al. (8)	• 11 kidney transplant recipients received an infusion of expanded autologous Tregs. Dosage design: three escalating doses: 0 (c), 0.5, 1, 2.5-3 x 10^6 Treg/Kg (n = 3-4 patients/study group). All groups received drug immunosuppression Follow-up: three years.	**Isolation:** 2 step immunomagnetic isolation from 40-50 ml of peripheral blood: -1^{st}: CD8 negative selection. -2^{nd}: CD25 positive selection. **Expansion:** 21-day culture with anti-CD3-CD28 paramagnetic beads, IL-2 and Rapamycin. **Preservation:** none.	>$1x10^9$ cells. 91.9% were $CD4^+$ $CD25^+$ $FoxP3^+$. (Post-expansion)	Peripheral IV infusion • Tracking TCR repertoire Monitoring Tregs: • Tregs group: significant increase in Tregs counts and favorable Tregs/Teffector ratio for up to eight weeks after infusion. • Control group: Decreased Treg levels compared to baseline for up to 12 weeks after kidney transplantation.	• Cell infusion well tolerated by all recipients • 100% patients and graft survival after 2 years. • Treg therapy was significantly associated with successful weaning of drug therapy ($p<0.001$ at three years). • 10 patients in Tregs therapy were successfully weaned to low-dose tacrolimus monotherapy within 48 weeks. 2 patients required temporal or continuous reversal to triple immunosuppression therapy.
Sanchez-Fueyo et al. (9)	9 liver transplant recipients received an infusion of expanded autologous Tregs 3-16 months after transplant. Patients were assigned to one of two escalating doses: 1 $x10^6$ Tregs/Kg (3pt) or 4.5 $x10^6$ Tregs/Kg (6 pt). All patients received standard immunosuppression. Follow-up: 12 months.	**Isolation:** 2 step immunomagnetic isolation from 40-50 ml of peripheral blood: -1^{st}: CD8 negative selection. -2^{nd}: CD25 positive selection. **Expansion:** 36-day culture with anti-CD3-CD28 paramagnetic beads, IL-2 and Rapamycin. **Preservation:** Expanded Treg product	61-92% of cells were $CD4^+$ $CD25^+$ $FoxP3^+$. Viability after thawing: 58-89% (Post-expansion).	• Peripheral IV infusion of expanded Treg cells thawed just before administration. • No tracking. • Monitoring: Six patients who received 4.5 $x10^6$ Tregs/Kg had an increase in circulating Tregs noticeable from day 3 to 1 month. This increase was not observed in patients receiving 1 $x10^6$ Tregs/Kg.	• No episodes of rejection during the follow-up period. • One patient receiving 4.5 $x10^6$ Tregs/Kg developed mild temporary adverse effects.

(Continued)

TABLE 1 | Continued

Study	Clinical setting	Manufacturing process	Phenotype and purity of infused Treg cells	Administration and Tracking	Outcomes and safety
Harden et al. (10, 11)	12 kidney transplant recipients received autologous Tregs infusion at post-operative d5 Control group: 19 kidney transplant recipients Dosage design: 3 + 3 dose-escalation (three patients at each dose). Doses: 1, 3, 6 or 10 x 10^6 Treg/Kg All patients received standard immunosuppression Follow up: four years	**Isolation:** immunomagnetic isolation. 2 steps: -1st: CD8 negative selection. -2nd: CD25 positive selection. **Expansion:** 36-day culture with anti-CD3-CD28 paramagnetic beads, IL-2 and Rapamycin. **Preservation:** Expanded Treg product	91.6% ± 9.3% of total cells were CD4+ CD25+ FoxP3+ After thawing, >70% of cells were $CD4^+$ $CD25^+$ $FoxP3^+$. Viability after thawing: 58-89%. (Post-expansion)	• Peripheral IV infusion. Premedication with acetaminophen and antihistamine. Unfractionated heparin for 48 hours beginning on the day of infusion. No tracking. • Monitoring: Two weeks after transplant, observed dose-dependent increase in circulating number of Treg cells.	• Cell infusion: well tolerated by all recipients • 100% patients and graft survival after 2 years in all groups. • 100% patients and graft survival after 2 years in all groups. • Lower incidence of composite opportunistic infection with polyomavirus or cytomegalovirus in the Treg group. • Four patients in the Treg therapy cohort had successful minimization of immunosuppression (100% of patients attempted).

Tregs, regulatory T cells; FoxP3, forkhead box P3; IV, intravenous.

immunoreceptor with Ig and ITIM domains (TIGIT) and Fc Receptor-like 3 (FCRL3, CD307c) in $Helios^+$ FoxP3 Treg cells (29) could facilitate reliable identification and selection of $Helios^+$ Treg cells.

A shortcoming in the published literature of Treg cell-based clinical trials is the inconsistency and poor definition of the initial population of isolated Treg cells. Further phenotypic characterization is needed to identify the most appropriate population from which to expand $FoxP3^+$ Tregs for use in adoptive transfer approaches, which will also help to determine whether a unique initial phenotypic Treg cell profile is well-suited to fulfill the specific demands of different target tissues.

LINEAGE STABILITY

Lineage stability refers to the capability of a Treg cell to sustain immunosuppressive function in different environments and generate a progeny with similar characteristics after replication. The stable expression of signature genes for Treg cell lineage commitment should be considered as a critical parameter in the clinical competent population of Treg cells. Epigenetic changes such as DNA methylation, histone modification, and non-coding RNA synthesis regulate gene expression and cellular differentiation (30). Despite the phenotypic variability of Tregs, the epigenetic pattern can be used to identify a cell lineage with a stable immunosuppressive function. Indeed, the type of Treg-specific CpG hypomethylation pattern (TrHMP) is regarded as a more specific biomarker of functionally stable Treg than mere FoxP3 expression (31). The TrHMP includes hypomethylation of signature genes such as FoxP3, CTLA, GITR, and Helios (32), is heritable, independent of FoxP3 expression, and persists after TCR stimulation and in different culture conditions (30, 32). TrHMP is also linked to the suppressive strength of Treg cells as observed with *in vitro* induced (iTregs) cells. Despite the expression of FoxP3, iTregs show a TrHMP similar to activated Tconv, are less suppressive and demonstrate less lineage commitment than natural, thymus-originated Tregs (nTregs) (32). The strong association between Treg lineage stability and specific epigenetic imprinting supports the use of TrHMP as biomarker for Treg lineage determination/stability and the inclusion among the most reliable parameters currently available as criterion for the identification of functional Treg cells in experimental settings. However, the methylation status pattern is not included in any reported clinical study as a release criterion for clinically competent Treg cells (**Table 1**). In zaddition, the capacity to track lineage stability of infused Tregs *in vivo* in the clinic is limited (7). In Chandran et al. study, the authors transferred deuterium-labeled Tregs into kidney transplant recipients. The fact that deuterium signals were only detected in the Treg population within three months post-infusion suggests the lineage stability of infused cells (6).

MANUFACTURING PROCESS

While the Treg ability to inhibit the effector immune reactions that trigger graft rejection has been demonstrated in numerous pre-clinical studies, their low concentration in peripheral blood has become a major obstacle to their clinical application (33). However, refinements in the manufacturing process under Good Manufacturing Practices (GMP)-compatible conditions now facilitate escalating the cellular yield up to 2,000-fold (8). This process includes three main steps: isolation, expansion, and preservation.

Isolation

The two most common methods for Treg isolation are Fluorescence-Activated Cell Sorting (FACS) and immuno-magnetic cell separation. FACS has been primarily used for research and analytical purposes, but recent adaptations to comply with GMP legislation have allowed its clinical use.

FACS can distinguish very specific cellular subpopulations, sort cells based on the degree of expression of particular markers and discriminate several subpopulations simultaneously. However, FACS-based isolation of Treg cells for human therapy relies only on extracellular markers to identify the target population (34). Another technical limitation of this method is that the sorting efficiency is reduced when the population of interest is rare, requiring lengthy processing times from a large initial cell population (35). Still, some groups have successfully isolated Treg cells for clinical interventions with FACS (6, 36, 37), and the progress towards using more complex membrane marker combinations to define the initial Treg population may help a broader use of this technology as cell isolation procedure.

Immunomagnetic cell separation is the current method of choice for Treg isolation in clinical trials. Biotechnology companies have developed closed, automatic systems to comply with GMP regulations. In this method, magnetized particles are conjugated with antibodies, and consecutive steps of negative and positive selection allow the isolation of a specific Treg cell population. The purity of the isolation can increase by selecting multiple markers during a single pass of negative selection (e.g., CD8 and CD19) (11, 38). Most published Treg-based clinical trials in SOT reported the use of magnetic immunoselection isolation technique as a two-step procedure with initial CD8 depletion and subsequent CD25 enrichment (7–10). A significant loss of targeted cells after each selection step, the necessity of fine-tune optimization to find the optimal balance between cell yield and purity, the lack of discriminatory capacity between low or high expression of cellular markers, and the elevated cost of the procedure (specific equipment and supplies) are some of the shortcomings associated with Treg isolation by magnetic immunoselection. As such, more versatile GMP technologies are needed to improve yield and purity of clinical-grade quality Treg cell isolates and facilitate the standard implementation of this technology in clinical practice.

Expansion

Tregs constitute 1-5% of the total circulating $CD4^+$ T lymphocyte population (39). These low numbers and favoring cell purity over yield in the isolation process make *ex vivo* expansion a critical step towards successful cell therapy implementation. The main strategy for *ex vivo* expansion is establishing cell culture conditions to preferentially activate and expand Treg cells while preventing the replication of other potential contaminant cell types. Expansion protocols can produce up to 2,000-fold amplification of Treg cell numbers (36) and are based on the concomitant engagement of the T cell receptor (TCR) and the costimulatory receptor CD28, and high doses of the T cell growth and survival factor IL-2. Addition of mTOR inhibitors (e.g., rapamycin, everolimus) promotes the selective expansion and suppressive activity of Tregs (40, 41) while preventing Tconv activation and growth. Mechanistic evidence supports that mTOR signaling pathway is a critical regulator of effector Tconv homeostasis and function but not of Tregs (42–44). In fact, PI3K/Akt/mTOR activation represses Treg differentiation, and the inhibition of the Akt pathway is crucial to promote the activation of FoxP3 (45–47). Metabolically, Tconv depends on the mTOR-driven glycolytic pathway for a rapid supply of energy and molecular precursors (48); in contrast, the energy demand of Treg cells is fulfilled by the constant crosstalk between glycolytic and oxidative mitochondrial metabolic arms (49–51).

Prolonged stimulation of Tregs triggers epigenetic changes leading to suboptimal TCR signaling and progressive hypermethylation of Treg-specific demethylated regions (52). These epigenetic alterations can change the quality of the final cell product by promoting Treg conversion to Tconv or reducing their suppressive function (52, 53). Upon activation, Tregs may undergo a progressive shift from $CD45RA^+$ to $CD45RA^-$ phenotype (54). Upon further expansion, the $CD45RA^-$ fraction experiences a decline in both FoxP3 expression and suppressive activity (54). As mentioned, adding an mTOR inhibitor such as rapamycin sustains the expansion and suppressive activity of Tregs (40), but also induces the conversion of conventional $CD4^+$ T cells into iTregs. However, these iTregs do not possess the TrHMP hypomethylated signature of Treg genes and can revert into non-suppressive cells in the absence of rapamycin. Therefore, as suggested by Battaglia et al. (40), careful attention and appropriate quality controls must be in place when mTOR inhibitors are included in the expansion protocol for Treg cell therapy. For clinical application, the initiation of the expansion phase with highly purified and well-defined population of Treg cells seems the appropriate strategy. Overall, these studies highlight the importance of optimizing cell culture conditions (composition and duration) and quality control assessments in the expansion protocols for Treg manufacturing (52, 54). The progress of Treg immunotherapy demands establishing relevant mechanistic links between pre- and post-expansion phenotype, suppressor function and epigenetic profile of Treg cell populations with corresponding clinically relevant outcomes of operational tolerance or reduced rejection.

Preservation

For far-reaching applications of Treg cell-based therapy, it is essential to ensure the stability of the cell product during storage, including optimal cell viability, recovery and functionality. Widening the window between the collection and application of Tregs for adoptive therapy would increase the flexibility of their clinical use. Because preservation techniques can potentially change the yield, viability, and activity of Tregs, they are considered a new therapeutic biological product from a regulatory point of view (55).

Tregs can be cryopreserved before isolation (as peripheral blood mononuclear cells, PBMCs), just after isolation, or after the expansion phase. Treg cell recovery rates from cryopreserved PBMCs fluctuate between 35 to 63% (55–61). Using isolated Tregs cryopreserved in liquid nitrogen for up to one year, Peters et al. reported a viability of 70-80%, with a suppressive capacity that was significantly impaired after thawing but recovered after activation (38). Kaiser et al. found better recovery rates and cellular viability by using cryogenic solution of 5% DMSO instead of 10% DMSO (55). Cryopreserved Tregs after three or four cycles of re-stimulation did not alter their original phenotype or

suppressive function (56). Different groups have reported using cryopreserved Treg cells in SOT patients. Mathew et al. cryopreserved the leukapheresis product approximately one month before kidney transplant, their expansion protocol lasted 21 days, and the infusion of Tregs was given 60 days after surgery (7). Harden et al. and Sanchez-Fueyo et al. cryopreserved the Treg product after isolation and expansion and thawing was performed at the bedside of the patient prior to administration (9, 10). Fraser et al. reported the feasibility of infusing pre-expanded cryopreserved Tregs, showing a reported cell recovery >90%, viability >75% and suppressive function of >80% (11).

Using fresh starting material may have recognized advantages, but the ability to cryopreserve also allows for a more flexible, convenient, efficient -and less expensive- manufacturing process that can be easily managed and scheduled in cellular therapy laboratories (9, 10). However, the effect of cryopreservation on Treg phenotypic and functional parameters and on subsequent clinical outcomes has to be properly established. Such assessments would have a profound logistical impact on clinical trial design, infusion timelines and testing requirements for future studies.

ADMINISTRATION ROUTE

Too often, the cell delivery method is an overlooked factor that may have a direct effect on treatment bioavailability to the target organ and, as such, a determining factor for assessments of feasibility, safety and efficacy outcomes of treatment (62, 63). There are two principal methods to introduce cells into the body: systemic delivery and local delivery into the organ. The most common method for Treg cell infusion is systemic intravenous (IV) injection. IV injection allows for wide distribution of cells throughout the body, and it has the advantage of being minimally invasive with low/minimal safety risks in early phase clinical studies. With this methodology, there are several hurdles to overcome in order to deliver cells to the target organ and have them engrafted. IV delivered cells have to pass through the lungs before they can distribute throughout the body. This pulmonary "first-pass" effect results in significant entrapment of cells (64) caused by the estimated size of Tregs (10–15 μm diameter) (65–68), as observed with microsphere particles of this size (64, 69). Similarly, clinical studies with IV-delivered stem cell infusion showed that the majority of cells get trapped in the lungs after intravenous administration (64, 69, 70). Likewise, systemic infusion of expanded tumor infiltrating lymphocytes (TILs) resulted in higher concentrations of cells in lung, liver, and spleen (71).

The optimal method of therapeutic cell delivery will always depend on the mechanism of action of the cell product. Since Tregs cannot exert their organ-protective effect distally, the delivery system must reach the target organ or allow Treg cells to migrate toward it. The alternative to systemic infusion is the direct local delivery into the organ. This approach can provide a high concentration of Tregs in a first passage where all injected cells have opportunity to interact with post-capillary endothelia of the target organ (72). Direct intra-arterial infusion of stem cells into the brain has proven to significantly enhance cellular engraftment and concentration in animal models of brain ischemia when compared to systemic IV administration (73–75). Also reported, infusion of radiolabeled TILs in the hepatic artery is followed by a rapid increase and slow decline in the intensity signal of the liver (76). However, a disadvantage of local injection is that it may cause further local damage in tissue that, such as a SOT, is already particularly sensitive. It has also been shown that, although direct injection increased localization, it did not necessarily increase engraftment or survival (77). Animal models using direct intra-arterial delivery of mesenchymal stromal cells to the kidney have shown retention of cells in the renal cortex (78) and induction of a favorable tolerogenic milieu after transplantation (79–85). To the best of our knowledge, all currently active clinical protocols using adoptive transferred Treg cells in transplantation are using systemic IV delivery of cells. As safety is the necessary focus of these phase I/(II) studies, alternative routes of cell administration have become an understudied area that remains to be properly addressed. Developing efficient cell delivery protocols could significantly improve the effective implementation and outcomes of Treg-based cell therapy in SOT.

MONITORING AND TRACKING INFUSED CELLS

Regardless of the infusion route, the success of any cell-based immunotherapy relies on efficacy of cell trafficking and recruitment to the targeted area where they must remain functional. Tracking these adoptive cells *in vivo* becomes critical to evaluate their delivery, biodistribution and therapeutic response. However, our ability to longitudinally interrogate the migration and fate of infused Treg cells throughout the body remains elusive. In fact, it has become one of the most challenging limitations in current Treg cell immunotherapy studies.

There are only a few studies reporting the *in vivo* assessment of the distribution and fate of infused Treg cells in humans. Oo et al. used single-photon emission computed tomography (SPECT) to track the distribution of autologous Tregs marked with ^{111}Indium tropolonate (^{111}In) in four patients with autoimmune hepatitis. At 24 hours, they detected a predominant distribution within the liver (22-44%), spleen (11-24%), and bone marrow (9-13%). Tregs persisted in the liver for 72 hours until the ^{111}In was no longer detectable (86). Bluestone et al. used non-radioactive labeling of deoxyribose with deuterium for tracking Treg cells after infusion in type-1 diabetes patients (37). They observed a peak concentration of circulating Tregs between days 7 and 14 with a subsequent decline. Ninety days later, the concentration was 25% of the maximum, and one year after, labeled Tregs were still detected. They reported an initial fast decay phase of infused cells with a half-life of 19.6 days, followed by a slower decay phase. Chandran et al. demonstrated similar kinetic and stability

pattern: Tregs peaked in the first week, had a bi-phasic decay, and were still detectable circulating one month after infusion, but were undetectable at the 3-month mark (6). However, a study in non-human primates reported strikingly different results: using carboxyfluorescein succinimidyl ester (CFSE)-labeled cells, Singh et al. observed a rapid decrease of Tregs in peripheral blood during the first three days after infusion, and were barely detectable after 16 days. The uptake and clearance of infused Tregs in bone marrow and lymph nodes followed a similar pattern as with concentrations in blood. They also reported a significant change in phenotype, with less than 30% of CFSE-labeled cells holding the $CD25^{+}FoxP3^{+}$ phenotype by day 16 (87). Although cell manufacturing and labeling protocols differed among studies, and accounting for possible inter-species variability, the inconsistent results among available studies underscore current limitations to assess *in vivo* trafficking, homing and fate of infused Treg cells to the transplanted organ.

Novel approaches for monitoring the biodistribution and organ trafficking efficacy of adoptive Treg cells after infusion are in dire need. In the absence of standard non-invasive modalities to assess treatment responses, allograft biopsy analyses of FoxP3 mRNA expression in the transplanted organ, either alone or as a ratio with *GranzymeB*, are used as surrogate markers for infiltrated Treg and Teff cells, respectively (88–101). New non-invasive imaging technologies such as SPECT, Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI) or hybrid modalities such as MRI-SPECT in combination with computational biology (102–104) still require validation and standardization. However, they are among emerging technologies that, once implemented into clinical practice, will significantly help improve the efficacy of current cell-based therapy protocols.

ANTIGEN-SPECIFIC TREG CELLS

The generation of antigen-specific Treg cells is a valuable new approach to provide local, more restricted, immune tolerance (105–109) with cells that are efficiently trafficking to tissues that express cognate antigens (110, 111). Efforts to generate antigen-specific Tregs are currently focused on two different strategies: *ex-vivo* induction of Tregs by stimulation of antigen-directed $CD4^{+}$ effector Tconv cells (112), and engineering synthetic T-cell receptors (TCRs) or chimeric antigen receptors (CARs) with target-tissue specificity (113). The reported lineage instability of iTregs cells under inflammatory conditions precludes the clinical use of these cells. To potentially overcome the limitations to generate clinically efficient antigen-directed iTreg cells, gene-editing or transgenic approaches are being applied to induce stable expression of FoxP3 or other Treg signature proteins, as well as to identify key gene targets and pathways involved in the regulation of Treg function and stability (112, 114–118).

On the other hand, the genetic introduction of engineered TCRs and CARs can provide antigen-specificity to polyclonal Tregs (106, 111, 113, 119–121). The ectopic expression of TCRs in Treg cells allows the targeting of processed intracellular antigens presented by HLA molecules. Several pre-clinical studies have demonstrated translational potential of this approach (120, 122–125). However, the HLA-restricted physiological activation limits the application of engineered TCRs and may acquire harmful specificities when mispaired with endogenous TCRs. Interestingly, enforcing the expression of MHC-I-restricted TCRs or not functional low affinity Tconv TCRs (126), enable human Treg cells to bypass the MHC requirement for antigen recognition. Also, instead of using exogenous TCRs isolated from Tconv cells, there is the option of using specific Treg TCRs, which have shown some structural differences (127–129). Another strategy may entail the creation of universal Treg donor cell lines by sequential genetic modifications of MHC molecules (130).

CARs are modular artificial receptors that combine an extracellular antigen-recognition domain and intracellular signaling and costimulatory domains. CAR-engineered effector T cells are being used to reprogram effector Tconv to target tumor cells in patients with blood cancers (131–134). The major advantage of CARs is their ability to recognize whole proteins expressed in target tissues unrestricted to MHC class I or II presentation. Therefore, unlike TCR-modified Treg, CAR-Tregs cells could be applicable to a larger number of patients. However, the design of the CAR should consider specific traits of the host Treg cell, such as the determination of optimal specificity and affinity/avidity of antigen recognition and identification of co-stimulatory signaling domains and accessory molecules that enhance suppressive activity without jeopardizing Treg lineage stability. Recent in-depth reviews comprehensively discuss current status and future prospects of engineered TCR- and CAR-Treg cells in different clinical settings (135, 136).

Currently, a multi-center clinical trial is investigating safety and tolerability of CAR-Treg therapy in HLA-A2 mismatched kidney transplant recipients (NCT04817774) (**Table 2**). These advanced genetic technologies in cell therapy should be implemented in clinical settings with restricted safety precautions and quality control assessments. Among the latter, the complex nature of Treg functional fitness needs careful attention to any TCR genetic manipulation as the maintenance of Treg identity depends on a fine-tuned strength of antigen-specific stimulation (113, 137, 138).

EFFICACY: ENDPOINTS AND BIOMARKERS

Traditional primary endpoints for treatment efficacy in transplant clinical trials include graft survival, death with a functioning graft, and quality of life (QoL). These 'patient-centered' endpoints are commonly evaluated by surrogate endpoints which, by definition, require adequate validation and should demonstrate robust ability to predict meaningful benefits. Effective use of surrogate endpoints offers the promise of more efficient assessment by providing earlier answers to questions of therapeutic efficacy. Common surrogate endpoints to assess transplant allograft survival include: subclinical, acute cellular, antibody-mediated and steroid-resistant rejection episodes.

TABLE 2 | Clinical trials evaluating Treg transfer therapy after solid organ transplantation*.

Study ID (Phase)	Treg product	Clinical settings
NCT04817774 (I/II)	Antigen-specific CAR-Tregs (TX200-TR101) Dose: not specified. Three single ascending dose cohorts and an additional expansion cohort.	Population: HLA-A2 mismatched living kidney donor transplant recipients. Intervention: IV infusion of autologous CAR-Tregs. Follow-up: 84 weeks after infusion.
NCT03943238 (I)	Autologous, polyclonal, *ex-vivo* expanded Tregs. Dose: starts at $25x10^6$ cells. Escalated doses of Tregs if the donor chimerism is less than 25% after 60 days.	Population: Kidney transplant recipients. Intervention: Two weeks after transplant, on separate days, IV infusion of: - Purified $CD34^+$ and T cells from the kidney donor. - Autologous Treg cells. Follow-up: 2 years
NCT03867617 (I/II)	Autologous, polyclonal, *ex-vivo* expanded Tregs. Dose (cells/kg): Target dose: $1x10^7$ Dose range: 0.3-1.5 $x10^7$	Population: HLA-mismatched living donor kidney transplant recipients. Intervention: IV infusion of autologous regulatory T cells + donor bone marrow + Tocilizumab. Follow-up: one year
NCT03284242 (I)	Autologous, polyclonal, *ex-vivo* expanded Tregs. Dose: 50-300$x10^6$	Population: Kidney transplant recipients Intervention: IV infusion of Treg cells Follow-up: 2 years
NCT02711826 (I/II)	Autologous, polyclonal, *ex-vivo* expanded Tregs. Dose: 100-1000 $x10^6$ cells.	Population: Kidney transplant recipients Intervention: IV infusion of autologous regulatory T cells 3-7 months after transplant. Follow-up: 405 days
NCT03577431 (I/II)	Donor alloantigen-specific autologous Tregs. Dose: Target dose: 2.5 x 10^6 cells. Dose range: 1-125 x 10^6 cells. Intent to treat analysis: 1-2.5 x 10^6cells.	Population: Liver transplant recipients. Intervention: IV infusion of Treg cells. Follow up: Until completion of study (At least 104 weeks, up to 4.5 years).
NCT03654040 (I/II)	Donor alloantigen-specific autologous Tregs. Dose: Target dose: 90x10^6 cells.	Population: Liver transplant recipients. Intervention: IV infusion of Treg cells. Follow up: Until completion of study (At least 104 weeks, up to 4.5 years).
NCT02474199 (I/II) (completed)	Donor alloantigen-specific autologous Treg cells. Dose: 400 x 10^6 cells (300-500 x 10^6)	Population: Liver transplant recipients (2 to 7 years post Tx) Intervention: IV infusion of Treg cells. Follow-up: 52 weeks.
NCT02188719 (I) (Terminated)	Donor alloantigen-specific autologous Treg cells. Dose: Cohort #1: No cells; #2: 25-60 million cells (target: 50 million); #3: 100-240 million cells (target: 200 million); #4: 400-960 million (target: 800 million).	Population: Liver transplant recipients Intervention: IV infusion of Treg cells. Follow up: 40 weeks after transplant.
NCT02091232(I) (completed)** (12)	Autologous, donor antigen reactive, *ex-vivo* expanded Tregs, stimulated with kidney donor PBMC in the presence of beltacept	Population: Kidney transplant recipients Intervention: IV infusion of Treg cells. Follow-up: 2 years

*Tregs, regulatory T cells; CAR-Tregs, chimeric antigen receptor Treg; HLA, human leukocyte antigens; IV, intravenous; ALT, Alanine amino-transferase; GGT, Gamma-Glutamyl transpeptidase. *Clinical trials with unknown status are not reported in the table. **Results are published as part of the ONE study.*

The use of biomarkers as surrogates has facilitated the assessment of treatment efficacy in numerous clinical studies. Currently, there is no validated biomarker for treatment efficacy in organ transplantation, which is a fundamental limitation in clinical studies. QoL is a complex endpoint difficult to evaluate because it includes multiple physical, emotional and intellectual parameters that are subjective in nature (139). Although Treg transfer therapy studies are early phase I/II safety and feasibility trials, the evaluation of clinical efficacy is still a critical unresolved issue. To aggravate this limitation, current good short-term

outcomes of standard drug immunosuppression regimens demand long-term evaluation of large cohorts to assess the efficacy of any novel therapeutic intervention (140, 141). Implementation of standardized measurable outcomes of direct relevance to patients (including graft function and QoL) is an obvious shortcoming of current SOT clinical trials. Multicentric trials such as *Treg Adoptive Therapy for Subclinical Inflammation in Kidney Transplantation* (TASK), *TReg Adoptive Cell Therapy* (TRACT), or the *ONE Study* are positive initial attempts to unify criteria of cell manufacturing and evaluation of SOT Treg-based clinical studies. However, current protocols still vary in essential criteria and further efforts are required to develop common designs in future clinical trials.

