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Regulatory T cell therapy in Crohn's disease: challenges and advances

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Regulatory T cell therapy in Crohn's disease: challenges and advances

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ABBREVIATIONS:

APC – antigen presenting cell
AREG – amphiregulin
ATP – adenosine triphosphate
CD – Crohn's disease
CDAI – Crohn's disease activity index
CTLA-4 – cytotoxic T lymphocyte antigen 4
DC – dendritic cell
DR5 – death receptor 5
DSS – dextran sulfate sodium
Ebi3 – Epstein Barr virus induced 3
EGF – epidermal growth factor
FACS – fluorescence-activated cell sorting

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3 Fgl2 – fibrinogen-like protein 2
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5 Foxp3 – Forkhead box P-3
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7 GALT – gut-associated lymphoid tissue
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9 GC-MS – gas chromatography mass spectrometry
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11 GI – gastrointestinal
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13 GMP – good manufacturing practice
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15 GvHD – graft versus host disease
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17 HEPA – high efficiency particulate air
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19 HSCT – haematopoietic stem cell transplant
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21 IBD – inflammatory bowel disease
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23 IFN γ – interferon γ
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25 IL – interleukin
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27 IPEX – ‘immune dysregulation, polyendocrinopathy, enteropathy, X-linked’
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29 LPMC – lamina propria mononuclear cell
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31 MACS – magnetic bead-activated cell sorting
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33 MAdCAM-1 – mucosal vascular addressin cell adhesion molecule 1
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35 MHC – major histocompatibility complex
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37 MMP – matrix metalloproteinase
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39 NK – natural killer
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41 NOD – non-obese diabetic
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43 PBMC – peripheral blood mononuclear cell
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45 RAR α – retinoic acid receptor α
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47 RORC – related orphan receptor C
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49 RPMI – Roswell Park Memorial Institute
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51 SCID – severe combined immunodeficiency
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53 SLE – systemic lupus erythematosus
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55 STAT – signal transducer and activator of transcription
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57 T1DM – type 1 diabetes mellitus
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59 TCR – T cell receptor
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61 Teff – effector T cell
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63 TGF- β – transforming growth factor β
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65 Th1 – T helper 1 cell

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3 Th17 – T helper 17 cell

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5 TIGIT – T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based
6 inhibitory motif (ITIM) domains

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8 TNBS – 2,4,6-trinitrobenzene sulfonic acid

9
10 TNF α – tumour necrosis factor α

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12 TRAIL – tumour necrosis factor-related apoptosis inducing ligand

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14 Treg – regulatory T cell

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16 pTreg – peripheral regulatory T cell

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18 tTreg – thymic regulatory T cell

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20 UC – ulcerative colitis

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23 **ABSTRACT:**

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27 The prevalence of inflammatory bowel disease is rising in the Western world. Despite an
28 increasing repertoire of therapeutic targets, a significant proportion of patients suffer
29 chronic morbidity. Studies in mice and humans have highlighted the critical role of
30 regulatory T cells in immune homeostasis, with defects in number and suppressive function
31 of regulatory T cells seen in Crohn's disease patients. We review the function of regulatory T
32 cells and the pathways by which they exert immune tolerance in the intestinal mucosa. We
33 explore the principles and challenges of manufacturing a cell therapy, and discuss clinical
34 trial evidence to date for their safety and efficacy in human disease, with particular focus on
35 the development of a regulatory T cell therapy for Crohn's disease.
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45 **Keywords:** Crohn's disease, Immunology, Immunoregulation, Intestinal T cell, T lymphocytes

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49 **INTRODUCTION:**

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52 Inflammatory bowel disease (IBD), chiefly comprising Crohn's disease (CD) and ulcerative
53 colitis (UC), is a chronic inflammatory group of disorders of the gastrointestinal (GI) tract
54 arising from overexuberant innate and adaptive immune responses to environmental
55 factors in genetically susceptible individuals. IBD affects at least 0.5% of the population in
56 the Western world with 1 million sufferers in USA and 2.5 million in Europe.¹ Global
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3 prevalence continues to increase, largely driven by rising numbers of patients in newly
4 industrialised regions including India and Asia.¹ The burden of disease is significant with 20-
5 25% of patients experiencing chronic continuous symptoms which contributes to higher
6 rates of unemployment, sick leave and permanent work disability.² Even with an aggressive
7 top-down approach to therapy, the majority of patients fail to achieve prolonged, steroid-
8 free remission and are at particular risk of requiring surgical intervention. Cumulative
9 surgery rates in CD are high in Europe with 30-50% of patients requiring surgical
10 intervention and up to 20% needing a reoperation 5-10 years from diagnosis.²
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20 As our understanding of the pathophysiology of IBD and its socioeconomic impact has
21 evolved, there has been great impetus to identify novel therapeutic targets to add to the
22 existing arsenal of immunomodulators and biologics. These have focussed on a variety of
23 areas including targeting lymphocyte trafficking (vedolizumab, ozanimod, anti-MAdCAM1)
24 and activation (anti-IL6, anti-IL12/IL23), modulating intestinal barrier function
25 (phosphatidylcholine), matrix remodelling (STNM-01, MMP9 blocker) and manipulation of
26 gut microbiota (faecal microbiota transplant).³ An important pathological process
27 increasingly recognised as driving intestinal inflammation and autoimmunity is the loss of
28 immune homeostasis secondary to qualitative or quantitative defects in the regulatory T cell
29 (Treg) pool.
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40 Tregs are CD4⁺ T cells that characteristically express the high affinity IL-2 receptor α -chain
41 (CD25), and master transcription factor Forkhead box P-3 (Foxp3), which is essential for
42 their suppressive phenotype and stability.⁴⁻⁶ As activated CD4⁺ T cells can upregulate CD25
43 expression, an additional defining feature of Tregs is the absence of IL-7 receptor α -chain
44 (CD127).⁷ Their primary function is as dominant controllers of self-tolerance, tissue
45 inflammation and long-term immune homeostasis. Despite making up only 5-10% of the
46 peripheral CD4⁺ T cell pool, Tregs exert powerful inhibitory effects on effector cells through
47 a variety of mechanisms including cytokine secretion, metabolic disruption, inhibition of
48 dendritic cells (DCs) and cytotoxicity. These mechanisms have been rigorously examined using
49 animal models and shown to protect against the development of intestinal inflammation.
50 Studies in patients with IBD have identified defects in the number and distribution of Tregs,
51 and their ability to traffic to the GI tract.⁸ Additionally, resistance to Treg-mediated
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3 suppression has been noted in lamina propria T effector cells (Teffs).⁹ These factors are
4 likely to be pivotal in driving intestinal inflammation.
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9 There is growing interest in the therapeutic potential of adoptively transferring healthy
10 Tregs into patients with a wide range of conditions, including IBD and autoimmune disease,
11 in an attempt to shift the balance in areas of active inflammation towards a more
12 tolerogenic microenvironment. Early phase clinical trials have already reported in the fields
13 of solid organ transplantation, graft-versus-host disease (GvHD) and type 1 diabetes mellitus
14 (T1DM) with reassuring safety data and potential signals of efficacy.
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21 This review provides a summary of the suppressive mechanisms utilised by Tregs and
22 highlights seminal work linking intestinal inflammation with loss of Treg function in both
23 animal models of disease and in humans. Additionally, we review ongoing clinical trials with
24 Treg therapy and outline an entirely novel therapeutic strategy for CD using Tregs expanded
25 under GMP (Good Manufacturing Practice) conditions that will be adoptively transferred to
26 patients in an attempt to ameliorate intestinal inflammation and restore immune
27 homeostasis.
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36 **TREGS IN HEALTH AND DISEASE:**

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40 Tregs can be broadly divided into two groups, thymic Tregs (tTregs) or peripherally induced
41 Tregs (pTregs) based on their developmental origin. Tregs generated in the thymus (tTregs)
42 in the early neonatal period migrate to peripheral organs where they maintain tolerance.
43 This was discovered in 1969 by Nishizuka and Sakakura who showed that in mice,
44 thymectomy 3 days after birth led to the depletion of Foxp3⁺ Tregs and development of
45 autoimmune oophoritis.¹⁰ In contrast, mice who had thymectomy at day 7 remained healthy
46 as the tTregs had already migrated to the periphery by this point.¹¹ Over a decade later,
47 Sakaguchi *et al* demonstrated that day-3 thymectomy autoimmune oophoritis could be
48 prevented with CD4⁺ T cell inoculation from healthy syngeneic donors. Conversely, the
49 adoptive transfer of T cells from these sick mice were capable of inducing autoimmune
50 disease in healthy T cell deficient mice.¹² Similar findings were noted in rats that underwent
51 adult thymectomy and irradiation resulting in lymphopenia, autoimmune diabetes and
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3 insulinitis. An injection of CD45RC(low) T cells from healthy donors were capable of
4 preventing disease.¹³ Mottet *et al* subsequently described CD25-expressing CD4⁺ T cells that
5 were able to cure established T cell transfer colitis.¹⁴ By the early 2000's it was clear that a
6 thymically-derived CD4⁺CD25⁺ T cell population possessed the ability to suppress
7 autoreactive T cells and eliminate autoimmunity.
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14 Peripherally induced Tregs (pTregs) were first described in 2003 where naïve CD4⁺CD25⁻ T
15 cells could be converted into Foxp3-expressing CD4⁺CD25⁺ Tregs by T cell receptor (TCR) co-
16 stimulation in the presence of TGF- β .¹⁵ pTreg conversion in gut-associated lymphoid tissues
17 (GALT) was enhanced when naïve CD4⁺ T cells encountered antigen in the presence of TGF-
18 β , IL-2 and retinoic acid (RA).^{16,17} This is facilitated by CD103⁺ DCs conditioned by the
19 intestinal microenvironment to produce or activate TGF- β and provide RA.^{18,19} In the
20 absence of CD103 expression, DCs fail to induce Treg development and produce
21 proinflammatory cytokines.^{18,20} Additionally, in patients with UC, CD103⁺ DCs appear to
22 have impaired ability to generate pTregs, but induce colitogenic T helper (Th) 1, Th2 and
23 Th17 responses suggesting CD103⁺ DC-mediated pTreg induction is functionally relevant in
24 IBD pathogenesis.²¹
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36 Distinguishing tTregs from pTregs can be difficult as no definitive markers exist. Recently,
37 the expression of the membrane protein neuropilin-1 (Nrp1) and the transcription factor
38 Helios by tTregs but not by pTregs has been used to differentiate Treg subsets.²² The
39 significance of this lies in the epigenetic differences in the *Foxp3* locus rendering pTregs less
40 stable and more likely to demonstrate plasticity towards a Th17 cell phenotype under
41 inflammatory conditions.²³ The developmental origin of Tregs selected for expansion as a
42 cell therapy product is therefore an important consideration and will be addressed in more
43 detail later in this review.
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53 The first study identifying Tregs in humans was published in 2001. Baecher-Allan *et al*
54 characterised CD4⁺CD25⁺ T cells in the thymus and peripheral blood which exhibited anti-
55 inflammatory and suppressive properties.²⁴ Subsequent work established Foxp3 as the
56 master transcription factor for Tregs.^{4,6,25} Foxp3 can however be expressed transiently in
57 non-regulatory CD4⁺ T cells upon TCR activation and the CD4⁺CD25⁺CD127^{lo} surface
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3 phenotype must be used to define Tregs.²⁶ Inactivating mutations in *Foxp3* clinically
4 manifest as severe autoimmunity with a scurfy phenotype in mice and IPEX syndrome
5 ('immune dysregulation, polyendocrinopathy, enteropathy, X-linked') in humans.²⁷⁻³⁰ With
6 autoimmune enteropathy (manifesting as chronic diarrhoea and malabsorption) a
7 predominant feature, attention was focussed on the functional role of Tregs within the GI
8 tract.
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16 Peripheral Tregs are found in abundance in the intestinal lamina propria where interactions
17 with environmental antigens can shape phenotypic differences and transcription factor
18 expression.³¹ The gut microbiota represents a substantial antigen load driving the expansion
19 of colonic pTregs that co-express the Th17 master transcription factor ROR γ t.³² These
20 Foxp3⁺ ROR γ t⁺ pTregs have a stable regulatory phenotype and provide tolerance against the
21 gut microbiota.^{33,34} Conversely, ROR γ t⁻ pTregs are found in the small intestine where they
22 are induced by dietary antigens and repress underlying Th1 cell responses to ingested
23 proteins.³⁵ Finally, an intestinal tTreg population that co-express the Th2 master
24 transcription factor, GATA3, has been shown to mediate repair of the intestinal mucosa.
25 GATA3⁺ tTregs express high levels of the IL-33 receptor, ST2, and amphiregulin, an
26 epidermal growth factor receptor ligand involved in tissue repair.^{36,37}
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38 Following on from the fundamental observations linking Treg dysfunction to an array of
39 autoimmune polyendocrine syndromes, studies began to emerge identifying defects in
40 either number or function of peripheral blood Tregs in autoimmune disorders including IBD,
41 type 1 diabetes, multiple sclerosis, systemic lupus erythematosus (SLE), myasthenia gravis
42 and rheumatoid arthritis.^{8,38-42} Maul *et al* observed that in patients with active IBD, the
43 intestinal lamina propria Treg pool was significantly smaller than that of a positive control,
44 namely diverticulitis.⁸ Additionally, in these patients, the peripheral blood Treg pool was
45 smaller than that of inactive IBD or diverticulitis.⁸ Interestingly, the peripheral blood Tregs
46 retained their suppressive capacity suggesting that disease may be driven by ineffective
47 trafficking to the gut and reduced numbers of Tregs. Furthermore, colitogenic T cells from
48 IBD patients appear to be resistant to TGF- β 1-mediated Treg suppression highlighting an
49 additional defect in immunological tolerance that may drive disease.⁴³
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TREG FUNCTION AND COLITIS:

Tregs function as key mediators of peripheral tolerance through direct cellular contact and paracrine actions on tissues where they reside.^{44,45} It is essential that Tregs effectively traffic to target organs where they promote a tolerogenic microenvironment. An important example is IL-10-secreting Tregs that reside in the GI mucosa and control inflammatory responses induced by environmental insults. Selective disruption of IL-10 expression in these Tregs has been shown to cause spontaneous colitis.⁴⁶ This is one of many modalities that Tregs can employ to maintain immune homeostasis at the mucosal interface. Others include inhibitory cytokine secretion, cytolysis of effector cells, metabolic disruption, neutralization of antigen presenting cells (APC) and promotion of tissue repair.⁴⁷ These functions will be reviewed in further detail outlining their associations with intestinal inflammation (see Figure 1).

