CLINICAL THERAPEUTIC TOLERANCE: FIRST-IN-HUMAN DATA: PROCEEDINGS OF THE 4th NEWCASTLE THERAPEUTIC TOLERANCE WORKSHOP

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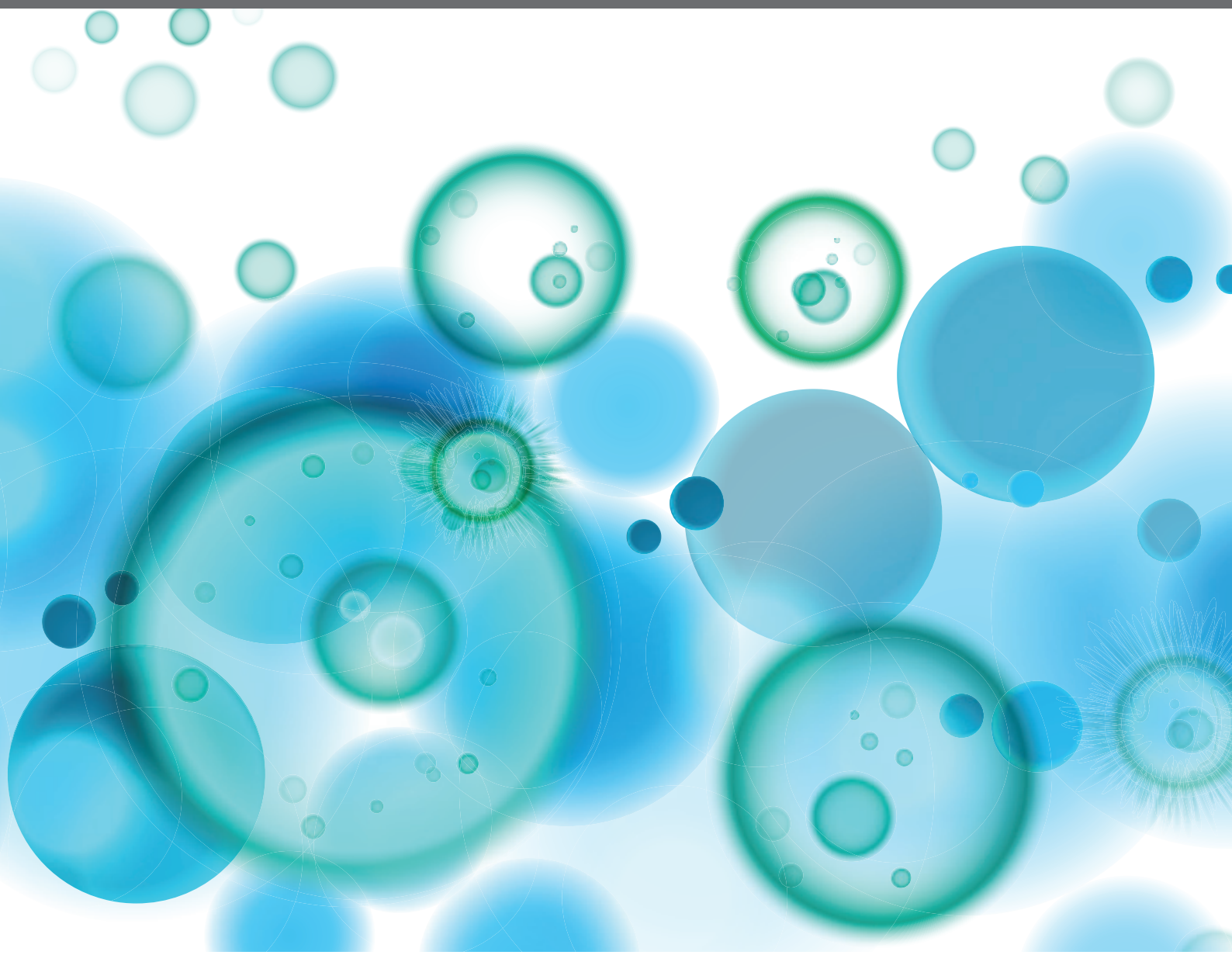

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MINI REVIEW
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Indoleamine 2,3-Dioxygenase and Tolerance: Where Are We Now?

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Cells expressing IDO suppress innate and adaptive immunity to promote tolerance by catabolizing the amino acid tryptophan (Trp) and other indole compounds. Interferon type I (IFN-I) and type II (IFN-II) produced at sites of inflammation or by activated immune cells are potent IDO inducers because mammalian IDO genes contain IFN response elements. Elevated IDO expression by dendritic cells (DCs) is of particular significance because IDO activity converts mature DCs into tolerogenic APCs that suppress effector T cells (Teff) and promote regulatory T cells (Tregs), thereby promoting tolerance. Local Trp depletion and production of immune suppressive Trp catabolites contribute to tolerogenic processes by activating metabolic pathways responsive to amino acid withdrawal and aryl hydrocarbon signaling, respectively. Sustained IDO elevation creates local immune privilege that protects tissues from immune-mediated damage and allows tissues to heal. This response occurs in lymphoid tissues when DNA released by dying tissue cells is sensed to induce specialized DC subsets to acquire tolerogenic phenotypes. The tolerogenic effects of IDO also promote tumorigenesis and help establish immune checkpoints in cancer, as malignant cells are protected from immune surveillance. Similar processes may attenuate host immunity to some pathogens that persist in immunocompetent individuals. However, if inflammation with IDO involvement is not resolved, chronic immune activation at such sites causes progressive tissue damage over time. Another effect of sustained IDO activity is enhanced pain sensitivity, as some Trp catabolites produced by cells expressing IDO are neuroactive. In this review, we summarize links between IDO and chronic inflammatory diseases and discuss prospects for exploiting IDO and Trp catabolism to suppress immunity and promote tolerance for clinical benefit, with particular emphasis on protecting tissues from destructive autoimmunity.