CONCLUSION

The indispensable role of Treg cells to immune homeostasis and sustaining self-tolerance has awakened an exciting field of research in SOT. Aiming at improving the QoL and long-term outcomes of transplant recipients, Treg cell therapy appears as an attractive alternative to current standard immunosuppressive treatments. Recent bench-to-bedside progress is paving the way towards the successful application of cellular therapies to achieve transplant tolerance. We hope this review will help the reader appreciate the enormous therapeutic potential and also the challenges of Treg cell-based immunotherapy in transplantation.

AUTHOR CONTRIBUTIONS

Conception and design of the study, RG and FM. GO, RG, and FM wrote the review. MG provided significant revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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Regulatory T Cells, a Viable Target Against Airway Allergic Inflammatory Responses in Asthma

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Asthma is a multifactorial disorder characterized by the airway chronic inflammation, hyper-responsiveness (AHR), remodeling, and reversible obstruction. Although asthma is known as a heterogeneous group of diseases with various clinical manifestations, recent studies suggest that more than half of the clinical cases are ''T helper type 2 (Th2)-high'' type, whose pathogenesis is driven by Th2 responses to an inhaled allergen from the environmental exposures. The intensity and duration of inflammatory responses to inhaled allergens largely depend on the balance between effector and regulatory cells, but many questions regarding the mechanisms by which the relative magnitudes of these opposing forces are remained unanswered. Regulatory T cells (Tregs), which comprise diverse subtypes with suppressive function, have long been attracted extensive attention owing to their capability to limit the development and progression of allergic diseases. In this review we seek to update the recent advances that support an essential role for Tregs in the induction of allergen tolerance and attenuation of asthma progression once allergic airway inflammation established. We also discuss the current concepts about Treg induction and Treg-expressed mediators relevant to controlling asthma, and the therapies designed based on these novel insights against asthma in clinical settings.

Keywords: regulatory T cells, allergic airway inflammation, asthma, airway epithelial repair, therapeutic strategies

INTRODUCTION

Asthma is a chronic airway inflammatory disease that affects more than 350 million individuals worldwide (1). The etiology underlying asthma includes both genetic predisposition and environmental exposures (2). In general, the airway inflammation in asthmatic setting arises from the reaction in response to allergens and/or other environmental factors, thereby leading to an aberrant airway Th2-type immune response (3). Although a great effort of studies had advanced the understanding of pathologic features of asthma, its mechanisms underlying the regulation of allergic

airway inflammation are yet to be fully elucidated. As a result, the development of novel therapeutic strategies against asthma is confronted with formidable challenges.

There is strong evidence in animals that regulatory T cells (Tregs) act as a key regulator of allergic diseases and are essential to limit antigen-specific immune responses. For example, mice deficient in $CD4^+CD25^+$ Tregs resulted from loss-of-function mutations in the *Foxp3* gene are featured by the development of spontaneous autoimmunity, lymphoproliferation, allergic airway inflammation, hyper IgE syndrome, and eosinophilia (4). Similarly, adoptive transfer of ovalbumin (OVA) peptide-specific $CD4^+CD25^+$ Tregs into the OVA-sensitized mice attenuated airway hyper-responsiveness (AHR) along with reduced number of eosinophils and production of Th2 cytokines in the lung following allergen challenge (5). $Foxp3^+$ Tregs also suppress chronic allergic inflammation to establish allergen-tolerance in the respiratory mucosa (6). Furthermore, manipulation of steroid responsiveness in Tregs could represent a novel strategy to treat steroid refractory asthma, as their responsiveness determines steroid sensitivity during allergic airway inflammation (7). Collectively, these studies underscore the significance of Tregs in the regulation of allergic airway inflammation in mouse models.

Unlike the impact observed in animal models, the role of Tregs in asthmatic patients is yet to be well defined. Studies revealed that adult asthmatic patients with either stable or exacerbated symptoms displayed lower percentage of Tregs along with impaired suppressive function in the blood and airway (8). Similarly, decreased pulmonary Treg number coupled with lower capability to suppress pulmonary Th2 responses were observed in asthmatic children (9). In sharp contrast, some studies also demonstrated that the amount of airway Tregs was increased in adult patients with moderate to severe asthma as compared to both mild asthmatic patients and healthy subjects (10), especially in response to bronchial allergen provocation (11). The discrepancy between these findings could be caused by the differences of study cohorts and the approaches for Treg analysis. Nevertheless, a consistent conclusion could probably be reached for the impaired Treg function in asthmatic patients, particularly for their capability to suppress Th2 responses. A recent study further suggested that the numerical and functional defects of Tregs may render the children and younger adults more susceptible to asthma, while the relationship between Tregs and asthma risk or severity in older patients is relatively weak (12). Although the contribution of Tregs in asthma is not fully addressed, clinical improvement following allergen immunotherapy (AIT) for asthma suggested an association with the induction of IL-10-, IL-35- and TGF-β-producing Tregs and $Foxp3^+$ Tregs (13). Therefore, in this review we seek to summarize the immunological features of allergic asthma, and then update the recent advances that support the role of Tregs in allergen tolerance induction and in limiting disease severity once allergic airway inflammation established. We also discuss the current concepts about Treg induction and Treg-expressed mediators relevant to controlling asthma, and the therapies designed based on these novel insights against asthma in clinical settings.

THE IMMUNOLOGICAL CHARACTERISTICS UNDERLYING ALLERGIC ASTHMA

Type 2 immunity has now been well recognized to be a critical feature relevant to a complex network of immunologic mechanisms in allergic asthma (14). Type 2 immune response involves an ever-expanding repertoire of immune cells, including Th2 cells, B cells, natural killer (NK) cells, NKT cells, basophils, eosinophils, mast cells, and group 2 innate lymphoid cells (ILC2s) and their associated cytokines (15). IL-4, IL-5, IL-9, and IL-13 are predominantly produced by immunocytes, while IL-25, IL-31, IL-33, and thymic stromal lymphopoietin (TSLP) are released from tissue cells, particularly epithelial cells (16).

The immunological mechanisms underlying allergic response can be classified into two main phases: (1) the sensitization and memory phase, and (2) the effector phase. The later can be subdivided into the immediate and late-phase reactions (17, 18). During the sensitization and memory phase, the differentiated and clonal expanded allergen-specific Th2 cells produce copious amount of IL-4 and IL-13 to drive the class switching of antibody isotypes to the ε heavy chain. The allergen-specific IgE then binds to the high-affinity FcεRI on the surface of mast cells and basophils, thereby contributing to the IgE sensitization of individuals against allergens. In this phase, a memory pool of allergen-specific Th2 and B cells is also generated, which is ready to act upon allergen encounters. The immediate reaction of allergic response is also termed as type 1 hypersensitive reaction. Upon the challenge from same allergens, crosslinking of the IgE-FcεRI complexes on the sensitized basophils and mast cells leads to the release of anaphylactogenic mediators (such as vasoactive amines, prostaglandin D, platelet-activating factor, leukotriene, chemokines, and other cytokines) responsible for the classical immediate symptoms of allergic disease. The late-stage reaction generally occurs following 4-6 hours of allergen stimulation and lasts for more than a few days, and is featured by the localized inflammatory responses mediated by the infiltrated eosinophils, neutrophils, macrophages, Th2 cells and basophils. The ongoing inflammation results in more severe clinical manifestations of allergy, such as chronic persistent asthma, allergic rhinitis, and in extreme cases, systemic anaphylactic reactions (18).

Recent studies also suggested the involvement of epithelial cells in allergic pathology. Barrier epithelial cells not only represent the very first line of defense against environmental insults, but also produce cytokines (e.g., IL-25, IL-31, IL-33, and TSLP) and alarmins (e.g., uric acid, ATP, HMGB1, and S100 proteins) following allergen exposures (19). These signals constitute important factors in the early phase of asthma and promote Th2 differentiation through their effect on tissue dendritic cells and ILC2s (20). In particular, there is evidence that a neutralizing mAb against IL-25 results in a significantly reduced production of IL-5 and IL-13 along with attenuated eosinophil infiltration, goblet cell hyperplasia, and serum IgE secretion, by which it prevents AHR following OVA-induced allergic airway inflammation in mice (21). More excitingly,

blocking antibodies against either TSLP or IL-33/ST2 signaling are currently at different stages of clinical trials, which could be promising candidates for asthma treatment in clinical settings (22, 23).

ILCs are defined as ILC type1, 2, and 3 cells based on their transcription factors and cytokine production patterns, and among which, ILC2s play a substantial role in the initiation, progression, and steroid resistance of allergic airway inflammation (20). It was noted that IL-33 targets ILC2 to produce IL-5 and IL-13, thereby enhancing eosinophil recruitment, goblet cell hyperplasia, macrophage M2 polarization, dendritic cell (DC) activation and Th2 differentiation (24–27). Studies revealed that the number of total and type 2 cytokine-expressing ILC2s is significantly higher in the peripheral blood and airway of patients with systemic steroid-dependent severe eosinophilic asthma than those of patients with mild asthma (28). Given that the intracellular cytokine expression by Th2 cells within the airways did not differ between the above two groups of patients, The observations support that uncontrolled ILC2s rather than Th2 cells, represent a steroid-insensitive population of cells to exacerbate the development of airway inflammation in patients with severe prednisone-dependent eosinophilic asthma (28). Other distinct types of effector T cells (Teffs) may also get involved in continuous allergic inflammation as well. For example, although Th1 and IFN-γ secreting NKT cells induce epithelial apoptosis through cell-mediated cytotoxicity, they also exert an inhibitory role in Th2 cells and suppress IgE isotype switching (29). While IL-17 producing Th17 cells mediate neutrophilic type inflammation other than exacerbating Th2-related allergic inflammation (30). Moreover, Th9 cells employ multiple mechanisms to orchestrate allergic inflammation, and particularly, their interaction with diverse cell types including mast cells, ILCs, and DCs, to promote coordinated regulation of allergic airway inflammation (31, 32). Other than secretion of their signature cytokine IL-9, Th9 cells from mice and humans also secret other cytokines such as IL-10, IL-17, IL-21, and IL-22, to facilitate immune responses in the setting of allergic asthma (33, 34). Furthermore, studies on atopic dermatitis demonstrated feasible evidence supporting that the expansion of Th2/Tc2 and Th22/Tc22 may also exert an important role in allergic inflammation (14, 35).

ORIGINS AND SUBTYPES OF TREGS

Tregs are one of the main bastions against inappropriate or over-exuberant inflammatory responses, and play an indispensable role in the maintenance of immune tolerance in asthma (36). However, subsets of $CD8^+$ T cells, $CD4^-CD8^-$ T cells, γδ T cells, regulatory B cells (Bregs), IL-10-producing DCs, IL-10-producing NK cells, and macrophage subsets with suppressive properties also contribute to the suppressive and regulatory events (37). Currently, two main subsets of Tregs have been defined: the thymus-derived naturally occurring $CD4^+CD25^{hi}$ $Foxp3^+$ Tregs, also called tTregs, and the peripherally induced adaptive Tregs (pTregs) (38, 39). pTregs are further divided into $Foxp3^+$ pTregs, $Foxp3^-$ IL-10-producing Tr1 cells, and $Foxp3^-$ TGF-β-expressing Th3 cells. Studies in animals suggest the $Foxp3^+$ pTregs and IL-10-producing Tr1 cells may contribute to the differences of asthma susceptibility associated with different genetic background (40). Generally, Foxp3 induction in tTregs can occur at the double positive (DP) stage or preferentially at the CD4 single positive (SP) stage or during the transition to this stage in the thymus (41). Interaction with antigen presented by either cortical or medullary thymic epithelial cells is sufficient to induce Foxp3 expression, thereby committing T-cell precursor to Treg lineage (42). pTregs are differentiated in the secondary lymphoid organs and tissues, and they are particularly enriched in the intestinal mucosa and lung during chronic inflammation, with specificities directed against food antigens, gut microflora, and environmental allergens (43). The induction of pTregs at the gastrointestinal tract is facilitated by the $CD103^+CD11c^+$ DCs in a TGF-β and retinoic acid-dependent manner (44–46), while lung tissue-resident macrophages constitutively coexpressing TGF-β and retinal dehydrogenases (RALDH1 and RALDH2) are the main subset of cells driving pTreg generation from naïve $CD4^+$ T cells (47). It is worthy of note that the classification of Tregs could vary based on the specific markers employed. For example, Tregs can be also classified into nTregs, iTregs, $ICOS^+$ Tregs, Tr1, $CD8^+$ Tregs and IL-17-producing Tregs (48); however, some of these Treg subsets could be functionally overlapping or synergizing each other.

tTregs and pTregs are phenotypically distinct, and display different specificities and complementary functions *in vivo* (49). Generally, TCR on tTregs primarily recognizes self-antigens, which is crucial for establishing self-tolerance and preventing autoimmunity, while pTregs are thought to predominantly govern tolerogenic responses against non-self-antigens, such as allergens, food, and the commensal microbiota (50, 51). In a mouse model of chronic asthma, passive transfer of pTregs efficiently suppressed all aspects of asthmatic phenotype, whereas equal amount of tTregs only manifested a modest impact in this model, indicating that pTreg are substantially more tolerogenic in this setting (52). Although both tTregs and pTregs attenuate the development of asthma in WT recipients, those cells, however, enhance lung allergic responses in $CD8^{-/-}$ recipients (53). The reprogramming pathways and enhancement appear to be distinct and cytokine specific, in which IL-13 production in nTreg depends on the GITR signaling, while IL-17 production in pTregs is induced by IL-6 signaling (53). There is evidence that tTregs stability in allergic settings is maintained by the epigenetic mechanisms and metabolites generated by themselves such as cyclic adenosine monophosphate (cAMP) (50). In contrast, the instability of Foxp3 expression and loss of suppressive function in pTreg are closely related to the methylation state of the Treg-specific demethylated region (TSDR) (50). Therefore, the regulatory mechanisms underlying the maintenance of Treg stability and functionality are essential to the development of effective strategies against allergic airway diseases.

CELLULAR AND MOLECULAR MECHANISMS UNDERLYING TREG ATTENUATION OF ASTHMA

The suppressive functions of Tregs in allergic inflammation are mediated by an ever-growing list of mechanisms (**Figure 1**). Tregs can suppress antigen presentation cells (APCs) to activate Teffs, while enhancing the function of tolerogenic DCs. Tregs inhibit the migration and functionality of Teffs including Th1, Th2, and Th17 cells. Tregs also repress the secretion of allergen-specific IgE from plasma cells and induce IgG4-secreting B cells and IL-10-producing Bregs. Other than Teffs, Tregs are able to suppress the activation of eosinophils, basophils, mast cells, NKT cells, and ILC2s (14, 54). The above-mentioned suppressive functions from Tregs are introduced by a number of soluble and membrane-bound molecules such as cytokines with inhibitory effect (e.g., IL-10, TGF-β and IL-35), enzymes or proteins relevant to cytolysis (e.g., granzymes A, B and K, and perforin), membrane proteins and signaling molecules relevant to metabolic homeostasis (e.g., CD25, CD39, CD73, cAMP, LAG3, adenosine receptor 2, and histamine receptor 2), and surface molecules (e.g., PD-1 and CTLA-4) for targeting DCs (38, 55–57).

The key role played by IL-10 and TGF-β in the context of allergic asthma is now well established (summarized in **Table 1**). Apart from Tregs, IL-10 is also released by Bregs, monocytes, a small fraction of NK cells, macrophages, DCs and ILCs (15). IL-10 exerts its effect on both innate and adaptive immune responses, thereby inducing immune tolerance and dampening tissue inflammation (90). For example, transfer of OVA peptide-specific Tregs to OVA-sensitized mice repressed AHR, eosinophil recruitment, and Th2 cytokine expression in the lung following allergen challenge (5), which was reversed by the application of IL-10R blocking antibody (5). It is worthy of note that the IL-10-producing Tr1 cells also represent an essential mechanism in immune tolerance to a high dose of allergens in nonallergic individuals, such as high dose bee venom exposure in beekeepers by natural bee stings (55).

TGF-β is a pleiotropic cytokine and Tregs are the major source of its secretion. The implication of TGF-β in allergic asthma is complicated, and confronting effects are observed (38). It has been recognized that TGF-β produced by Tregs is indispensable for the *in vivo* pTreg expansion and immunomodulatory functionality in an autocrine manner, which is crucial for the induction of immune tolerance and design of alternative mucosal vaccination strategies. However, TGF-β has also been noted to increase mucus production, promote airway remodeling and fibrosis in asthmatic settings, which could be prevented by the anti-TGF-β therapy in allergen challenged animals (91). Therefore, further studies are required to elucidate the paradoxical role of TGF-β in the regulation of allergic asthma.

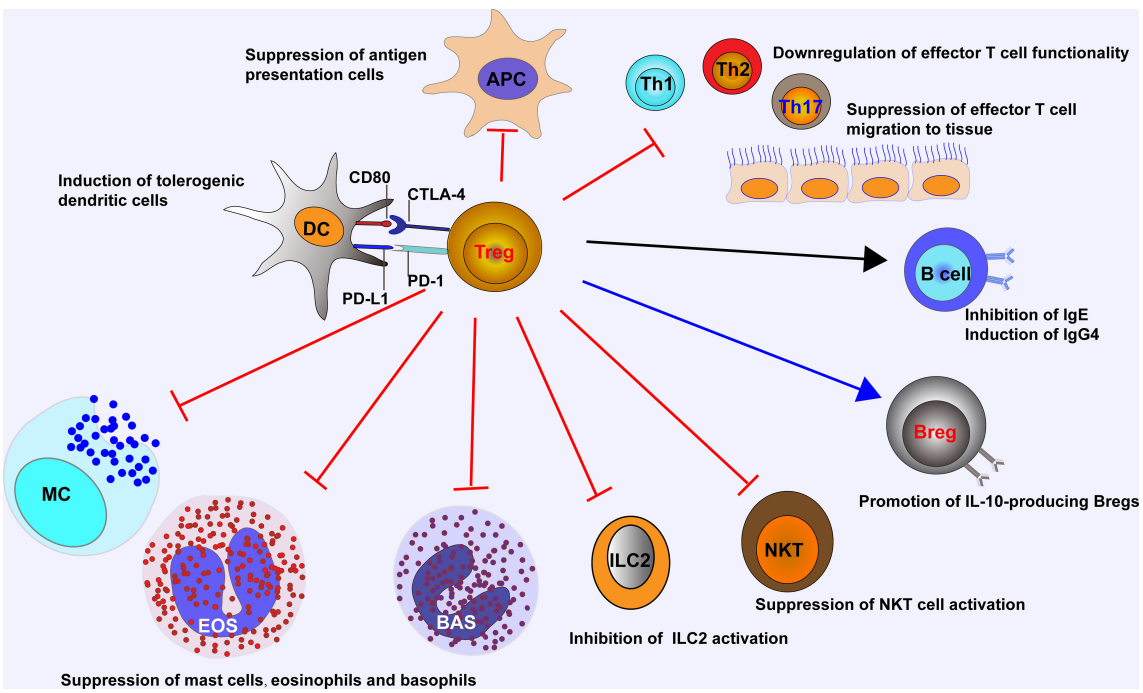

FIGURE 1 | Tregs control ongoing inflammation by acting on major cells that drive allergic reaction, including antigen presentation cells, Teffs, NKT cells, ILC2s, eosinophils, basophils, and mast cells. Tregs suppress IgE-producing B cells, while induce IgG4-producing B cells and IL-10-producing Bregs, and promote the generation of tolerogenic dendritic cells. APC, antigen presentation cell; EOS, eosinophil; BAS, basophil; MC, mast cell.

TABLE 1 | Summarized functions of IL-10 and TGF-β in allergic asthma.

IL-10	TGF-β
Inhibits antigen present cell (APC) maturation, antigen presentation and pro-inflammatory cytokine secretion (58) Induces IL-10-producing DCs (59)	Inhibits DC maturation and antigen presentation; promotes Langerhans cell development (60, 61) Stimulates cells at the resting state (monocytes), whereas activated cells (macrophages) are inhibited (62) Inhibits expression of FcεRI (65) Promotes chemotaxis of neutrophils, eosinophils, and mast cells (66–68)
Inhibits mast cell activation and release of pro-inflammatory cytokines (63) Inhibits eosinophil and basophil cytokine production (5) Suppresses ILC2 activation and cytokine production (64) Suppresses allergen-specific Teffs (69)	Suppresses allergen-specific Teffs (70, 71) Associates with CTLA-4 expression on T cells (72) Promotes T cell survival (73)
Suppresses IgE (74) Induces IgG4 and IgA (75)	Suppresses class switching to the majority of IgG isotypes (76, 77) Suppresses IgE (78) Induces IgA (79)
Enhances B cell survival (75, 80)	Inhibits B cell proliferation (81) Promotes apoptosis of naïve or immature B cells (82, 83)
Promotes the generation of Tr1 cells (84)	Induces Foxp3 and suppressive function of Tregs (72, 85) Induces Th9, Th17 and Tfh cells under different conditions (86–88)
Synergistic effect in *in vivo* suppression with CTLA-4, PD-1 and TGF-β (89)	Synergistic effect in *in vivo* suppression with CTLA-4, PD-1 and IL-10 (89)

THE IMPLICATION OF TREGS IN AIRWAY REMODELING

Airway remodeling refers to the pathological restructuring of the small and large airways in asthma, including neovascularization, subepithelial fibrosis, loss of epithelial integrity, goblet cell and mucus gland enlargement, and increased airway smooth muscle (ASM) mass (92). These pathophysiological changes result in alterations in the composition and structural organization of molecular and cellular components that constitute the airway. As a consequence, the asthmatic patients manifest the presence of airway narrowing and edema, AHR, and mucus hypersecretion, which is relevant to the poor clinical outcomes in asthmatic patients (93).

Airway remodeling is induced by factors from both inflammatory cells and structural cells. In general, the change of structural cells is under the influence of inflammatory cells (94). A variety of inflammatory cells presented in the asthmatic airways is able to produce mediators that have the potential to induce airway remodeling, such as cysteinyl leukotrienes (CysLTs), IL-13, endothelins, TGF-β, and epidermal growth factor (EGF). Vascular endothelial growth factor (VEGF) is an angiogenic factor, which has also been shown to induce airway remodeling and enhance Th2-mediated lung inflammation (95). Secretion of VEGF by cultured ASM cells is upregulated in response to the proinflammatory cytokines, TNF-α and IL-1β (96), or the Th2 cytokines, IL-13 and IL-4 (97). Therefore, the infiltrated immune cells are considered as "amplifiers" of airway remodeling (98). Indeed, sustained immune responses are key drivers in exacerbating the development of airway remodeling. For example, cytokines produced by the infiltrated Th2 cells, such as IL-4 and IL-13 enhance subepithelial fibrosis, mucous hyperplasia, and collagen deposition to promote airway remodeling (99–101). Similarly, alveolar macrophages contribute to airway remodeling through the release of matrix metalloproteinases to alter the extracellular matrix (ECM) and airway structure (102). Although the role of Th17 cells in airway remodeling remains controversial, a synergistic effect of DCs together with Th17 cytokines to promote the accumulation of fibrotic matrix components that correlate with TGF-β expression had been observed (103). It is noteworthy that all of above mentioned immune responses are relevant to the suppressive function of Tregs, and therefore, the role of Tregs in airway remodeling has recently been highly appreciated.