Inhibitory Cytokines:

The Treg cytokine repertoire includes the anti-inflammatory molecules IL-10, TGF- β and IL-35. The expression of IL-10 and IL-35 requires TCR signalling, suggesting that Treg function in part relies on antigen encounter in the local microenvironment.⁴⁸ Pioneering work by Powrie *et al* over 20 years ago showcased the potent inhibitory ability of IL-10, where recombinant IL-10 therapy ameliorated established T cell transfer colitis.⁴⁹ Subsequently, the co-transfer of CD45RB(low) T cells were shown to prevent colitis and IL-10 was identified as an essential mediator for this *in vivo* suppression.⁵⁰ The suppressive effects of Treg-derived IL-10 in mice appear to be specific for mucosal surfaces rather than controlling systemic autoimmunity.⁴⁶ Further studies have demonstrated that IL-10 induces robust activation of a STAT3-dependent Th17 suppression program in Tregs, downstream of IL-10R.⁵¹ This suppresses pathogenic Th17 cell responses and ablation of IL-10R in Tregs has been shown to cause colitis. It is therefore plausible that disordered IL-10 signalling may contribute to aberrant Th17 activity, which is implicated in IBD.⁵² In fact, there have been several cases of homozygous loss-of-function mutations in *Il-10* and *Il-10r* arising in individuals from consanguineous marriages. These resulted in infantile severe, progressive, intractable Crohn's-like colitis.⁵³

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5 TGF- β plays an important role inducing pTreg formation upon antigen encounter in GALT
6 and has a functional role in suppressing pro-inflammatory pathways.⁵⁴ Tregs are capable of
7 producing TGF- β , which profoundly suppresses the proliferation of Teffs.⁵⁵ Treg-derived
8 TGF- β 1 inhibits Th1-cell differentiation and IBD in a transfer model of colitis.⁵⁶ Conversely,
9 Tregs from TGF- β 1-deficient mice fail to suppress intestinal inflammation in a SCID transfer
10 model of colitis.⁵⁵ Human studies have supported these early findings; a study on healthy
11 human colonic biopsies and lamina propria mononuclear cells (LPMC) treated with anti-TGF-
12 β neutralising antibody showed that TGF- β is a critical suppressor of T-bet-dependent Teff
13 proliferation and Th1 cytokine expression.⁵⁷ This suggests a role for TGF- β in suppressing
14 intestinal inflammation in humans. Indeed, MacDonald *et al* have shown that colonic tissue
15 and isolated T cells from patients with CD overexpress Smad7, an inhibitor of TGF- β 1
16 signalling.⁵⁸ Furthermore, colonic LPMCs from CD patients were resistant to Treg-mediated
17 suppression, a phenomenon that could be reversed with Smad7 antisense treatment.⁴³
18 Smad 7 antisense therapy (Mongersen) was subsequently evaluated in CD but, despite
19 promising early phase data, a phase III clinical trial was terminated early due to lack of
20 benefit.^{59,60} Although Mongersen may overcome Teff resistance to TGF- β , it is possible in CD
21 there are insufficient numbers of functional Tregs in the mucosal environment to produce
22 TGF- β explaining the disappointing trial outcome .
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40 IL-35 is a heterodimer of Ebi3 and IL-12 α that is constitutively expressed in Foxp3⁺ Tregs but
41 not Teffs. It was first described in 2007 where *Ebi3*^{-/-} and *IL-12 α* ^{-/-} Tregs were shown to have
42 significantly reduced regulatory activity *in vitro* and failed to cure T cell transfer colitis *in*
43 *vivo*.⁶¹ Additionally, IL-35 can induce the generation of a regulatory population from naïve
44 mouse or human CD4⁺ T cells. These so-called iT(R)35 cells mediate suppression via IL-35
45 alone, do not express Foxp3, and are strongly suppressive and stable *in vivo*.⁶² In both
46 dextran sulphate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis,
47 recombinant IL-35 therapy can treat disease through downregulation of the Th1 and Th17
48 master transcription factors, T-bet and RORC, respectively, and through inhibition of IFN- γ ,
49 IL-6 and IL-17.⁶³
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Inhibition of Metabolic Processes:

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5 While Tregs are not known to produce IL-2, their development and function is critically
6 dependent on this cytokine. IL-2 and the transcription factor STAT5, downstream of IL-2
7 receptor (IL-2R), induce the expression of Foxp3 and differentiation of tTregs.⁶⁴
8 Furthermore, STAT5 activation driven by IL2R signalling enhances the suppressor function of
9 differentiated Tregs.⁶⁵ An absence of IL-2 signalling has been shown to reduce the number
10 and functional activity of Tregs, predisposing to autoimmunity and inflammation.^{66,67} The
11 structural conformation of IL-2R in Tregs provides a competitive advantage for IL-2-receptor
12 engagement over alternative cell subsets. Tregs abundantly express IL-2 receptor α -chain
13 (CD25), which together with the common γ -chain (γ c, CD132) and IL-2 receptor β -chain
14 (CD122) form a characteristic three subunit receptor configuration. This confers a ~1000-
15 fold increase in receptor affinity for IL-2 over Teffs.⁶⁸ In a pro-inflammatory environment
16 dominated by actively dividing effector cells, Tregs have the ability to “consume” local IL-2,
17 starving effector cells of this essential cytokine for survival and proliferation.^{45,69} Moreover,
18 this mechanism has been shown to induce the apoptosis of effector cells.⁷⁰ This highlights
19 an important TCR-independent paracrine mode of suppression in local tissues, facilitated
20 through the constitutive expression of high affinity IL-2R (containing CD25). There have
21 been a handful of cases of CD25 deficiency in humans often manifesting in an IPEX-like
22 syndrome.⁷¹⁻⁷³ A notable case who presented with autoimmune enteropathy at 6 months
23 had Foxp3⁺ Tregs with defective IL-10 expression suggesting that IL-2 responsiveness is
24 important for Treg-mediated IL-10 production.⁷⁴
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44 Tregs can also interfere with adenosine triphosphate (ATP) metabolism to dampen pro-
45 inflammatory responses. Tregs co-express the ectoenzymes CD39 and CD73 responsible for
46 the degradation of ATP and generation of pericellular adenosine.⁷⁵ Adenosine stimulates the
47 A2A receptor on Teffs exerting potent inhibitory effects. Activation of the A2A receptor also
48 inhibits IL-6 expression while enhancing the production of TGF- β .⁷⁶ This promotes the
49 development of adaptive induced Tregs and simultaneously inhibits pro-inflammatory Th17
50 cell formation. Furthermore, signalling through the A2A receptor appears to control *in vivo*
51 murine colitis.⁷⁷
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Neutralisation of Dendritic Cell Function:

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5 The activation of T cells requires TCR-antigen/MHC engagement in the context of a
6 secondary signal, namely T cell-derived CD28 binding the DC B7 ligands, CD80 and CD86.
7 This process is negatively regulated through the production of cytotoxic T lymphocyte
8 antigen 4 (CTLA-4) which is constitutively expressed in Foxp3⁺ Tregs.⁷⁸ CTLA-4-expressing
9 cells can capture CD80 and CD86 by a process of trans-endocytosis and degrade these
10 ligands, resulting in impaired co-stimulation via CD28.⁷⁹ This is a functionally significant
11 process with Treg-conditioned DCs inducing poor T cell proliferation.⁸⁰ An additional
12 mechanism mediated through the interaction of CTLA-4 and CD80/CD86 is the upregulation
13 of indoleamine 2, 3-deoxygenase in DCs. This is a potent regulatory molecule which
14 catabolises the essential amino acid tryptophan to the pro-apoptotic metabolite kynurenine
15 leading to suppression of Teff function.⁶⁴ *In vivo* models have demonstrated that CTLA-4 is
16 essential in preventing autoimmunity. Selective deletion of CTLA-4 in Tregs of BALB/c mice
17 results in fatal T cell mediated autoimmune disease at just 20 days of age.⁸¹ Additionally,
18 several cases of germline heterozygous mutations in CTLA-4 have been identified in
19 humans.⁸² CTLA-4 haploinsufficiency resulted in dysregulation of Tregs, hyperactivation of
20 Teffs and lymphocytic infiltration of target organs including the GI tract. It was recently
21 discovered that LRBA (lipopolysaccharide-responsive and beige-like anchor protein)
22 regulates CTLA-4 expression, where mutations in LRBA lead to reduced levels of CTLA-4.⁸³
23 These mutations are commonly associated with primary immunodeficiency, reduced Treg
24 numbers and susceptibility to IBD.^{84,85}

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43 Recently, the coinhibitory molecule TIGIT has been described as an inhibitor of autoimmune
44 responses through its interactions with DCs and T cells. TIGIT interacts with its ligand CD155
45 on DCs to induce IL-10 and suppress IL-12 production, thereby inhibiting Th1 responses.⁸⁶ As
46 Tregs are the primary cell type that constitutively express TIGIT, it has been suggested that
47 the observed effects on DCs are mediated by TIGIT⁺ Tregs. Furthermore, Tregs expressing
48 TIGIT have been shown to directly suppress Th1 and Th17 responses through the production
49 of the effector molecule fibrinogen-like protein 2 (Fgl2).⁸⁷

50 51 52 53 54 55 56 57 58 **Cytotoxic Activity:** 59 60

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3 Historically, cytotoxic activity has been associated with natural killer (NK) cells and cytotoxic
4 T lymphocytes (CD8⁺ T cells). In 2004, Grossman *et al* first described granzyme-B expressing
5 CD4⁺ Tregs capable of killing target cells in a perforin-dependent, but TCR-independent
6 manner.⁸⁸ Boissonnas *et al* subsequently showed that in a mouse tumour model, Foxp3⁺ T
7 cells can kill antigen-specific DCs. Treg cytotoxicity has also been observed against CD4⁺ T
8 cells in both *in vitro* and *in vivo* models. Activated Tregs upregulate tumour necrosis factor-
9 related apoptosis inducing ligand (TRAIL) which enhances suppressive activity as well as
10 cytotoxicity against CD4⁺ T cells. This is entirely dependent on the TRAIL/death receptor 5
11 (DR5) pathway.⁸⁹ Galectin-1, a β -galactoside-binding protein known to induce T cell
12 apoptosis has also been implicated in Treg cytotoxic function. Galectin-1 was found to be
13 overexpressed in Tregs and galectin-1 knockout models were shown to possess reduced
14 regulatory activity.⁹⁰

25 26 27 **Tissue Repair:**

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30 Aside from limiting mucosal damage through the suppression of pro-inflammatory cells
31 following environmental insults like infection, Tregs may also promote tissue repair.
32 Recently, the epidermal growth factor (EGF)-like molecule amphiregulin (AREG) has gained
33 attention as an important regulator of tissue repair and regeneration. In a murine model of
34 influenza, selective Treg deficiency in AREG leads to severe acute lung damage without any
35 alterations in Treg suppressor function. This suggests that Tregs play a direct role in tissue
36 repair and maintenance that is distinct from their suppressive function.⁹¹ Treg production of
37 AREG is dependent on IL-18 or IL-33 which function as endogenous danger signals or
38 alarmins, in response to tissue damage.⁹¹ Studies in humans have revealed high levels of IL-
39 33 in inflamed lesions of IBD patients, and Tregs expressing the IL-33 receptor, ST2, are
40 enriched in the colon.⁹²⁻⁹⁴ IL-33-Treg signalling may therefore represent an important
41 pathway in both disease pathogenesis and recovery.

42 43 44 45 46 47 48 49 50 51 52 53 54 **TREGS AS A THERAPEUTIC PRODUCT:**

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58 In light of the vast array of preclinical data showcasing how a multitude of defects in Treg
59 function contribute to autoimmunity and inflammation, including IBD, there has been great
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3 interest in harnessing the suppressive ability of Tregs as a therapeutic product.
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5 Consequently, there are over 50 registered trials of Treg therapy that are either completed
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7 or ongoing (clinicaltrials.gov). Most of these trials involve adoptive cell transfer, although
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9 the dose of Tregs given is highly variable. In the setting of autoimmune disease and
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11 transplantation, the goals of treatment are the restoration of peripheral self tolerance, the
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13 suppression of inflammation and promotion of tissue repair.⁹⁵
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16 In order to become a successful therapeutic product, Tregs must home to sites of
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18 inflammation and secondary lymphoid tissues, and must undergo TCR engagement. It has
19
20 been demonstrated in solid organ transplantation that alloantigen-specific Tregs provide
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22 higher therapeutic benefits than polyclonal Tregs, without delivering a systemic
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24 immunosuppressive effect.⁹⁶ Directing Tregs against a specific alloantigen also permits
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26 immunomodulatory functions to be concentrated at the site of the alloantigen source,
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28 circumventing the relative paucity of Tregs. An early study demonstrated that peripheral
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30 Treg expansion in mice could be driven by prolonged low dose subcutaneous infusion of a
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32 specific peptide.⁹⁷ The induced Tregs had suppressive abilities, and demonstrated high
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34 levels of Foxp3 expression indicating a stable Treg phenotype. However, in IBD, a specific
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36 antigen has yet to be identified.
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39 The relative paucity of Tregs in peripheral blood represents an obstacle to the development
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41 of a cellular therapy, though the optimum number of Tregs to be infused remains unclear. It
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43 has been suggested that the number of Tregs given should be at least as great as the
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45 number of T cells in the body,⁹⁸ though Tregs also exhibit the ability to confer suppressive
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47 ability on conventional T cells through 'infectious tolerance'.⁹⁶ In this process, the direct
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49 secretion of TGF- β , IL-10 and IL-35 by Tregs, and indirect induction via DCs, can generate a
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51 regulatory microenvironment which may partially circumvent the problem of low absolute
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53 numbers of Tregs.⁹⁹
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56 Several groups have developed protocols in line with GMP requirements to permit *ex vivo*
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58 cell expansion of Tregs.^{98,100,101} GMP-manufactured Tregs delivered in some early trials were
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60 only around 50% pure, but the development of plastic beads coated with stimulatory
antibodies and the discovery of additional surface markers for Treg phenotyping mean that