Keywords: indoleamine 2,3-dioxygenase, tolerance, autoimmunity, nociceptive pain, transplant, transplantation immunology

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INTRODUCTION

Higher mammals possess two closely linked and homologous genes encoding indoleamine 2,3-dioxygenase (IDO1, IDO2), which catabolizes compounds containing indole rings, including tryptophan (Trp) and the neurotransmitter serotonin, aka 5-hydroxytryptamine (5HT). IDO1 gene expression is responsive to interferons (IFNs), explaining why IDO activity is elevated in many inflammatory settings, including infectious, allergic and autoimmune (AI) diseases, tumorigenesis, and pregnancy. In this brief review, we highlight key aspects of IDO1 immunobiology and summarize prospects for exploiting this pathway to protect tissues from immune-mediated damage in

patients with AI syndromes and transplanted tissues. The concise format of this review precludes a comprehensive overview and readers are directed elsewhere for detailed discussions of the current state of this highly active field. The ability of cells expressing IDO1 genes to suppress immune responses was first described almost 20 years ago (1). Since then, research on IDO has blossomed and IDO inhibitors are promising immune checkpoint inhibitor drugs to enhance cancer immunotherapy (2, 3).

IFNs, IDO, AND IMMUNE BALANCE

Interferons released at sites of inflammation stimulate hundreds of downstream genes, known collectively as IFN-stimulated genes (ISGs). Historically, ISGs that activate immune cells to incite immunity to infections have been the major focus of research. However, some ISGs activate immune regulatory cells to promote tolerogenic, rather than immunogenic processes. The local balance of immunogenic and tolerogenic responses to IFNs is a key factor driving the effects of local inflammation on immune responses and tissue functions.

IDO1 is an example of a tolerogenic ISG because IDO suppresses immune responses and the IDO1 gene is responsive to both IFN type I (IFN-I) and type II (IFN-II). IFN response elements called ISRE and GAS sequences, which confer responsiveness to IFN-I and IFN-II signaling, respectively, are located in mammalian IDO1 gene promoters. Though most cell types express IFN receptors, IFNs induce IDO only in select cell types because IFN signaling is regulated in some cells. For example, discrete subsets of dendritic cells (DCs) from humans and mice are competent to express IDO when exposed to IFNs (4). Importantly, IDO1 gene transcription may not enhance IDO enzyme activity due to posttranslational controls, such as limited access to hemin, an IDO enzyme co-factor, local redox status, and nitric oxide, which inhibits heme/O_2 conjugation needed to break indole structures (5, 6). IDO can also promote tolerance *via* non-catalytic signaling to induce TGFβ release by some DCs (7). Thus, whether IDO activity manifests in inflamed tissues depends on many factors, linked to the complexity of biochemical pathways and cell interactions driving responses to IFNs in tissues. Thus, it is important to study responses to IFNs in physiologic settings, where multiple effects are integrated to generate particular responses to inflammatory insults. Though much can be learned by studying how cultured or physiologic cells and isolated tissues respond to IFNs, these approaches cannot predict physiologic outcomes accurately in the absence of the full panoply of biochemical processes and cellular interactions that exist in inflamed tissues.

IDO AND Trp CATABOLISM

IDO catalyzes the initial, rate-limiting step in oxidative catabolism of compounds containing indole rings (**Figure 1**), including Trp and 5HT. Cells expressing IDO1 genes deplete Trp and generate bioactive catabolites, known as kynurenines, after the catabolite kynurenine (Kyn). Enzymes downstream of IDO further degrade Kyn to generate kynurenic acid (KA), 3-hydroxy-anthranilic acid (HAA), quinolinic acid (QA), niacin, and other catabolites. Some immune cells can sense Trp depletion

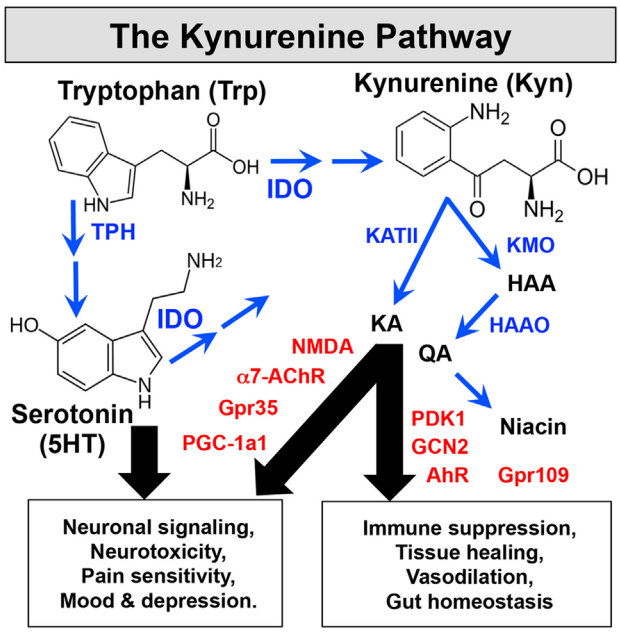

FIGURE 1 | The Kynurenine pathway. IDO catabolizes indole compounds, including Trp and 5-hydroxytryptamine. Enzymes (blue) downstream of IDO generate bioactive catabolites called kynurenines. Immune and CNS cells express sensors (red) that detect these metabolic changes to impact immunologic and neurologic processes.