Indeed, data collected from animal studies characterized signaling molecules and transcription factors implicated in airway remodeling, which are closely related to Treg function (94, 104, 105). Specifically, transfer of $CD4^+CD25^+$ Tregs at the peak of acute inflammation before the onset of airway remodeling reversed established airway inflammation and prevented the development of airway remodeling (106), supporting a role of Tregs in the prevention of airway remodeling. Similarly, adoptive transfer of tTregs caused a substantial reduction in bronchoalveolar lavage eosinophil composition and suppressed airway remodeling and T cell migration into the lung of STAT6 and RAG2 double knockout mice, confirming the role of Tregs in repressing allergic airway inflammation and remodeling (107). There is also feasible evidence that Th17 responses in chronic allergic airway inflammation abrogate Treg-mediated tolerance, and thereby contributing to airway remodeling (108). More interestingly, adoptive transfer of Tregs into OVA-induced asthmatic mice at the chronic stage selectively reduced the vessel numbers in both peritracheal and peribronchial regions and the lung parenchyma (109), which indicate a potential role of Tregs in the regulation of structural cells such as endothelial cells, smooth muscle cells and mesenchymal cells during the development of airway remodeling in asthmatic settings other than immune cells. Therefore, the exact impact of Tregs on airway remodeling in asthmatic setting is worthy of further investigations.

THE ROLE OF TREGS IN AIRWAY EPITHELIAL REPAIR

Asthmatic patients generally manifest different levels of chronic airway inflammation with airway epithelial damage that occurs even in mild, early and nonfatal asthma (110, 111). Damage and shedding of airway epithelial cells are important pathological features of asthma, and altered epithelium in the airway is more susceptible to injury and apoptosis than those from non-asthmatic individuals (112). Specifically, epithelial cells derived from asthmatic patients collected by bronchial brushing seem to be more hyperreactive and less viable (113), which likely results from inflammatory damage. Furthermore, the airway of asthmatic patients is characterized by the dysregulation of airway epithelial repair, leading to a chronic cycle of wound repair coupled with bronchial remodeling (110).

Except for the immunosuppressive function and capacity to limit the intensity and sustained time of immune responses, Tregs also participate in non-immunological processes such as tissues repair during extensive inflammation. The presence of Tregs has been documented in several non-lymphoid tissues, including lung, skin, placenta, intestinal mucosa, adipose tissue, and atherosclerotic plaques (114). Tregs rapidly accumulate in the acutely injured skeletal muscle of mice. Ablation of Tregs impairs muscle repair due to decreased amphiregulin, an epidermal growth factor family member known to promote healing and tissue regeneration (115). Another study demonstrates that amphiregulin deficiency in Tregs results in severe acute lung damage and a rapid decline in lung function during influenza virus infection. In addition, anti-viral immune responses and suppressive function of Tregs are unaltered, suggesting these two functions are invoked by separable cues (116). Nevertheless, implication of Tregs in airway epithelial repair in the context of asthma has not yet been reported, which is necessary for further investigations.

DYSREGULATION OF TREGS

Increasing clinical evidence supports that dysregulated Tregs play an important role in the pathogenesis and chronicity of allergic asthma. In patients with asthma and other allergic diseases, the expression of *FOXP3* is reduced as compared to that of healthy controls (117). In atopic children, tTreg maturation is significantly delayed as compared to that of age-matched nonatopic children (118). Additionally, Tregs in patients with allergic asthma exhibit impaired suppressive function compared to those Tregs from healthy controls (11, 119).

Several subsets of dysfunctional Tregs are relevant to allergic asthma. Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) is one of the functional prostaglandin D2 (PGD2) receptors, and regarded as a potent inducer of type 2 cytokine secretion (48). The allergic asthma patients have more $CRTH2^+$ Tregs in the peripheral blood than healthy controls (120). These $CRTH2^+$ Tregs produce greater amounts of IL-4 and show less suppressive function than that of $CRTH2^-$ Tregs in the PGD2-stimulated cultures (120). Another dysregulated Treg subpopulation is the ILT3 (also known as gp49B or CD85k)-expressing Tregs. Flow cytometry detected a substantially elevated percentage of $ILT3^+$ Tregs in mice with massive asthma-like airway pathologies, which promoted the maturation of $IRF4^+PD\text{-}L2^+$ DCs to activate Th2 cells (121). Simultaneously, $ILT3^+$ Tregs displayed a compromised suppressive function owing to low expression of FOXP3 and Helios (122). In mice, the expression of IL-33 receptor ST2 has been identified in $Foxp3^+$ Tregs in the lung (123). In the presence of IL-33, Tregs display upregulated expression of canonical Th2 transcription factor GATA3 and IL-33 receptor ST2 along with enhanced secretion of type 2 cytokines (122). Furthermore, Tregs lose their ability to suppress Teffs once exposed to IL-33 (122). However, *in vitro* studies suggest that $ST2^+$ Tregs are highly activated and superior to $ST2^-$ Tregs in suppressing $CD4^+$ T cell proliferation through IL-10 and TGF-β release (124). Although those *in vitro* data may not mimic the *in vivo* situation, further investigations would be necessary to fully address this question. Pro-inflammatory cytokine-secreting Tregs such as IFN-γ-producing $FOXP3^+$ cells, IL-4-producing $FOXP3^+$ cells, and IL-17-producing $FOXP3^+$ cells are also noticed in asthmatic patients, which are strongly correlated with the severity of asthma and might be insensitive to corticosteroids (125, 126).

TREG-BASED STRATEGIES FOR ASTHMA THERAPY

It is generally accepted that Tregs are a promising candidate for developing effective therapies to treat immune disorders such as allergic asthma. Current preclinical studies and clinical trials of Treg-based therapies are mainly on the basis of two approaches: one of which is to directly boost Treg number and functionality *in vivo*, and the other is re-administration of purified, *ex vivo* modified, GMP (good manufacturing practice)-compliant Tregs back to patients (127).

Several approaches have been employed to promote the *in vivo* expansion of Tregs or depletion of Teffs, leading to an increased Treg/Teff ratio. These therapies include the administration of IL-2 or IL-2/anti-IL-2 mAb complex, anti-CD3 mAbs, mTOR inhibitors, and dietary or microbe-derived pro-tolerogenic stimulators (127). Although high dose of IL-2 has been used for immunotherapy against metastatic cancer (128), low-dose of IL-2, however, preferentially stimulates Tregs and has shown a great potential of success in Treg-based immunosuppressive strategies against autoimmune and inflammatory diseases (129). Advances in the knowledge of the functional, biophysical and structural characteristics of IL-2 have promoted the generation of IL-2 formulations, such as IL-2/anti-IL-2 mAb complexes (130). Depending on the clone of the anti-IL-2 mAb, IL-2/anti-IL-2 mAb complex exerts differential effect on the expansion of T cell subsets (131). Studies in mice revealed

that IL-2/anti-IL-2 mAb complexed with S4B6 clone induces the preferential proliferation of $CD8^+$ T cells, while the IL-2/JES6-1 complex preferentially induces the expansion of Tregs by blocking the interaction of IL-2 with IL-2Rβ (CD122) and IL-2Rγ (CD132), and promoting the interaction of IL-2 with IL-2Rα (CD25) (131, 132). Particularly, the IL-2/JES6-1 complexes have already manifested exciting results in terms of suppressing organ transplant rejection (133), autoimmune and inflammatory diseases in mice such as type 1 diabetes (134), dextran sodium sulfate-induced colitis (132), experimental myasthenia (135), collagen-induced arthritis (136), experimental autoimmune encephalomyelitis (133), and allergic airway disease (137). In the model of established airway allergy, treatment with IL-2/JES6-1 complex dampens eosinophilia and airway inflammation, and inhibits the production of eotaxin-1 and IL-5 (137). Mucus production, AHR to methacholine, and parenchymal tissue inflammation are also dramatically reduced following IL-2/anti-IL-2 mAb complex administration, which is dependent on Treg-derived IL-10 (137). Interestingly, administration of IL-2/JES6-1 complex also improves some manifestations of metabolic diseases, such as obesity related chronic inflammation and insulin resistance, which are characterized by the inflammatory infiltration of immune cells in the adipose tissues that are amenable to Treg modulation (138).

In neoplastic diseases, adoptive cell therapies (ACT) use T cells engineered to express either Ag-specific TCRs or chimeric Ag receptors (CARs) targeting specific tumor antigens to selectively eliminate target cells, which have been approved for the treatment of acute lymphoblastic leukemia and advanced lymphomas (127). In addition to killing cancerous cells, ACT can also be used to regain appropriate Treg function in the inflammatory context. Polyclonal expansion of Tregs *via* TCR represents the initial strategy for ACT. Unlike other type of Tregs, antigen-specific Tregs are more potent in controlling local inflammation and inhibiting T cell priming in secondary lymphoid tissues (139). More recently, a number of publications demonstrate the utility of CARs in Tregs (140). In this case, Tregs are reinfused after engineering with chimeric TCR of different types. CAR-Tregs have several characteristics versus TCR-Tregs: (1) non-MHC-restriction and less dependent on IL-2; (2) the hinge region provides flexibility, which enables CARs binding to antigen in various orientations; (3) higher antigen affinity than TCRs; and (4) more precise control of the type of antigen-stimulated response (141).

Current preclinical studies and clinical trials for Treg ACTs in inflammatory disorders have indicated the efficacy and technical feasibility of these methods (142, 143). In experimentally induced allergic asthma, CAR-redirected Tregs suppressed allergic airway inflammation, prevented excessive pulmonary mucus production, and attenuated the increase of allergen-specific IgE and Th2 cytokine levels (144). Over the past few decades, autoimmune involvement in the pathogenesis of asthma has been proposed due to the presence of circulating autoantibodies against diverse self-antigens/structures (145). Some patients with severe asthma have autoantibodies against eosinophil peroxidase (EPX) and autologous cellular components in the sputum, which may necessitate an increase for the maintenance of corticosteroids (146). These findings raise the potential of utilizing CAR-Treg ACT in severe and therapy-refractory asthmatics. However, many important issues such as managing the stability and plasticity of Tregs, directing their homing to the desired sites, and safety concerns are still waiting to be worked out.

CONCLUSION REMARKS

Allergic asthma involves complex innate and adaptive immune responses to environmental allergens, resulting in airway inflammation predominately mediated by Th2-type cells and allergen-specific IgE (147). Both human and animal studies show that Tregs are essential for the maintenance of self-tolerance and immune homeostasis, and therefore, Tregs defects are observed in asthmatic individuals as compared to healthy controls in terms of their functionality. These discoveries promoted the development of technologies with Treg-based therapies, such as Treg expansion and CAR-Tregs, which may represent a viable approach for curative therapy of allergic diseases. Despite the current achievements, some critical issues, such as how to improve the safety of Tregs, increase the stability of Tregs, and direct their homing to the desired sites, are yet to be elucidated. As a result, additional in-depth studies are necessary to improve current therapeutic approaches against asthma in clinical settings.

AUTHOR CONTRIBUTIONS

JZ, YZ, and LC wrote the manuscript and prepared the figure. QX collected and analyzed the information. YW, MX, and XL reviewed the manuscript. JPZ and C-YW supervised the conception and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Costimulation blockade and Tregs in solid organ transplantation

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Regulatory T cells (Tregs) play a critical role in maintaining self-tolerance and in containing allo-immune responses in the context of transplantation. Recent advances yielded the approval of the first pharmaceutical costimulation blockers (abatacept and belatacept), with more of them in the pipeline. These costimulation blockers inhibit effector cells with high clinical efficacy to control disease activity, but might inadvertently also affect Tregs. Treg homeostasis is controlled by a complex network of costimulatory and coinhibitory signals, including CD28, the main target of abatacept/belatacept, and CTLA4, PD-1 and ICOS. This review shall give an overview on what effects the therapeutic manipulation of costimulation has on Treg function in transplantation.

KEYWORDS

Treg - regulatory T cell, transplantation, costimulation blockade, CTLA4 Ig IL-2, immunosuppressant, costimulation

Introduction

Tregs

Natural, thymus-derived regulatory T cells express CD4 and are characterized by the surface expression of the high affinity chain (alpha-unit) of the IL-2 receptor (CD25) and a diminished expression of the alpha-unit of the IL-7 receptor (CD127). They are further defined by the expression of the X-chromosome encoded transcription factor forkhead box P3 (FoxP3), which controls Treg development, plasticity and stability (1). Of note, FoxP3 is not absolutely required for a suppressive T cell phenotype. There are defined subsets of T cells which do not express FoxP3$^-$ but have suppressive function (2, 3). Tr1cells are a prominent example of FoxP3- regulatory cells, with critical roles in suppression of inflammation (4) and with therapeutic potential (5). There are two main types of FoxP3+ Tregs that can be distinguished with overlapping features but also

distinct properties. The main proportion of Tregs consists of thymic-derived Tregs (tTregs, formerly classified as natural Tregs (nTregs)). tTregs develop in the thymus and are typically characterized by the expression of helios (6) and neuropilin 1 (nrp1) (7, 8). In contrast to that, peripheral Tregs (pTregs) develop from $CD4^+$ $FoxP3^-$ cells upon antigen stimulation in the presence of distinct anti-inflammatory cytokines. pTregs develop primarily in the intestinal system (9) and the placenta (10). Whereas tTregs are supposed to be essential to control systemic and tissue specific autoimmunity, pTregs control commensal microbiota composition and Th2 responses (11).

Tregs are crucial to maintain self-tolerance and control an overall immune response (12). It has been shown, that absence or mutation of FoxP3 leads to severe autoimmune disease in mice (scurfy phenotype) (13, 14) and humans respectively (IPEX immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (3, 15). In humans, approximately 1-3% of all $CD4^+$ T cells are regulatory T cells (16, 17), however, the numbers may vary substantially between individuals, and further show a distinct distribution in various tissues in the human body. The pleiotropic mechanisms of action of Treg mediated immune modulation include the production of anti-inflammatory cytokines (IL-10, TGFβ, IL-35) (18), expression of co-inhibitory molecules (CTLA4, PD-1, LAG3) (19, 20) and cytotoxic suppression *via* granzyme A, B and perforin (18, 21). Additionally, IL-2 consumption *via* the high affinity unit of the IL-2 receptor (CD25) contributes to a down regulation of an overall immune response. Moreover, Tregs remove peptide-MHC complexes from the surface of dendritic cells (DC), thereby leading to antigen-specific regulation (22). Several of these mechanisms are often contributing to regulation. Notably, the expression of CTLA4 seems particularly important as Treg-specific CTLA4 deficiency results in an impaired *in vivo* and *in vitro* suppressive Treg function (20, 23). A main mechanism of CTLA4 is the removal of B7 molecules from the surface of antigen presenting cells (mainly migratory dendritic cells) by CTLA4-mediated trans-endocytosis (19, 24).

Several costimulation blockers have been approved for clinical use and many more are currently investigated in preclinical studies. Given the tight interplay between Tregs and costimulatory signals, knowledge about these interactions is crucial especially in diseases where Tregs play an important role (e.g. transplantation, auto-immune disease, cancer…).

Tregs in transplantation

As a pivotal part in regulation of the immune system, Tregs also play a major role in allogeneic transplantation (25, 26). In this context, Tregs can intercept at several critical steps during allo-immune responses: Tregs can prevent priming of indirectly alloreactive T cells by removing peptide-MHC complexes and B7 molecules from the surface of dendritic cells. Furthermore, Tregs can restrict expansion of allo-antigen specific follicular T and B-cells and thereby confine humoral allo-immunity (27). Within the allograft itself, Tregs can create a privileged environment through consumption of IL-2 and secretion of immunosuppressive cytokines and metabolites like IL-10 and Adenosine (28). Through *infectious tolerance*, new generations of Tregs can be recruited to and induced within the allograft. Thereby Treg-mediated intra-graft regulation might be self-sustaining (29).

Accordingly, operationally tolerant patients with a liver allograft display significantly higher levels of Tregs than matched control patients (30, 31). Tregs were also shown to be indispensable for deliberate induction and maintenance of donor-specific transplantation tolerance in several models (32).

Given the important role in immune regulation, the exploitation of Tregs has become an attractive aim in order to reduce life-long immunosuppression. In preclinical studies the therapeutic use of Tregs prolongs allograft survival (33–35). Currently, the potential of adoptive Treg therapy in solid organ transplantation is explored in several clinical trials (36–39) with the first preliminary evidence emerging for the efficacy of Treg therapy (40).

Homeostatic control of Tregs

Several factors contribute to Treg homeostasis to maintain numbers within a physiological range. One major stimulus is signaling *via* the IL-2 receptor and activation of the STAT5 signaling pathway. As Tregs are incapable of self-producing IL-2, abundance of this cytokine is crucial for Tregs survival especially in mature FoxP3 positive regulatory T cells (41, 42). Interestingly, FoxP3 induces a pro-apoptotic protein signature and a reduced expression of pro survival Bcl-2 molecules, leading to FoxP3 induced death in most newly arising Tregs. This lethality can be prevented in presence of (the limited) IL-2 signaling *via* the common gamma chain (43). As Tregs consume IL-2, Treg depletion leads to higher levels of IL-2 underlying the importance of Tregs in controlling the abundance of IL-2 (44).

However, Treg homeostasis and function is tightly regulated *via* numerous costimulatory signals in order to keep the fine balance between immunosuppression (potentially resulting in infection or malignancy) and avoiding excessive immune activation and autoimmunity.

The complex crosstalk between PD1-PDL1 and CD28/CTLA4-B7

On conventional T-cells, PD-1 is upregulated upon T-cell receptor (TCR) mediated stimulation (45). Interaction with its

ligands, PD-L1 and PD-L2, restricts further activation and proliferation of T-cells, thereby providing a central immune checkpoint to contain excessive immune responses (46, 47). This co-inhibitory signal is (at least partly) conveyed through downregulation of the PI3K pathway, providing direct antagonism to CD28-mediated costimulation (resulting in PI3K activation). On regulatory T-cells however, PD-1 seems to take on a distinctive role, which might be at least partly depending on the activity of CD28.

With an unaltered CD28 pathway, a conditional PD-1 knockout specifically in Tregs has been shown to enhance their suppressive capacity. In this context, Tregs lacking PD-1 out-proliferated conventional Tregs *in vitro*, protected NOD mice from diabetes and mitigated the severity of induced autoimmune encephalitis (48). In contrast, when the CD28-B7 interaction was disrupted (using CTLA4Ig) in transgenic mice overexpressing PD-1 on T-cells (including Tregs), PD-1^{high} Tregs demonstrated greater suppressive function, allowing for long-term survival of fully mismatched cardiac allografts (49). Transgenic PD-1^{high} Tregs under costimulation blockade expressed greater amounts of cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and inducible T-cell costimulator (ICOS). Interestingly, active ICOS signaling in transgenic PD-1^{high} Tregs was required for the survival benefit in cardiac transplantation in this model. Although some of these differential results might also be explained by differences in the models used (auto vs. allo-immunity), these data indicate a complex interconnection between CD28, PD-1 and ICOS signaling in regulatory T-cells. In this context, our group has demonstrated that both PD-1 and CTLA4 are indispensable for maintaining chimerism and transplantation tolerance in a murine mixed chimerism model employing Treg-cell therapy and costimulation blockade with CTLA4Ig (50).

PD-1 upregulation has also been observed upon interleukin-2 (IL-2) stimulation of regulatory Tregs for *in vitro* and *in vivo* expansion. Asano and colleagues demonstrated an increased surface expression of PD-1 in Tregs during *in vitro* expansion in mice (with recombinant IL-2) and *in vivo* expansion using low-dose IL-2 in mice as well as human GvHD patients (51). Interestingly, when PD-1 signaling in Tregs was intercepted during expansion (in murine *in vitro* and *in-vivo* expansion) using PD-1 knockout Tregs or anti-PD-1 antibodies, Treg proliferation initially spiked, but then rapidly diminished due to FAS-dependent apoptosis induction and reduced BCL-2 expression on Tregs. These data indicate a central role for PD-1 as modulator of Treg homeostasis in clinically relevant Treg-expansion protocols.

Similar observations regarding the upregulation of PD-1 during *in vivo* Treg expansion have been made by our group using IL-2 complexes (IL-2 cplxs: IL-2 complexed with an anti-IL-2 antibody to sterically inhibit the binding to CD122 on CD8 T-cells and NK-cells while selectively expanding regulatory T-cells *via* CD25) for *in vivo* Treg expansion in a murine mixed chimerism model. In addition to PD-1, Tregs also upregulated ICOS and CTLA4 upon stimulation with IL-2 complexes (52). Together with CTLA4Ig these *in vivo* expanded Tregs facilitated long-term survival of fully mismatched cardiac allografts in mice (34).

The group of Robert Negrin has recently engineered an orthogonal interleukin-2 that selectively binds to an orthogonal IL-2 receptor (but not the native IL-2 receptor), that was introduced in regulatory T-cells (53). This model elegantly allows to provide IL-2 stimulation exclusively to transferred Tregs expressing the orthogonal IL-2 receptor. Also, in this context, stimulation with orthogonal IL-2 *in vitro* and *in vivo* was accompanied with an upregulation of ICOS in the transfected Tregs (PD-1 and CTLA4 were not assessed). Transferred orthogonal IL-2R Tregs facilitated induction of long-term mixed chimerism and subsequent donor-specific tolerance towards cardiac allografts.

These models, while using different strategies to deliver IL-2 selectively to regulatory T-cells, commonly demonstrate that IL-2 stimulation of Tregs is accompanied by an upregulation of PD-1, ICOS and CTLA4. In most of these reports and especially under costimulation blockade with CTLA4Ig, PD-1 upregulation was associated with enhanced suppressive function of regulatory T-cells. Another possible explanation for the observed disparity in Treg functionality with or without intact PD-1 signaling between autoimmunity and transplantation might be the PD-1 ligand (PD-L1) expression within the graft itself. PD-L1 is expressed by vascular endothelial cells and rapidly upregulated upon pro-inflammatory stimuli *via* interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNF-α) (54). Reduced PD-L1 expression within cardiac allografts has been associated with an increased incidence of acute T-cell mediated rejections (55). Mechanistically, endothelial PD-L1 has been shown to reduce graft infiltration of CD8 T-cells expressing a memory phenotype (56, 57). Beyond this, endothelial PD-L1 might also interact with PD-1 on regulatory T-cells. A recent report suggests a novel role for PD-1 expressing Tregs in regulating endothelial trans-migration of lymphocytes through interaction with endothelial PD-L1 on lymphatic endothelial cells (58).

In this context, the effect that endothelial PD-L1 within the allograft itself has on PD-1 expressing regulatory T-cells could be of great interest and yet needs to be elucidated.

Also, on antigen-presenting cells, the PD-1 and CD28/CTLA4 pathways are strongly interconnected. Experimental data have suggested that CD80 (B7.1) and PD-L1 (CD274) can bind each other (59). The original assumption was that this interaction involves CD80 and PD-L1 expressed by two different cells (*trans*). Recent reports however suggest that CD80 and PD-L1 rather interact in a *cis* structure, forming CD80:PD-L1 heterodimers on the same cell (60). In this heterodimerized form, PD-L1 cannot be accessed by PD-1 on T-cells. This has been identified as one key mechanism by which PD-1 activity is

restricted during T-cell activation to yield optimal T-cell responses (61).

Heterodimerization also impacts the accessibility of CD80 to its trans-ligands CD28 and CTLA4. While the binding of CD28 to CD80 is preserved, even in the cisCD80:PD-L1 form, CTLA4 cannot engage with heterodimerized CD80 [likely due to its multivalent zipper-like binding structure (62)]. Consequently, heterodimerized CD80 has been shown to be protected from CTLA4-mediated trans-endocytosis (63).

Thereby, upregulation of CD80 and increased CD80:PD-L1 heterodimerization on APC might lead to repression of co-inhibition by PD-1 (by reducing available PD-L1) and CTLA4 (by restraining trans-endocytosis of CD80) while preserving CD28 co-stimulation. In turn this might result in increased T-cell activation. Recent work by the group of Shimon Sakaguchi has demonstrated how regulatory T-cells can influence the balance between heterodimerized and "free" PD-L1 on APC (64): Through trogocytosis, Tregs can deplete (non-heterodimerized) CD80 from the APC's surface, resulting in less cisCD80:PD-L1 heterodimerization and more "free" PD-L1 available to inhibit PD-1 expressing T-cells. These reports highlight the complex link between CTLA4-and PD1-mediated suppression of T-cell responses by regulatory T-cells.

Under steady-state conditions, Tregs control the amount of available CD80 on antigen-presenting cells (APC) *via* competitive inhibition and removal through trans-endocytosis. Both mechanisms rely on CTLA4 binding to CD80. This tight restriction on free CD80 results in limited formation of CD80:PD-L1 heterodimers and a high abundance of *free* PD-L1 (homodimers) providing co-inhibitory signals to engaging T-cells through PD-1 (Figure 1, left).

Under inflammatory conditions, upregulation of CD80 results in a higher rate of CD80:PD-L1 heterodimerization. While CD80 within those heterodimers is not accessible for CTLA4, it maintains affinity for CD28. Thus, in a heterodimerized form, CD80 can evade Treg-mediated control. As heterodimerized PD-L1, on the other side, loses affinity to its ligand PD-1 (on T-cells), in this setting, co-stimulatory signaling *via* CD28 prevails (Figure 1, right).

Cis-heterodimerization to PD-L1 might not only allow CD80 to evade Treg mediated control (via CTLA4), but potentially also costimulation blockade with CTLA4Ig. Experimental data investigating the effect of CTLA4-Ig (or other pharmacological interventions) on CD80:PDL1 heterodimer formation would therefore be of great interest to the field.

CD28/CTLA4-B7 pathway and its blockade

The CD28/CTLA4 pathway is one of the most thoroughly studied costimulatory pathways. CD28 ligation *via* B7 molecules expressed on antigen presenting cells (signal 2) is crucial for T cell activation in combination with TCR/MHC interaction (signal 1). As absence of signal 2 in the presence of signal 1 renders T cells anergic (65) the concept of selective blockade of signal 2 has become attractive in order to therapeutically modulate immune responses in the clinical setting. Of note, CD28 engagement by B7 (CD80; CD86) is not only required for conventional T cells but also for Treg homeostasis (66, 67). Interestingly, CD86 appears to be the dominant ligand for Treg proliferation in spite of its approximately 10-times lower affinity to CD28 than CD80 (68). This can be explained by a constitutive high surface expression of CTLA4 on Tregs that selectively impair CD80/CD28 interaction (69).