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3 a product with purity greater than 90% is now achievable.⁹⁸ Contamination of the expansion
4 product with Tregs hampers expansion,¹⁰² but the inclusion of rapamycin in cell culture
5 blocks expansion of Tregs without affecting Treg proliferation, leading to the preferential
6 promotion of Treg proliferation.^{98,103}
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12 Tregs are first isolated from peripheral blood by surface marker expression
13 (CD4⁺CD25^{hi}CD127^{lo}). This can be performed using stream in air fluorescence-activated cell
14 sorters (FACS) which yield a highly pure starting population, but the necessary air exposure
15 requires high efficiency particulate air (HEPA) enclosures, and single use sample lines to be
16 compatible with manufacturing GMP cell products. Closed system magnetic bead-activated
17 cell sorting (MACS) can be adapted for large-scale isolation of human Tregs, but unlike FACS
18 cannot easily distinguish surface marker expression density. A recently developed
19 microfluidic chip fluorescence-activated cell sorter, the MACSQuant Tyto (Miltenyi Biotech,
20 Germany) surmounts the problems of stream in air sorters, as the cells remain in a closed
21 system throughout the sorting process. Expansion of the sorted cells is achieved through
22 polyclonal TCR activation with anti-CD3/anti-CD28 beads.¹⁰⁴ Tregs are sampled and checked
23 for sterility and phenotype throughout the expansion process. With optimised conditions, a
24 500-fold expansion can be anticipated over a 14 day period.¹⁰¹
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Uncertainty about the plasticity of Tregs in culture and following infusion means there is a theoretical concern about the development of a pro-inflammatory phenotype, which could lead to transplant rejection or aggravation of inflammation. However, rapamycin-expanded Tregs are not contaminated by IL-17-producing Th17 cells, and these cells maintain a stable phenotype on transfer *in vivo* to mice.¹⁰⁵ Canavan *et al.* found that the starting population for Treg expansion from the peripheral blood of CD patients has a critical effect on the phenotype of the expanded cell population.¹⁰⁰ Tregs from a highly pure FACS-sorted 'naïve' CD4⁺CD25^{hi}CD127^{lo}CD45RA⁺ precursor population demonstrated enhanced suppressive ability and reduced Th17 plasticity *in vitro* compared to a FACS-sorted CD4⁺CD25^{hi}CD127^{lo}CD45RA⁻ or MACS-enriched CD8⁻CD25⁺ population. Rapamycin appears to imprint a fixed CD4⁺CD25^{hi} phenotype to cells expanded from a 'naïve' CD45RA⁺ population, as evidenced by the retention of demethylation at the Foxp3 locus.

TREG THERAPY IN OTHER CONDITIONS:

There is an increasing body of evidence for the use of Tregs as cellular therapy in autoimmune disease and transplantation (see Table 1). Adoptive transfer of Tregs to prevent GvHD was the first illustration of the potent therapeutic potential of Tregs in experimental transplantation.

Study	Clinical context	Enrichment protocol	Expansion protocol	Dose	Study outcome
Trzonkowski <i>et al.</i> (2009)	Treatment of acute and chronic GvHD N=2	Tregs from allogenic buffy coat. CD4 ⁺ negative bead selection followed by FACS-based sorting of CD4 ⁺ CD25 ^{hi} CD127 ^{lo} cells	RPMI 1640 with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) 3 weeks	Acute GvHD: 1x10 ⁶ /kg Chronic GvHD: 3x10 ⁶ /kg	Transient improvement in acute GvHD; alleviation of symptoms and reduction of immunosuppression in chronic GvHD
Brunstein <i>et al.</i> (2011)	Prevention of GvHD following umbilical cord blood transplantation N=23	CD25 ⁺ bead positive selection	X-Vivo 15 with 10% human AB serum IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:2) 18±1 days	0.1-30x10 ⁵ /kg	Well –tolerated; reduced incidence of grade II-IV GvHD in Treg recipients
Marek-Trzonkowska <i>et al.</i> (2012)	Safety of autologous <i>in vitro</i> expanded Tregs in paediatric type 1 diabetes N=10	FACS-based sorting of CD3 ⁺ CD4 ⁺ CD25 ^{hi} CD127 ^{lo} cells	CellGro medium with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) Up to 2 weeks	10-20x10 ⁶ /kg	Well-tolerated; decreased insulin requirements and C-peptide levels in Treg recipients
Desreumaux <i>et al.</i> (2012)	Safety and efficacy in Crohn's disease N=20	Culture of PBMCs with ovalbumin, IL-2 and IL-4 followed by cloning of ovalbumin-specific T cells	X-Vivo 15 IL-2 (200IU/ml) Anti-CD3/anti-CD28 beads (1:1) Ova-Tregs selected based on ovalbumin-specific IL-10 production 12 to 15 weeks	1x10 ⁶ -1x10 ⁹	Well-tolerated; dose-related efficacy
Bluestone <i>et al.</i> (2015)	Safety in adults with type 1 diabetes (N=14)	FACS-based sorting of CD4 ⁺ CD25 ^{hi} CD127 ^{lo} cells	X-Vivo 15 with 10% human AB serum and deuterated glucose IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:1) 14 days	0.05x10 ⁸ -26x10 ⁸	Well-tolerated, no significant adverse events. Stable C-peptide levels and insulin use in recipients for up to two years post infusion
Mathew <i>et al.</i> (2018)	Safety in living donor kidney transplant N=9	CliniMACS plus GMP enrichment system (Miltenyi)	IL-2 (1000IU/ml) MACS © GMP expansion beads 1:1-4:1 3 weeks	0.5-5x10 ⁹	Well-tolerated, no infections or rejection up to two years post transplant

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3 **Table 1: Summary of clinicaltrials.gov listings for reported trials using *in vitro* expanded**
4 **regulatory T cell (Treg) therapy**
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7 **Graft vs. host disease (GvHD):**
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11 The risk of developing GvHD following haematopoietic stem cell transplantation (HSCT) is
12 associated with low numbers of Tregs in the periphery,¹⁰⁶ and *in vivo* expansion of Tregs
13 post-HSCT using low dose IL-2 has demonstrated efficacy against GvHD.^{107,108} Studies in mice
14 involving infusion of cultured CD4⁺CD25⁺ T cells resulted in a significantly reduced GvHD
15 phenotype,¹⁰⁹ and in humans it was found that infusion of freshly isolated donor Tregs given
16 at the same time as haplotype mismatched HSCT prevented the development of GvHD.¹¹⁰
17 Five trials of *ex vivo* expanded Tregs have to date involved small numbers of patients only,
18 but suggest therapy can prevent or delay the onset of chronic GvHD^{111,112}. Treg therapy
19 seems to be effective only in the chronic form of GvHD, but this may be because of the time
20 requirements to expand the cellular product which makes it difficult to administer in a
21 timely manner in acute GvHD.¹¹³
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33 **Solid organ transplant:**
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37 Adoptive Treg therapy has been trialled following renal and liver transplantation, with the
38 aim of inducing tolerance to the allograft and reducing the burden of long-term
39 immunosuppression.¹¹⁴ Tregs have been shown to control immune responsiveness to
40 alloantigens and contribute to 'operational tolerance' in preclinical transplantation
41 models.^{115,116} Recipient-derived Tregs expanded for direct and indirect pathway
42 allospecificity *in vitro* were able to mediate effective protection against acute and chronic
43 rejection in skin and heart allografts in mice,¹¹⁷ and could be used to induce tolerance of a
44 murine skin transplant following thymectomy and T cell depletion.¹¹⁸ In these models,
45 alloantigen reactive Tregs were more effective at preventing graft rejection than
46 polyclonally expanded Tregs¹⁰⁴.
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57 A phase I study in renal transplantation recruited nine living donor transplant recipients, and
58 used the product of leukapheresis as the basis for *ex vivo* expansion of polyclonal
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3 autologous Tregs.¹¹⁴ Alemtuzumab was given at induction to achieve lymphodepletion, on
4 the basis of previous experiments suggesting a reduction in circulating Tregs worked
5 synergistically with Treg infusion to prolong allograft survival.¹¹⁶ Recipients were switched
6 from traditional immunosuppression with tacrolimus, which blocks IL-2 production, to
7 sirolimus (rapamycin), which has Treg promoting activity.¹¹⁹
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14 An enhanced suppressive ability of the expanded Tregs was demonstrated when compared
15 to Tregs taken directly *ex vivo*.¹¹⁴ There were no adverse infusion-related side effects,
16 infections or rejection up to two years post-transplant, and there was a 5-20 fold increase in
17 the number of circulating Tregs seen up to one year post-transplant. Transplant biopsies
18 taken at three months did not show rejection and recipients had not developed peripheral
19 donor-specific antibodies. An additional important outcome from trials in transplantation is
20 that they have demonstrated that it is possible to expand Tregs from immunocompromised
21 patients.¹²⁰
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30 A trial of Treg immunotherapy in liver transplantation is currently underway.¹²¹ This is
31 predicated on the observation that when liver allografts in mice were infiltrated with Tregs,
32 loss of Treg numbers was associated with a loss of tolerance.¹²² Increased frequencies of
33 Tregs are also seen in human subjects who acquire 'operational tolerance' to their liver
34 transplant.¹²³
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41 **Type 1 diabetes mellitus:**

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45 The development of T1DM is associated with deficits in the number and suppressive activity
46 of Tregs.¹²⁴ Accelerated diabetes onset is seen in both scurfy mice²⁹ and children with
47 IPEX,¹²⁵ highlighting the role of Tregs in protecting pancreatic islet cells from destruction.
48 Tregs have been implicated in the pathogenesis of diabetes in the non-obese diabetic (NOD)
49 mouse model,^{126,127} and anti-CD3 antibodies have been efficacious in the treatment of
50 diabetes in both mouse^{128,129} and human trials.^{130,131} Subjects exhibited lower insulin
51 requirements and higher C-peptide levels at least 18 months after a short course of
52 intravenous treatment, with evidence of anti-CD3 treatment inducing expansion of a
53 CD4⁺CD25⁺ T cell population.¹²⁹ A trial of ten children treated with expanded polyclonal Tregs
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3 within two months of their diagnosis demonstrated statistically lower insulin requirements
4 and C peptide levels compared with matched controls up to six months post infusion, with
5 two patients remaining insulin-independent.¹²⁴ There were no serious adverse events up to
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7 one year following infusion.
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12 In a phase I open-label trial of 14 adult patients infused with *ex vivo* expanded Tregs in
13 escalating doses, 7 of 14 patients had stable C peptide levels and insulin use for up to two
14 years following infusion.¹⁰¹ However, the study was not powered to detect significant
15 clinical improvement. There were no infusion reactions or therapy-related serious adverse
16 events. Phenotypic analysis of the cell product after expansion and after infusion identified
17 stable surface marker expression, demonstrating that the infused Tregs did not acquire a
18 pathological phenotype. High throughput TCR- β sequencing analysis indicated that
19 expanded Tregs retained a high degree of diversity.
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29 Adoptively transferred Tregs were tagged by labelling the deoxyribose moiety of replicating
30 DNA during expansion *ex vivo*, through addition of deuterated [6,6-²H₂] glucose to Treg
31 culture throughout the 14 day expansion period.¹⁰¹ Patient samples were analysed by gas
32 chromatography mass spectrometry (GC-MS) for deuterium enrichment to create
33 pharmacokinetic curves. Adoptively transferred T cell numbers peaked at two weeks
34 following infusion, but were still detectable at up to 25% of the peak level at one year in
35 peripheral blood. Significantly, deuterium labelling was never found in non-Tregs, indicating
36 the stability of infused Tregs. However, due to the nature of this study, the stability of these
37 cells was not assessed within the target tissue.
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47 **TREG THERAPY IN INFLAMMATORY BOWEL DISEASE:**

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51 A local imbalance between Treg and Teff responses plays a key role in the development of
52 gut inflammation in IBD.⁸ T cell gut homing is mediated by specific interaction between
53 integrin $\alpha 4\beta 7$ and its ligand MAdCAM-1.^{132,133} Several groups have shown that transfer of
54 Tregs into mice leads to clinical and histological improvement in colitis,^{14,134,135} and
55 rapamycin-expanded Tregs ameliorated established colitis in a SCID mouse model.¹³⁶
56 Polyclonality of the TCR is likely to be an important requirement for Tregs to maintain
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3 intestinal homeostasis *in vivo*. Mice which express a restricted TCR repertoire develop
4 spontaneous colitis due to a loss of tolerance to intestinal microbiota.¹³⁷
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9 Several groups have demonstrated that it is feasible to extract Tregs from patients, and
10 expand them *in vitro* under GMP conditions, including from subjects receiving thiopurines
11 and anti-TNF α medications.^{98,100,103,138} Even after prolonged culture, these Tregs maintained
12 Foxp3 expression and demonstrated enhanced suppression of autologous T cells.
13
14 Uncertainty regarding the potential for adoptively transferred Tregs to express IL-17 and
15 exacerbate CD lesions is a concern. However, the administration of pro-inflammatory
16 cytokines (IL-1, IL-2, IL-6, IL-21, IL-21 and TGF- β) failed to induce IL-17 production by
17 CD45RA⁺ expanded Tregs *in vitro*.¹⁰⁰
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25 **Antigen specific vs. polyclonal Treg cell products for Crohn's disease:**