or catabolites to suppress innate and adaptive immunity and promote tolerogenic responses. Limiting access to Trp activates the ribosomal kinase GCN2, which senses binding of uncharged tRNA to ribosomes. Activated GCN2 triggers the integrated stress response (ISR) to amino acid withdrawal, which induces CHOP gene expression but shuts down most gene transcription to promote cell autophagy. The ISR blocks cell cycle entry by TCR-activated T cells and activates resting Foxp3-lineage regulatory CD4 T cells (Tregs) to promote tolerogenic responses to inflammatory signals from immune adjuvants and tumor vaccines (8). Some catabolites in the Kyn pathway bind to receptors expressed by immune cells to promote tolerogenic responses. For example, HAA, Kyn, and KA suppress T cell responses by binding to PDK1 or aryl hydrocarbon receptors (AhR) in T cells, APCs, or other immune cells. As well as influencing inflammatory and immunologic processes, the Kyn pathway also drives neurologic comorbidities such as pain, depression, and fatigue, commonly associated with chronic inflammatory disease. By consuming Trp, the substrate for 5HT and melatonin synthesis, IDO impacts mood and promotes depression. Moreover, receptors such as NMDA, α7AChR, and Gpr35 expressed by neuronal cells can sense neuroactive catabolites such as KA and QA. An intriguing link between physical exercise and reduced depression was reported due to increased uptake of circulating Kyn by PGC1a1 receptors expressed by active skeletal muscles (9). Niacin, another product of the kynurenine pathway that binds to the Gpr109a receptor, also suppresses colonic inflammation and carcinogenesis to promote gut homeostasis and health (10).

Collectively, these points reveal pivotal roles for the Kyn pathway as a critical modifier of immunologic and neurologic responses to inflammation *via* the biochemical effects of catabolizing indole compounds such as Trp and 5HT.

CHRONIC INFLAMMATORY DISEASE AND IDO

Elevated IDO activity manifests in many chronic inflammatory syndromes, including cancer, infections, AI and allergic diseases, transplant rejection, and pregnancy (11). A link between IDO and immune regulation was first described in pregnancy. IDO inhibitors applied to pregnant mice induced allogeneic fetal rejection by maternal T cells, while sparing syngeneic fetus' from the same fate (1), indicating that IDO stops maternal T cells from attacking fetal allografts during pregnancy. Further studies revealed that complement activation drove fetal allograft rejection when IDO was inhibited, indicating that IDO blocks complement activation by maternal T cells responsive to fetal alloantigens (12).

IDO and Hypo-Immune Syndromes

IDO expression is often elevated in inflamed tumor microenvironments in mice and humans. Tumor cells or tumor-associated cells in malignant lesions may express IDO. Elevated IDO may also manifest in local lymph nodes draining sites of tumor growth. The paradigm that IDO promotes tolerogenic responses suggests that elevated IDO activity during tumorigenesis contributes to robust tumor resistance to natural and vaccine-induced anti-tumor immunity. Consistent with this notion, IDO inhibitors enhance tumor immunity to retard tumor growth in several mouse models, identifying IDO as a cancer immune checkpoint. Consequently, IDO inhibitors are under scrutiny as potential immune checkpoint inhibitor drugs in ongoing clinical trials in cancer patients. Early indications are that IDO inhibitors are well tolerated and may enhance clinical response rates to some cancers. However, heightened risk of inciting autoimmunity may be an undesirable side effect, as for other immune checkpoint inhibitors. Similarly, IDO induced by infectious pathogens may attenuate host immune responses. Most infections induce rapid IDO upregulation since IFN-I is a common innate response to many bacterial, viral, fungal, and parasitic infections. Largely, this is because Toll-like receptors (TLRs) and nucleic acid sensors that recognize pathogen-associated molecular patterns stimulate IFN-I production at sites of infection. Some pathogens that cause chronic infections may exploit the Kyn pathway to promote persistence in immunocompetent individuals. Consistent with this paradigm, IDO inhibitors reduced *Leishmania major* burdens in mice, even when applied at the peak of infection (13, 14). IDO inhibitors also reduced lentiviral HIV-1 burdens in a mouse model of HIV-1 encephalitis, suggesting that robust IDO induction on HIV-1 infection attenuates host immunity (15). However, opposing protective effects of IDO for hosts and pathogens were observed in distinct models of infection in mice. For example, IDO inhibition induced uniform mortality of mice infected with the parasite *Toxoplasma gondii* (14), consistent with earlier work showing that IDO contributes to innate host resistance to some pathogens such as parasitic Toxoplasma and fungal Candida infections. Moreover, despite >100-fold increase in lung IDO activity during influenza infections in mice, IDO ablation had no impact on virus burdens and only nuanced effects on host T cell responses to influenza infection (16). Thus, interventions to boost host immunity to pathogens by manipulating the Kyn pathway must be investigated thoroughly to avoid potential undesirable consequences.