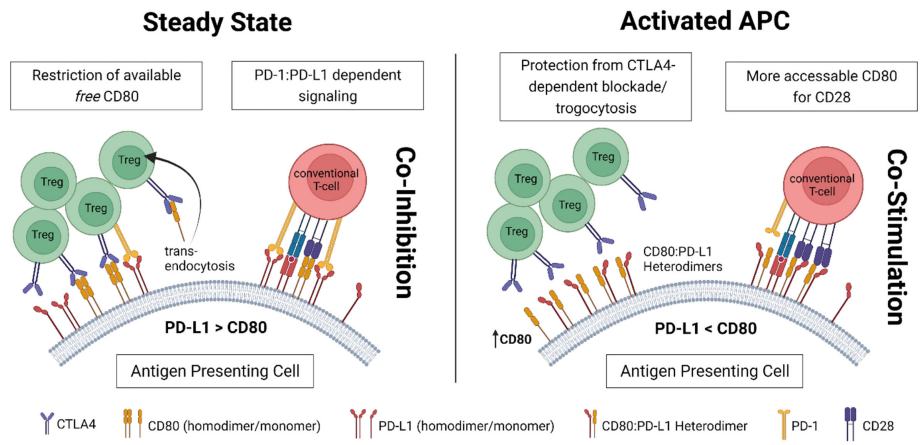

FIGURE 1
Proposed concept for the complex interconnection between Tregs and CD80:PD-L1 cis-heterodimers on antigen-presenting cells (APC).

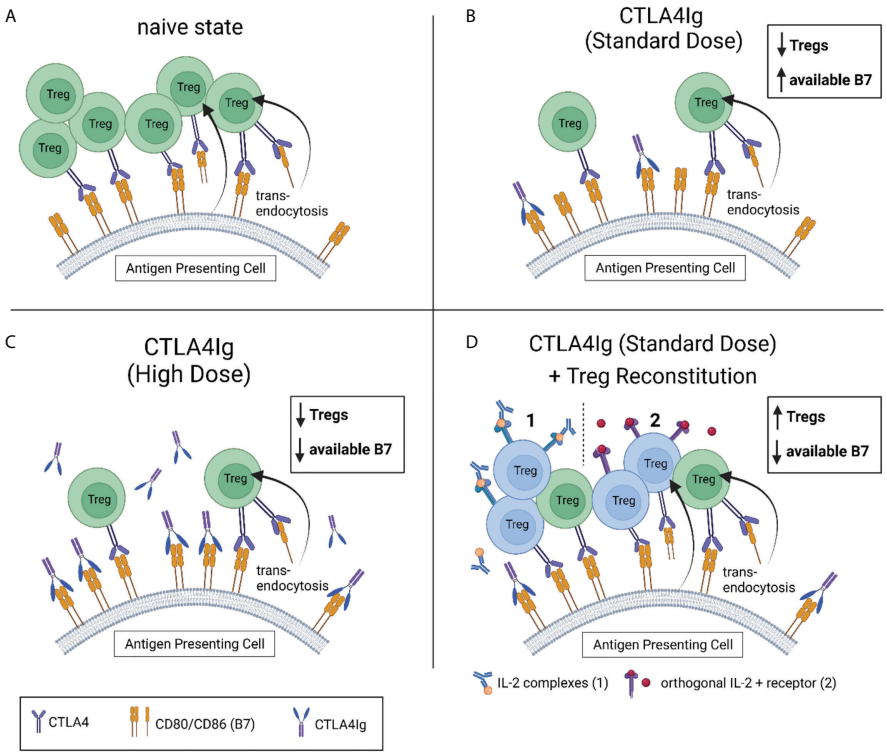

FIGURE 2
Strategies to compensate for reduced Treg levels under CTLA4Ig. **(A)** Tregs control the amount of available of CD80 and CD86 (B7) on antigen presenting cells (APC) through competitive inhibition and trans-endocytosis. **(B)** CTLA4Ig causes a dose-independent drop in Treg frequencies, contradicting their restriction on surface B7. Subsequently, more B7 is available for CD28-mediated co-stimulation. Standard doses of CTLA4Ig (10mg/kg) are not sufficient to bind all available B7. This can be compensated by administering higher doses of CTLA4Ig (50mg/kg) in the experimental setting **(C)**, or by reconstituting the recipient's Treg levels (to or beyond naïve levels). Two promising strategies to reconstitute Tregs under costimulation blockade are depicted in **(D)** (1). Interleukin-2 complexed with an anti-IL-2 monoclonal antibody (IL-2 complexes) has been successfully used to *selectively* expand Tregs under costimulation blockade *in vivo*. (2) Engineered Tregs expressing a modified *orthogonal* IL-2 receptor that exclusively binds a modified (*orthogonal*) IL-2 have successfully been used for adoptive cell therapy in a mixed chimerism model. In both models, the re-established control of B7 expression on APCs by reconstituted Tregs has permitted sufficient immunosuppression with CTLA4Ig in standard dosing.

In order to control conventional T cell activation, different approaches were used to inhibit B7/CD28 binding with the most promising strategy being the use of the fusion protein CTLA4Ig. Experimental research led to the development of abatacept, which now is approved for treating rheumatoid arthritis and recently also as GvHD prophylaxis by the FDA (70), and ultimately belatacept. Belatacept is a modified CTLA4Ig with a higher binding avidity to human B7 molecules (2-fold higher avidity for B7.1 and 4-fold higher avidity for B7.2 resulting in a 10 fold higher biological potency compared to conventional CTLA4Ig) (71), which has been approved for treating kidney transplant recipients. The main benefit of belatacept is probably the absence of nephrotoxic side effects compared to the conventionally used Calcineurin inhibitors (CNI) and in addition the improved patient adherence due to the monthly i.v. application of the drug. Long-term studies highlight the excellent allograft (kidney-) function which is preserved over time (72). However, in spite of the initial success, enthusiasm was dampened by higher rates of T-cell mediated rejections, especially in the early phase after transplantation, observed under belatacept compared to CNIs (73).

We have shown previously that the immunosuppressive capacity of CTLA4Ig is Treg-dependent at low but not high doses (74). However, the relationship between CTLA4Ig and Tregs remains incompletely understood. On the one hand there is a well-established negative impact of CTLA4/CD28-targeted costimulation blockade on Tregs (75, 76). Even though the exact mechanism of action has not been fully discovered it is likely that the negative effect on Treg numbers results from less available IL-2 (42) and decreased CD28 signaling which is essential for intrathymic Treg generation (67) and proper Treg function (77). This concept is further supported by the observation of a higher dependency on CD28 than conventional T cells (78). The negative effect of CTL4Ig on the number of Tregs is dose-independent and

the main proportion of Tregs affected are helios$^+$, nrp1$^+$ tTregs (74). However, despite a reduction in Treg numbers *in vivo*, CTLA4Ig might also have a beneficial effect on Treg function and/or generation depending on the context (79).

For instance, murine iTreg generation and suppressor function was improved by CTLA4Ig *in vitro* (80). These findings are underlined by the observation that the addition of belatacept might enhance Treg mediated *in vitro* suppression of allogeneic immune response without affecting viability, proliferation or expression of functional Treg markers (81).

In a clinical study of kidney transplantation, there is a positive impact of costimulation blockade combined with mTor inhibition on Treg numbers with a sustained anti-donor suppressive activity compared to patients with a CNI-based immunosuppressive regimen (82). Similarly, belatacept treatment had no short or long-term effect on regulatory T-cell frequencies and *in vitro* functionality when compared to CNI in a *post hoc* analysis of the BENIFIT trials (83). Interestingly, there is evidence that costimulation blockade with CTLA4Ig might negatively affect CD44^{high} memory phenotype Tregs but not CD44^{low} naïve phenotype Tregs (84).

Some of the observed negative effects of CTLA4Ig on regulatory T-cells and the higher incidence of TCMR episodes might be attributed to the unintended interception of physiological CTLA-B7 binding. CTLA4 is upregulated on activated T-cells and delivers a co-inhibitory signal upon ligation with B7 (85). This co-inhibitory signaling is prohibited by CTLA4Ig. Directly targeting CD28 through non-crosslinking compounds might be a potential strategy to overcome this problem. Furthermore, CD28 blockade and CTLA4 on Tregs might synergize in their control over CD28 as they target the CD28-B7 interaction from two different angles. This has been shown experimentally by the group of Kathryn Wood in a humanized mouse model, where they demonstrated that direct CD28 blockade enhances Treg function and is superior to CTLA4Ig in prevention of allograft rejection (86). Two agents for direct CD28 blockade are currently under clinical evaluation in a phase I trial (NCT05238493) and a phase I/II trial in kidney transplantation (NCT04837092).

The effect of CTLA4 interaction with B7 molecules on APCs remains disputed. It has been suggested that CTLA4 might induce indolamine 2,3 Dioxygenase (IDO) *via* reverse signaling through B7 expressed on antigen-presenting cells (87). IDO is a tryptophan-catabolizing enzyme which leads to the production of pro-apoptotic metabolites (88). However, this concept has been challenged by the lack of IDO induction of CTLA4Ig in dendritic cells (89). The CTLA4Ig effect promoting chimerism in a murine model was also found to be independent of IDO (90). Moreover, the intracellular domains of CD80 and CD86 are short and due to their amino acid sequence are unlikely to transmit reverse signals (91). Notably, no IDO induction was detectable in liver transplant recipients treated with belatacept (92).

As Tregs constantly deplete B7 molecules from the surface of APCs, and CTLA4Ig reduces the numbers of Tregs, B7 expression on APCs is increased in mice under costimulation therapy compared to untreated animals.

We suggested, that low dose CTLA4Ig might only insufficiently bind all available B7 receptors (93). Thus, there are two distinct strategies to overcome the resulting immune activation: 1) The administration of higher doses of CTLA4Ig (to bind all available B7 molecules (74); or 2) by increasing Treg numbers to ultimately decrease the number of B7 molecules expressed on APCs (34) (Figure 2).

Increasing Treg numbers by adoptive cell transfer of *in vitro* activated Tregs was insufficient in a mouse model of heart transplantation. Even though 3x10^6 transferred Tregs were traceable for up to 16 weeks, we observed only a modest increase in Treg numbers that was absent in mice under costimulation blockade. We suggested that homeostatic control *via* the restricted availability of IL-2 might have limited the effect of adoptively transferred Tregs on overall Treg numbers.

Next, we aimed at increasing Treg numbers through IL-2 complexes (IL-2 cplxs). Thereby, we could successfully increase the number of regulatory T cells but also showed a synergistic effect of IL-2 cplxs and CTLA4Ig in reducing the expression of B7 molecules on dendritic cells (34).

However, there are further possible interpretations that can explain the observed beneficial effect of IL-2 cplxs on allograft survival under costimulation blockade. In a murine model of FoxP3 deficiency treatment with IL-2 cplxs can – at least partly - compensate the deleterious effect of the defective Treg compartment indicating other suppressive cells may be supported by IL-2 cplxs (3).

The close relationship between Tregs and CTLA4Ig is further underlined by the observation that in patients with DEF6 deficiency (an inherited syndrome characterized by immunodeficiency and systemic autoimmunity cause by an aberrant CTLA4 homeostasis) CTLA4Ig can improve the clinical phenotype (94).

CD40-CD154 blockade

The interaction of CD40 on B cells and its ligand, CD154 (CD40L) is crucial for B-T cell crosstalk and activation. Consequently, great efforts have been taken to target this pathway therapeutically in transplantation and autoimmunity (95). In several models, blocking CD154 has shown to be superior compared to targeting CD40. This might be due to CD11b acting as alternative receptor for CD154, partially compensating for CD40 (96).

Blocking CD154 yielded promising pre-clinical results in several experimental models (97). However, clinical translation was hampered by thromboembolic complications during phase I

testing (98). The originally developed monoclonal antibody targeting CD40L (hu5c8) caused immune complex-mediated platelet activation *via* FcγRIIa resulting in thromboembolic complications (NCT02273960). Recently, novel Fc-silenced constructs, devoid of any FcγRIIa binding have shown promising pre-clinical results (99).

Contrary to CTLA4Ig, blocking CD154 has been associated with an increase in Tregs across several murine (100–102) and non human primate models (99). Mechanistically, it seems that naïve CD4 FoxP3$^-$ T cells are induced to become pTregs following transplantation under CD154 blockade (100). This effect might be one of many explanations for the observed synergy between CTLA4Ig and anti-CD154 in experimental transplant models (103).

Outlook and future perspectives

Current challenges in transplant medicine including chronic allograft rejection and adverse side effects caused by conventional immunosuppressive regimens demand for novel strategies in order to further improve transplant outcome. Tregs are a powerful subset of immune cells that provide prompt and selective fine tuning of immune responses. The close association between costimulation blockade and Tregs observed in preclinical and clinical studies indicate a synergistical potential that merits further efforts in order to delineate the complex network between immune activation and regulation.

Several strategies are currently investigated in prospective trials including adoptive Treg transfer or Treg expansion by using IL-2 complexes. Also, new costimulation blockers are tested in preclinical and clinical studies. However, whether these efforts ultimately will result in reduced immunosuppression or even in donor-specific tolerance remains unclear.

Author contributions

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Conflict of interest

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

Publisher's note

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CD1d-independent NK1.1$^+$ Treg cells are IL2-inducible Foxp3$^+$ T cells co-expressing immunosuppressive and cytotoxic molecules

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Regulatory T cells (Treg) play pivotal roles in maintaining self-tolerance and preventing immunological diseases such as allergy and autoimmunity through their immunosuppressive properties. Although Treg cells are heterogeneous populations with distinct suppressive functions, expression of natural killer (NK) cell receptors (NKR) by these cells remains incompletely explored. Here we identified that a small population of Foxp3$^+$CD4$^+$ Treg cells in mice expresses the NK1.1 NKR. Furthermore, we found that rare NK1.1$^+$ subpopulations among CD4$^+$ Treg cells develop normally in the spleen but not the thymus through CD1d-independent pathways. Compared with NK1.1$^-$ conventional Treg cells, these NK1.1$^+$ Treg cells express elevated Treg cell phenotypic hallmarks, pro-inflammatory cytokines, and NK cell-related cytolytic mediators. Our results suggest that NK1.1$^+$ Treg cells are phenotypically hybrid cells sharing functional properties of both NK and Treg cells. Interestingly, NK1.1$^+$ Treg cells preferentially expanded in response to recombinant IL2 stimulation *in vitro*, consistent with their increased IL2Rαβ expression. Moreover, DO11.10 T cell receptor transgenic NK1.1$^+$ Treg cells were expanded in an ovalbumin antigen-specific manner. In the context of lipopolysaccharide-induced systemic inflammation, NK1.1$^+$ Treg cells downregulated immunosuppressive molecules but upregulated TNFα production, indicating their plastic adaptation towards a more pro-inflammatory rather than regulatory phenotype. Collectively, we propose that NK1.1$^+$ Treg cells might play a unique role in controlling inflammatory immune responses such as infection and autoimmunity.

KEYWORDS

Treg cells, NK1.1, IFNγ, CD1d-independent NKT-like cells, IL2

Introduction

Immune responses must be tightly regulated in multiple layers, one of which is mediated by regulatory T (Treg) cells. Treg cells regulate various immune responses such as autoimmunity, hypersensitivity, infection, cancer, and organ transplantation (1–4). Treg cells are characterized by the expression of CD4, CD25 (IL2Rα) and transcription factor Foxp3 (forkhead box P3). In addition, these cells play a critical role in maintaining self-tolerance and immune homeostasis by regulating the effector functions of various immune cells such as T cells, dendritic cells (DCs), B cells, macrophages, natural killer (NK) cells, and natural killer T (NKT) cells (5, 6).

Based on their developmental locations, Treg cells are classified into two major subsets, thymus-derived Treg (tTreg) cells mediating central tolerance to self-antigens and peripheral Treg (pTreg) cells mediating peripheral tolerance to foreign antigens (7, 8). In addition, Treg cells can be called either natural Treg (nTreg) or inducible Treg (iTreg), depending on the inducibility of Foxp3 expression. For instance, nTreg cells express Foxp3 constitutively while iTreg cells are differentiated from naive conventional $CD4^+$ T cells *via* transforming growth factor β (TGFβ)-mediated Foxp3 induction upon stimulation with antigen in the periphery (9). In addition, a few studies have demonstrated that TNFR2 signaling is also involved in inducing and maintaining the functions of Treg cells (10, 11). Furthermore, Treg cells have been subclassified into Th1-, Th2-, and Th17-types, depending on their distinct transcription factor profiles such as T-bet, GATA3, and RORγt, respectively (8). Treg cells exert immunosuppressive activity *via* multiple mechanisms: 1) the secretion of soluble anti-inflammatory factors (e.g., IL35, IL10, and TGFβ); 2) cell-to-cell contact-dependent suppression through inhibitory receptors (e.g., cytotoxic T-lymphocyte-associated protein 4 (CTLA4), glucocorticoid-induced TNFR family-related gene (GITR)); and 3) the release of cytolytic granules containing granzymes and perforin (12, 13).

Foxp3 (also known as scurfin) is a critically important transcription factor for controlling the development and functions of Treg cells (14). Although it is well known that Foxp3 protein is expressed in the classical $CD4^+$ Treg cell population, several studies revealed that Foxp3-expressing populations are present in other cell types, including γδ T cells (15), $CD8^+$ T cells (16), invariant NKT (iNKT) cells (17), and B cells (18) and these cells exert suppressive functions similar to classical $CD4^+$ Treg cells.

iNKT cells are one of the innate-like T cells expressing a semi-invariant T cell receptor (Vα14Jα18 in mice and Vα24Jα18 in humans), and their development and activation are dependent on MHC I-like CD1d molecules (19, 20). Moreover, previous studies provide evidence that iNKT cells can express Foxp3 upon TGFβ and glycolipid antigen (e.g., α-GalCer) stimulation and that these cells acquire NK and Treg cell functions (17, 21). In addition to CD1d-dependent iNKT cells, CD1d-independent NKT-like cells exist that display a hybrid phenotype of NK and T cells (22). However, it remains unclear whether CD1d-independent NKT-like cells can become Treg cells. Thus, we characterized CD1d-independent $NK1.1^+$ Treg cells that are not originated from iNKT cells. We demonstrated that these $NK1.1^+$ Treg cells express Foxp3 and other Treg-related markers including CTLA4, GITR, and CD25.

Materials and methods

Study design

This study was designed to determine the phenotype of $NK1.1^+$ Treg cells using Foxp3 green fluorescence protein (GFP) knockin (KI) reporter (hereafter Foxp3(GFP)) mice. To address this issue, splenocytes were harvested and further analyzed by flow cytometry. Furthermore, to explore CD1d dependency of $NK1.1^+$ Treg cell development, we established Foxp3(GFP) CD1d knockout (KO) B6 mice and measured the $NK1.1^+$ Treg cell population.

Mice and reagents

Wild type (WT) C57BL/6 (B6) mice were purchased from Jung Ang Lab Animal Inc. (Seoul, Korea). CD1d KO B6 mice were provided by Dr. A. Bendelac (University of Chicago, IL, USA). Foxp3(GFP) WT B6 mice were obtained from Dr. Rho H. Seong (Seoul National University, Seoul, Korea). Foxp3(GFP) WT B6 mice were further crossed with CD1d KO B6 mice to obtain Foxp3(GFP) CD1d KO B6 mice. DO11.10 OVA-specific TCR transgenic (Tg) Balb/c × B6 F1 mice were generated by intercrossing DO11.10 Balb/c mice and B6 mice. All mice used in this study were maintained at Sejong University and used for experiments at 6-12 weeks of age. Mice were maintained on a 12-hour light/12-hour dark cycle in a temperature-controlled barrier facility with free access to food and water. Mice were fed a γ-irradiated sterile diet and provided with autoclaved tap water. Age- and sex-matched mice were used for all experiments. The animal experiments were approved by the Institutional Animal Care and Use Committee at Sejong University (protocol code SJ-20180804, approved on 4 August 2018). Lipopolysaccharide (LPS) derived from *Escherichia coli* (serotype 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ovalbumin (OVA) peptide$_{323-339}$ (ISQAVHAAHAEINEAGR) was synthesized by Peptron Inc. (Daejeon, Korea).

Cell isolation and culture

Splenic $CD4^+$ T cells were isolated from B6 mice using a magnetically activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions (23). $CD4^+$ T cells were enriched >97% after MACS. Primary cells were cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) culture media supplemented with 10% FBS, 10mM HEPES, 2mM L-glutamine, 100 units/mL penicillin-streptomycin, and 5μM 2-mercaptoethanol. Total splenocytes (2×10^5/well) were cultured with or without 5, 10, and 20 ng/ml of recombinant mouse IL2 (R&D Systems, Minneapolis, MN, USA).

Calculation of $NK1.1^+$ Treg cell number

Splenocytes were prepared after removing red blood cells (RBCs) using RBC lysis buffer and these cells were subsequently stained with trypan blue for counting viable cells under the microscope. After staining splenocytes with mAbs, the percentage of $NK1.1^+$ Treg cells was evaluated by flow cytometry. The total cell number of $NK1.1^+$ Treg cells was calculated by multiplying their percentage value with the splenocyte cell number. In addition, for experiments with $CD4^+$ T cells purified using MACS, total splenocyte number (A) were counted under the microscope after trypan blue staining. Second, after staining splenocytes with mAbs, the percentage (B) of $CD4^+$ T cells ($CD3^+CD4^+$) among total splenocytes and the percentage (C) of $NK1.1^+Foxp3^+$ cells among MACS-purified total $CD4^+$ T cells were evaluated by flow cytometry. The total cell number of $NK1.1^+$ Treg cells was calculated by multiplying the percentage value (B) of $CD4^+$ T cells ($CD3^+CD4^+$) and percentage value (C) of $NK1.1^+Foxp3^+$ cells with the splenocyte cell number (A).

Flow cytometry

The following monoclonal antibodies (mAbs) from BD Biosciences (San Jose, CA, USA) were used: Phycoerythrin (PE)- or allophycocyanin (APC)-conjugated anti-NK1.1 (clone PK-B6); fluorescein isothiocyanate (FITC)- or PE-Cy7-conjugated anti-TCRβ (clone H57-597); FITC-, PE-Cy7-, or APC-conjugated anti-CD3ϵ (clone 145-2C11); FITC-, PE-, or APC-conjugated anti-CD4 (clone RM4-5); PE- or APC-conjugated anti-CD25 (clone PC61); PE-conjugated anti-FasL (clone MFL3); PE-conjugated anti-CD152 (CTLA4) (clone UC10-4B9); PE-conjugated anti-NKG2D (clone C7); PE-conjugated anti-FR4 (clone FBP, FRd); PE-Cy7-conjugated anti-GITR (clone DTA-1); PE-conjugated anti-CD103 (clone M290); PE-conjugated anti-TNFα (clone MP6-XT22); and FITC- or PE-conjugated anti-IgG1 (isotype control) (clone R3-34). In addition, the following mAbs from Thermo Fisher Scientific were used: PE-Cy7-conjugated anti-KJ1-26 (clone KJ1-26); FITC-conjugated anti-Foxp3 (clone NRRF-30); PE-conjugated anti-IFNγ (clone XMG1.2); PE-conjugated anti-γc (clone TUGm2); PE-conjugated anti-IL4R (clone BVD6-24G2); PE-conjugated anti-IL2Rα (clone PC61); PE-conjugated anti-IL2Rβ (clone 5H4); PE-conjugated anti-IL15Rα (clone DNT15Ra); PE-conjugated anti-perforin (clone eBioOMAK-D); PE-conjugated anti-LAP (TGFβ) (clone TW7-16B4); and PE-conjugated anti-TRAIL (clone N2B2). To perform surface staining, cells were harvested and washed twice with cold 0.5% BSA-containing PBS (FACS buffer). To block Fc receptors, the cells were incubated with anti-CD16/CD32 mAbs on ice for 10 min and subsequently stained with fluorescently-labeled mAbs. Flow cytometric data were acquired using a FACSCalibur flow cytometer (Becton Dickson, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Intracellular cytokine staining

For intracellular staining, splenocytes were incubated with brefeldin A, an intracellular protein transport inhibitor (10 μg/ml), in RPMI medium for 2 hrs at 37°C. The cells were stained for cell surface markers, fixed with 1% paraformaldehyde, washed once with cold FACS buffer, and permeabilized with 0.5% saponin. The permeabilized cells were then stained for an additional 30 min at room temperature with the indicated mAbs (PE-conjugated anti-IFNγ, anti-TNFα, anti-perforin, anti-CTLA4, anti-LAP (TGFβ), anti-IL10; or PE-conjugated isotype control rat IgG mAbs). Fixation and permeabilization were performed using a Foxp3 staining kit (eBioscience, San Diego, CA, USA) with the indicated mAbs (FITC-conjugated anti-Foxp3 or isotype control rat IgG mAbs). More than 5,000 cells per sample were acquired using a FACSCalibur, and the data were analyzed using the FlowJo software package (Tree Star, Ashland, OR, USA).

CD1d/α-GalCer dimer staining for iNKT cells

To stain iNKT cells specifically, mCD1d/Ig fusion proteins (CD1d dimer; mouse CD1d dimerX, BD Biosciences, San Jose, CA, USA) were incubated overnight at 37°C with a 40-fold molar excess of α-GalCer (in PBS containing 0.5% Tween 20). The staining cocktail was prepared by mixing α-GalCer-loaded mCD1d/Ig proteins with FITC- or APC-conjugated anti-mouse IgG1 Ab (clone A85-1, BD PharMingen, San Diego, CA, USA) at a 1:2 ratio of dimer to anti-mouse IgG1 Ab. Subsequently, the mixture was incubated for 2 h at room temperature.