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29 No antigens have yet been verified as causal in CD. Attempts have been made to identify
30 shared TCRs between CD sufferers with the aim of discovering target antigens.^{139–141} This
31 work has observed that the CD4 TCR repertoires are significantly more diverse in patients
32 with CD and UC than healthy controls.¹⁴² This may be explained by GI barrier disruption
33 increasing the number of antigen presentation events in comparison to a healthy gut.
34
35 Resolving a target from the GI peptidome is challenging due to the heterogenous nature of
36 the environment. Developments in the understanding of non-conventional epitopes are also
37 increasing the magnitude and complexity of the peptidome itself.¹⁴³ In the absence of a
38 known target, the broad reactivity of a polyclonal Treg product may be advantageous, as the
39 cell product will recognise millions of putative epitopes, increasing the likelihood of TCR
40 engagement and subsequent Treg activation. Sequencing of isolated Tregs from GI biopsies
41 post transfer may yield novel targets, upon which chimeric antigen receptor technology
42 could be readily implemented.¹⁴⁴
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54 For Treg therapy to be effective in IBD, expanded Tregs must have the ability to home to the
55 gut.¹⁴⁵ A French group reported the results of an open label multicentre phase I/IIa trial of
56 ovalbumin-specific Tregs in 20 patients with refractory CD.¹⁴⁶ Ovalbumin is a common food
57 antigen, and is not implicated in intestinal inflammation in animal models or in patients with
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3 CD. Its distribution along the digestive tract can be used to activate Tregs locally. In the
4 study, this was facilitated through ingestion of meringue cakes by subjects.¹⁴⁶
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9 The cell product was cultured in the presence of ovalbumin, and trial subjects received a
10 dose of 10^6 - 10^9 Tregs.¹⁴⁶ Patients enrolled in the study had at least moderately active CD,
11 with a Crohn's Disease Activity Index (CDAI) greater than or equal to 220 within six months
12 of screening, and a washout period was required for immunosuppression and anti-TNF α
13 therapy. The infusion was well tolerated, with mild GI symptoms and CD flares being the
14 most commonly reported adverse effects. Two patients experienced thrombotic events, but
15 these are known to occur more frequently in inflammatory conditions including active CD.¹⁴⁷
16 Eight (40%) patients had a significant CDAI response at weeks five and eight after treatment,
17 with two patients experiencing sustained remission. Overall, the results suggested good
18 tolerability in this disease group with possible signals of efficacy.
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29 In the absence of a known antigen, other methods must be used to direct the Tregs to the
30 areas of inflammation. A recent study has shown that a highly specific retinoic acid receptor
31 α (RAR α) agonist induces expression of Integrin $\alpha 4\beta 7$ (the ligand of MAdCAM-1) on the Treg
32 surface. Adoptive transfer of RAR α agonist-treated Tregs leads to improved Treg trafficking
33 to gut tissue in a humanised mouse model of colitis.¹⁰⁰ Supporting this mechanism for
34 resolving inflammation, another group have demonstrated that DCs can be engineered *de*
35 *novo* to produce high concentrations of RA.¹⁴⁸ When transferred to mice, the RA-secreting
36 DCs were able to augment the expression of Foxp3 and the gut-homing receptor CCR9 in
37 native Tregs with the subsequent suppression of colitis.
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47 The RAR α agonist treated cell product forms the basis of the TRIBUTE trial (ClinicalTrials.gov
48 Identifier: NCT03185000), a double-blinded placebo-controlled phase I/IIa trial of adoptive
49 Treg therapy in CD.
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54 **FUTURE DEVELOPMENTS IN TREG THERAPY:**

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58 The potential therapeutic benefits of adoptive cell therapy are being explored in numerous
59 autoimmune conditions. In SLE, adoptive transfer of *ex vivo* expanded Tregs in mice delayed
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3 the onset of renal complications and prolonged survival,^{149,150} and a pilot study of low dose
4 IL-2 in 37 patients led to increased circulating peripheral Treg numbers and decreased SLE
5 disease activity scores.¹⁵¹ Adoptive Treg transfer in a single patient with cutaneous lupus did
6 not lead to clinical benefit, but increased percentages of highly activated Tregs were
7 identified in biopsies taken from diseased skin.¹⁵² Treg accumulation in skin was associated
8 with a marked attenuation of IFN- γ , which was more pronounced relative to peripheral
9 blood.

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11 Preliminary results from mouse models suggest a role for Treg therapy in conditions as
12 diverse as pemphigus vulgaris,¹⁵³ autoimmune hepatitis,¹⁵⁴ multiple sclerosis,¹¹³ asthma,
13 and allergy, in which antigen-specific Tregs may represent a viable therapeutic option.^{155,156}

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15 Many ongoing challenges exist for the advancement of Treg therapy. Uncertainties remain
16 about the optimal timing of *ex vivo* Treg expansion, and whether IL-2 administration would
17 be a useful adjunct to support a Treg population *in vivo*.^{101,107} In addition, concomitant
18 treatment of autoimmune disease with immunosuppressive drugs may affect the function
19 of adoptively transferred cells.⁹⁵

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21 The optimal dosing strategy for Treg therapy also remains unclear, although data tracking
22 the survival of deuterium-labelled Tregs *in vivo* could be invaluable in informing a suitable
23 dosing regimen.¹⁰¹ A two-phase decay in numbers of deuterium-labelled Tregs has been
24 seen, with 75% of the peak level lost at three months. However, levels stabilised at one
25 year, with up to 25% of peak Treg numbers remaining in the peripheral circulation. The
26 decrease in labelled Tregs may represent cell death, trafficking to lymphoid tissue and sites
27 of inflammation, or proliferation of the Treg compartment leading to dilution of deuterium
28 enrichment. Reassuringly, at no point during the trial was deuterium detected in cell
29 populations other than Tregs, suggesting a stable phenotype *in vivo*.¹⁰¹

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31 Tracking of TCR clonotypes may also provide useful data on Treg kinetics and dispersal.
32 Analysis of the TCR repertoire has suggested that the kinetics of transferred Tregs in
33 peripheral blood varies significantly between individuals¹⁵⁷. In a descriptive study, the TCR
34 V α chain was sequenced in two patients receiving donor Treg infusion¹⁵⁷. Treg therapy

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3 altered the patients' peripheral TCR repertoire considerably towards that of the infused cell
4 product, but to different degrees in each patient. Importantly, the degree of alteration of
5 the TCR repertoire appeared to correlate with clinical response. This suggests that
6 monitoring TCR repertoires following adoptive cell transfer may provide clinically
7 meaningful information.
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16 **CONCLUSION:**

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19 There is now robust evidence of the therapeutic potential of Treg therapy in Crohn's
20 disease. Trials in multiple autoimmune diseases and results from use of ovalbumin-specific
21 Tregs in IBD show promising early signs of efficacy. The safety signal is reassuring, with
22 evidence that the adoptively transferred Treg phenotype is stable *in vivo*. Results from
23 deuterium labelling suggest that infused Tregs may be able to exert a long-lasting systemic
24 effect with labelled cells detectable up to a year after infusion. It is hoped that upcoming
25 early phase clinical trials in patients with Crohn's disease will inform safety, dosing, and Treg
26 kinetics and dispersal allowing further development of a novel therapeutic option in this
27 hard-to-treat population.
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38 **FIGURE LEGEND:**

39 **Figure 1. Mechanisms of Treg mediated suppression**

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42 Tregs utilize a multitude of mechanisms to promote a tolerogenic microenvironment and
43 tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL-10, TGF- β and IL-35, not
44 only inhibit Teff proliferation, but also suppress Th1 and Th17 effector function, both of
45 which are key mediators of IBD. (B) Tregs express the high affinity IL-2 receptor α -chain
46 (CD25) consuming local IL-2 with greater affinity than effector cells. Teffs which are 'starved'
47 of IL-2 exhibit restricted proliferation and undergo apoptosis. (C) Tregs co-expressing CD39
48 and CD73 disrupt metabolic processes in effector cells by converting ATP into peri-cellular
49 adenosine, a potent inhibitor of Teff function. Additionally, adenosine stimulates TGF- β
50 production, promoting development of pTregs. (D) Tregs are capable of secreting perforin,
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3 granzyme B and galectin-1 which are directly cytotoxic against Teffs. Activated Tregs also
4 express TRAIL, inducing apoptosis of Teffs through the TRAIL/DR5 pathway. (E) Expression of
5 CTLA-4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-
6 stimulation of T cells. DC function is further inhibited through the interaction of Treg-
7 derived TIGIT and CD155 on DCs. This induces IL-10 production and suppresses IL-12. (F) In
8 response to alarmins, Tregs produce AREG, an important regulator of tissue repair and
9 regeneration. AREG, amphiregulin; ATP, adenosine tri-phosphate; CTLA-4, cytotoxic T
10 lymphocyte associated protein 4; DC, dendritic cell; DR5, death receptor 5; IBD,
11 inflammatory bowel disease; pTregs, peripheral regulatory T cells; Teff, T effector
12 lymphocyte; TGF- β , transforming growth factor beta; Th1, T helper 1 cell; Th17, T helper 17
13 cell; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TRAIL, TNF-related apoptosis-
14 inducing ligand; Treg, regulatory T cell. Figure generated using BioRender© illustration
15 software.
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41 of the NHS, the NIHR, or the Department of Health.
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53 **COMPETING INTERESTS:**

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56 PI is the Chief Investigator and GL is the Chief Scientific Investigator on the MRC-funded
57 TRIBUTE trial of regulatory T cell immunotherapy in Crohn's disease (ClinicalTrials.gov
58 NCT03185000).
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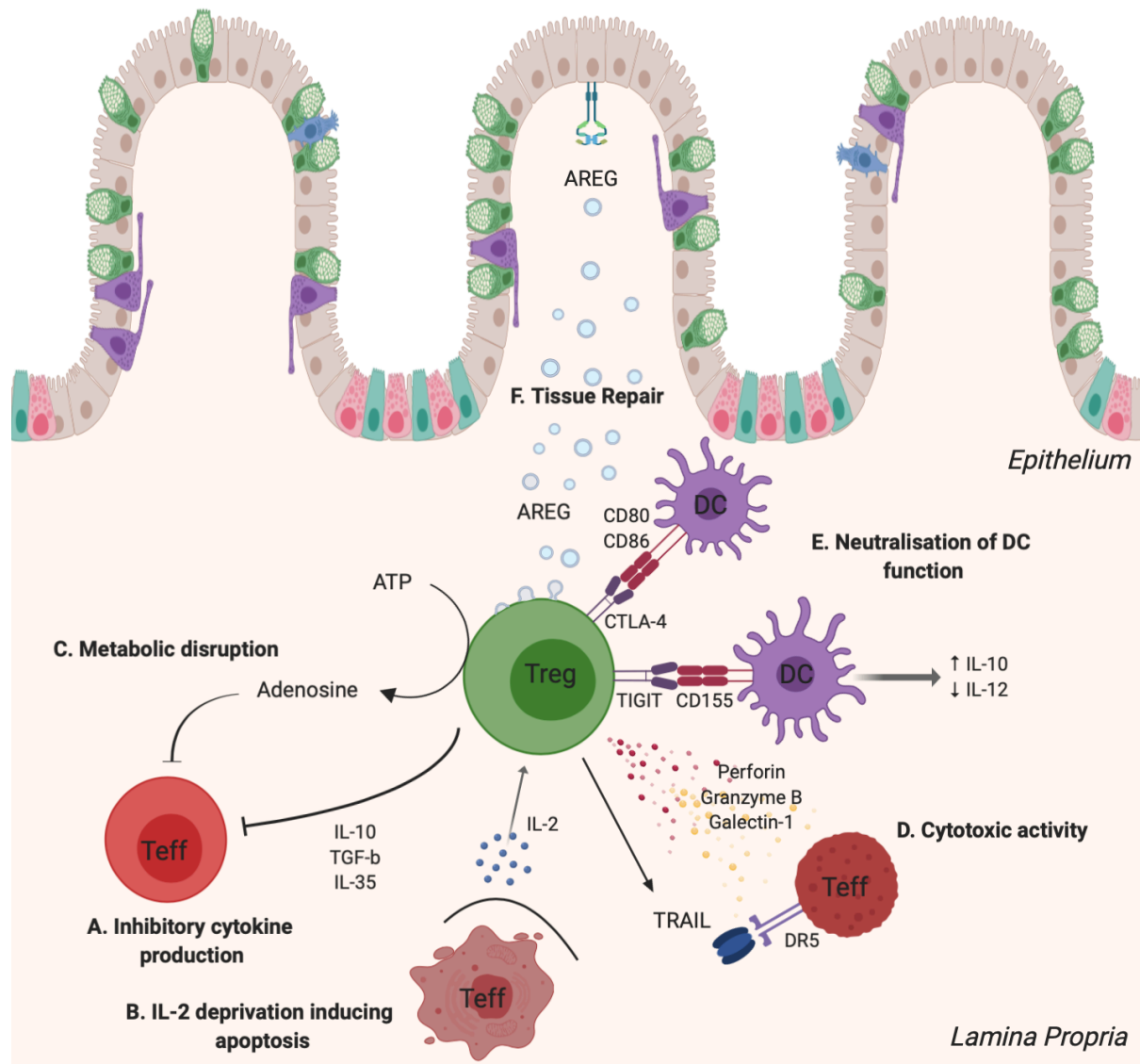


Figure 1. Mechanisms of Treg mediated suppression

Tregs utilize a multitude of mechanisms to promote a tolerogenic microenvironment and tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL-10, TGF- β and IL-35, not only inhibit Teff proliferation, but also suppress Th1 and Th17 effector function, both of which are key mediators of IBD. (B) Tregs express the high affinity IL-2 receptor α -chain (CD25) consuming local IL-2 with greater affinity than effector cells. Teffs which are 'starved' of IL-2 exhibit restricted proliferation and undergo apoptosis. (C) Tregs co-expressing CD39 and CD73 disrupt metabolic processes in effector cells by converting ATP into peri-cellular adenosine, a potent inhibitor of Teff function. Additionally, adenosine stimulates TGF- β production, promoting development of pTregs. (D) Tregs are capable of secreting perforin, granzyme B and galectin-1 which are directly cytotoxic against Teffs. Activated Tregs also express TRAIL, inducing apoptosis of Teffs through the TRAIL/DR5 pathway. (E) Expression of