IDO and Hyper-Immune Syndromes

Elevated IDO activity also manifests in many AI and allergic syndromes in mice and humans. IDO attenuates AI progression in several mouse models of type I diabetes, multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus, and graft-versus-host disease since IDO1 gene ablation potentiated disease onset and severity mediated by T cells, while enhancing IDO1 gene expression attenuated disease progression and severity (2). One exception is the KxBN arthritis model driven by AI B cells, potentially because IDO and IL6 (aka B cell growth factor) co-promote B cell autoimmunity in this model (17). IDO may also have equivocal roles in allergic diseases, as T_h2-mediated allergic airway inflammation in mice was attenuated by treating mice with TLR9 ligands (CpGs) to induce IDO and by ablating IDO1 genes (18, 19). The reason for this discrepancy is unknown, though distinct cell types expressing IDO may promote or attenuate allergic disease. This point is important when considering potential roles for IDO in immune responses, as several cell types may express IDO and mediate diametric effects on immune responses in particular inflammatory settings.

USING IDO INDUCERS TO PROMOTE TOLERANCE

The tolerogenic effects of cells expressing IDO have prompted efforts to boost IDO activity, particularly in DCs, as novel strategies to alleviate AI disease and prolong transplant survival. A number of strategies can be envisaged to exploit these properties for clinical benefit, as described below.

IDO1 Gene Transduction

In transplantation, proof-of-principle emerged from several studies on rodent models of lung engraftment showing that pre-transplant IDO1 gene transduction promoted robust lung allograft survival in the absence of global immune suppressants (20–22). Moreover, $CD8^+$ T cells infiltrating IDO1-transduced lungs exhibited impaired effector functions due to selective IDO-mediated inhibition of mitochondrial electron transfer (20), a finding reminiscent of functional impairment of tumor-infiltrating T cells in cancer. It is unclear from these reports if lung allograft survival was dependent on sustained IDO activity to suppress alloimmunity or if stable tolerance to donor alloantigens not requiring sustained IDO1 gene expression was induced post-transplantation. Nevertheless, as in pregnancy, these studies reveal that elevated IDO activity protects healthy tissues from immune-mediated destruction. However, as gene transduction in clinical settings is complicated technically and raises serious

risks, a key question is how to elevate and, as may be necessary, to sustain IDO in order to protect healthy tissues in patients.

Soluble CTLA4

A potential alternative strategy is to use drugs to induce IDO. Several IDO inducing drugs are known, including soluble CTLA4, TLR ligands, and DNA. The immunosuppressant drug Abatacept (Orencia™) is a soluble CTLA4 molecule that blocks CD28-mediated T cell co-stimulation. Abatacept is used to alleviate RA in some patients and may help alleviate other AI syndromes, though treatments are expensive. In mice, soluble CTLA4 reagents induced specialized DC subsets to express IDO1 genes and acquire potent tolerogenic functions *via* "reverse signaling" mediated by CD80/86 (B7) surface ligands on DCs that bind CD28 and CTLA4 on T cells (23, 24). It is unclear how B7 molecules stimulate DCs to express IDO1 genes but IFN-I signaling and critical functions in the Fc (Ig) domain of soluble CTLA4 were required for this response. Commercial products like Orencia were optimized for CD28 binding and bio-availability. Consequently, these reagents may have lost the ability to induce IDO if critical Fc (Ig) domain functions were engineered out. Reverse signaling *via* other ligand-receptor pathways (e.g., GITR, ICOS, CD200) to induce DCs to express IDO has also been shown to cause DCs to adopt tolerogenic phenotypes (25–27).

TLR Ligands

TLR4 (LPS) and TLR9 (CpGs) ligands also induce DCs to express IDO. Thus, CpGs given systemically to mice activated resting Tregs to suppress effector T cell responses *via* a mechanism dependent on IDO1 gene expression by discrete splenic DC subsets. In contrast, CpG treatments induced resting Tregs to convert into T_h17 T cells when IDO1 genes were ablated, identifying DCs as pivotal regulators of tolerogenic and immunogenic responses to TLR9 ligands contingent on if DCs were induced to express functional IDO or not, respectively. However, co-induced pro-inflammatory and consequent immunogenic responses to TLR ligands may overcome tolerogenic responses, especially in inflamed tissues.

Stimulator of IFN Genes (STING) Agonists

Systemic treatments with DNA nanoparticles (DNPs) induced IDO in many mouse tissues. In spleen, myeloid DCs ingested DNPs and sensed cargo DNA to activate the STING adaptor, a potent IFN-I inducer (28, 29). Consistent with these findings DNPs alleviated AI disease progression in mouse models of RA and MS (EAE) and therapeutic responses to DNPs depended on STING-IFN-I signaling to induce IDO in DCs (28, 30). Moreover, synthetic cyclic dinucleotides (CDNs) that mimic natural STING agonists generated by the cytosolic DNA sensor cyclic guanyl-adenyl diphosphate (cGAMP) synthase (cGAS) also alleviated AI syndromes in mice (30). Paradoxically, CDNs administered directly into developing tumors stimulated potent anti-tumor immunity that attenuated tumor growth in mice (31). Diametric responses to CDNs in AI and tumor models emphasize the critical importance of dosing since systemic and intra-tumoural delivery of CDNs was required to induce tolerogenic and immunogenic responses, respectively, in these distinct inflammatory settings.

Interferons

In principle, IFN-I could also be used to induce IDO1-dependent tolerogenic responses since IFN-I mediated such responses to B7, TLR, and STING ligands. Indeed, some therapeutic effects of IFN-I (IFNβ) in MS patients may accrue from tolerogenic responses mediated *via* IDO, though it is unclear if this does occur. As with TLR ligands, undesirable pro-inflammatory (immunogenic) responses to IFN-I (or IFN-II) may predominate and overcome tolerogenic responses mediated by IDO, making the clinical use of IFNs to induce tolerance high risk.