Immunization protocols

OVA peptide$_{323-339}$ (ISQAVHAAHAEINEAGR) was synthesized by Peptron Inc. (Daejeon, Korea). DO11.10 TCR Tg Balb/c × B6 F1 mice were immunized *via* subcutaneous injection with 20μg of the OVA peptide emulsified in complete Freund's adjuvant (CFA) containing 5mg/mL of the heat-killed H37Ra strain of *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, USA) into the lower back. Ten days after immunization, these groups were sacrificed by CO_2 inhalation for experiments.

Statistical analysis

Statistical significance was determined using Excel (Microsoft, USA). Student's t-test was performed for the comparison of two groups. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered significant in the Student's t-test. Two-way ANOVA analysis was carried out using the VassarStats (http://vassarstats.net/anova2u.html). $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ were considered to be significant in the two-way ANOVA.

Results

CD1d-independent NK1.1$^+$ Foxp3$^+$ cells are present in the spleen but not in the thymus

Previous studies have reported that treatment of human and murine CD1d-dependent iNKT cells with TGFβ and rapamycin can induce Foxp3 expression, leading to an increase in their suppressive capacities (17, 21). Thus, we wondered whether Foxp3-expressing NKT cells exist in the thymus and spleen under steady-state conditions. First, we examined NK1.1 expression of Foxp3$^+$ cells in Foxp3(GFP) WT B6 mice. We found that NK1.1-expressing Foxp3$^+$ cells were detected in the spleen (about 2% out of total Foxp3$^+$ Treg cells) but not the thymus, implying that these cells might be pTreg cells rather than tTreg cells (Figure 1A). Moreover, we further confirmed the existence of these rare NK1.1$^+$Foxp3$^+$ Treg cells using isotype control mAb staining (Figure S1). Next, we decided to examine whether the developmental origin of these cells is the iNKT cell lineage. To address this issue, we generated Foxp3(GFP) CD1d KO B6 mice by crossing Foxp3(GFP) reporter B6 mice with CD1d KO B6 mice and subsequently evaluated the Foxp3 expression by iNKT cells. We confirmed that iNKT cells do not express Foxp3(GFP) during steady-state conditions. Interestingly, however, we found that both Foxp3(GFP) WT B6 and Foxp3(GFP) CD1d KO B6 mice had comparable numbers of NK1.1$^+$ Treg cells expressing Foxp3(GFP), strongly indicating that NK1.1$^+$ Treg cells do not derive from iNKT cells (Figures 1B, C). Thus, NK1.1$^+$ Treg cells are not CD1d-restricted, although they are considered NKT-like cells expressing NK1.1 (Figure S2). Taken together, our findings suggest that NK1.1$^+$ Treg cells develop in the peripheral tissue in a CD1d-independent manner.

NK1.1$^+$ Treg cells display a hybrid phenotype of Treg cells and NK cells

Treg cell-related molecules (e.g., CTLA4, GITR, CD103, and FR4) are constitutively expressed by Treg cells and are essential for maintaining Foxp3$^+$ Treg cells (24). In particular, it has been previously demonstrated that CD103 (αEβ7) is an established marker for activated Treg cells with effector memory phenotypes (25). Thus, we investigated whether NK1.1 expression affects Treg marker expression of Foxp3$^+$ Treg cells. To explore this possibility, we compared CTLA4, GITR, CD103, and FR4 expression of either NK1.1$^+$ or NK1.1$^-$Foxp3$^+$ Treg cells under normal conditions. Unexpectedly, we found that NK1.1$^+$ Treg cells expressed markedly higher levels of Treg-related molecules than NK1.1$^-$ Treg cells, indicating that NK1.1$^+$ Treg cells might display enhanced suppressive effector functions (Figure 2A). Since NKT cells rapidly produce various cytokines that play critical roles in immune responses (20, 26), we examined expression profiles of either pro-inflammatory or anti-inflammatory cytokines of NK1.1$^-$ and NK1.1$^+$ Treg cells. Compared with NK1.1$^-$ Treg cells, NK1.1$^+$ Treg cells expressed higher levels of both pro-inflammatory (IFNγ and TNFα) and anti-inflammatory (TGFβ) cytokines. However, anti-inflammatory IL10 expression was not significantly different between NK1.1$^-$ and NK1.1$^+$ Treg cells (Figure 2B). Because NKT cells can produce cytolytic molecules and NK cell stimulatory receptors (27, 28), we investigated whether NK1.1$^+$ Treg cells also display these properties. The expression of cytolytic molecules such as perforin, FasL, and TRAIL was markedly higher in NK1.1$^+$ Treg cells than NK1.1$^-$ Treg cells. In addition, most NK1.1$^+$ Treg lacked NKG2D expression, similar to NK1.1$^-$ Treg cells (Figure 2C). Our immune profiling results support the notion that NK1.1$^+$ Treg cells are endowed with hybrid functional properties of both NK cells and Treg cells.

IL2 induces the expansion of NK1.1$^+$ Treg cells

It has been reported that the signaling pathway of the common gamma chain (γc) cytokines (e.g., IL2, IL4, and IL15) influences the homeostasis and function of Treg cells in the periphery (29). Thus, to investigate whether the distinct phenotypes of NK1.1$^+$ and NK1.1$^-$ Treg cells are related to altered cytokine receptor (γc, IL4Rα, IL2Rα, IL2Rβ, and IL15Rα) expression, we compared their surface levels in these

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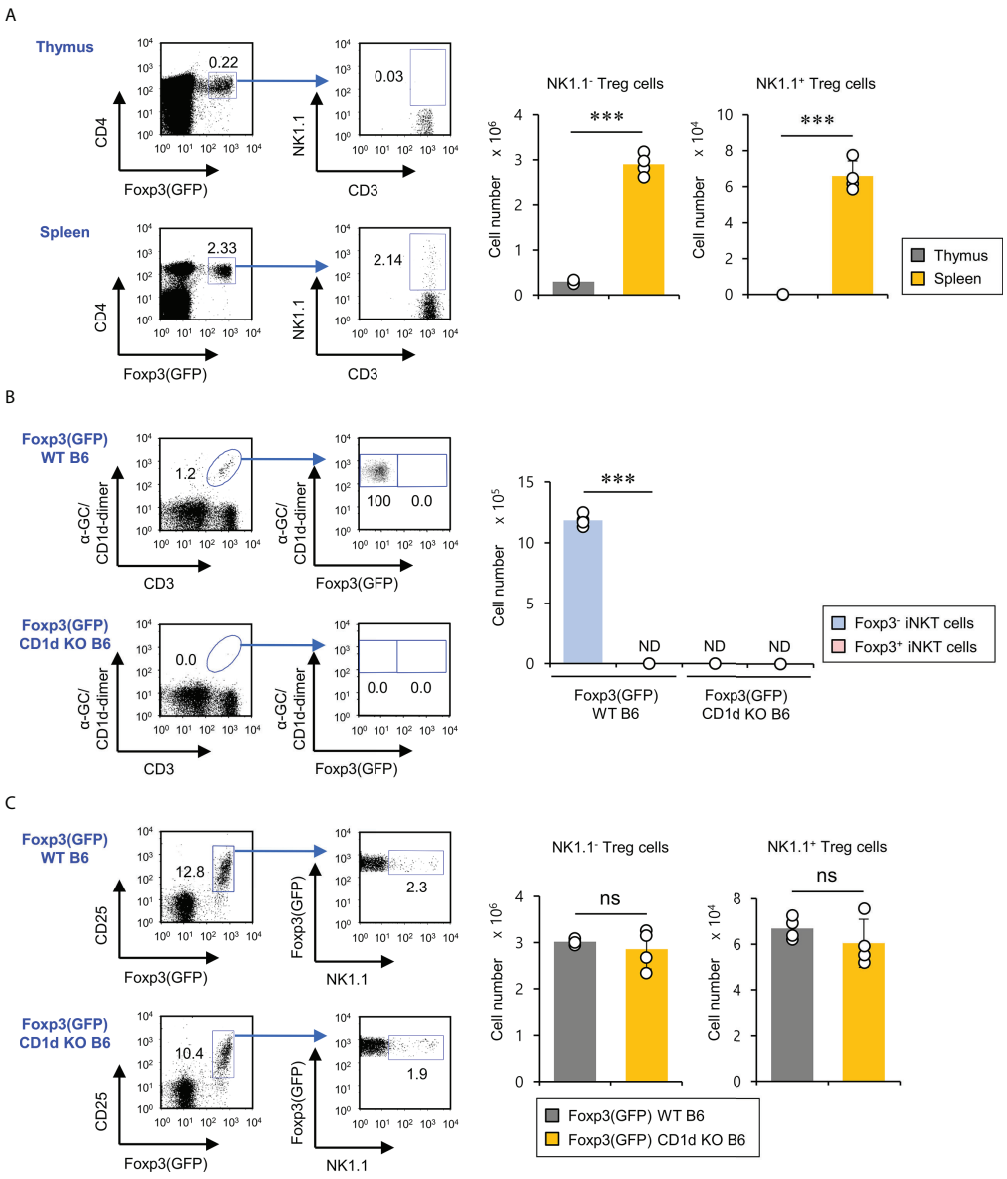

FIGURE 1
CD1d-independent NK1.1$^+$ Treg cells exist in the spleen but not in the thymus. **(A)** Splenocytes and thymocytes were prepared from 8-week-old Foxp3(GFP) WT B6 mice. Left, the percentages of NK1.1$^+$ subpopulations among splenic and thymic Treg cells (CD3$^+$CD4$^+$Foxp3(GFP)$^+$) were plotted. Right, the absolute cell number of NK1.1$^-$ Treg and NK1.1$^+$ Treg cells was assessed by flow cytometry. **(B)** Left, the percentages of Foxp3 (GFP)$^+$ subpopulations among splenic iNKT cells (α-GC/CD1d dimer$^+$CD3$^+$) from 8-week-old Foxp3(GFP) WT or Foxp3(GFP) CD1d KO B6 mice were determined by flow cytometry. Right, the absolute cell numbers of splenic Foxp3$^+$ iNKT or Foxp3$^-$ iNKT cells from 8-week-old Foxp3(GFP) B6 or Foxp3(GFP) CD1d KO B6 were assessed by flow cytometry. **(C)** The percentages of NK1.1$^+$ subpopulations among splenic Treg cells (CD3$^+$CD4$^+$Foxp3(GFP)$^+$) (left) and the absolute cell number of NK1.1$^-$ Treg and NK1.1$^+$ Treg cells (right) from 8-week-old Foxp3(GFP) WT or Foxp3(GFP) CD1d KO B6 mice were determined by flow cytometry. The mean values ± SD (n = 4; per group in the experiment; Student's t-test; ***p < 0.001) are shown. One representative experiment of three experiments is shown. ns, not significant; ND, not detected.

two populations. Although both NK1.1$^+$ and NK1.1$^-$ Treg cells expressed high levels of γc, NK1.1$^+$ Treg cells expressed significantly higher levels of IL2Rα compared with NK1.1$^-$ Treg cells. Furthermore, we found that NK1.1$^+$ Treg cells displayed slightly but significantly higher levels of IL2Rβ expression than NK1.1$^-$ Treg cells. However, IL4Rα and IL15Rα, which were only expressed by a small subset of NK1.1$^-$ Tregs, were even lower on NK1.1$^+$ Treg cells than NK1.1$^-$ Treg cells (Figures 3A, B). Collectively, these results revealed that IL2 but not IL4 and IL15 cytokines might be closely

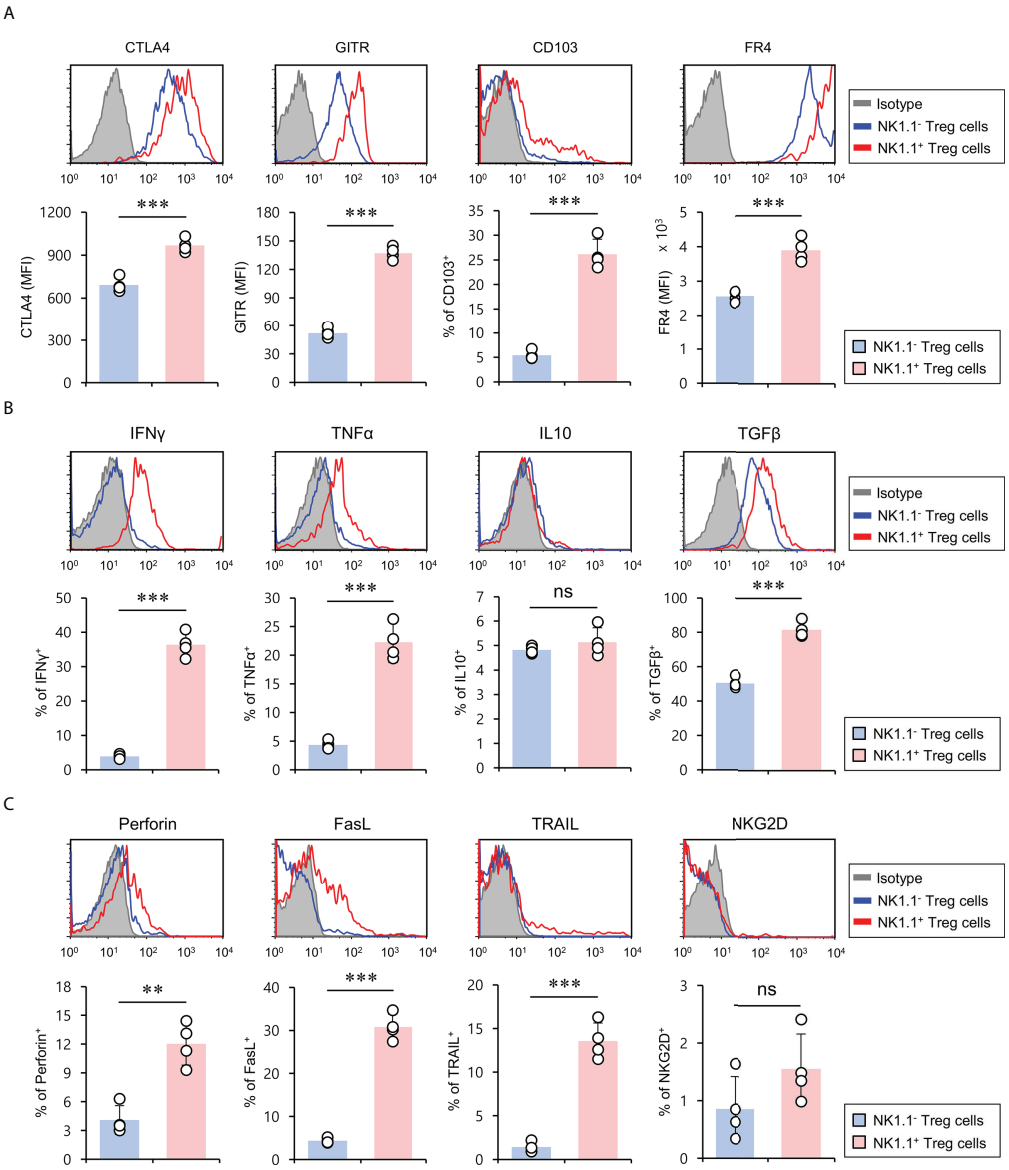

FIGURE 2
NK1.1$^+$ Treg cells have the hybrid phenotype of Treg cells and NK cells. Splenic CD4$^+$ T cells were isolated from 8-week-old CD1d KO B6 mice. **(A)** The expression of Treg cell-associated molecules (i.e., CTLA4, GITR, CD103, and FR4), **(B)** cytokines (i.e., IFNγ, TNFα, IL10, and TGFβ), and **(C)** NK cell-associated molecules (i.e., perforin, FasL, TRAIL, and NKG2D) on NK1.1$^-$ Treg (NK1.1$^-$CD4$^+$Foxp3$^+$) and NK1.1$^+$ Treg (NK1.1$^+$CD4$^+$Foxp3$^+$) cells were evaluated by flow cytometry. Upper, representative FACS histogram; lower, summary figures. The mean values ± SD (n = 4; per group in the experiment; Student's t-test; $^{**}p < 0.01$, $^{***}p < 0.001$) are shown. One representative experiment of two experiments is shown. ns, not significant.

associated with the differentiation of NK1.1$^+$ Treg cells. Furthermore, it has been established that IL2 plays a pivotal role in the expansion and activation of NK cells and NKT cells (30–32) and in maintaining and developing Treg cells (33). Therefore, we considered that IL2, a well-known NK and Treg cell activator, participates in the expansion of NK1.1$^+$ Treg cells expressing high levels of IL2R. To address this issue further, we examined immune responses of NK1.1$^+$ Treg cells upon IL2 stimulation. We found that IL2 treatment significantly increased NK1.1 expression of Treg cells in a dose-dependent manner and greatly enhanced the expression of CD25 on NK1.1$^+$ Treg cells, which was CD1d-independent (Figures 4A, B). In addition, IL2 stimulation significantly increased CTLA4 expression and IFNγ secretion in an iNKT cell-independent manner, suggesting that

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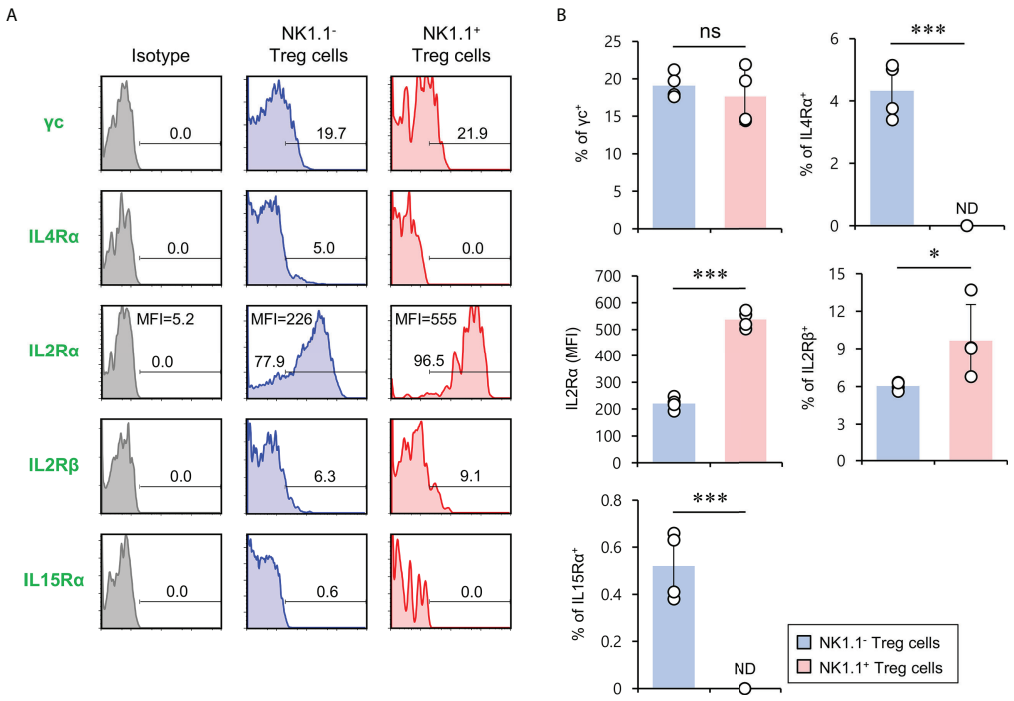

FIGURE 3
The expression of members of the common cytokine receptor γ-chain (γc) family of cytokine receptors on NK1.1$^+$ Treg cells. Splenic CD4$^+$ T cells were isolated from 8-week-old Foxp3(GFP) CD1d KO B6 mice. **(A, B)** Cell surface expression of the common cytokine receptor γ-chain (γc) family cytokine receptors (i.e., IL2Rγ, IL4Rα, IL2Rα, IL2Rβ, and IL15Rα) on NK1.1$^-$ Treg (NK1.1$^-$CD4$^+$Foxp3(GFP)$^+$) and NK1.1$^+$ Treg (NK1.1$^+$CD4$^+$Foxp3(GFP)$^+$) cells were evaluated by flow cytometry. **(A)** Representative FACS histogram; **(B)** summary figures. The mean values ± SD (n = 4; per group in the experiment; Student's t-test; *p < 0.05, ***p < 0.001) are shown. One representative experiment of three experiments is shown. ns, not significant; ND, not detected.

IL2 signaling contributes to inducing NK and Treg cells (Figures 4C and S3). Overall, these results provide strong evidence that IL2 signaling plays a pivotal role in expanding NK1.1$^+$ Treg cells and inducing dual effector functions of NK and Treg cells in them.

Expansion of antigen-specific NK1.1$^+$ Treg cells

pTreg cells can be induced upon antigenic stimulation in the periphery (34). Thus, we wondered whether NK1.1$^+$ Treg cells could be generated similarly to conventional pTreg cells. To address this issue, we employed ovalbumin (OVA)-specific DO11.10 TCR transgenic system and KJ1-26 mAb (specific for DO11.10 TCR-expressing T cells). Since the Balb/c strain does not express the NK1.1 marker, we used (Balb/c × B6) F1 mice to monitor NK1.1$^+$ Treg cells expressing OVA-specific DO11.10 TCR and compared them with NK1.1$^-$ conventional pTreg cells (Figure 5A). We found that OVA peptide-immunized mice displayed a significantly increased number of KJ1-26$^+$CD4$^+$ Treg cells (Figure 5B). Moreover, OVA stimulation significantly expanded both KJ1-26$^+$NK1.1$^-$ and KJ1-26$^+$NK1.1$^+$ Treg subpopulations compared with unimmunized mice (Figure 5C). Together, these results suggest that NK1.1$^+$ Treg cells can be stimulated and expanded in an antigen-specific manner *in vivo*.

LPS-induced systemic inflammation downregulates suppressive marker expression but upregulates TNFα production in NK1.1$^+$ Treg cells

Since it has been demonstrated that acute LPS-induced systemic inflammation limits the suppressive capacity of conventional Treg cells (35), we wondered if inflammatory stimulation can affect the differentiation and/or activation of NK1.1$^+$ Treg cells. To explore this possibility, LPS was intraperitoneally (i.p.) injected into WT B6 mice once a day for a total of 3 days. First, we measured the frequencies and absolute cell numbers of both total and NK1.1$^+$ Treg populations

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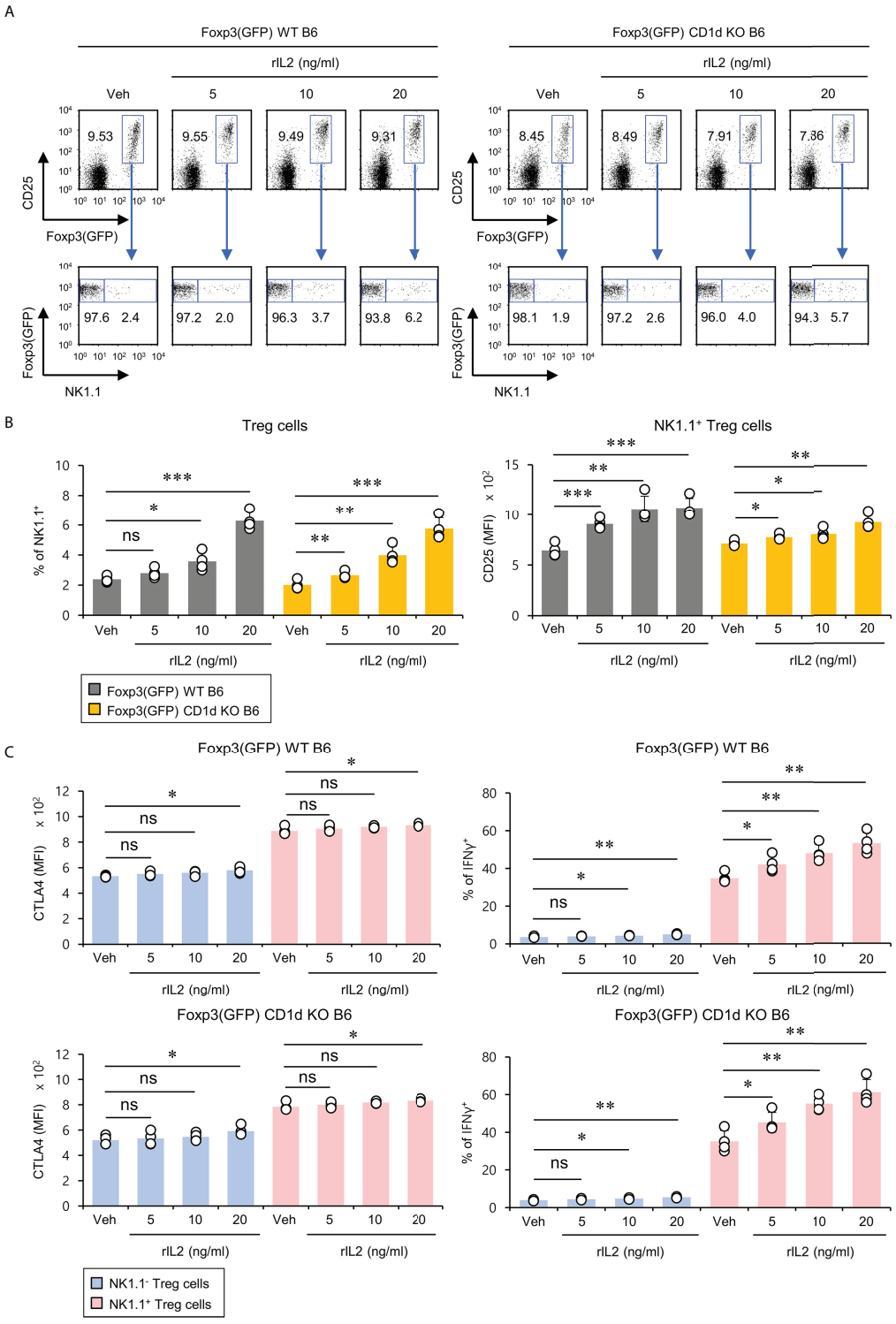

FIGURE 4
IL2 induces the expansion of NK1.1$^+$ Treg cells. Splenocytes were prepared from 8-week-old Foxp3(GFP) WT and Foxp3(GFP) CD1d KO B6 mice. Subsequently, these cells were cultured with rIL2 (5, 10, or 20 ng/ml) for 5 days *in vitro*. **(A, B)** The frequency of NK1.1 and CD25 expression on NK1.1$^-$ Treg (NK1.1$^-$CD4$^+$Foxp3(GFP)$^+$) and NK1.1$^+$ Treg (NK1.1$^+$CD4$^+$Foxp3(GFP)$^+$) cells were determined by flow cytometry. **(C)** CTLA4 expression and IFNγ production by NK1.1$^-$ Treg (NK1.1$^-$CD4$^+$Foxp3$^+$) and NK1.1$^+$ Treg (NK1.1$^+$CD4$^+$Foxp3$^+$) cells were determined by flow cytometry. The mean values ± SD (n = 4 per group in the experiment; Student's t-test; *p < 0.05, **p < 0.01, ***p < 0.001). One representative experiment of two experiments is shown. ns, not significant.