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3 CTLA-4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-
4 stimulation of T cells. DC function is further inhibited through the interaction of Treg-derived
5 TIGIT and CD155 on DCs. This induces IL-10 production and suppresses IL-12. (F) In response
6 to alarmins, Tregs produce AREG, an important regulator of tissue repair and regeneration.
7 AREG, amphiregulin; ATP, adenosine tri-phosphate; CTLA-4, cytotoxic T lymphocyte
8 associated protein 4; DC, dendritic cell; DR5, death receptor 5; IBD, inflammatory bowel
9 disease; pTregs, peripheral regulatory T cells; Teff, T effector lymphocyte; TGF- β ,
10 transforming growth factor beta; Th1, T helper 1 cell; Th17, T helper 17 cell; TIGIT, T-
11 cell immunoreceptor with Ig and ITIM domains; TRAIL, TNF-related apoptosis-inducing
12 ligand; Treg, regulatory T cell. Figure generated using BioRender© illustration software.
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3 **1 Regulatory T cell therapy in Crohn's disease: challenges and advances**
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34 **Word Count: 5981**
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38 **ABBREVIATIONS:**
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- 40 APC – antigen presenting cell
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42 AREG – amphiregulin
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44 ATP – adenosine triphosphate
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46 CD – Crohn's disease
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48 CDAI – Crohn's disease activity index
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50 CTLA-4 – cytotoxic T lymphocyte antigen 4
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52 DC – dendritic cell
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54 DR5 – death receptor 5
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56 DSS – dextran sulfate sodium
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58 Ebi3 – Epstein Barr virus induced 3
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60 EGF – epidermal growth factor
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62 FACS – fluorescence-activated cell sorting

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3	33 Fgl2 – fibrinogen-like protein 2
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5	34 Foxp3 – Forkhead box P-3
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7	35 GALT – gut-associated lymphoid tissue
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9	36 GC-MS – gas chromatography mass spectrometry
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11	37 GI – gastrointestinal
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13	38 GMP – good manufacturing practice
14	
15	39 GvHD – graft versus host disease
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17	40 HEPA – high efficiency particulate air
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19	41 HSCT – haematopoietic stem cell transplant
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21	42 IBD – inflammatory bowel disease
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23	43 IFN γ – interferon γ
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25	44 IL – interleukin
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27	45 IPEX – ‘immune dysregulation, polyendocrinopathy, enteropathy, X-linked’
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29	46 LPMC – lamina propria mononuclear cell
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31	47 MACS – magnetic bead-activated cell sorting
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33	48 MAdCAM-1 – mucosal vascular addressin cell adhesion molecule 1
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35	49 MHC – major histocompatibility complex
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37	50 MMP – matrix metalloproteinase
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39	51 NK – natural killer
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41	52 NOD – non-obese diabetic
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43	53 PBMC – peripheral blood mononuclear cell
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45	54 RAR α – retinoic acid receptor α
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47	55 RORC – related orphan receptor C
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49	56 RPMI – Roswell Park Memorial Institute
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51	57 SCID – severe combined immunodeficiency
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53	58 SLE – systemic lupus erythematosus
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55	59 STAT – signal transducer and activator of transcription
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57	60 T1DM – type 1 diabetes mellitus
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59	61 TCR – T cell receptor
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	62 Teff – effector T cell
	63 TGF- β – transforming growth factor β
	64 Th1 – T helper 1 cell

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3 65 Th17 – T helper 17 cell
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5 66 TIGIT – T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based
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7 67 inhibitory motif (ITIM) domains
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9 68 TNBS – 2,4,6-trinitrobenzene sulfonic acid
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11 69 TNF α – tumour necrosis factor α
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13 70 TRAIL – tumour necrosis factor-related apoptosis inducing ligand
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15 71 Treg – regulatory T cell
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17 72 pTreg – peripheral regulatory T cell
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19 73 tTreg – thymic regulatory T cell
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21 74 UC – ulcerative colitis
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76 **ABSTRACT:**

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78 The prevalence of inflammatory bowel disease is rising in the Western world. Despite an
79 increasing repertoire of therapeutic targets, a significant proportion of patients suffer
80 chronic morbidity. Studies in mice and humans have highlighted the critical role of
81 regulatory T cells in immune homeostasis, with defects in number and suppressive function
82 of regulatory T cells seen in Crohn's disease patients. We review the function of regulatory T
83 cells and the pathways by which they exert immune tolerance in the intestinal mucosa. We
84 explore the principles and challenges of manufacturing a cell therapy, and discuss clinical
85 trial evidence to date for their safety and efficacy in human disease, with particular focus on
86 the development of a regulatory T cell therapy for Crohn's disease.

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88 **Keywords:** Crohn's disease, Immunology, Immunoregulation, Intestinal T cell, T lymphocytes

89

90 **INTRODUCTION:**

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92 Inflammatory bowel disease (IBD), chiefly comprising Crohn's disease (CD) and ulcerative
93 colitis (UC), is a chronic inflammatory group of disorders of the gastrointestinal (GI) tract
94 arising from overexuberant innate and adaptive immune responses to environmental
95 factors in genetically susceptible individuals. IBD affects at least 0.5% of the population in
96 the Western world with 1 million sufferers in USA and 2.5 million in Europe.¹ Global

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3 97 prevalence continues to increase, largely driven by rising numbers of patients in newly
4 98 industrialised regions including India and Asia.¹ The burden of disease is significant with 20-
5 99 25% of patients experiencing chronic continuous symptoms which contributes to higher
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9 100 rates of unemployment, sick leave and permanent work disability.² Even with an aggressive
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11 101 top-down approach to therapy, the majority of patients fail to achieve prolonged, steroid-
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13 102 free remission and are at particular risk of requiring surgical intervention. Cumulative
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15 103 surgery rates in CD are high in Europe with 30-50% of patients requiring surgical
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17 104 intervention and up to 20% needing a reoperation 5-10 years from diagnosis.²
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20 106 As our understanding of the pathophysiology of IBD and its socioeconomic impact has
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22 107 evolved, there has been great impetus to identify novel therapeutic targets to add to the
23
24 108 existing arsenal of immunomodulators and biologics. These have focussed on a variety of
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26 109 areas including targeting lymphocyte trafficking (vedolizumab, ozanimod, anti-MAdCAM1)
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28 110 and activation (anti-IL6, anti-IL12/IL23), modulating intestinal barrier function
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30 111 (phosphatidylcholine), matrix remodelling (STNM-01, MMP9 blocker) and manipulation of
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32 112 gut microbiota (faecal microbiota transplant).³ An important pathological process
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34 113 increasingly recognised as driving intestinal inflammation and autoimmunity is the loss of
35
36 114 immune homeostasis secondary to qualitative or quantitative defects in the regulatory T cell
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38 115 (Treg) pool.
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40 117 Tregs are CD4⁺ T cells that characteristically express the high affinity IL-2 receptor α -chain
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42 118 (CD25), and master transcription factor Forkhead box P-3 (Foxp3), which is essential for
43
44 119 their suppressive phenotype and stability.⁴⁻⁶ As activated CD4⁺ T cells can upregulate CD25
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46 120 expression, an additional defining feature of Tregs is the absence of IL-7 receptor α -chain
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48 121 (CD127).⁷ Their primary function is as dominant controllers of self-tolerance, tissue
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50 122 inflammation and long-term immune homeostasis. Despite making up only 5-10% of the
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52 123 peripheral CD4⁺ T cell pool, Tregs exert powerful inhibitory effects on effector cells through
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54 124 a variety of mechanisms including cytokine secretion, metabolic disruption, inhibition of
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56 125 dendritic cells (DCs) and cytotoxicity. These mechanisms have been rigorously examined using
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58 126 animal models and shown to protect against the development of intestinal inflammation.
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60 127 **Studies in patients with IBD have identified defects in the number and distribution of Tregs,**
128 **and their ability to traffic to the GI tract.⁸ Additionally, resistance to Treg-mediated**

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3 129 suppression has been noted in lamina propria T effector cells (Teffs).⁹ These factors are
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5 130 likely to be pivotal in driving intestinal inflammation.
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9 132 There is growing interest in the therapeutic potential of adoptively transferring healthy
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11 133 Tregs into patients with a wide range of conditions, including IBD and autoimmune disease,
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13 134 in an attempt to shift the balance in areas of active inflammation towards a more
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15 135 tolerogenic microenvironment. Early phase clinical trials have already reported in the fields
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17 136 of solid organ transplantation, graft-versus-host disease (GvHD) and type 1 diabetes mellitus
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19 137 (T1DM) with reassuring safety data and potential signals of efficacy.
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22 139 This review provides a summary of the suppressive mechanisms utilised by Tregs and
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24 140 highlights seminal work linking intestinal inflammation with loss of Treg function in both
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26 141 animal models of disease and in humans. Additionally, we review ongoing clinical trials with
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28 142 Treg therapy and outline an entirely novel therapeutic strategy for CD using Tregs expanded
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30 143 under GMP (Good Manufacturing Practice) conditions that will be adoptively transferred to
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32 144 patients in an attempt to ameliorate intestinal inflammation and restore immune
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34 145 homeostasis.
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37 147 **TREGS IN HEALTH AND DISEASE:**

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40 149 Tregs can be broadly divided into two groups, thymic Tregs (tTregs) or peripherally induced
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42 150 Tregs (pTregs) based on their developmental origin. Tregs generated in the thymus (tTregs)
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44 151 in the early neonatal period migrate to peripheral organs where they maintain tolerance.
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46 152 This was discovered in 1969 by Nishizuka and Sakakura who showed that in mice,
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48 153 thymectomy 3 days after birth led to the depletion of Foxp3⁺ Tregs and development of
49
50 154 autoimmune oophoritis.¹⁰ In contrast, mice who had thymectomy at day 7 remained healthy
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52 155 as the tTregs had already migrated to the periphery by this point.¹¹ Over a decade later,
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54 156 Sakaguchi *et al* demonstrated that day-3 thymectomy autoimmune oophoritis could be
55
56 157 prevented with CD4⁺ T cell inoculation from healthy syngeneic donors. Conversely, the
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58 158 adoptive transfer of T cells from these sick mice were capable of inducing autoimmune
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60 159 disease in healthy T cell deficient mice.¹² Similar findings were noted in rats that underwent
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160 160 adult thymectomy and irradiation resulting in lymphopenia, autoimmune diabetes and

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3 161 insulinitis. An injection of CD45RC(low) T cells from healthy donors were capable of
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5 162 preventing disease.¹³ Mottet *et al* subsequently described CD25-expressing CD4⁺ T cells that
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7 163 were able to cure established T cell transfer colitis.¹⁴ By the early 2000's it was clear that a
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9 164 thymically-derived CD4⁺CD25⁺ T cell population possessed the ability to suppress
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11 165 autoreactive T cells and eliminate autoimmunity.

12
13 166
14 167 Peripherally induced Tregs (pTregs) were first described in 2003 where naïve CD4⁺CD25⁻ T
15
16 168 cells could be converted into Foxp3-expressing CD4⁺CD25⁺ Tregs by T cell receptor (TCR) co-
17
18 169 stimulation in the presence of TGF- β .¹⁵ pTreg conversion in gut-associated lymphoid tissues
19
20 170 (GALT) was enhanced when naïve CD4⁺ T cells encountered antigen in the presence of TGF-
21
22 171 β , IL-2 and retinoic acid (RA).^{16,17} This is facilitated by CD103⁺ DCs conditioned by the
23
24 172 intestinal microenvironment to produce or activate TGF- β and provide RA.^{18,19} In the
25
26 173 absence of CD103 expression, DCs fail to induce Treg development and produce
27
28 174 proinflammatory cytokines.^{18,20} Additionally, in patients with UC, CD103⁺ DCs appear to
29
30 175 have impaired ability to generate pTregs, but induce colitogenic T helper (Th) 1, Th2 and
31
32 176 Th17 responses suggesting CD103⁺ DC-mediated pTreg induction is functionally relevant in
33
34 177 IBD pathogenesis.²¹

35 178
36 179 Distinguishing tTregs from pTregs can be difficult as no definitive markers exist. Recently,
37
38 180 the expression of the membrane protein neuropilin-1 (Nrp1) and the transcription factor
39
40 181 Helios by tTregs but not by pTregs has been used to differentiate Treg subsets.²² The
41
42 182 significance of this lies in the epigenetic differences in the *Foxp3* locus rendering pTregs less
43
44 183 stable and more likely to demonstrate plasticity towards a Th17 cell phenotype under
45
46 184 inflammatory conditions.²³ The developmental origin of Tregs selected for expansion as a
47
48 185 cell therapy product is therefore an important consideration and will be addressed in more
49
50 186 detail later in this review.

51 187
52
53 188 The first study identifying Tregs in humans was published in 2001. Baecher-Allan *et al*
54
55 189 characterised CD4⁺CD25⁺ T cells in the thymus and peripheral blood which exhibited anti-
56
57 190 inflammatory and suppressive properties.²⁴ Subsequent work established Foxp3 as the
58
59 191 master transcription factor for Tregs.^{4,6,25} Foxp3 can however be expressed transiently in
60
192 non-regulatory CD4⁺ T cells upon TCR activation and the CD4⁺CD25⁺CD127^{lo} surface

1
2
3 193 phenotype must be used to define Tregs.²⁶ Inactivating mutations in *Foxp3* clinically
4
5 194 manifest as severe autoimmunity with a scurfy phenotype in mice and IPEX syndrome
6
7 195 ('immune dysregulation, polyendocrinopathy, enteropathy, X-linked') in humans.²⁷⁻³⁰ With
8
9 196 autoimmune enteropathy (manifesting as chronic diarrhoea and malabsorption) a
10
11 197 predominant feature, attention was focussed on the functional role of Tregs within the GI
12
13 198 tract.