IDO PAIN AND DEPRESSION

As stated above, the Kyn pathway has profound effects on neurologic, as well as inflammatory and immunologic, processes. The KA:QA balance is a major factor in neuro-inflammatory syndromes, as high QA levels correlate with neuro-toxicities and dementia, while KA ameliorates these damaging effects. Heightened and sustained IDO activity during chronic inflammatory diseases may contribute to enhanced pain sensitivity, depression, and fatigue, which are common comorbidities associated with many of these syndromes. These effects complicate efforts to exploit the Kyn pathway because beneficial therapeutic effects that suppress hyper-immunity and promote tolerance may come at the cost of increased pain, depression and fatigue, as consequences of therapy. Nevertheless, using IDO inducers to reduce disease-associated inflammation and hyper-immunity in the short term may attenuate innate IDO activity that promotes debilitating neurologic comorbidities. For example, in the EAE model of MS in mice, IDO activity was elevated in CNS tissues during EAE induction but STING agonists treatments to induce IDO in peripheral lymphoid tissues and suppress autoimmunity abolished IDO1 expression in the CNS (30). Thus, overall beneficial effects in attenuating disease progression and reducing comorbidities may accrue from the diametric effects of IDO activity in distinct neuronal and lymphoid tissues. Furthermore, it may be possible to use drugs that modify the Kyn pathway to enhance production of immune suppressive Trp catabolites and reduce production of neurotoxic Trp catabolites. Thus, using HAAO inhibitors to block neurotoxic QA production and KATII inhibitors to promote immune suppressive HAA production may enhance therapeutic responses and reduce neurologic comorbidities, especially if combined with reagents that stimulate the Kyn pathway.

SUMMARY AND FUTURE PROSPECTS

Elevated IDO activity manifests in many settings of high clinical significance, driving substantial interest in manipulating IDO and the Kyn pathway for clinical benefit. Extensive research on mouse models of chronic inflammatory syndromes supports the hypothesis that IDO activity suppresses innate and adaptive immunity by depleting Trp and generating bioactive Trp catabolites. Though IDO-mediated tolerogenic responses to sustained inflammation are particularly prominent, elevated IDO activity may also manifest rapidly to overcome co-induced immunogenic responses. It remains to be seen how many observations from

mouse models of clinical syndromes can be translated into clinical practice. Promising initial results from recently completed or ongoing experimental clinical trials on cancer patients using several different IDO inhibitors will be the first potential clinical application to be tested fully. If outcomes support the use of IDO inhibitors in cancer, future research will focus on identifying optimal drug combinations to halt tumor growth and disrupt immune checkpoints in the widest range of patients, without stimulating autoimmunity or other toxic responses. Since immune checkpoints may also promote pathogen persistence and many infections induce IDO activity, there may also be opportunities to use IDO inhibitors to reduce pathogen burdens and promote pathogen clearance in patients with chronic infections. However, more research is needed to understand the balance between anti-microbial and (host) tolerogenic effects of IDO, as the risk of inducing undesirable toxic effects is high until the role of IDO in specific chronic infections is elucidated fully. On the flip side, enhancing IDO activity as novel strategies to prevent or alleviate hyper-immune syndromes are supported by many proof-of-concept studies in mouse models of transplantation and AI syndromes. Translating these outcomes into clinical application will take some time, in part due to potential for many IDO inducing drugs to elicit tissue-damaging immunogenic responses as well as co-inducing tissue-protective tolerogenic responses. Thus, it may not be straightforward to satisfy ethical and corporate requirements for specific molecular targets driving unequivocal outcomes, unless more precise ways are found to manipulate the immune balance for clinical benefit. Finally, the combined immunologic and neurologic effects of bioactive Trp catabolites emphasizes the key roles of IDO and the Kyn pathway as drivers or regulators of many chronic conditions and common comorbidities, including pain depression and fatigue. Clearly, while more research is needed to understand the complex roles of IDO and the Kyn pathway in chronic diseases, the prospects are good for major novel insights and advances leading to clinical practice.

AUTHOR CONTRIBUTIONS

AM wrote the review. HL and LH read and critiqued the review.

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Novel Concept of CD4-Mediated Activation of Regulatory T Cells for the Treatment of Graft-Versus-Host Disease

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Allogeneic hematopoietic stem cell transplantation is the only curative treatment option for several hematological malignancies and immune deficiency syndromes. Nevertheless, the development of a graft-versus-host disease (GvHD) after transplantation is a high risk and a severe complication with high morbidity and mortality causing therapeutic challenges. Current pharmacological therapies of GvHD lead to generalized immunosuppression followed by severe adverse side effects including infections and relapse of leukemia. Several novel cell-based immunomodulatory strategies for treatment or prevention of GvHD have been developed. Herein, thymus-derived regulatory T cells (tTreg), essential for the maintenance of peripheral immunologic tolerance, are in the focus of investigation. However, due to the limited number of tTreg in the peripheral blood, a complex, time- and cost-intensive *in vitro* expansion protocol is necessary for the production of an efficient cellular therapeutic. We demonstrated that activation of tTreg using the CD4-binding human immunodeficiency virus-1 protein gp120 leads to a substantially increased suppressor activity of tTreg without the need for additional expansion. Gp120-activated tTreg prevent GvHD development in a preclinical humanized mouse model. In addition, gp120 is not only effective in prevention but also in therapy of GvHD by suppressing all clinical symptoms and improving survival of treated mice. These data indicate that tTreg activation by gp120 is a feasible and potent strategy for significant functional improvement of tTreg as cellular therapeutic for GvHD treatment without the need of complicated, time-intensive, and expensive *in vitro* expansion of isolated tTreg.