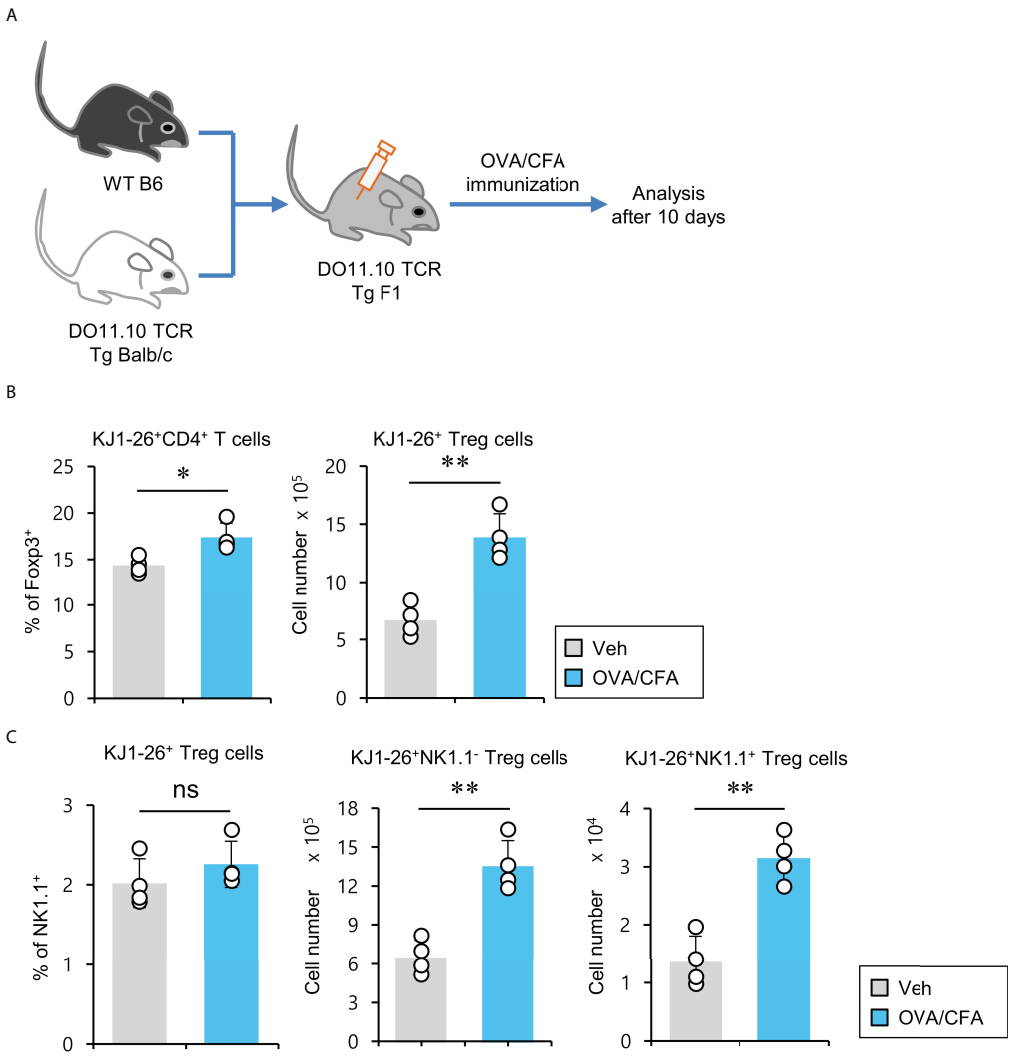

FIGURE 5
Expansion of antigen-specific NK1.1$^+$ Treg cells after immunization with CFA/OVA **(A)** DO11.10 OVA-specific TCR transgenic (Tg) Balb/c × B6 F1 mice were immunized *via* subcutaneous injection with the OVA peptide emulsified in CFA. Ten days after immunization, splenic CD4$^+$ T cells were enriched from these mice using MACS. **(B)** The percentage of Foxp3$^+$ cells among splenic KJ1-26$^+$CD4$^+$ T cells and the absolute cell number of KJ1-26$^+$ Treg cells were assessed by flow cytometry. **(C)** The percentage of NK1.1$^+$ subpopulation among splenic KJ1-26$^+$ Treg cells and the absolute cell numbers of both KJ1-26$^+$NK1.1$^-$ Treg and KJ1-26$^+$NK1.1$^+$ Treg cells were assessed by flow cytometry. The mean values ± SD (n = 4 per group in the experiment; Student's t-test; *p < 0.05, **p < 0.01). One representative experiment of two experiments is shown. ns, not significant.

24 hrs after the last injection. (Figure 6A). LPS treatment resulted in a modest but significant decrease in the frequency but not absolute cell number of splenic Treg cells. However, it did not significantly change the frequency and cell number of NK1.1$^+$ Treg cells (Figures 6B, C). Next, we examined the expression of Treg cell-related molecules and pro-inflammatory cytokines in splenic NK1.1$^+$ Treg cells. We found that NK1.1$^+$ Treg cells from LPS-treated mice displayed a Treg phenotype with significantly decreased expression of CTLA4, CD103, FR4 but not GITR. In addition, LPS stimulation induced NK1.1$^+$ Treg cells to increase TNFα but not IFNγ secretion (Figures 6D, E). Overall, these results provide strong evidence that LPS-induced systemic inflammation promotes the conversion of NK1.1$^+$ Treg cells from a regulatory towards a pro-inflammatory phenotype, which indicates the potential plasticity of these regulatory NKT-like cells.

Discussion

We have identified a small subset of CD4$^+$ Treg cells expressing NK1.1 NKR (NK1.1$^+$ Treg cells) in mice. Thymic Treg (tTreg) cells

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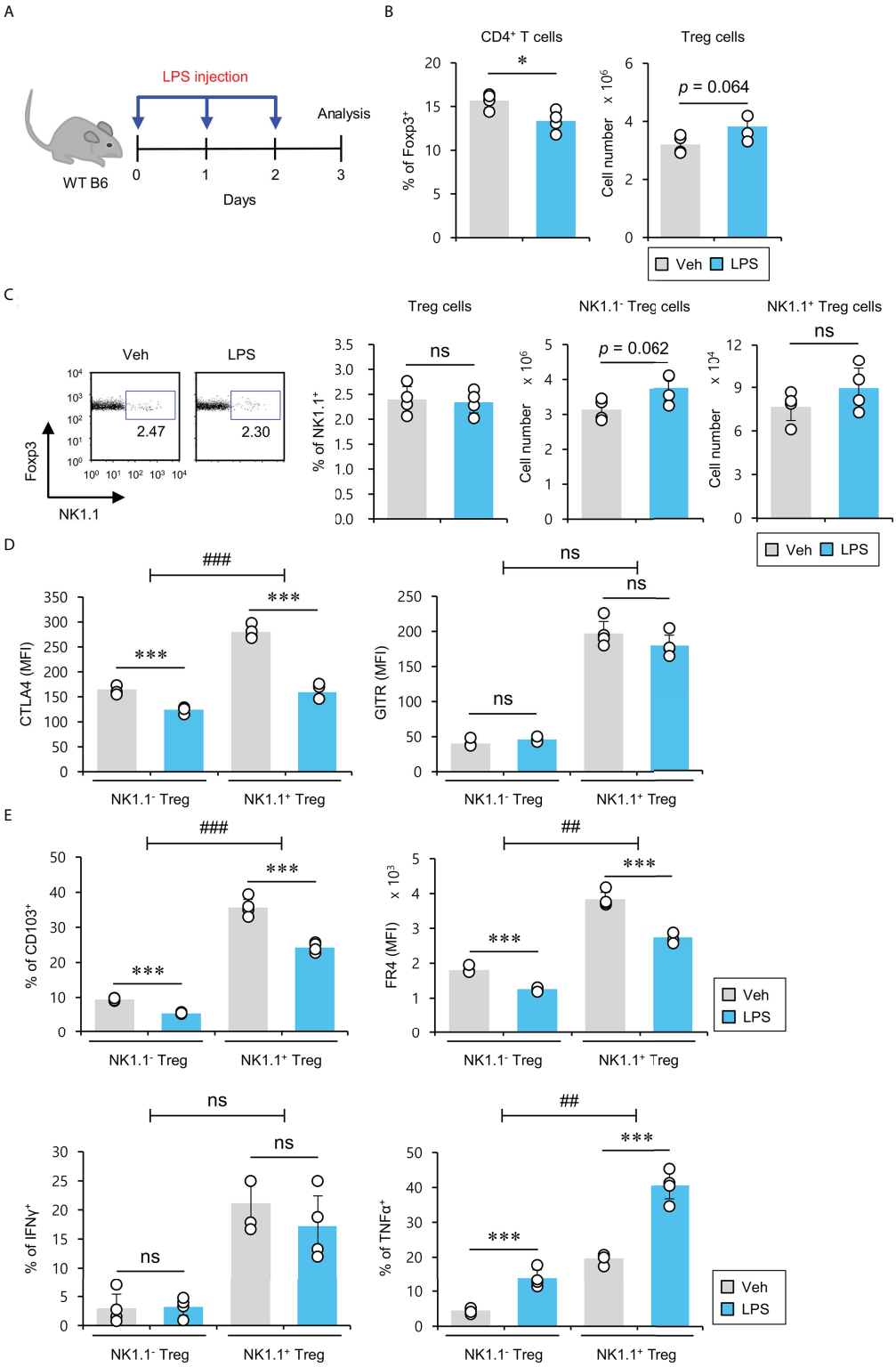

FIGURE 6
LPS-induced systemic inflammation downregulates immunosuppressive marker expression but upregulates TNFα production in NK1.1$^+$ Treg cells. **(A)** WT B6 mice were i.p. injected either LPS (2 mg/kg) or vehicle (Veh) once per day for a total of 3 days, and total splenocytes from these mice were prepared one day after the last injection. **(B)** The percentage and absolute cell number of Treg cells were assessed by flow cytometry. **(C)** The percentage of NK1.1$^+$ cells among splenic Treg cells and the absolute cell number of both NK1.1$^-$ Treg cells and NK1.1$^+$ Treg cells were assessed by flow cytometry. **(D, E)** The expression of Treg cell-associated molecules (i.e., CTLA4, GITR, CD103, and FR4) and cytokines (i.e., IFNγ and TNFα) on NK1.1$^-$ Treg (NK1.1$^-$CD4$^+$Foxp3$^+$) and NK1.1$^+$ Treg (NK1.1$^+$CD4$^+$Foxp3$^+$) cells were evaluated by flow cytometry. The mean values ± SD (n = 4 per group in the experiment; Student's t-test; *p < 0.05, ***p < 0.001). One representative experiment of two experiments is shown. Two-way ANOVA (cell type × treatment) showed an interaction between these two factors ($^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001). ns, not significant.

develop in the thymus through high-affinity TCR-MHC peptide interactions. As NK1.1$^+$ Treg cells are not detectable in the thymus, our data demonstrate that these cells likely differentiate in the periphery. Previously, Monteiro *et al.* showed that Foxp3$^+$ Vα14 TCR iNKT cells detected in the draining lymph node of the central nervous system could protect mice from experimental autoimmune encephalomyelitus by α-GalCer treatment. However, these cells were undetectable in the steady-state (17), which indicates that iNKT cell-derived Treg cells also differentiate in the periphery. Furthermore, although NK1.1$^+$ Treg cells are a minor population (approximately 2% of total pTreg cells) in WT mice, they were unchanged in CD1d KO mice, indicating their CD1d-independence.

Treg cells maintain immunological tolerance and organ homeostasis. Specifically, pTregs participate in the control of immunity at sites of inflammation (36) and subsequently, these cells play an important role in repairing tissue damage after inflammation. Several cell types have been reported to facilitate the differentiation of pTreg cells. For example, tissue-resident macrophages in the lung (37) and CD103-expressing DCs in the lamina propria secrete retinoic acid through retinal dehydrogenase (RALDH) enzyme expression to promote Treg cell differentiation (38). In addition, mesenchymal stem cells (MSCs), a primary source for tissue regeneration, contribute to inducing Treg cell differentiation by secreting prostaglandin E2, IL10, and TGFβ (39–41). Recent studies have shown that MSCs can deliver various bioactive molecules (i.e., growth factors, cytokines, and microRNAs) *via* exosomes to neighboring immune cells for maintaining an anti-inflammatory environment (39, 42). As there is currently no information available on the cell types that can facilitate the differentiation of NK1.1$^+$ Treg cells, it will be exciting to investigate the interaction between MSCs and NK1.1$^+$ Treg cells during inflammation in future studies. Such information may be relevant to the development of immune therapies aimed at promoting tissue regeneration.

NK1.1$^+$ Treg cells respond to recombinant IL2 treatment relatively better than NK1.1$^-$ Treg cells. In addition, we found that NK1.1$^+$ Treg cells increased upon antigen stimulation, indicating their antigen-specific proliferation. However, since pTreg cells can migrate from peripheral blood to tissues or vice versa under certain immunostimulatory conditions, the possibility of cell migration into the spleen rather than only proliferation cannot be completely ruled out (43). Therefore, examining the *in vivo* kinetics of NK1.1$^+$ Treg cells upon antigen stimulation will be worthwhile in future studies.

IFNγ derived from NK cells is generally thought to play a pathogenic role in allograft rejection (44). In addition, NK cells activated by NKG2D ligands can cause lung ischemia-reperfusion injury, which occurs after lung transplantation (45). Moreover, MHC class I chain-related molecules (MICA and MICB) upregulated in allografts could trigger acute rejection through increased infiltration of NKG2D$^+$NK1.1$^+$ cells and CD8$^+$ T cells into heart allografts (46). However, it has also been reported that IFNγ produced by allogenic Treg cells contributes to the prevention of graft-versus-host disease (GVHD) (47). Moreover, TNFα/TNFR2 signaling plays a pivotal role in Treg cell-mediated regulation of GVHD (10). Furthermore, inducible T-cell co-stimulator (ICOS) is indispensable for optimal survival and development of iTreg cells during chronic GVHD (48). Since NKG2D expression was not detectable on NK1.1$^+$ Treg cells, which are capable of secreting high amounts of IFNγ and TNFα, it will be interesting to further investigate whether these cells play an essential role in the resolution of allograft rejection *via* TNFR2 and ICOS signaling-related mechanisms. Moreover, since NK1.1 is not expressed in Balb/c mice, alternative markers for NK1.1$^+$ Treg cells applicable to all mouse strains will be essential to understanding the immunological significance of NK1.1$^+$ Treg cells in the setting of mouse transplantation experiments such as GVHD. Pan-NK cell markers such as CD49a and DX5 can be tested first to determine whether they are expressed by NK1.1$^+$ Treg cells (49). In addition, it will be intriguing to explore the putative human immune cells that may represent the counterpart to murine NK1.1$^+$ Treg cells. A prior study identified Foxp3$^+$ Treg cells expressing a characteristic human NK cell marker (i.e., CD56) in cancer tissues of hepatocellular carcinoma patients (50). In addition, previous studies have reported that a subpopulation of Treg cells expresses CD161 in humans, and these CD161$^+$ Treg cells possess classic Treg signatures and pro-inflammatory phenotypes (51). Consistent with NK1.1$^+$ Treg cells described in our study, human CD161$^+$ Treg cells express significantly higher levels of Treg-associated molecules (e.g., CTLA4 and GITR) than CD161$^-$ Treg cells in the blood of healthy adults (52). Based on these previous reports, it will be interesting to investigate whether CD56$^+$ and CD161$^+$ Treg cells might represent the human analogues to murine NK1.1$^+$ Treg cells.

Previous studies demonstrated that Th1-type T-bet$^+$ Treg cells develop in a STAT1-dependent manner and can produce IFNγ upon IL12 stimulation (53). Moreover, *in vitro* treatment with IL12 and TGFβ potentially promotes the generation of IFNγ$^+$ Treg cells in the presence of IL2 (54). Although IL15 contributes to inducing NK1.1$^+$ expression in CD8$^+$ T cells (55) and enhancing Foxp3 expression in Treg cells (56), it is still unclear what factors can induce both NK markers (i.e., NK1.1) and Foxp3 in conventional CD4$^+$ T cells. In future studies, it will be worthwhile to examine what signaling pathway can participate in NK1.1$^+$ Treg cell differentiation.

Our results suggested the functional plasticity of NK1.1$^+$ Treg cells with both regulatory and pro-inflammatory phenotypes under inflammatory conditions in mice. It has previously been shown that human NK-like Treg cells can alter their functions from pro-inflammatory to immunosuppressive phenotypes, indicating their functional adaptation depending on their microenvironment (50). Likewise, NK1.1$^+$ Treg cells with dual phenotypes during steady-state conditions may potentially display biased phenotypes depending on the niche they belong to. For example, IFNγ$^+$ NK1.1$^+$ Treg cells might lose their pro-inflammatory functions in the immunosuppressive tumor microenvironment. In contrast, it has also been reported that IFNγ$^+$ Treg cells induced by IL12/TGFβ effectively suppress inflammatory disease such as colitis (54). Thus, it will be exciting to investigate whether NK1.1$^+$ Treg cells exhibit protective or pathogenic effects in acute and chronic inflammatory diseases.

It has been reported that LPS directly activates Treg cells, ultimately resulting in the inhibition of neutrophil inflammatory responses (57). However, another study showed that acute LPS-induced inflammation rapidly suppresses STAT5 signaling and proliferation of Treg cells (35). These discordant findings regarding the effects of LPS on Treg cell development and functions may suggest the need for temporal control of Treg cell function during infection and inflammation. For example, Treg cells should be inhibited during the early phase of infections (i.e., LPS stimulation) to maximize cytotoxic immune responses against infectious agents. After pathogens are eradicated (or immune responses have subsided), Treg cells come to the frontline to participate in tissue repair and wound healing. Thus, such a regulatory immune homeostatic mechanism might explain why NK1.1$^+$ Treg cells downregulate suppressive molecules but upregulate pro-inflammatory molecules in response to acute LPS exposure. In addition, previous studies showing that the TNFα/TNFR2 signaling pathway is required to stabilize Treg cells (10, 11) can support our contention that the higher TNFα-producing characteristics of NK1.1$^+$ Treg cells during inflammation may reflect a crucial role of autocrine TNFα/TNFR2 signaling pathway in maintaining the stability of NK1.1$^+$ Treg cells.

Furthermore, depending on NK1.1 expression, Treg cells consist of two subpopulations with distinct roles. NK1.1$^+$ Treg cells might exist to regulate the anti-inflammatory function of conventional Treg cells in an antigen-specific manner to promote immune homeostasis. In our previous studies, we showed that an increase in exogenous IFNγ signaling negatively regulates pTreg cell development by repressing Foxp3 expression in the presence of NKT cells and natural killer dendritic cells (NKDC) (28, 58). Since Th1-type effector molecules and transcription factors can inhibit pTreg generation (59, 60), NK1.1$^+$ Treg cells with IFNγ-producing and cytotoxic properties might be excellent candidates to re-establish immune competence by inhibiting overt conventional pTreg cells. Several previous studies have reported that NK cell marker expression associates with IFNγ-producing and cytotoxic properties in DCs and γδ T cells (28, 61, 62), suggesting their immunoregulatory roles. Therefore, we propose that NK1.1$^+$ Treg cells are antigen-specific regulators of pTreg cells, and it will be of interest to investigate this possibility in the future.

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 Institutional Animal Care and Use Committee (Sejong University).

Author contributions

HP: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis, obtained funding. SL: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis, obtained funding. YP: acquisition of data, analysis and interpretation of data. T-CK: acquisition of data, analysis and interpretation of data. LVK: interpretation of data and drafting of the manuscript, review of the manuscript. SH: study concept and design, acquisition of data, analysis and interpretation of data, drafting manuscript, statistical analysis, obtained funding, administrative, and material study supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

LVK is a member of the scientific advisory board of Isu Abxis Co., Ltd. (South Korea).

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.951592/full#supplementary-material

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IL-2 availability regulates the tissue specific phenotype of murine intra-hepatic Tregs

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CD4+CD25+Foxp3+ Tregs are known to acquire tissue-specific features and exert cytoprotective and regenerative functions. The extent to which this applies to liver-resident Tregs is unknown. In this study, we aimed to explore the phenotypic and functional characteristics of adult murine liver resident Tregs during homeostasis. Additionally, we investigated their role in ameliorating liver inflammation and tissue damage. Quantification of Foxp3+CD4+CD25+ cells comparing different tissues showed that the liver contained significantly fewer resident Tregs. A combination of flow cytometry phenotyping and microarray analysis of intra-hepatic and splenic Tregs under homeostatic conditions revealed that, although intra-hepatic Tregs exhibited the core transcriptional Treg signature, they expressed a distinct transcriptional profile. This was characterized by reduced CD25 expression and increased levels of pro-inflammatory Th1 transcripts *Il1b* and *Ifng*. *In vivo* ablation of Tregs in the Foxp3-DTR mouse model showed that Tregs had a role in reducing the magnitude of systemic and intra-hepatic inflammatory responses following acute carbon tetrachloride (CCl_4) injury, but their absence did not impact the development of hepatocyte necrosis. Conversely, the specific expansion of Tregs by administration of IL-2 complexes increased the number of intra-hepatic Tregs and significantly ameliorated tissue damage following CCl_4 administration in C57BL/6 mice. The cytoprotective effect observed in response to IL-2c was associated with the increased expression of markers known to regulate Treg suppressive function. Our results offer insight into the transcriptome and complex immune network of intra-hepatic Tregs and suggest that strategies capable of selectively increasing the pool of intra-hepatic Tregs could constitute effective therapies in inflammatory liver diseases.

KEYWORDS

regulatory T cells (Tregs), liver, acute inflammation, Treg depletion, CCl_4 induced liver injury, tissue specific

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Introduction

Forkhead box P3 positive (Foxp3+) regulatory T cells (Tregs) are a subset of T helper cells with immunosuppressive properties that are central to the maintenance of immune homeostasis and peripheral tolerance, both in mice and humans, and are being developed as novel immunomodulatory therapies for autoimmune diseases and transplantation (1, 2). Due to their constitutive expression of CD25, the IL-2 receptor alpha chain (IL-2RA), Tregs respond to even very low concentrations of IL-2, a cytokine secreted by effector T cells (Teffs) (3) that is essential for Treg survival and suppressive function *via* STAT5 signalling (4). Furthermore, the administration of exogenous IL-2, either in the form of low dose recombinant IL-2 (LDIL-2) therapy in humans or as IL-2 complexes (IL-2c) consisting of soluble IL-2 conjugated with the IL-2 specific monoclonal antibody JES6-1A12, has been shown to increase the pool of Tregs *in vivo* and exert anti-inflammatory effects (5–7).

While all Tregs maintain common gene signatures attaining peripheral tolerance, they are far from being a homogenous cell lineage (8, 9). Thus, increasing evidences indicate that Tregs acquire unique phenotypic features depending on the tissue compartment they reside in (10). Ablation of Foxp3-expressing cells in animal models of lung, skeletal muscle, heart muscle, skin, bone, and central nervous system injury shows that the tissue-resident Tregs exhibit distinct cytoprotective and pro-regenerative properties (11–17). Differential tissue-specific features of Tregs include active involvement in cell functional reprogramming, such as the inhibition of M1 macrophage inflammatory activity and the promotion of M2 macrophages polarisation (18). This is because tissue-resident Tregs express the epidermal growth factor family member amphiregulin (Areg) which is known to promote tissue repair and regeneration under inflammatory conditions. Distinct from signals eliciting suppressor function, Areg production is independent of T-cell receptor (TCR) engagement and induced by IL-33 or IL-18 released from activated endothelial cells in response to tissue injury and inflammation. As tissue resident Tregs respond directly to alarmins like IL-33, they play a reparative role in ST2/IL-33/Areg mediated regeneration and differentiation following tissue injury (19, 20). Different non-lymphoid tissue resident Treg populations that display adaptation to tissue microenvironments were reported. For instance, visceral adipose tissue Tregs exhibit distinct phenotype exhibiting high levels of chemokine receptors CCR1, CCR2, CCR6 and CCL6 and play a regulatory role in insulin resistance and sensitivity (21, 22). On the other hand, muscle Tregs exhibit higher expression of CTLA-4, TIM-3 and ST2, as well as chemokine receptor CCR1 and activate satellite cell proliferation and muscle regeneration through Areg (11, 12).