14 199
15
16 200 Peripheral Tregs are found in abundance in the intestinal lamina propria where interactions
17
18 201 with environmental antigens can shape phenotypic differences and transcription factor
19
20 202 expression.³¹ The gut microbiota represents a substantial antigen load driving the expansion
21
22 203 of colonic pTregs that co-express the Th17 master transcription factor ROR γ t.³² These
23
24 204 Foxp3⁺ ROR γ t⁺ pTregs have a stable regulatory phenotype and provide tolerance against the
25
26 205 gut microbiota.^{33,34} Conversely, ROR γ t⁻ pTregs are found in the small intestine where they
27
28 206 are induced by dietary antigens and repress underlying Th1 cell responses to ingested
29
30 207 proteins.³⁵ Finally, an intestinal tTreg population that co-express the Th2 master
31
32 208 transcription factor, GATA3, has been shown to mediate repair of the intestinal mucosa.
33
34 209 GATA3⁺ tTregs express high levels of the IL-33 receptor, ST2, and amphiregulin, an
35
36 210 epidermal growth factor receptor ligand involved in tissue repair.^{36,37}

37 211
38 212 Following on from the fundamental observations linking Treg dysfunction to an array of
39
40 213 autoimmune polyendocrine syndromes, studies began to emerge identifying defects in
41
42 214 either number or function of peripheral blood Tregs in autoimmune disorders including IBD,
43
44 215 type 1 diabetes, multiple sclerosis, systemic lupus erythematosus (SLE), myasthenia gravis
45
46 216 and rheumatoid arthritis.^{8,38-42} Maul *et al* observed that in patients with active IBD, the
47
48 217 intestinal lamina propria Treg pool was significantly smaller than that of a positive control,
49
50 218 namely diverticulitis.⁸ Additionally, in these patients, the peripheral blood Treg pool was
51
52 219 smaller than that of inactive IBD or diverticulitis.⁸ Interestingly, the peripheral blood Tregs
53
54 220 retained their suppressive capacity suggesting that disease may be driven by ineffective
55
56 221 trafficking to the gut and reduced numbers of Tregs. Furthermore, colitogenic T cells from
57
58 222 IBD patients appear to be resistant to TGF- β 1-mediated Treg suppression highlighting an
59
60 223 additional defect in immunological tolerance that may drive disease.⁴³

225 TREG FUNCTION AND COLITIS:

226

227 Tregs function as key mediators of peripheral tolerance through direct cellular contact and
228 paracrine actions on tissues where they reside.^{44,45} It is essential that Tregs effectively traffic
229 to target organs where they promote a tolerogenic microenvironment. An important
230 example is IL-10-secreting Tregs that reside in the GI mucosa and control inflammatory
231 responses induced by environmental insults. Selective disruption of IL-10 expression in
232 these Tregs has been shown to cause spontaneous colitis.⁴⁶ This is one of many modalities
233 that Tregs can employ to maintain immune homeostasis at the mucosal interface. Others
234 include inhibitory cytokine secretion, cytotoxicity of effector cells, metabolic disruption,
235 neutralization of antigen presenting cells (APC) and promotion of tissue repair.⁴⁷ **These**
236 **functions will be reviewed in further detail outlining their associations with intestinal**
237 **inflammation (see Figure 1).**

238

239 Inhibitory Cytokines:

240

241 The Treg cytokine repertoire includes the anti-inflammatory molecules IL-10, TGF- β and IL-
242 35. The expression of IL-10 and IL-35 requires TCR signalling, suggesting that Treg function in
243 part relies on antigen encounter in the local microenvironment.⁴⁸ Pioneering work by
244 Powrie *et al* over 20 years ago showcased the potent inhibitory ability of IL-10, where
245 recombinant IL-10 therapy ameliorated established T cell transfer colitis.⁴⁹ Subsequently,
246 the co-transfer of CD45RB(low) T cells were shown to prevent colitis and IL-10 was
247 identified as an essential mediator for this *in vivo* suppression.⁵⁰ The suppressive effects of
248 Treg-derived IL-10 in mice appear to be specific for mucosal surfaces rather than controlling
249 systemic autoimmunity.⁴⁶ Further studies have demonstrated that IL-10 induces robust
250 activation of a STAT3-dependent Th17 suppression program in Tregs, downstream of IL-
251 10R.⁵¹ This suppresses pathogenic Th17 cell responses and ablation of IL-10R in Tregs has
252 been shown to cause colitis. It is therefore plausible that disordered IL-10 signalling may
253 contribute to aberrant Th17 activity, which is implicated in IBD.⁵² **In fact, there have been**
254 **several cases of homozygous loss-of-function mutations in *Il-10* and *Il-10r* arising in**
255 **individuals from consanguineous marriages. These resulted in infantile severe, progressive,**
256 **intractable Crohn's-like colitis.⁵³**

257

258 TGF- β plays an important role inducing pTreg formation upon antigen encounter in GALT
259 and has a functional role in suppressing pro-inflammatory pathways.⁵⁴ Tregs are capable of
260 producing TGF- β , which profoundly suppresses the proliferation of Teffs.⁵⁵ Treg-derived
261 TGF- β 1 inhibits Th1-cell differentiation and IBD in a transfer model of colitis.⁵⁶ Conversely,
262 Tregs from TGF- β 1-deficient mice fail to suppress intestinal inflammation in a SCID transfer
263 model of colitis.⁵⁵ Human studies have supported these early findings; a study on healthy
264 human colonic biopsies and lamina propria mononuclear cells (LPMC) treated with anti-TGF-
265 β neutralising antibody showed that TGF- β is a critical suppressor of T-bet-dependent Teff
266 proliferation and Th1 cytokine expression.⁵⁷ This suggests a role for TGF- β in suppressing
267 intestinal inflammation in humans. Indeed, MacDonald *et al* have shown that colonic tissue
268 and isolated T cells from patients with CD overexpress Smad7, an inhibitor of TGF- β 1
269 signalling.⁵⁸ Furthermore, colonic LPMCs from CD patients were resistant to Treg-mediated
270 suppression, a phenomenon that could be reversed with Smad7 antisense treatment.⁴³
271 Smad 7 antisense therapy (Mongersen) was subsequently evaluated in CD but, despite
272 promising early phase data, a phase III clinical trial was terminated early due to lack of
273 benefit.^{59,60} Although Mongersen may overcome Teff resistance to TGF- β , it is possible in CD
274 there are insufficient numbers of functional Tregs in the mucosal environment to produce
275 TGF- β explaining the disappointing trial outcome .

276

277 IL-35 is a heterodimer of Ebi3 and IL-12 α that is constitutively expressed in Foxp3⁺ Tregs but
278 not Teffs. It was first described in 2007 where *Ebi3*^{-/-} and *IL-12 α* ^{-/-} Tregs were shown to have
279 significantly reduced regulatory activity *in vitro* and failed to cure T cell transfer colitis *in*
280 *vivo*.⁶¹ Additionally, IL-35 can induce the generation of a regulatory population from naïve
281 mouse or human CD4⁺ T cells. These so-called iT(R)35 cells mediate suppression via IL-35
282 alone, do not express Foxp3, and are strongly suppressive and stable *in vivo*.⁶² In both
283 dextran sulphate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis,
284 recombinant IL-35 therapy can treat disease through downregulation of the Th1 and Th17
285 master transcription factors, T-bet and RORC, respectively, and through inhibition of IFN- γ ,
286 IL-6 and IL-17.⁶³

287

288 **Inhibition of Metabolic Processes:**

289

290 While Tregs are not known to produce IL-2, their development and function is critically
291 dependent on this cytokine. IL-2 and the transcription factor STAT5, downstream of IL-2
292 receptor (IL-2R), induce the expression of Foxp3 and differentiation of tTregs.⁶⁴
293 Furthermore, STAT5 activation driven by IL2R signalling enhances the suppressor function of
294 differentiated Tregs.⁶⁵ An absence of IL-2 signalling has been shown to reduce the number
295 and functional activity of Tregs, predisposing to autoimmunity and inflammation.^{66,67} The
296 structural conformation of IL-2R in Tregs provides a competitive advantage for IL-2-receptor
297 engagement over alternative cell subsets. Tregs abundantly express IL-2 receptor α -chain
298 (CD25), which together with the common γ -chain (γ c, CD132) and IL-2 receptor β -chain
299 (CD122) form a characteristic three subunit receptor configuration. This confers a ~1000-
300 fold increase in receptor affinity for IL-2 over Teffs.⁶⁸ In a pro-inflammatory environment
301 dominated by actively dividing effector cells, Tregs have the ability to “consume” local IL-2,
302 starving effector cells of this essential cytokine for survival and proliferation.^{45,69} Moreover,
303 this mechanism has been shown to induce the apoptosis of effector cells.⁷⁰ This highlights
304 an important TCR-independent paracrine mode of suppression in local tissues, facilitated
305 through the constitutive expression of high affinity IL-2R (containing CD25). There have
306 been a handful of cases of CD25 deficiency in humans often manifesting in an IPEX-like
307 syndrome.⁷¹⁻⁷³ A notable case who presented with autoimmune enteropathy at 6 months
308 had Foxp3⁺ Tregs with defective IL-10 expression suggesting that IL-2 responsiveness is
309 important for Treg-mediated IL-10 production.⁷⁴

310

311 Tregs can also interfere with adenosine triphosphate (ATP) metabolism to dampen pro-
312 inflammatory responses. Tregs co-express the ectoenzymes CD39 and CD73 responsible for
313 the degradation of ATP and generation of pericellular adenosine.⁷⁵ Adenosine stimulates the
314 A2A receptor on Teffs exerting potent inhibitory effects. Activation of the A2A receptor also
315 inhibits IL-6 expression while enhancing the production of TGF- β .⁷⁶ This promotes the
316 development of adaptive induced Tregs and simultaneously inhibits pro-inflammatory Th17
317 cell formation. Furthermore, signalling through the A2A receptor appears to control *in vivo*
318 murine colitis.⁷⁷

319

320 **Neutralisation of Dendritic Cell Function:**

321

322 The activation of T cells requires TCR-antigen/MHC engagement in the context of a
323 secondary signal, namely T cell-derived CD28 binding the DC B7 ligands, CD80 and CD86.

324 This process is negatively regulated through the production of cytotoxic T lymphocyte
325 antigen 4 (CTLA-4) which is constitutively expressed in Foxp3⁺ Tregs.⁷⁸ CTLA-4-expressing

326 cells can capture CD80 and CD86 by a process of trans-endocytosis and degrade these
327 ligands, resulting in impaired co-stimulation via CD28.⁷⁹ This is a functionally significant

328 process with Treg-conditioned DCs inducing poor T cell proliferation.⁸⁰ An additional
329 mechanism mediated through the interaction of CTLA-4 and CD80/CD86 is the upregulation

330 of indoleamine 2, 3-deoxygenase in DCs. This is a potent regulatory molecule which
331 catabolises the essential amino acid tryptophan to the pro-apoptotic metabolite kynurenine

332 leading to suppression of Teff function.⁶⁴ *In vivo* models have demonstrated that CTLA-4 is
333 essential in preventing autoimmunity. Selective deletion of CTLA-4 in Tregs of BALB/c mice

334 results in fatal T cell mediated autoimmune disease at just 20 days of age.⁸¹ Additionally,
335 several cases of germline heterozygous mutations in CTLA-4 have been identified in

336 humans.⁸² CTLA-4 haploinsufficiency resulted in dysregulation of Tregs, hyperactivation of
337 Teffs and lymphocytic infiltration of target organs including the GI tract. It was recently

338 discovered that LRBA (lipopolysaccharide-responsive and beige-like anchor protein)
339 regulates CTLA-4 expression, where mutations in LRBA lead to reduced levels of CTLA-4.⁸³

340 These mutations are commonly associated with primary immunodeficiency, reduced Treg
341 numbers and susceptibility to IBD.^{84,85}

342

343 Recently, the coinhibitory molecule TIGIT has been described as an inhibitor of autoimmune
344 responses through its interactions with DCs and T cells. TIGIT interacts with its ligand CD155

345 on DCs to induce IL-10 and suppress IL-12 production, thereby inhibiting Th1 responses.⁸⁶ As
346 Tregs are the primary cell type that constitutively express TIGIT, it has been suggested that

347 the observed effects on DCs are mediated by TIGIT⁺ Tregs. Furthermore, Tregs expressing
348 TIGIT have been shown to directly suppress Th1 and Th17 responses through the production

349 of the effector molecule fibrinogen-like protein 2 (Fgl2).⁸⁷

350

351 **Cytotoxic Activity:**

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1
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3 353 Historically, cytotoxic activity has been associated with natural killer (NK) cells and cytotoxic
4 354 T lymphocytes (CD8⁺ T cells). In 2004, Grossman *et al* first described granzyme-B expressing
5 355 CD4⁺ Tregs capable of killing target cells in a perforin-dependent, but TCR-independent
6
7 356 manner.⁸⁸ Boissonnas *et al* subsequently showed that in a mouse tumour model, Foxp3⁺ T
8
9 357 cells can kill antigen-specific DCs. Treg cytotoxicity has also been observed against CD4⁺ T
10
11 358 cells in both *in vitro* and *in vivo* models. Activated Tregs upregulate tumour necrosis factor-
12
13 359 related apoptosis inducing ligand (TRAIL) which enhances suppressive activity as well as
14
15 360 cytotoxicity against CD4⁺ T cells. This is entirely dependent on the TRAIL/death receptor 5
16
17 361 (DR5) pathway.⁸⁹ Galectin-1, a β -galactoside-binding protein known to induce T cell
18
19 362 apoptosis has also been implicated in Treg cytotoxic function. Galectin-1 was found to be
20
21 363 overexpressed in Tregs and galectin-1 knockout models were shown to possess reduced
22
23 364 regulatory activity.⁹⁰
24

25 365

26 366 **Tissue Repair:**

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28
29 368 Aside from limiting mucosal damage through the suppression of pro-inflammatory cells
30
31 369 following environmental insults like infection, Tregs may also promote tissue repair.
32
33 370 Recently, the epidermal growth factor (EGF)-like molecule amphiregulin (AREG) has gained
34
35 371 attention as an important regulator of tissue repair and regeneration. In a murine model of
36
37 372 influenza, selective Treg deficiency in AREG leads to severe acute lung damage without any
38
39 373 alterations in Treg suppressor function. This suggests that Tregs play a direct role in tissue
40
41 374 repair and maintenance that is distinct from their suppressive function.⁹¹ Treg production of
42
43 375 AREG is dependent on IL-18 or IL-33 which function as endogenous danger signals or
44
45 376 alarmins, in response to tissue damage.⁹¹ Studies in humans have revealed high levels of IL-
46
47 377 33 in inflamed lesions of IBD patients, and Tregs expressing the IL-33 receptor, ST2, are
48
49 378 enriched in the colon.⁹²⁻⁹⁴ IL-33-Treg signalling may therefore represent an important
50
51 379 pathway in both disease pathogenesis and recovery.
52

53 380

54 381 **TREGS AS A THERAPEUTIC PRODUCT:**

55 382

56
57 383 In light of the vast array of preclinical data showcasing how a multitude of defects in Treg
58
59 384 function contribute to autoimmunity and inflammation, including IBD, there has been great

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2
3 385 interest in harnessing the suppressive ability of Tregs as a therapeutic product.
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5 386 Consequently, there are over 50 registered trials of Treg therapy that are either completed
6
7 387 or ongoing (clinicaltrials.gov). Most of these trials involve adoptive cell transfer, although
8
9 388 the dose of Tregs given is highly variable. In the setting of autoimmune disease and
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11 389 transplantation, the goals of treatment are the restoration of peripheral self tolerance, the
12
13 390 suppression of inflammation and promotion of tissue repair.⁹⁵

14 391

15
16 392 In order to become a successful therapeutic product, Tregs must home to sites of
17
18 393 inflammation and secondary lymphoid tissues, and must undergo TCR engagement. It has
19
20 394 been demonstrated in solid organ transplantation that alloantigen-specific Tregs provide
21
22 395 higher therapeutic benefits than polyclonal Tregs, without delivering a systemic
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24 396 immunosuppressive effect.⁹⁶ Directing Tregs against a specific alloantigen also permits
25
26 397 immunomodulatory functions to be concentrated at the site of the alloantigen source,
27
28 398 circumventing the relative paucity of Tregs. An early study demonstrated that peripheral
29
30 399 Treg expansion in mice could be driven by prolonged low dose subcutaneous infusion of a
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32 400 specific peptide.⁹⁷ The induced Tregs had suppressive abilities, and demonstrated high
33
34 401 levels of Foxp3 expression indicating a stable Treg phenotype. However, in IBD, a specific
35
36 402 antigen has yet to be identified.