Keywords: regulatory T cell, graft-versus-host disease, cellular therapy, tolerance, CD4 stimulation

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a potentially curative therapy for many patients with hematological malignancies or immune deficiencies. However, the development of graft-versus-host disease (GvHD) dramatically limits the efficacy of aHSCT, and is the leading cause of long-term morbidity and mortality (1). The report of the Worldwide Network for Blood and Marrow Transplantation in 2015 revealed an exponential increase in the use of HSCT, from the first transplant in 1957 to more than one million worldwide by now, with the highest number of HSCT reported in Europe [501,315 (52%), of which 45% were aHSCT] (2).

Current therapeutic treatments for GvHD after aHSCT are primarily based on broadly immune suppressive agents such as corticosteroids and calcineurin inhibitors (3). However, GvHD still occurs in 40–60% of recipients with 50% of the patients developing steroid resistance and thus remains a major reason of non-relapse mortality. There is a high need for the development of more effective immunomodulatory therapies to prevent and treat GvHD. In this review, we are focusing on advantages and recent challenges of using thymus-derived regulatory T cells (tTreg) to suppress GvHD development.

GvHD PATHOGENESIS

In 1966, Billingham initially defined GvHD as a syndrome in which donor immune cells (the allograft) recognize the recipient's cells and tissues (the host) as foreign, leading to a complex interaction between donor and recipient adaptive immunity followed by massive host tissue destruction (4). The clinical forms of GvHD include acute and chronic GvHD (cGvHD). Acute GvHD (aGvHD) is characterized by a strong systemic inflammation and tissue destruction of multiple organs, particularly the liver, lung, gut, and skin, whereas cGvHD often imitates autoimmune diseases with massive fibrosis of target organs (3, 5). Both GvHD syndromes involve distinct pathological processes. The risk of GvHD development starts during the conditioning phase of the recipient, even before the allograft is infused. Chemotherapy or total-body irradiation of the patient can cause severe tissue damages which activate host antigen-presenting cells (APC). Following antigen presentation, host APC activate $CD4^+$ donor T cells in the graft which differentiate into IFN-γ and IL-17 producing T effector cells (6, 7). A strong cytokine response is initiated promoting the recruitment and activation of further effector cells, including NK cells, $CD8^+$ T effector cells, and macrophages, leading to organ damages, clinically indicated by a strong aGvHD in the skin, gut, lung, and liver. The occurrence of aGVHD after aHSCT as well as the conditioning regimen itself can furthermore cause tissue destruction of thymic epithelium, resulting in a reduced negative selection of alloreactive $CD4^+$ T cells. The release of fibrogenic cytokines such as IL-2, IL-10, and TGF-β activates macrophages which then stimulate the proliferation of tissue fibroblast, leading to massive fibrosis of target organs in cGvHD. In addition, chronic inflammation and the continuous production of inflammatory cytokines such as IL-6 and TNF-α inhibits the generation of induced regulatory T cells (iTreg) as the naïve $CD4^+$ T cells preferentially differentiate into T effector cells, and inflammatory cytokines block the suppressive function of tTreg (8–11). Therefore, tTreg should be used as a cellular drug as early as possible in order to achieve the greatest possible therapeutic effect by restoring the immunological balance.

Treg CAN PREVENT GvHD DEVELOPMENT

Up to now, systemic corticosteroid therapy remains the first line treatment for GvHD. The need for new or improved therapies based on manipulating immune responses has extremely increased in the last decades, especially in cases of steroid-refractory GvHD patients. Targeting and modulating T cell responses, the etiological factors in GvHD induction, seems to be a promising strategy.

Thymus-derived Treg, comprising 2–5% of all peripheral blood cells in humans (12), are key players in the modulation of immune responses and play an important role in self-tolerance (13–15). Additionally, tTreg are a mandatory cell type for the maintenance of immune tolerance and thus prevention of overshooting immune responses such as of GvHD after aHSCT (16). The therapeutic efficacy of adoptively transferred tTreg in promoting tolerance has been shown in mouse models of aHSCT (17, 18) and the function of tTreg in reducing the risk of GvHD development has been furthermore demonstrated in humans (8, 19). A high number of tTreg in blood stem cell transplants is associated with reduced risk of GvHD development and patients with active cGvHD show reduced tTreg frequencies compared with healthy volunteers. Therefore, adoptive tTreg transfer to enhance tTreg frequencies in transplanted patients that suppress GvHD development is an attractive therapeutic option and protocols for effective generation of such cellular therapies have been developed.

A significant challenge in the development of efficient tTreg cellular therapies is the low rate of these cells in the peripheral blood. Since in murine models high numbers of tTreg are needed for a marked reduction of GvHD, it has been postulated that ratios of nearly 1:1 tTreg to T effector cells are necessary for successful GvHD prevention (17, 20). However, such high cell numbers of tTreg cannot be isolated from normal blood products including leukapheresis. As one approach, an expansion of isolated tTreg is necessary in order to be able to produce sufficient cell numbers for GvHD prevention in patients.