The liver is an organ with unique regenerative capacity, which is key to ensure that hepatic functions are maintained following acute tissue damage/loss (23). A previous study characterised the transcriptomic profile of thymus-derived Tregs in neonatal livers from 1–2-week-old-mice under homeostatic conditions and showed that their accumulation is critical to maintain self-tolerance and liver maturation (24). However, the extent to which adult mice livers harbour tissue-resident Tregs with distinct phenotypic characteristics has not been previously investigated. Likewise, the broader functional role of intra-hepatic Tregs in the initiation and modulation of liver inflammation remains to be fully explored. To answer these questions, in the current study we aimed to delineate the specific adaptations of liver resident Tregs by analysing their molecular profile during homeostatic conditions. Further, using the well-described acute liver inflammation model induced by the hepatotoxin carbon tetrachloride (CCl_4), we investigated the local and systemic effects of both Treg depletion and Treg augmentation *via* IL-2c administration. Our results highlight how the abundancy and distinct phenotype of intra-hepatic Tregs influence their ability to modulate liver inflammation and they suggest that therapies selectively increasing the intra-hepatic Treg pool could be exploited to regulate inflammatory liver diseases.

Materials and methods

Animals

All procedures and experiments were approved by the Animal Welfare and Ethical Review Body of King's College London. All animals had unrestricted access to food and water and were kept according to the standards of the Animals (Scientific Procedures) Act 1986. Wild-type C57BL/6 mice were purchased from Harlan and kept in our specific pathogen-free animal facility at King's College London. Foxp3-tm4(YFP/icre) Ayr (Foxp3/YFP-cre) and Foxp3-tm3(DTR/GFP)Ayr (Foxp3-DTR) mice were provided as a kind gift of G. Lombardi, King's College London. All transgenic mice were bred in-house for the experiments. 8-weeks old male mice were used for all experiments to avoid gender differences.

Statistics

Unless otherwise stated, statistical analyses were performed with GraphPad Prism 8.0 software. Student's t test was used for comparison between two groups, and one or two-way ANOVA analysis with Tukey's *post hoc* correction for pairwise comparisons was used to compare more than two groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$). Results are reported as mean ± SEM.

CCL_4 acute inflammation model

Induction of acute inflammatory liver damage by the hepatotoxin CCL_4 is a widely reported model due to its hepatotoxic capacities impairing key cellular processes and leading to fatty degeneration and steatosis as an effect of trichloromethyl radical metabolised by the cytochrome p450.

Treg depletion model

DTR-eGFP mice express a diphtheria toxin receptor fused to an enhanced green fluorescent protein) transgene under the control of the FOXP3 promoter to enable the specific depletion of Tregs at any desired time point by application of diphtheria toxin (DT).

IL-2c, CCl_4, DT treatments

IL-2 complexes (IL-2c) were formed by incubating 1 µg recombinant mouse IL-2 (eBioscience) and 5 µg of purified anti-mouse IL-2 (clone JES6-1A12) (eBioscience) for 30 min at 37°C and were diluted with 75 µl of PBS before injection. Animals received 3 doses of IL-2c overall on consecutive days administered as intraperitoneal injections. Diphtheria toxin (DT) was administered intraperitoneally at a dose of 1µg diluted in 100µl of phosphate-buffered saline on 3 consecutive days. Carbon tetrachloride (CCl_4) was administered intraperitoneally as a single dose (25% CCL_4 in corn oil and 2 µl/g weight of mouse) either 2 days after the last dose of IL-2c or 3 days after the last dose of DT. Mice were sacrificed 24 h after CCL_4 administration by CO_2 inhalation.

Isolation of spleen and non-parenchymal intra-hepatic mononuclear cells

Murine spleen and livers were harvested into 1xPBS with 2% FBS. Livers were excised, washed and flushed multiple times with 1xPBS and mechanically homogenised to obtain single cell suspensions (we processed the whole livers in their entirety except for two small fragments employed for RNA extraction and histological analyses, respectively). Previous experiments conducted in our laboratory demonstrated that mechanical homogenisation provided a similar yield of intra-hepatic leukocytes than an alternative method that included liver perfusion and digestion with collagenase (data not shown). Mechanical homogenisation was achieved using a 1 ml syringe plunger and gently passing the tissue through a sterile 70µm cell strainer. Hepatic single-cell suspensions were centrifuged at 100 x g for 2 minutes to eliminate hepatocytes. Cells of the supernatant were retained and washed at 400 x g for 7 minutes and mononuclear cells were isolated by Ficoll®-Paque (GE Healthcare) density gradient sedimentation. Spleens were homogenised in a similar manner followed by red blood cell lysis with ammonium chloride potassium (ACK) lysing buffer (Life Technologies) and a wash at 400 x g for 7 minutes.

RT-PCR analysis

RNA isolation for RT-PCR was prepared by homogenization liver biopsies in TRIzol® Reagent (Ambion by Life Technologies, UK) using Tissuelyser II (Qiagen, UK). The RNA was purified by chloroform and isopropanol extraction and reversed transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) and a T9800 Fast Thermal Cycler (Applied Biosystems, UK). The reaction mixture for SYBR Green RT-PCR assay contained cDNA, 2x Fast SYBR Green PCR master mix (Applied Biosystems, UK) and 10µM of forward and reverse primers (IDT, UK). All amplifications and detections were carried out in a MicroAmp optical 384-well reaction plate covered with an optical adhesive film (Applied Biosystems™, UK) and detected on QuantStudio™ 7 Sequence Detection System (Applied Biosystems™, UK). For statistical analysis, relative fold changes (R) were calculated with the function ($R = 2^{-\Delta\Delta CT}$), where $\Delta\Delta Ct$ is the normalized difference in threshold cycle (Ct) number between the control and test samples. Each CT was calculated from duplicate replicates.

Primer sequences were as follows:

GAPDH Forward 5'- CCCATCACCATCTTCCAGGAGC -3'
Reverse 5'- CCAGTGAGCTTCCCGTTCAGC -3'
TNFa Forward 5'-CGAGTGACAAGCCTGTAGCC -3'
Reverse 5'-AGATAGCAAATCGGCTGACG -3'
IL-6 Forward 5'-GAGGATACCACTCCCAACAGACC -3'
Reverse 5'-AAGTGCATCATCGTTGTTCATACA -3'

Microarray analysis

A small liver biopsy, always from the same lobe to avoid sampling variability, was taken for RNA extraction. RNA extracted from liver biopsies and sorted cells using TRIzol® (Thermo Fisher) was quantified using Qubit HS and its integrity assessed in a Bioanalyzer (Agilent). Samples were then reverse transcribed and amplified before hybridization to Affymetrix Mouse Gene 2.1 ST arrays. Microarray expression data were processed using quantile normalization using the Affy Bioconductor package. A conservative probe-filtering step was conducted next to exclude probes with a coefficient of variation >5%. To identify genes differentially expressed between groups, we used significant analysis of microarray (SAM) statistical

significance of differentially expressed transcripts was defined at a fold change higher than 2 and a p value <0.05. To assess the deregulation of sets of genes associated with specific functional pathways, we computed an enrichment score for each of the predefined gene sets included in the MSigDB (https://www.broadinstitute.org/gsea/msigdb/index.jsp) with a p-value <0.05 using Gene set enrichment analysis (GSEA) method using the Gene Ontology (GO) gene sets. All microarray data discussed in this article have been deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO) (accession no. GSE80814).

Definition of core Treg transcriptional signature

Pfoertner et al. constructed and validated a unique microarray (Human Treg Chip) containing 350 Treg associated genes based on whole human and mouse genome transcription data in the literature and identified 62 differentially expressed genes in mouse and human Treg cells. We defined the core Treg transcriptional signature using these reported comprehensive set of genes.

Cell sorting

For cell sorting, Foxp3 $^{YFP/cre}$ reporter mice were used. Splenocytes and liver single cell suspensions were enriched for CD4+ cells (Stemcell Technologies Cat.# 19772). Following enrichment, samples were counted, washed with PBS and a viability staining was performed using LIVE/DEAD Fixable violet dead cell stain according to manufacturer's protocol (Life Technologies). Following viability staining, samples were washed with PBS 2% FBS, and the pellets were resuspended in 2% FBS with CD3, CD4 and CD25 antibodies, incubated in the dark, at 4°C for 20 minutes. Samples were then washed, and pellets resuspended in 2% FBS and kept on ice to be sorted and analysed by flow cytometry (FACSAria, BD Biosciences). CD4+CD25+Foxp3^{YFP+} Treg and CD4+CD25-Foxp3^{YFP-} Teff populations were then washed, and cell pellets were transferred to an ultra-high recovery RNAse-free 1.5 ml Eppendorf. After the final wash, the pellets were resuspended in TRIzol (Invitrogen) and were stored in -80°C for subsequent RNA extraction.

Flow cytometry analysis

Flow cytometry analysis was performed on 1-2 x 10^6 freshly isolated mouse cells to immunophenotype isolated intra-hepatic and splenic regulatory and effector T cells, macrophages and monocytes. For experiments involving Foxp3-DTR mice, Cells were stained with LIVE/DEAD^{TM} Fixable Violet Dead Cell stain kit (1:1000 dilution with PBS) (Life Technologies). Otherwise, LIVE/DEAD Fixable Green Dead cell stain was used (Life Technologies). Following Live/Dead staining, the cells were washed and resuspended in 100 µl surface staining mastermix (Table S1). Intracellular staining was performed using the Foxp3 Fixation/Permeabilization Kit (eBioscience) according to the manufacturer's instructions. Cells were immunophenotyped using BD LSR Fortessa (BD Bioscience) and analysis was performed using FlowJo software (TreeStar, Inc).

Cytokine quantification

Immediately prior to sacrifice, mouse blood samples were collected *via* cardiac puncture. Serum was separated from the coagulated blood by centrifugation at 3000 x g for 15 minutes at 4°C. Analysis was performed by mouse ELISA kits for IFN-g, IL-6 and IL-10 (Biolegend, ELISA MAX^{TM}). Cytokine concentrations were calculated using linear regression after the generation of a standard curve.

Serum alanine and aspartate aminotransferase levels

Serum AST and ALT levels were measured using a clinical bioanalyzer (King's College Hospital). Enzyme activities were shown in international units per litre (IU/L).

Liver histology

Liver tissue was collected from the same lobe in all mice to avoid sampling variability and placed in 10x formalin and washed in PBS 24 hours later. Fixed liver tissue samples were then placed in plastic cassettes and embedded in paraffin blocks. Liver biopsies were stained with haematoxylin and eosin (H&E). The microscope slides were then analysed under a Nikon Eclipse TE2000 light microscope at 20x magnification.

Results

Tissue-resident Tregs are less abundant in the liver than in other organs and exhibit a distinct molecular profile

Flow cytometric analysis of mononuclear cells isolated from liver, secondary lymphoid organs, blood, thymus, lung and adipose tissue revealed that the liver exhibited the lowest proportion of Foxp3++CD4+CD25+ T cells (3.299 +/- 0.693) (Figure 1A). To understand the molecular pathways underpinning this finding, we

FACS sorted intra-hepatic and splenic Foxp3+YFP+Tregs of Foxp3-YFP reporter mice and compared their transcriptional profiles using whole-genome transcriptional analysis. While both intra-hepatic and splenic Tregs exhibited a core Treg transcriptional signature, liver Tregs differed from splenic Tregs in the downregulation of immunoregulatory transcripts (*Gpr83, Il2ra, Lrrc32*) while showing upregulation of pro-inflammatory Th1 (IFNg, IL1b) related transcripts (Figure 1B). Liver Tregs also exhibited up-regulation of *Pde3b* (cyclic nucleotide phosphodiesterase 3B, CGMP-inhibited), an enzyme required by non-regulatory T cells for survival and expansion (9, 25), and whose transcriptional repression promotes lineage stability and Foxp3 expression in Tregs. The higher expression of *Pde3b* in liver-resident Tregs suggests therefore increased lineage instability as compared to peripheral Tregs. Furthermore, Tregs displayed increased expression of chemokine receptors (*Cxcr6, Cxcr3, Cx3cr1*, *Ccr2, Ccr4*), other tissue homing mediators (*Gpr15*), cell cycle regulators (*Cdk1, Ube2c*), as well as genes associated with effector T cell phenotype (*Il1b, Ifng, Il4, Rora*, *Ccl3)* (Figure 1C). Intra-hepatic Tregs also expressed increased levels of transcripts linked to tissue repair and regeneration, such as *Ace* and *Fn1* (both reported to promote regeneration and reparative cell response following ischaemic tissue damage (26, 27)), and *Il1rl1* (IL-33 receptor or ST2). Gene set enrichment analysis (GSEA) confirmed that intra-hepatic Tregs engaged in the regulation of IL-1 and TNF production in Th1 immune responses. Additionally, GSEA showed that the intra-hepatic Treg transcriptome was enriched in pathways involved in the regulation of lymphocyte proliferation, and in metabolic processes such as lipid homeostasis, reactive oxygen species generation and DNA repair (Figure 1D).

In agreement with the transcriptional analyses, flow cytometry experiments conducted in parallel showed that, as compared to spleen Tregs, intra-hepatic Tregs exhibited increased expression of tissue-residency marker ITGAE (CD103), high expression of CD39, and high KI67 levels indicating increased proliferation (Figures 1E, F). Taken together, these data indicate that although intra-hepatic Tregs retain the Treg-specific core transcriptional signature, they exhibit distinct transcriptional and phenotypic features that imply adaptation to the liver tissue microenvironment.

Depletion of Tregs exacerbates acute liver inflammation but has no effect on the degree of hepatocyte necrosis

Having established that intra-hepatic Tregs exhibit a distinct molecular profile, we next investigated their role in controlling acute inflammatory liver damage induced by the hepatotoxin CCL_4 (Figure 2A). We employed DTR-Foxp3 mice, in which DT administration resulted in the almost complete depletion of Tregs both in the liver and the spleen (reduction of Treg percentages from 2.29% to 0.26% in liver and 8.06% to 0.34% in spleen) (Figures 2B, C). Following CCL_4 administration, Treg-deficient mice exhibited increased serum levels of IL-6 and reduced levels of IL-10 (Figure 2D). The increase in systemic inflammatory mediators was not associated however with an increase in serum aspartate transaminase (AST) as a marker of hepatocyte necrosis (Figure 2E). Based on these results, we speculate that Tregs are involved in controlling the systemic effects induced by CCL_4 administration but are not capable of counteracting the direct hepatotoxic effects responsible for hepatocyte necrosis.

IL-2c expand intra-hepatic Tregs and enhance their expression of immunoregulatory transcripts

Our next step was to determine the effects of IL-2c, an established therapeutic strategy to expand the Treg compartment, on the numbers and phenotypic characteristics of intra-hepatic Tregs. IL-2c administration increased the proportion of Tregs in all tissue compartments analysed, with the largest increased being observed in the liver (7.7-fold increase, from 3.65%to 28.14%; Figure 3A). Microarray gene expression analyses of intra-hepatic Tregs revealed that, in addition to promoting Treg expansion, IL-2c administration resulted in increased expression of various Treg-specific genes, such as *Il2ra* (CD25), *Lrrc32*, *Gpr83* and *Areg* (Figures 3B, C). GSEA revealed that IL-2c treatment resulted in an enrichment in pathways involved in the regulation of T cell activation, proliferation, and migration, along with pathways associated with mitochondrial integrity, biogenesis, and lipid homeostasis (Figure 3D). These transcriptional changes induced by IL-2c in intra-hepatic Tregs were not observed in the splenic Tregs isolated from the same animals. Flow cytometry experiments showed that, upon administration of IL-2c, intra-hepatic Tregs increased the expression of ST2, CD39 (ENTPD1) and KI67 as compared to splenic Tregs, which is in keeping with the transcriptional results outlined above. In contrast, CD25 expression increased both in intra-hepatic and in splenic Tregs (Figures 3E, F), although CD25 MFI was noted to be higher in the intra-hepatic Treg compartment. Taken together, these results are suggestive exogenous IL-2c administration led to increases in intra-hepatic Treg pool and reverted low expression of key immunoregulatory molecules of these cells.

The expansion of intra-hepatic Tregs in response to IL-2c administration is associated with reduced inflammatory liver damage

Next, we explored the impact of acute liver inflammation induced by CCL_4, with or without IL-2c administration, on the

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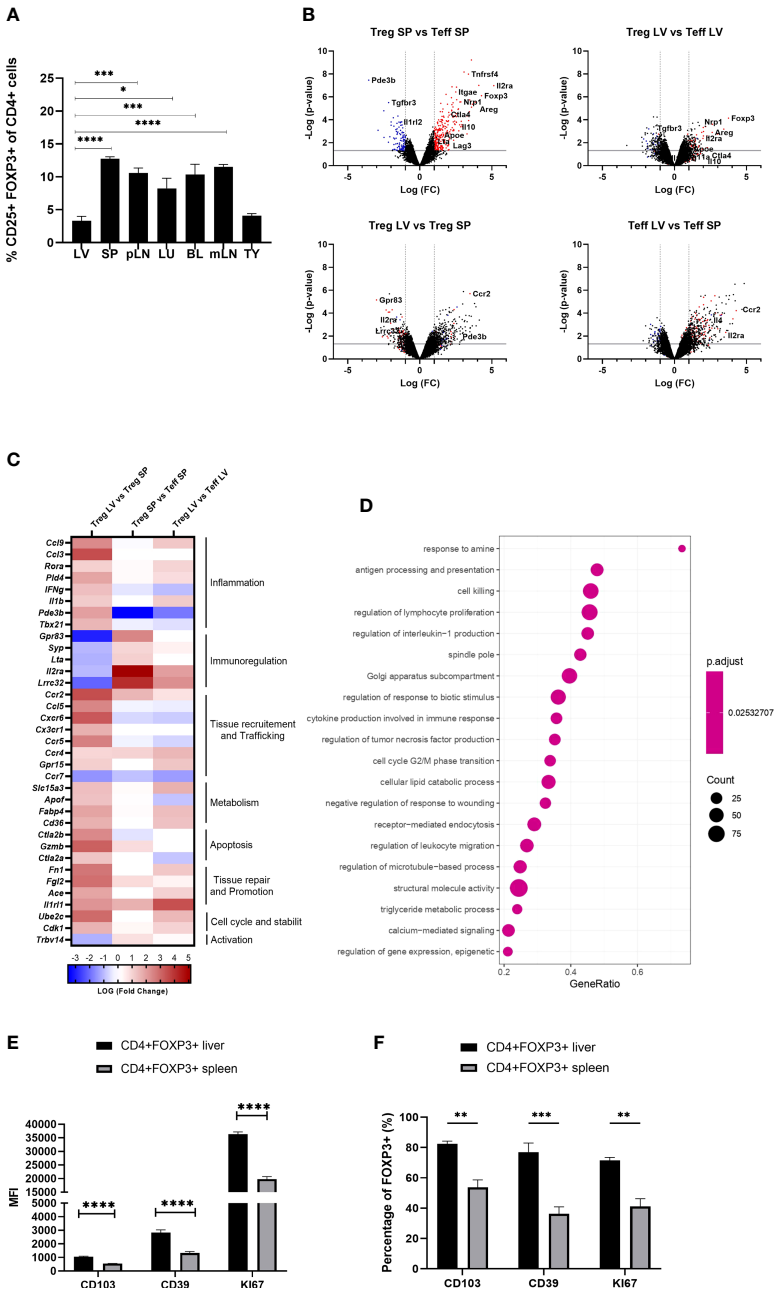

FIGURE 1
Intra-hepatic Tregs are less abundant but remain to exhibit a core Treg transcriptional signature and have a distinct molecular profile. **(A)** Quantification of the proportion of Foxp3+ CD4+CD25+ cells in the homogenised tissues of liver (LV) (n>8), spleen (SP) (n>8), peripheral lymph nodes (pLN), lung (LU), adipose tissue (AT), blood (BL), mesenteric lymph nodes (mLN) and thymus (TY) in homeostasis (n=4). **(B–D)** Whole genome microarray analysis in intra-hepatic and splenic Tregs versus T-effectors in Foxp3-YFP B6 mice (n=3). **(B)** Volcano plots showing difference in the transcriptome profile of intra-hepatic and splenic Tregs using normalised p-value versus fold change of all 20515 genes and splenic "Treg signature" is defined by up-regulated (red) and down-regulated (blue) genes above p-value of 0.05 and Fold change 2. **(C)** Heatmap exhibits pre-selected differentially expressed genes relevant to Treg function, tissue residency, tissue repair and metabolism between intra-hepatic and splenic Treg population and the significance is determined above p-value of 0.05 and Fold-change 2 **(D)** Gene set enrichment analysis of intra-hepatic Tregs compared with the splenic Tregs. Dot plot shows the top 20 up-regulated GO pathways of biological processes, molecular functions and cellular components with an FDR adjusted q-value of 0.020506. Dot size represents the number of genes enriched in the pathway, and gene ratio represents the ratio of the count of core enrichment genes to count of pathway genes. **(E)** CD4+Foxp3 + cells were gated from homogenised liver and spleen tissue to quantify the MFI of CD103, CD39 and KI67 (n=5). **(F)** Percentage of Foxp3+ cells expressing CD103, CD39 and KI67. Significance was determined by ordinary one-way ANOVA with Sidak's multiple comparison test for **(A)** and two-way ANOVA with Tukey multiple comparison test for **(E, F)** and the values are shown as the mean ± SEM. For B-D, p<0.05 and Fold Change >2. MFI, geometric mean fluorescence intensity. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

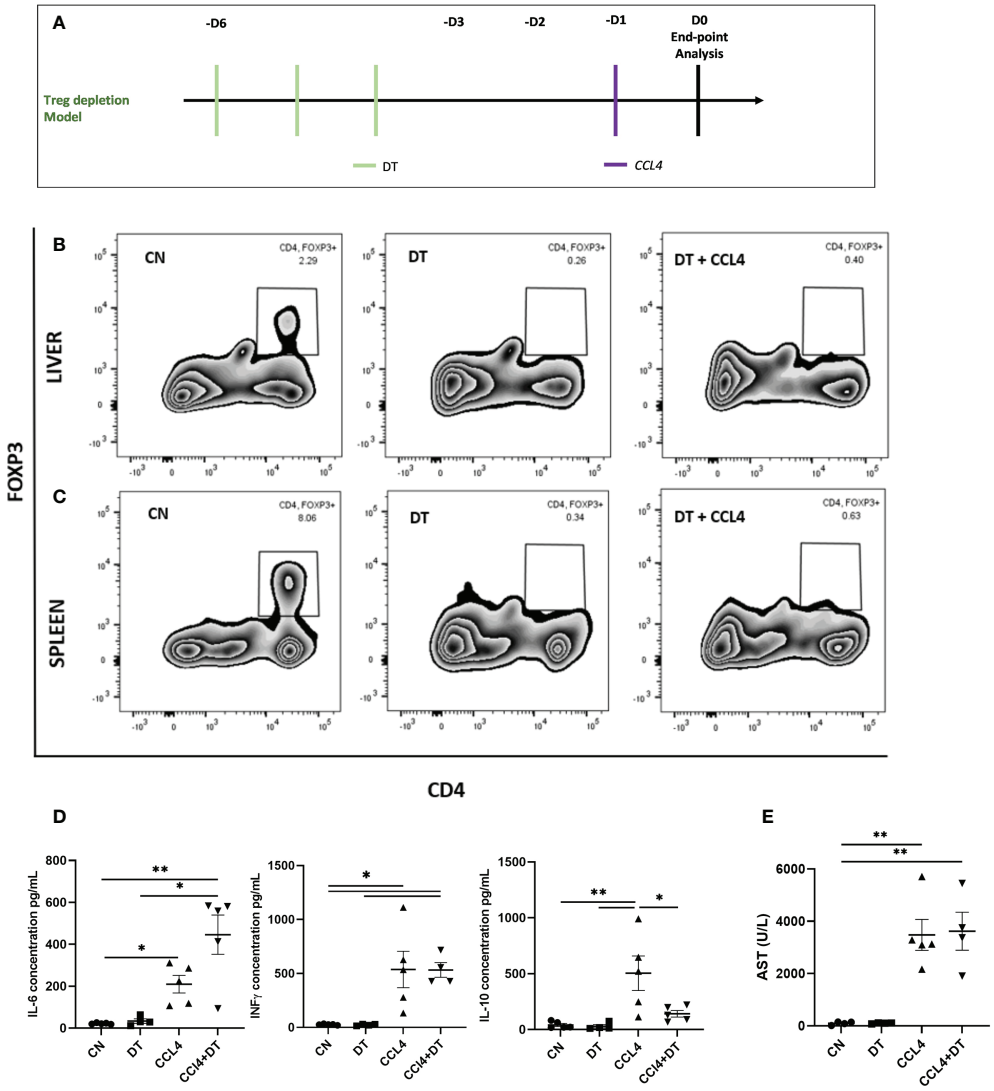

FIGURE 2
Hepatocyte necrosis is unaltered by Treg depletion. **(A)** Schematic representation of the experimental design for DT induced Treg depletion, 1ug diphtheria toxin (DT) was injected in Foxp3-DTR mice, 3 days prior to CCL_4 administration. Depletion of Foxp3+ cells was analysed by flow cytometry in **(B)** liver and **(C)** spleen and the results are shown as percentages from fixable live/dead stained viable CD4+ cells and representative of 3 individual experiments. **(D)** Serum concentration of IL-6, IFNg and IL-10 cytokines (pg/ml) were measured at 24h in the serum obtained by cardiac puncture following DT and CCL_4 treatment, determined by ELISA (n=5) along with **(E)** AST levels (n=4). The values are shown as the mean ± SEM and one-way ANOVA with Tukey's multiple comparison test has been performed to show statistical significance, *P < 0.05, **P < 0.01.

phenotype of intra-hepatic Tregs (Figures 4A, B). The number of intra-hepatic Tregs increased 2-fold (from 3.65 ± 0.45% to 7.45 ± 0.99%) following injection of a single dose of CCl_4, with a further increase up to 8.9-fold when IL-2c was administered prior to CCL_4 (Figure 4C). CCL_4 did not influence the number of CXCR3+ Tregs or the expression levels of CD25, CD39, ST2 and CTLA4 (Figures 4D–H), but it increased intra-hepatic Treg proliferation (Figure 4I). In contrast, treatment with IL-2c prior to CCL_4 administration did not modify KI67 levels but it resulted in increased intra-hepatic Treg expression of CD25, CTLA4, CD39 and ST2, as well as in a marked expansion of intra-hepatic CXCR3+ Tregs (Figures 4D–I). We also observed that combined IL-2c plus CCL_4 treatment resulted in an increase in the intra-hepatic proportion of NUR-77-positive Tregs (Figure 4J), a marker of immediate TCR stimulation (28).