37 403

38 404 The relative paucity of Tregs in peripheral blood represents an obstacle to the development
39
40 405 of a cellular therapy, though the optimum number of Tregs to be infused remains unclear. It
41
42 406 has been suggested that the number of Tregs given should be at least as great as the
43
44 407 number of T cells in the body,⁹⁸ though Tregs also exhibit the ability to confer suppressive
45
46 408 ability on conventional T cells through 'infectious tolerance'.⁹⁶ In this process, the direct
47
48 409 secretion of TGF- β , IL-10 and IL-35 by Tregs, and indirect induction via DCs, can generate a
49
50 410 regulatory microenvironment which may partially circumvent the problem of low absolute
51
52 411 numbers of Tregs.⁹⁹

53 412

54 413 Several groups have developed protocols in line with GMP requirements to permit *ex vivo*
55
56 414 cell expansion of Tregs.^{98,100,101} GMP-manufactured Tregs delivered in some early trials were
57
58 415 only around 50% pure, but the development of plastic beads coated with stimulatory
59
60 416 antibodies and the discovery of additional surface markers for Treg phenotyping mean that

1
2
3 417 a product with purity greater than 90% is now achievable.⁹⁸ Contamination of the expansion
4
5 418 product with Teffs hampers expansion,¹⁰² but the inclusion of rapamycin in cell culture
6
7 419 blocks expansion of Teffs without affecting Treg proliferation, leading to the preferential
8
9 420 promotion of Treg proliferation.^{98,103}

10 421
11
12 422 Tregs are first isolated from peripheral blood by surface marker expression
13
14 423 (CD4⁺CD25^{hi}CD127^{lo}). This can be performed using stream in air fluorescence-activated cell
15
16 424 sorters (FACS) which yield a highly pure starting population, but the necessary air exposure
17
18 425 requires high efficiency particulate air (HEPA) enclosures, and single use sample lines to be
19
20 426 compatible with manufacturing GMP cell products. Closed system magnetic bead-activated
21
22 427 cell sorting (MACS) can be adapted for large-scale isolation of human Tregs, but unlike FACS
23
24 428 cannot easily distinguish surface marker expression density. A recently developed
25
26 429 microfluidic chip fluorescence-activated cell sorter, the MACSQuant Tyto (Miltenyi Biotech,
27
28 430 Germany) surmounts the problems of stream in air sorters, as the cells remain in a closed
29
30 431 system throughout the sorting process. Expansion of the sorted cells is achieved through
31
32 432 polyclonal TCR activation with anti-CD3/anti-CD28 beads.¹⁰⁴ Tregs are sampled and checked
33
34 433 for sterility and phenotype throughout the expansion process. With optimised conditions, a
35
36 434 500-fold expansion can be anticipated over a 14 day period.¹⁰¹

37 435
38 436 Uncertainty about the plasticity of Tregs in culture and following infusion means there is a
39
40 437 theoretical concern about the development of a pro-inflammatory phenotype, which could
41
42 438 lead to transplant rejection or aggravation of inflammation. However, rapamycin-expanded
43
44 439 Tregs are not contaminated by IL-17-producing Th17 cells, and these cells maintain a stable
45
46 440 phenotype on transfer *in vivo* to mice.¹⁰⁵ Canavan *et al.* found that the starting population
47
48 441 for Treg expansion from the peripheral blood of CD patients has a critical effect on the
49
50 442 phenotype of the expanded cell population.¹⁰⁰ Tregs from a highly pure FACS-sorted 'naïve'
51
52 443 CD4⁺CD25^{hi}CD127^{lo}CD45RA⁺ precursor population demonstrated enhanced suppressive
53
54 444 ability and reduced Th17 plasticity *in vitro* compared to a FACS-sorted
55
56 445 CD4⁺CD25^{hi}CD127^{lo}CD45RA⁻ or MACS-enriched CD8⁻CD25⁺ population. Rapamycin appears to
57
58 446 imprint a fixed CD4⁺CD25^{hi} phenotype to cells expanded from a 'naïve' CD45RA⁺ population,
59
60 447 as evidenced by the retention of demethylation at the Foxp3 locus.

448

449 **TREG THERAPY IN OTHER CONDITIONS:**

450

451 There is an increasing body of evidence for the use of Tregs as cellular therapy in
 452 autoimmune disease and transplantation (see Table 1). Adoptive transfer of Tregs to
 453 prevent GvHD was the first illustration of the potent therapeutic potential of Tregs in
 454 experimental transplantation.

455

Study	Clinical context	Enrichment protocol	Expansion protocol	Dose	Study outcome
Trzonkowski <i>et al.</i> (2009)	Treatment of acute and chronic GvHD N=2	Tregs from allogenic buffy coat. CD4 ⁺ negative bead selection followed by FACS-based sorting of CD4 ⁺ CD25 ^{hi} CD127 ^{lo} cells	RPMI 1640 with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) 3 weeks	Acute GvHD: 1x10 ⁶ /kg Chronic GvHD: 3x10 ⁶ /kg	Transient improvement in acute GvHD; alleviation of symptoms and reduction of immunosuppression in chronic GvHD
Brunstein <i>et al.</i> (2011)	Prevention of GvHD following umbilical cord blood transplantation N=23	CD25 ⁺ bead positive selection	X-Vivo 15 with 10% human AB serum IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:2) 18±1 days	0.1-30x10 ⁵ /kg	Well –tolerated; reduced incidence of grade II-IV GvHD in Treg recipients
Marek-Trzonkowska <i>et al.</i> (2012)	Safety of autologous <i>in vitro</i> expanded Tregs in paediatric type 1 diabetes N=10	FACS-based sorting of CD3 ⁺ CD4 ⁺ CD25 ^{hi} CD127 ^{lo} cells	CellGro medium with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) Up to 2 weeks	10-20x10 ⁶ /kg	Well-tolerated; decreased insulin requirements and C-peptide levels in Treg recipients
Desreumaux <i>et al.</i> (2012)	Safety and efficacy in Crohn's disease N=20	Culture of PBMCs with ovalbumin, IL-2 and IL-4 followed by cloning of ovalbumin-specific T cells	X-Vivo 15 IL-2 (200IU/ml) Anti-CD3/anti-CD28 beads (1:1) Ova-Tregs selected based on ovalbumin-specific IL-10 production 12 to 15 weeks	1x10 ⁶ -1x10 ⁹	Well-tolerated; dose-related efficacy
Bluestone <i>et al.</i> (2015)	Safety in adults with type 1 diabetes (N=14)	FACS-based sorting of CD4 ⁺ CD25 ^{hi} CD127 ^{lo} cells	X-Vivo 15 with 10% human AB serum and deuterated glucose IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:1) 14 days	0.05x10 ⁸ -26x10 ⁸	Well-tolerated, no significant adverse events. Stable C-peptide levels and insulin use in recipients for up to two years post infusion
Mathew <i>et al.</i> (2018)	Safety in living donor kidney transplant N=9	CliniMACS plus GMP enrichment system (Miltenyi)	IL-2 (1000IU/ml) MACS © GMP expansion beads 1:1-4:1 3 weeks	0.5-5x10 ⁹	Well-tolerated, no infections or rejection up to two years post transplant

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2
3 457 **Table 1: Summary of clinicaltrials.gov listings for reported trials using *in vitro* expanded**
4 458 **regulatory T cell (Treg) therapy**
5 459

7 460 **Graft vs. host disease (GvHD):**
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9 461
10
11 462 The risk of developing GvHD following haematopoietic stem cell transplantation (HSCT) is
12 associated with low numbers of Tregs in the periphery,¹⁰⁶ and *in vivo* expansion of Tregs
13 463 post-HSCT using low dose IL-2 has demonstrated efficacy against GvHD.^{107,108} Studies in mice
14 464 involving infusion of cultured CD4⁺CD25⁺ T cells resulted in a significantly reduced GvHD
15 465 phenotype,¹⁰⁹ and in humans it was found that infusion of freshly isolated donor Tregs given
16 466 at the same time as haplotype mismatched HSCT prevented the development of GvHD.¹¹⁰
17 467 Five trials of *ex vivo* expanded Tregs have to date involved small numbers of patients only,
18 468 but suggest therapy can prevent or delay the onset of chronic GvHD^{111,112}. Treg therapy
19 469 seems to be effective only in the chronic form of GvHD, but this may be because of the time
20 470 requirements to expand the cellular product which makes it difficult to administer in a
21 471 timely manner in acute GvHD.¹¹³
22 472
23 473

33 474 **Solid organ transplant:**
34

35 475
36
37 476 Adoptive Treg therapy has been trialled following renal and liver transplantation, with the
38 477 aim of inducing tolerance to the allograft and reducing the burden of long-term
39 478 immunosuppression.¹¹⁴ Tregs have been shown to control immune responsiveness to
40 479 alloantigens and contribute to 'operational tolerance' in preclinical transplantation
41 480 models.^{115,116} Recipient-derived Tregs expanded for direct and indirect pathway
42 481 allospecificity *in vitro* were able to mediate effective protection against acute and chronic
43 482 rejection in skin and heart allografts in mice,¹¹⁷ and could be used to induce tolerance of a
44 483 murine skin transplant following thymectomy and T cell depletion.¹¹⁸ In these models,
45 484 alloantigen reactive Tregs were more effective at preventing graft rejection than
46 485 polyclonally expanded Tregs¹⁰⁴.
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57 487 A phase I study in renal transplantation recruited nine living donor transplant recipients, and
58 488 used the product of leukapheresis as the basis for *ex vivo* expansion of polyclonal
59
60

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3 489 autologous Tregs.¹¹⁴ Alemtuzumab was given at induction to achieve lymphodepletion, on
4
5 490 the basis of previous experiments suggesting a reduction in circulating Tregs worked
6
7 491 synergistically with Treg infusion to prolong allograft survival.¹¹⁶ Recipients were switched
8
9 492 from traditional immunosuppression with tacrolimus, which blocks IL-2 production, to
10
11 493 sirolimus (rapamycin), which has Treg promoting activity.¹¹⁹

12 494

14 495 An enhanced suppressive ability of the expanded Tregs was demonstrated when compared
15
16 496 to Tregs taken directly *ex vivo*.¹¹⁴ There were no adverse infusion-related side effects,
17
18 497 infections or rejection up to two years post-transplant, and there was a 5-20 fold increase in
19
20 498 the number of circulating Tregs seen up to one year post-transplant. Transplant biopsies
21
22 499 taken at three months did not show rejection and recipients had not developed peripheral
23
24 500 donor-specific antibodies. An additional important outcome from trials in transplantation is
25
26 501 that they have demonstrated that it is possible to expand Tregs from immunocompromised
27
28 502 patients.¹²⁰

29 503

31 504 A trial of Treg immunotherapy in liver transplantation is currently underway.¹²¹ This is
32
33 505 predicated on the observation that when liver allografts in mice were infiltrated with Tregs,
34
35 506 loss of Treg numbers was associated with a loss of tolerance.¹²² Increased frequencies of
36
37 507 Tregs are also seen in human subjects who acquire 'operational tolerance' to their liver
38
39 508 transplant.¹²³

40 509

41 510 **Type 1 diabetes mellitus:**

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45 512 The development of T1DM is associated with deficits in the number and suppressive activity
46
47 513 of Tregs.¹²⁴ Accelerated diabetes onset is seen in both scurfy mice²⁹ and children with
48
49 514 IPEX,¹²⁵ highlighting the role of Tregs in protecting pancreatic islet cells from destruction.
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51 515 Tregs have been implicated in the pathogenesis of diabetes in the non-obese diabetic (NOD)
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53 516 mouse model,^{126,127} and anti-CD3 antibodies have been efficacious in the treatment of
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55 517 diabetes in both mouse^{128,129} and human trials.^{130,131} Subjects exhibited lower insulin
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57 518 requirements and higher C-peptide levels at least 18 months after a short course of
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59 519 intravenous treatment, with evidence of anti-CD3 treatment inducing expansion of a
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520 CD4⁺CD25⁺ T cell population.¹²⁹ A trial of ten children treated with expanded polyclonal Tregs

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3 521 within two months of their diagnosis demonstrated statistically lower insulin requirements
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5 522 and C peptide levels compared with matched controls up to six months post infusion, with
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7 523 two patients remaining insulin-independent.¹²⁴ There were no serious adverse events up to
8
9 524 one year following infusion.