IN VITRO EXPANDED tTreg AS CELLULAR THERAPEUTIC FOR GvHD SUPPRESSION

Human tTreg do not express an exclusive surface marker which allows their isolation without contamination with conventional T cells. However, to achieve a high-quality product for cell expansion, high purity of tTreg is needed as a starting population. The most extensively used method to isolate human tTreg is based on the use of anti-CD25 immunomagnetic beads (21–23). By using this technique, tTreg purities of 50–80% can be achieved. Since CD25 is expressed on tTreg as well as on activated T effector cells, contamination with these cells cannot be completely prevented using anti-CD25 beads and the risk that these T cells will also be activated and expanded after anti-CD3/CD28 antibody stimulation cannot be excluded. The additional use of CD127 as a marker can improve the purity of tTreg ($CD4^+CD25^+CD127^{low}$) and their efficacy *in vivo* (24, 25). Nevertheless, expansion results in significant changes of many marker molecules used for tTreg characterization. As an example, a part of the isolated cells loose Foxp3 expression during expansion (26). Furthermore, Voo et al. have shown that human $Foxp3^+$ T cell populations also contain Th17 precursors that expand after polyclonal

stimulation (27). Therefore, it is difficult to define the real ratio of functionally stable and active tTreg after *ex vivo* expansion. To increase tTreg purity, several groups improved their expansion protocols by adding rapamycin in order to reduce the unwanted proliferation of T effector cells and to increase the stability of expanded tTreg (22, 28).

Despite the difficulties with the functional stability of expanded tTreg and the potential contamination with T effector cells, the adoptive transfer of tTreg for treatment of GvHD is very attractive in order to address the high unmet medical need. Within the last decade, three trials of adoptive tTreg therapy in GvHD patients have been carried out. In 2009, Trzonkowski et al. reported the first-in-man trial which included two GvHD patients (29). The first patient suffered from cGvHD two years after aHSCT and received high doses of corticosteroids [triple-drug therapy; prednisone, tacrolimus, and mycophenolate mofetil (MMF)]. After infusing a single dose of 1×10^5/kg *ex vivo* expanded tTreg, MMF therapy was completely withdrawn, and lung function, blood hemoglobin, and body weight improved. The second patient, however, continued to suffer from aGvHD despite three infusions of 3×10^6/kg expanded tTreg. Finally, the condition further deteriorated and the patient died from multiorgan dysfunction. Di Ianni et al. published clinical data demonstrating the effect of tTreg infusion in the prevention of GvHD in 26 patients with high-risk hematological malignancies (30). In contrast to Trzonkowski et al., Di Ianni used freshly isolated donor tTreg without *ex vivo* expansion. Cellular tTreg infusions started four days prior transplantation with haploidentical $CD34^+$ stem cells added with donor T effector cells without any post-transplant immunosuppressive drugs. tTreg to T effector cell ratios of 2:1 were infused with no observable toxicities. Adoptive tTreg transfer was safe and did not diminish the graft versus leukemia effect of co-transferred effector T cells. Only 2 of 26 patients developed an aGvHD and no cGvHD was observed in the first year after treatment. Interestingly, the authors found an improvement in the immunological reconstitution of patients after tTreg transfer, and the relapse rate in this study was only 5% compared with 30–35% seen normally in patients with high-risk leukemia. These impressive results demonstrated that transfer of freshly isolated tTreg is safe and can significantly suppress GvHD development without hampering/affecting the graft versus tumor response.

Compared with peripheral blood, umbilical cord blood contains significant lower amounts of $CD4^+CD25^+$ T effector cells and a greater percentage of $CD4^+CD25^+CD45RA^+$ tTreg, a subset with a higher stability upon expansion. This makes cord blood an attractive starting product for tTreg expansion (31). Brunstein et al. completed the first clinical trial using two infusions with cord blood expanded tTreg for treatment of 23 patients early after transplantation (32). The partial HLA-matched tTreg were infused on days 1 and 15. All patients underwent additional GvHD prophylaxis with MMF combined with cyclosporine or sirolimus. Patients were treated with doses from 3 to 100×10^6 expanded tTreg/kg with a median purity of $Foxp3^+CD127^-$ cells of 87%. To evaluate the impact of tTreg infusions, the authors compared the risk of graft failure, general mortality, and GvHD development to 108 historical patients with identical transplant regimes but without tTreg therapy. Again, no infusional toxicities were observed but a significant reduction of aGvHD from 61% in historical controls to 43% in patients with tTreg infusions. No enhanced risks in relapse, opportunistic infections or early mortality were observed. These data confirm that adoptive transfer of Treg cellular products as a prophylaxis against GvHD is safe and effective. Nevertheless, one has to keep in mind that the source of cord blood for the large-scale preparation of Treg remains limited.

Theil et al. firstly described the outcome of a tTreg infusion therapy for a limited number of patients with existing cGvHD (33). They infused an average of 2.4×10^6 Treg/kg with an average purity of 84.1% of $Foxp3^+CD127^-$ cells. Treg were infused after a median time of 35 months after transplantation with continued prednisolone treatment. No infusional toxicity or other adverse effects were observed. Two of five patients showed a clinical response with improvement of GvHD symptoms, the other three a stable disease for up to 21 months. These data suggest that cellular Treg therapy may be clinically effective even after years of immunosuppressive therapy and cGvHD.

Nevertheless, functional stability of expanded tTreg and their persistence and distribution following infusions in patients are not sufficiently characterized. The reproducible generation of sufficient quantities of tTreg with high quality and purity currently requires specialized expertise which limits its general applicability to a few specialized transplant centers. Thus, if the immunosuppressive activity of the tTreg product is significantly increased and the complex and uncertain *in vitro* expansion could be avoided, the applicability of the tTreg therapy would be significantly improved.