In contrast to the effects of observed on intra-hepatic Tregs, neither IL2c nor CCL_4 increased the absolute numbers of intra-splenic and intra-hepatic CD4+Foxp3- Teffs, and in line with our transcriptome data, NUR-77, CTLA-4, CXCR3 and ST2 expression were not modified in CD4+Foxp3- Teffs following

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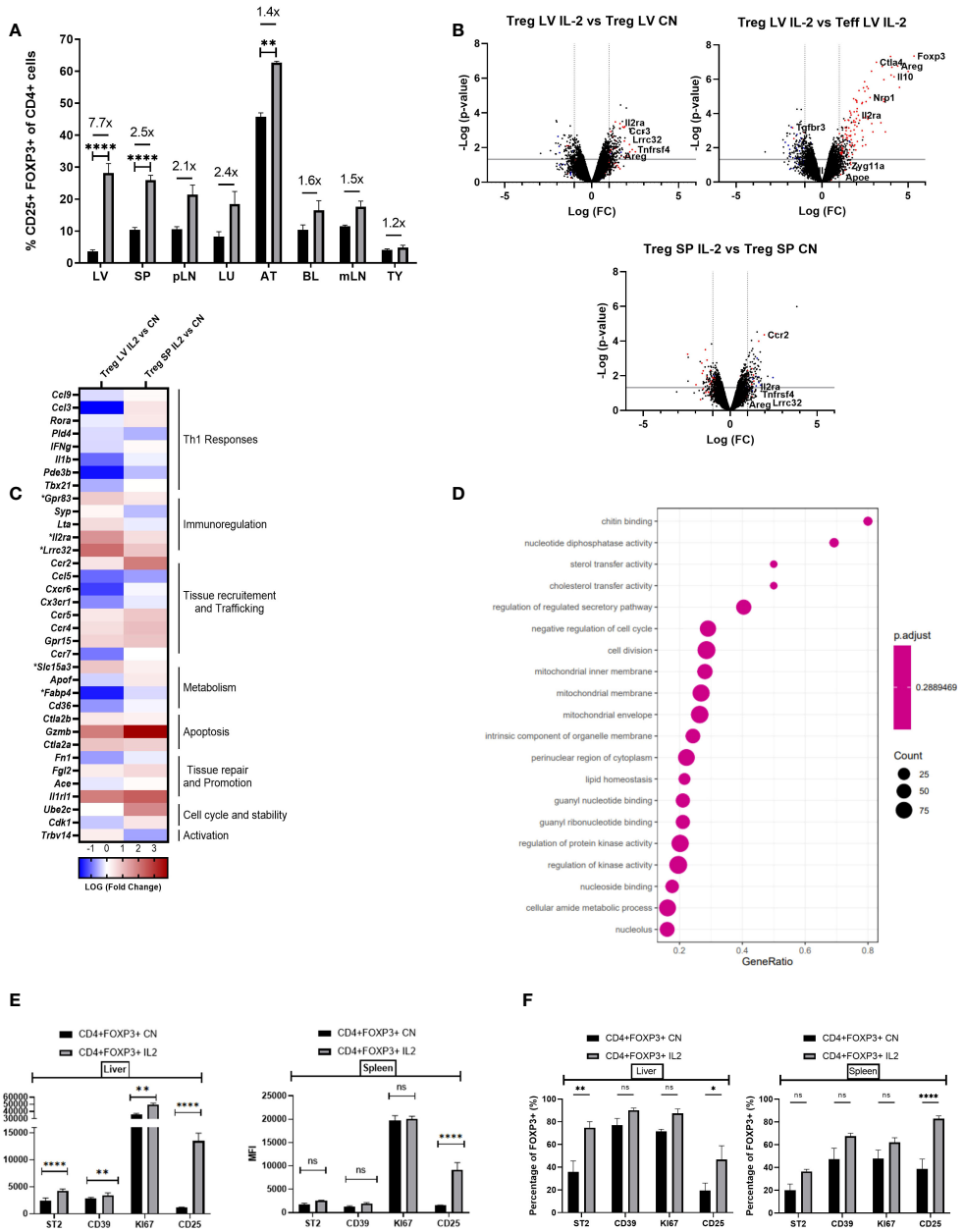

FIGURE 3
IL-2 administration increases Foxp3+ cells in the liver and enhances Treg signature transcriptional characteristics. **(A)** Quantification of the proportion of Foxp3+ CD4+CD25+ cells in the liver (LV) (n>8), spleen (SP) (n>8), peripheral lymph nodes (pLN), lung (LU), adipose tissue (AT), blood (BL), mesenteric lymph nodes (mLN) and thymus (TY) (n=4). **(B)** Volcano plot shows difference in the transcriptome profile of intra-hepatic Tregs and T-effectors when IL-2 is administered in Foxp3YFP mice (n=3) using normalised p-value versus fold change of all 20515 genes and splenic "Treg signature" is defined by up-regulated (red) and down-regulated (blue) genes above p-value of 0.05 and Fold change 2. **(C)** Heatmap exhibits differences in the expression of pre-defined differentially expressed genes relevant to Treg function, tissue residency, tissue repair and metabolism between intra-hepatic and splenic Treg population following IL-2c administration, and the significance is determined above p-value of 0.05 and Fold-change 2 and represented by a *. **(D)** Gene set enrichment analysis of IL-2c treated intra-hepatic Tregs compared with the intra-hepatic Tregs in homeostasis. Dot plot shows the top 20 up-regulated GO pathways of biological processes, molecular functions and cellular components with an FDR adjusted q-value of 0.020506. Dot size represents the number of genes enriched in the pathway, and gene ratio represents the ratio of the count of core enrichment genes to count of pathway genes. **(E, F)** Flow cytometric analysis of the effect of IL-2 administration on the previously defined differentially expressed transcripts of CD4+Foxp3+ cells in liver and spleen tissue, expressed as MFI and percentage. Significance was determined by ordinary one-way ANOVA with Sidak's multiple comparison test for **(A)** and two-way ANOVA with Tukey multiple comparison test for **(E, F)** and the values are shown as the mean ± SEM. For B-D, p<0.05 and Fold Change >2. MFI, geometric mean fluorescence intensity. Ns, non-significant *P < 0.05, **P < 0.01 and ****P < 0.0001.

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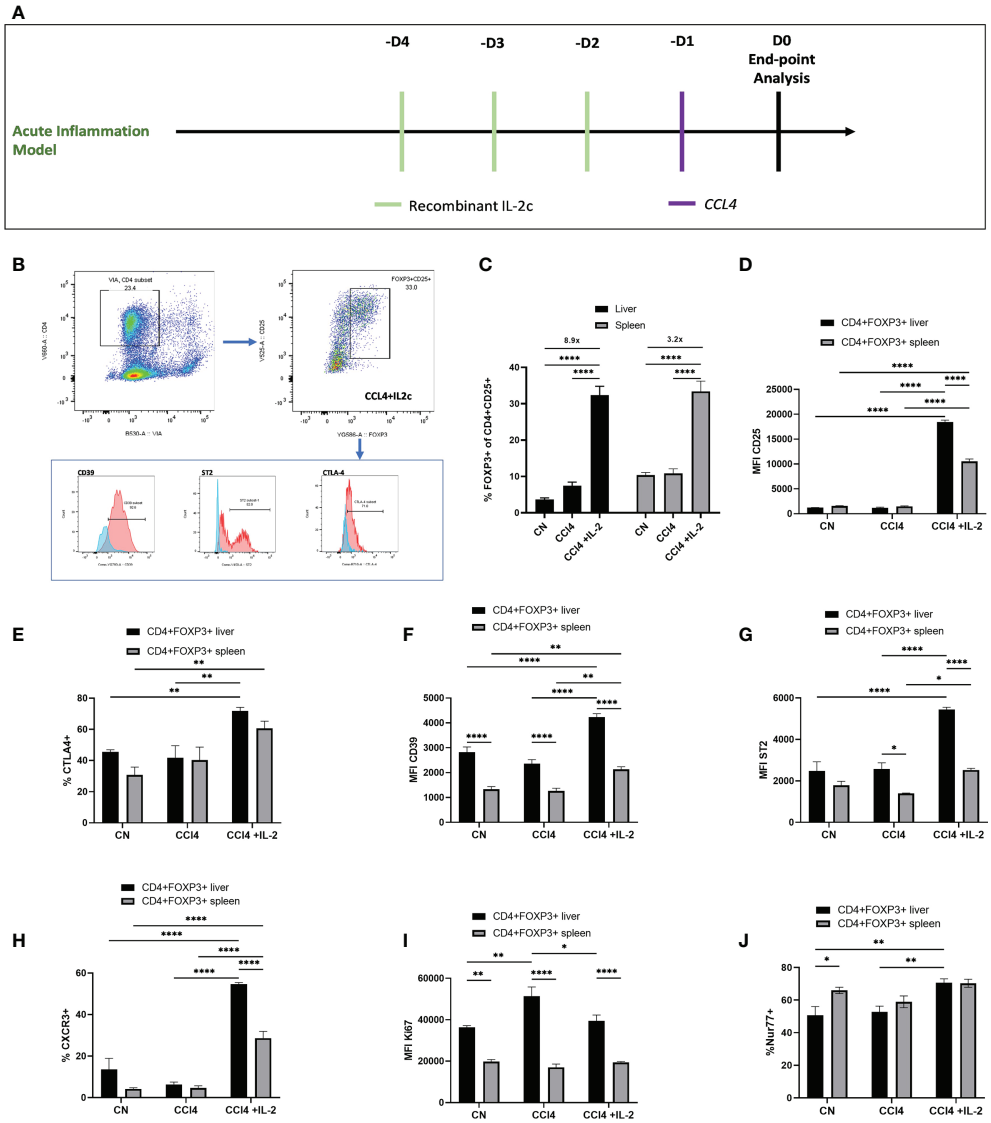

FIGURE 4
IL-2c expanded intra-hepatic Tregs exhibit enhanced expression of immunoregulatory transcripts in the inflammation rich microenvironment. **(A)** Schematic representation of the experimental design to generate CCL_4 induced acute liver inflammation; IL-2c administered for 3 consecutive days, following by a single dose of CCL_4 injection and animals were sacrificed 24h later for the tissue analysis. **(B)** Gating strategy employed to identify CD4+CD25+Foxp3+ cells and their expression profile, including the staining controls. **(C)** Percentage of CD4+CD25+Foxp3+ cells were quantified in liver and spleen in homeostasis, following IL-2c administration, CCL_4 induced inflammation and IL-2c administration prior to CCL_4 induced inflammation (n=9) along with **(D)** the changes in the MFI of CD25. Immunoregulatory profile and stability of these CD4+Foxp3+ cells in liver and spleen were characterised using flow cytometry by quantifying percentage of **(E)** CTLA4 expression and MFI of cell surface markers **(F)** CD39 and **(G)** ST2. **(H)** Percentage of Chemokine receptor CXCR3 positive cells regulating the homing of cells and **(I)** MFI of proliferating cells expressed by KI67+. **(J)** Additionally, percentage expression of NUR77 was compared between liver resident and splenic CD4+Foxp3+ cells. All data were quantified using flow cytometry (n=4). The values are shown as the mean ± SEM and one-way ANOVA with Tukey's multiple comparison test has been performed to show statistical significance, Ns= non-significant *P < 0.05, **P < 0.01 and ****P < 0.0001.

IL-2c administration (Figures 5A–C). Characterization of cell subsets (Figure 5D) showed that, neither IL-2c nor CCL_4 increased the number of intra-hepatic CD8+ cells, however a decrease in the splenic CD8+ percentage was observed with IL-2c administration, while an increase in NK and NKT cells was seen with IL-2c injection alone in spleen. Additionally, NK and NKT cells were increased by IL-2c administration in the inflamed liver compartment. (Figure 5E). Additionally, a shift to the pro-repair CD11b+Ly6C^{lo} M2 macrophage phenotype was observed in liver injury with the expansion of intra-hepatic Tregs. CCL_4 induced liver injury increased the percentage of pro-inflammatory CD11b+Ly6C^{hi} M1 macrophages, and this

increase was reversed with IL-2c administration. Moreover, a decrease in the anti-inflammatory pro-restorative M2 macrophages was seen in the CCL_4 induced inflammation and this population was restored with IL-2c injections. Furthermore, we observed an increase in the percentage of CD11b+F4/80+ Kupffer cells in mice treated with IL-2c in the presence of CCL_4 induced inflammation. (Figure 5F).

The changes in immune cell subsets induced by IL2c administration were associated with reduced expression of the pro-inflammatory cytokines *Il-6*, *Ifng*, and *Tnfa*, both in serum and in liver tissue, (Figures 6A, B), as well as with decreased AST/ALT serum levels (Figure 6C). These results were consistent with the histology analyses, which revealed that, in mice treated with CCL_4, IL-2c administration reduced both intra-hepatic leukocyte infiltration and hepatocyte necrosis (Figure 6D).

Discussion

The cytokine IL-2, which is secreted by activated CD4 and CD8 T cells, is known to influence the differentiation, homeostasis, and effector properties of multiple immune cell subsets, but in particular of Tregs, whose function and survival are critically dependent on IL-2 availability (29). We previously described that in humans receiving calcineurin inhibitors, low IL-2 levels are responsible for the reduced number of circulating Tregs, which exhibit both increased proliferation and increased apoptosis (30). Likewise, lack of IL-2 has also been proposed as an explanation for the impaired function and pro-apoptotic tendency of Tregs isolated from human livers with end-stage inflammatory diseases (3). The results of our current study indicate that even under non-inflammatory quiescent conditions, intra-hepatic Tregs are reduced in number as compared to what is found in other organs and exhibit increased proliferation and features suggestive of lineage instability. Of note, while in the liver the proportion of Tregs among CD4+ T cells was the lowest of all tissues sampled, following administration of exogenous IL-2c intra-hepatic Tregs exhibited the greatest relative increase. This was associated with overexpression of molecules traditionally associated with Treg function and lineage stability. Taken together, these findings support the hypothesis that IL-2 deprivation is a major driver of the distinct features exhibited

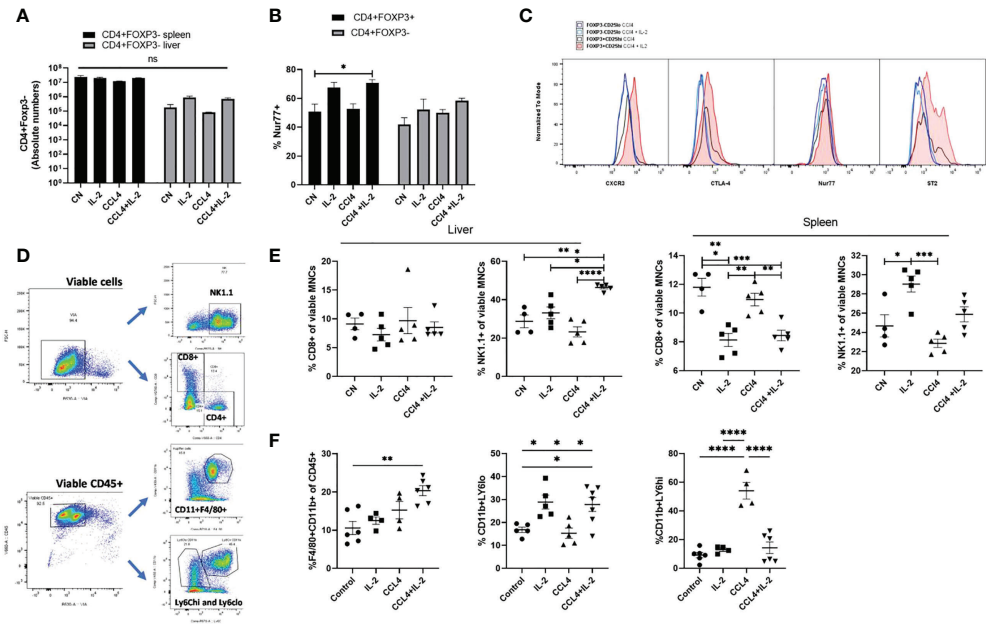

FIGURE 5
Characterisation of intra-hepatic cell subsets impacted by IL-2 administration and CCL_4 induced inflammation. **(A)** Absolute numbers of CD4+Foxp3- cells were quantified in liver and spleen in homeostasis, following IL-2c administration, CCL_4 induced inflammation and IL-2c administration prior to CCL_4 induced inflammation (n=5). **(B)** Percentage expression of NUR77 was compared between CD4+CD25+FOXP3+ Tregs and CD4+CD25+Foxp3- Effector T cells (n=4). **(C)** Additionally, these cell subsets were compared for changes in activation characterised by CTLA-4 and NUR77 expression and liver-specific regulation and homing seen by differences in ST2 and CXCR3 expression following CCL_4 alone or with IL-2 treatment, the plot is representative of 4 individual experiments **(D)** Gating strategy employed to characterise cell subsets. **(E)** Effect of CCL_4 induced liver injury with or without exogenous IL-2c on the percentage of CD8+ Effector T and NK cells in liver and spleen were summarised (n=4). **(F)** Changes in the hepatic non-parenchymal cell subsets were quantified by looking at the percentage abundance of Kupffer cells, Ly6C^{hi} pro-inflammatory and Ly6C^{lo} restorative macrophages following CCL_4 induced inflammation and exogenous IL-2c availability. The values are shown as the mean ± SEM and one-way ANOVA with Tukey's multiple comparison test has been performed to show statistical significance. Ns, non-significant *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

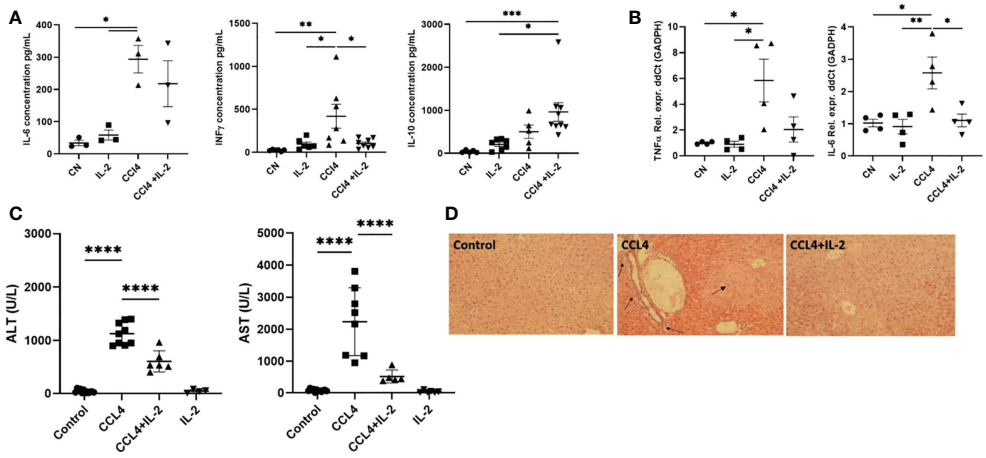

FIGURE 6
Impact of IL-2 administration on the modulation of inflammatory state of liver. Inflammation of liver was quantified by performing qPCR analysis on the RNA isolated from liver biopsies, assessing the distinctive cytokine profile and liver infiltrate in liver inflammation. **(A)** The quantification of pro and anti-inflammatory cytokines secreted in the serum (n=3) and **(B)** RNA extracted from the inflamed liver (n=4). **(C)** Levels of serum ALT and AST were measured at the study endpoint at 24h post-injection (n=5). **(D)** H&E staining of liver sections examined *via* light microscopy at 20x magnification. Black arrows indicate regions of excessive immune infiltration, and greater intensity of pink reveal regions of hepatocyte necrosis. The values are shown as the mean ± SEM and one-way ANOVA with Tukey's multiple comparison test has been performed to show statistical significance *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

by intra-hepatic Tregs. The insignificant increase observed in the Treg numbers under inflammatory conditions could be associated with increased IL-2 production by the activated T cell infiltrate, demonstrating importance of IL-2 accessibility in controlling the frequency and survival of Tregs (31)

The liver microenvironment is known to be biased towards immune tolerance. Multiple mechanisms contribute to this state , including clonal deletion, clonal anergy and T cell exhaustion. The liver is known to trap activated T cells (e.g., autoreactive T cells), promoting their apoptotic clearance (32–34). Both parenchymal (e.g., hepatocytes, endothelial cells) and non-parenchymal cells contribute to this phenomenon (35–38). The extent to which different cell types and tolerogenic mechanisms are non-redundant and operate according to a universal pre-defined hierarchy, as opposed to being model dependant, remain unanswered questions. The data described in our report suggest that, in steady-state conditions, Tregs are unlikely to play a dominant role in maintaining intra-hepatic immune tolerance, given their low number and molecular programme. Likewise, the results of the DTR-Foxp3 CCL_4-induced liver damage model indicate that the small size of the intra-hepatic Treg pool exerts limited immunoregulatory effects in the setting of acute liver inflammation.

On the other hand, the increased expression levels of the IL-33 receptor (ST2 or IL1rl1) in intra-hepatic Tregs, both in homeostasis and following acute liver injury, suggests that Treg might be involved in liver regeneration and tissue repair, as previously described in muscle, lung, and intestine (12, 39, 40). IL-33 is released by activated endothelial cells in response to tissue damage. This results in the stimulation of ST2-positive Tregs, which then secrete amphiregulin, responsible for promoting tissue regeneration at least in part through the polarisation of monocytes towards an M2-like phenotype (41).

The expansion of the intra-hepatic Treg compartment in response to IL-2c significantly ameliorated liver damage following CCL_4 administration. The expansion was associated with increased transcript levels of *Ctla4* and CD39, whose expression is typically correlated with Treg function and fitness (42, 43). An important question to address is whether this expansion results from increased trafficking of circulating Tregs, or, alternatively, is secondary to the heightened proliferation and longevity of pre-existing tissue-resident Tregs. The fact that the administration of IL-2c to CCL_4-treated mice markedly increase the number of intra-hepatic CXCR3+ Tregs without modifying their KI67 levels, suggests that trafficking is the most likely mechanism. We hypothesize that this process is TCR-dependent, given the changes observed in NUR-77 levels, a marker of recent TCR stimulation (28). These data are in keeping with published literature demonstrating that the accumulation of Tregs in tissues is mostly dependent on their antigen specificity (44–46).

Our results are consistent with a recent report in a murine model of autoimmune hepatitis in which IL-2c ameliorated chronic inflammatory liver damage (47). Beyond animal models, low-dose recombinant IL-2 can effectively increase the number of circulating Tregs in humans and exert anti-inflammatory effects, as shown in multiple clinical trials in autoimmunity and in GVHD. Our group and others previously described the use of short courses

of low-dose recombinant IL-2 to increase circulating Tregs in patients with autoimmune hepatitis (5) (48). In contrast to the results observed in autoimmunity, an additional study from our group in human liver transplantation revealed that low-dose IL-2 increased the immunogenicity of the liver allograft, facilitating rather than preventing allograft rejection (49). These somehow unexpected clinical results observed in liver transplant recipients are reminiscent of what has been described in murine models of intra-hepatic T cell priming, in which IL-2 reverts the inactivation of CD8+ T cells that takes place when they recognize cognate antigens expressed by hepatocytes (50). Recent data indicate that this process is regulated by NK and ILC1 cells, which constitute a significant proportion of intra-hepatic immune cells and compete with T cells for IL-2 (51). Altogether, these data indicate that IL-2 administration constitutes a double-edged sword in what regards controlling intra-hepatic inflammation.

Our study has a number of limitations that need to be taken into consideration to avoid over-interpretations. First, our experimental system does not allow to track antigen-specific Tregs or effector T cells. Second, the preferential binding of the of IL-2c composed of recombinant IL-2 and clone JES6-1A12 to the IL-2RA/B chains rather than the IL-2RBG is different than what is observed with recombinant IL-2 in humans. Third,we intentionally chose to investigate the role of Tregs in a model of acute inflammatory damage. While it would have been desirable to replicate similar experiments in a model of chronic liver damage, such a model would have been confounded by the marked systemic inflammatory effects mediated by auto-reactive T cells that results from chronic Treg depletion (52, 53). Previous work reported that the transient DT-induced Treg depletion in the late phase of chronic CCL_4 treatment resulted in aggravated liver damage and fibrosis (54), suggestive that with chronic damage, the situation could be different to our observations in acute damage. Additionally, our finding of a reduced proportion of Tregs in intra-hepatic CD4+ T cells would need to be confirmed in other murine strains and across a range of different ages. With these caveats in mind, we believe our report provides relevant novel insight into the complex immune network of liver microenvironment and the potential of intra-hepatic Tregs in modulating liver inflammation. These results will need to be taken into consideration when interpreting studies investigating strategies to boost or ameliorate liver inflammation both in animal models and in the clinic.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE80814.

Ethics statement

The animal study was reviewed and approved by Animal Welfare and Ethical Review Body of King's College London.

Author contributions

MM-L supervised the project. AK, KS, PR-M, GO, TC, LD, KW, EG, SM, and EK performed and analyzed experiments. MM-L, NS and AS-F contributed to data analysis. AK, MM-L, AS-F and NS prepared the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

The reviewer JH declared a shared consortium with one of the authors, AS-F, to the handling editor.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1040031/full#supplementary-material

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