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11
12 526 In a phase I open-label trial of 14 adult patients infused with *ex vivo* expanded Tregs in
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14 527 escalating doses, 7 of 14 patients had stable C peptide levels and insulin use for up to two
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16 528 years following infusion.¹⁰¹ However, the study was not powered to detect significant
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18 529 clinical improvement. There were no infusion reactions or therapy-related serious adverse
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20 530 events. Phenotypic analysis of the cell product after expansion and after infusion identified
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22 531 stable surface marker expression, demonstrating that the infused Tregs did not acquire a
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24 532 pathological phenotype. High throughput TCR- β sequencing analysis indicated that
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26 533 expanded Tregs retained a high degree of diversity.

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28
29 535 Adoptively transferred Tregs were tagged by labelling the deoxyribose moiety of replicating
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31 536 DNA during expansion *ex vivo*, through addition of deuterated [6,6-²H₂] glucose to Treg
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33 537 culture throughout the 14 day expansion period.¹⁰¹ Patient samples were analysed by gas
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35 538 chromatography mass spectrometry (GC-MS) for deuterium enrichment to create
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37 539 pharmacokinetic curves. Adoptively transferred T cell numbers peaked at two weeks
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39 540 following infusion, but were still detectable at up to 25% of the peak level at one year in
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41 541 peripheral blood. Significantly, deuterium labelling was never found in non-Tregs, indicating
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43 542 the stability of infused Tregs. However, due to the nature of this study, the stability of these
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45 543 cells was not assessed within the target tissue.

46 544

47 545 **TREG THERAPY IN INFLAMMATORY BOWEL DISEASE:**

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49
50 547 A local imbalance between Treg and Teff responses plays a key role in the development of
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52 548 gut inflammation in IBD.⁸ T cell gut homing is mediated by specific interaction between
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54 549 integrin $\alpha 4\beta 7$ and its ligand MAdCAM-1.^{132,133} Several groups have shown that transfer of
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56 550 Tregs into mice leads to clinical and histological improvement in colitis,^{14,134,135} and
57
58 551 rapamycin-expanded Tregs ameliorated established colitis in a SCID mouse model.¹³⁶
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60 552 Polyclonality of the TCR is likely to be an important requirement for Tregs to maintain

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3 553 intestinal homeostasis *in vivo*. Mice which express a restricted TCR repertoire develop
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5 554 spontaneous colitis due to a loss of tolerance to intestinal microbiota.¹³⁷
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8
9 556 Several groups have demonstrated that it is feasible to extract Tregs from patients, and
10
11 557 expand them *in vitro* under GMP conditions, including from subjects receiving thiopurines
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13 558 and anti-TNF α medications.^{98,100,103,138} Even after prolonged culture, these Tregs maintained
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15 559 Foxp3 expression and demonstrated enhanced suppression of autologous T cells.
16
17 560 Uncertainty regarding the potential for adoptively transferred Tregs to express IL-17 and
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19 561 exacerbate CD lesions is a concern. However, the administration of pro-inflammatory
20
21 562 cytokines (IL-1, IL-2, IL-6, IL-21, IL-21 and TGF- β) failed to induce IL-17 production by
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23 563 CD45RA⁺ expanded Tregs *in vitro*.¹⁰⁰
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25 564

25 565 **Antigen specific vs. polyclonal Treg cell products for Crohn's disease:**

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29 567 No antigens have yet been verified as causal in CD. Attempts have been made to identify
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31 568 shared TCRs between CD sufferers with the aim of discovering target antigens.^{139–141} This
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33 569 work has observed that the CD4 TCR repertoires are significantly more diverse in patients
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35 570 with CD and UC than healthy controls.¹⁴² This may be explained by GI barrier disruption
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37 571 increasing the number of antigen presentation events in comparison to a healthy gut.
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39 572 Resolving a target from the GI peptidome is challenging due to the heterogenous nature of
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41 573 the environment. Developments in the understanding of non-conventional epitopes are also
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43 574 increasing the magnitude and complexity of the peptidome itself.¹⁴³ In the absence of a
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45 575 known target, the broad reactivity of a polyclonal Treg product may be advantageous, as the
46
47 576 cell product will recognise millions of putative epitopes, increasing the likelihood of TCR
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49 577 engagement and subsequent Treg activation. Sequencing of isolated Tregs from GI biopsies
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51 578 post transfer may yield novel targets, upon which chimeric antigen receptor technology
52
53 579 could be readily implemented.¹⁴⁴
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54 580

55 581 **For Treg therapy to be effective in IBD, expanded Tregs must have the ability to home to the**
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57 582 **gut.**¹⁴⁵ A French group reported the results of an open label multicentre phase I/IIa trial of
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59 583 ovalbumin-specific Tregs in 20 patients with refractory CD.¹⁴⁶ Ovalbumin is a common food
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584 antigen, and is not implicated in intestinal inflammation in animal models or in patients with

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2
3 585 CD. Its distribution along the digestive tract can be used to activate Tregs locally. In the
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5 586 study, this was facilitated through ingestion of meringue cakes by subjects.¹⁴⁶
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8
9 588 The cell product was cultured in the presence of ovalbumin, and trial subjects received a
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11 589 dose of 10^6 - 10^9 Tregs.¹⁴⁶ Patients enrolled in the study had at least moderately active CD,
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13 590 with a Crohn's Disease Activity Index (CDAI) greater than or equal to 220 within six months
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15 591 of screening, and a washout period was required for immunosuppression and anti-TNF α
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17 592 therapy. The infusion was well tolerated, with mild GI symptoms and CD flares being the
18
19 593 most commonly reported adverse effects. Two patients experienced thrombotic events, but
20
21 594 these are known to occur more frequently in inflammatory conditions including active CD.¹⁴⁷
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23 595 Eight (40%) patients had a significant CDAI response at weeks five and eight after treatment,
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25 596 with two patients experiencing sustained remission. Overall, the results suggested good
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27 597 tolerability in this disease group with possible signals of efficacy.
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29 598

30
31 599 In the absence of a known antigen, other methods must be used to direct the Tregs to the
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33 600 areas of inflammation. A recent study has shown that a highly specific retinoic acid receptor
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35 601 α (RAR α) agonist induces expression of Integrin $\alpha 4\beta 7$ (the ligand of MAdCAM-1) on the Treg
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37 602 surface. Adoptive transfer of RAR α agonist-treated Tregs leads to improved Treg trafficking
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39 603 to gut tissue in a humanised mouse model of colitis.¹⁰⁰ Supporting this mechanism for
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41 604 resolving inflammation, another group have demonstrated that DCs can be engineered *de*
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43 605 *novo* to produce high concentrations of RA.¹⁴⁸ When transferred to mice, the RA-secreting
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45 606 DCs were able to augment the expression of Foxp3 and the gut-homing receptor CCR9 in
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47 607 native Tregs with the subsequent suppression of colitis.
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51 609 The RAR α agonist treated cell product forms the basis of the TRIBUTE trial (ClinicalTrials.gov
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53 610 Identifier: NCT03185000), a double-blinded placebo-controlled phase I/IIa trial of adoptive
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55 611 Treg therapy in CD.
56

57 612

58 613 **FUTURE DEVELOPMENTS IN TREG THERAPY:**

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61 615 The potential therapeutic benefits of adoptive cell therapy are being explored in numerous
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63 616 autoimmune conditions. In SLE, adoptive transfer of *ex vivo* expanded Tregs in mice delayed

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3 617 the onset of renal complications and prolonged survival,^{149,150} and a pilot study of low dose
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5 618 IL-2 in 37 patients led to increased circulating peripheral Treg numbers and decreased SLE
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7 619 disease activity scores.¹⁵¹ Adoptive Treg transfer in a single patient with cutaneous lupus did
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9 620 not lead to clinical benefit, but increased percentages of highly activated Tregs were
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11 621 identified in biopsies taken from diseased skin.¹⁵² Treg accumulation in skin was associated
12
13 622 with a marked attenuation of IFN- γ , which was more pronounced relative to peripheral
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15 623 blood.

16 624
17
18 625 Preliminary results from mouse models suggest a role for Treg therapy in conditions as
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20 626 diverse as pemphigus vulgaris,¹⁵³ autoimmune hepatitis,¹⁵⁴ multiple sclerosis,¹¹³ asthma,
21
22 627 and allergy, in which antigen-specific Tregs may represent a viable therapeutic option.^{155,156}

23 628
24
25 629 Many ongoing challenges exist for the advancement of Treg therapy. Uncertainties remain
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27 630 about the optimal timing of *ex vivo* Treg expansion, and whether IL-2 administration would
28
29 631 be a useful adjunct to support a Treg population *in vivo*.^{101,107} In addition, concomitant
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31 632 treatment of autoimmune disease with immunosuppressive drugs may affect the function
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33 633 of adoptively transferred cells.⁹⁵

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35
36 635 The optimal dosing strategy for Treg therapy also remains unclear, although data tracking
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38 636 the survival of deuterium-labelled Tregs *in vivo* could be invaluable in informing a suitable
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40 637 dosing regimen.¹⁰¹ A two-phase decay in numbers of deuterium-labelled Tregs has been
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42 638 seen, with 75% of the peak level lost at three months. However, levels stabilised at one
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44 639 year, with up to 25% of peak Treg numbers remaining in the peripheral circulation. The
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46 640 decrease in labelled Tregs may represent cell death, trafficking to lymphoid tissue and sites
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48 641 of inflammation, or proliferation of the Treg compartment leading to dilution of deuterium
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50 642 enrichment. Reassuringly, at no point during the trial was deuterium detected in cell
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52 643 populations other than Tregs, suggesting a stable phenotype *in vivo*.¹⁰¹

53 644
54
55 645 Tracking of TCR clonotypes may also provide useful data on Treg kinetics and dispersal.
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57 646 Analysis of the TCR repertoire has suggested that the kinetics of transferred Tregs in
58
59 647 peripheral blood varies significantly between individuals¹⁵⁷. In a descriptive study, the TCR
60
648 V α chain was sequenced in two patients receiving donor Treg infusion¹⁵⁷. Treg therapy

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3 649 altered the patients' peripheral TCR repertoire considerably towards that of the infused cell
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5 650 product, but to different degrees in each patient. Importantly, the degree of alteration of
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7 651 the TCR repertoire appeared to correlate with clinical response. This suggests that
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9 652 monitoring TCR repertoires following adoptive cell transfer may provide clinically
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11 653 meaningful information.

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13 655

14 656 **CONCLUSION:**

15 657

16 658 There is now robust evidence of the therapeutic potential of Treg therapy in Crohn's
17 659 disease. Trials in multiple autoimmune diseases and results from use of ovalbumin-specific
18 660 Tregs in IBD show promising early signs of efficacy. The safety signal is reassuring, with
19 661 evidence that the adoptively transferred Treg phenotype is stable *in vivo*. Results from
20 662 deuterium labelling suggest that infused Tregs may be able to exert a long-lasting systemic
21 663 effect with labelled cells detectable up to a year after infusion. It is hoped that upcoming
22 664 early phase clinical trials in patients with Crohn's disease will inform safety, dosing, and Treg
23 665 kinetics and dispersal allowing further development of a novel therapeutic option in this
24 666 hard-to-treat population.

25 667

26 668 **FIGURE LEGEND:**

27 669

28 670 **Figure 1. Mechanisms of Treg mediated suppression**

29 671 Tregs utilize a multitude of mechanisms to promote a tolerogenic microenvironment and
30 672 tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL-10, TGF- β and IL-35, not
31 673 only inhibit Teff proliferation, but also suppress Th1 and Th17 effector function, both of
32 674 which are key mediators of IBD. (B) Tregs express the high affinity IL-2 receptor α -chain
33 675 (CD25) consuming local IL-2 with greater affinity than effector cells. Teffs which are 'starved'
34 676 of IL-2 exhibit restricted proliferation and undergo apoptosis. (C) Tregs co-expressing CD39
35 677 and CD73 disrupt metabolic processes in effector cells by converting ATP into peri-cellular
36 678 adenosine, a potent inhibitor of Teff function. Additionally, adenosine stimulates TGF- β
37 679 production, promoting development of pTregs. (D) Tregs are capable of secreting perforin,

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2
3 680 granzyme B and galectin-1 which are directly cytotoxic against Teffs. Activated Tregs also
4
5 681 express TRAIL, inducing apoptosis of Teffs through the TRAIL/DR5 pathway. (E) Expression of
6
7 682 CTLA-4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-
8
9 683 stimulation of T cells. DC function is further inhibited through the interaction of Treg-
10
11 684 derived TIGIT and CD155 on DCs. This induces IL-10 production and suppresses IL-12. (F) In
12
13 685 response to alarmins, Tregs produce AREG, an important regulator of tissue repair and
14
15 686 regeneration. AREG, amphiregulin; ATP, adenosine tri-phosphate; CTLA-4, cytotoxic T
16
17 687 lymphocyte associated protein 4; DC, dendritic cell; DR5, death receptor 5; IBD,
18
19 688 inflammatory bowel disease; pTregs, peripheral regulatory T cells; Teff, T effector
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21 689 lymphocyte; TGF- β , transforming growth factor beta; Th1, T helper 1 cell; Th17, T helper 17
22
23 690 cell; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TRAIL, TNF-related apoptosis-
24
25 691 inducing ligand; Treg, regulatory T cell. Figure generated using BioRender© illustration
26
27 692 software.

28 693

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30 695

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47
48 704 Foundation Trust. The views expressed are those of the author(s) and not necessarily those
49
50 705 of the NHS, the NIHR, or the Department of Health.

51 706

52 707 **COMPETING INTERESTS:**

53 708

54
55
56 709 PI is the Chief Investigator and GL is the Chief Scientific Investigator on the MRC-funded
57
58 710 TRIBUTE trial of regulatory T cell immunotherapy in Crohn's disease (ClinicalTrials.gov
59
60 711 NCT03185000).

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