TOLERANCE INDUCTION BY ANTI-CD4 STIMULATION

Like conventional T cells, tTreg require T cell receptor (TCR) stimulation and costimulation for functional activation. Without this stimulation, only the few percent alloreactive tTreg are functionally activated and effective suppressor cells. Reagents that allow a polyclonal activation of tTreg without stimulation of conventional T cells would shift the balance in favor of the tTreg and significantly reduce the necessary number of tTreg needed for GvHD suppression. CD4-mediated activation of tTreg is such a possibility (34).

It is a well-known phenomenon first described by Waldmann's group that short-time therapy with non-depleting anti-CD4 antibodies can induce long-lasting tolerance (35, 36). They showed that a co-receptor blockade by anti-CD4 antibodies induce the conversion of naïve $CD4^+$ T cells into induced $Foxp3^+$ Treg. The induced tolerance by such treatment is "dominant, transferable to naïve recipients, and transferred $CD4^+$ T cells have the ability to "infect" naïve T cells to acquire a tolerant state" (36). The effect of anti-CD4 antibody treatment on tTreg was not clear at that time. Therefore, we investigated anti-CD4 stimulation on tTreg and found that human $CD4^+CD25^+Foxp3^+$ tTreg can be functionally activated by anti-CD4 stimulation in a dose-dependent manner (34). CD4-activated tTreg suppress

the proliferation and cytokine production of $CD4^+$ and $CD8^+$ T effector cells. In contrast, anti-CD4 stimulation did not induce suppressive activity in conventional $CD4^+$ T cells. The CD4 signal induces a specific phosphorylation of TCR associated signaling molecules (37), sufficient to activate the suppressive function of tTreg but inefficient in activating T effector cells. In contrast to TCR stimulation, the CD4 signal induces no proliferation of tTreg. These findings suggested also a direct activation of tTreg in the course of an anti-CD4 treatment *in vivo*. Additional studies by Kendal et al. firstly demonstrated that tTreg are essential for infectious tolerance induced by non-depleting anti-T cell antibodies (38).

Gp120 FOR THERAPEUTIC ACTIVATION OF Treg PREVENTS GvHD

One molecule that binds with particularly high affinity to human CD4 is the human immunodeficiency virus-1 envelope protein gp120 (**Figure 1**). We demonstrated that gp120 upon binding to and signaling through CD4 efficiently activates human tTreg (39). Gp120-stimulated tTreg up-regulate cyclic adenosine monophosphate (cAMP), a key event of tTreg activation, and tTreg-mediated suppression (40). Blocking of adenylate cyclases repressed cAMP up-regulation and abrogated suppressor activity in gp120-stimulated tTreg, demonstrating that cAMP up-regulation is crucial for the CD4-mediated suppressive capacity of human tTreg (39).

To investigate the potential tTreg-stimulating properties of gp120 *in vivo*, we used a well-established xenogeneic GvHD model based on the transfer of human peripheral immune cells into immunodeficient mice. Intraperitoneal injection of human PBMC into newborn NOD/*Scid* or $Rag2\gamma c^{-/-}$ mice resulted in development of a lethal GvHD leading to death of mice after 20–90 days, depending on the mouse strain, and the number of transferred PBMC (39, 41). GvHD in mice was characterized by decelerated growth, reduced body weight, and chronic inflammation of skin, liver, and colon thus resembling to symptoms in human GvHD patients. GvHD is induced by the activation and expansion of $CD4^+$ T cells which differentiate into IFN-γ and IL-17-forming T effector cells, respectively. The limited number of intrinsic tTreg within the PBMC cannot prevent GvHD development. Transfer of increased ratios of tTreg (PBMC:tTreg 4:1–10:1) blocked the activation, differentiation, and expansion of $CD4^+$ T cells and subsequently prevented all signs of GvHD, demonstrating that this human/mouse chimeric animal model is applicable for the analysis of human tTreg function, and tTreg cellular therapeutics *in vivo*. In accordance with published observations, transferred tTreg ratios lower than 10:1 were not effective in GvHD prevention. These results confirm the necessity of high tTreg numbers for successful suppression of GvHD.

Our *in vitro* experiments showed that CD4-mediated stimulation of tTreg by gp120 significantly improves their suppressor function. Consequently, significantly lower tTreg ratios were sufficient to inhibit a mixed leukocyte reaction *in vitro*. Therefore, we postulated that lower tTreg ratios should also be sufficient to prevent GvHD development *in vivo* after CD4-mediated activation of Treg. We investigated this possibility by directly using gp120 as tTreg activator in the humanized GvHD mouse model. Indeed, a single injection of 5 µg gp120 with 5×10^6 PBMC, without additional transfer of tTreg, was able to completely suppress the formation of GvHD (39). Activation of the limited number of intrinsic tTreg within the PBMC was efficient to prevent the activation of pathologic $CD4^+$ T cells. Furthermore, gp120 therapy blocked their differentiation into T effector cells, suppressed all signs of GvHD, and induced a long-lasting state of tolerance. However, gp120 therapy is strictly dependent on the presence of tTreg and showed no effect when the small number of intrinsic tTreg were depleted before transfer of PBMC. These results confirmed the *in vitro* data that binding of gp120 to conventional $CD4^+$ T cells did not block their activation directly.

The data suggest that gp120-activated tTreg are at least 20 times more effective than resting tTreg. For a cellular tTreg therapy, this means that significantly less tTreg must be transferred in order to achieve the same suppressive effect *in vivo*. Since with today's protocols more than 10^8 tTreg can be isolated from a single apheresis, the gp120 stimulation could replace a time- and cost-intensive expansion of the cellular tTreg product.

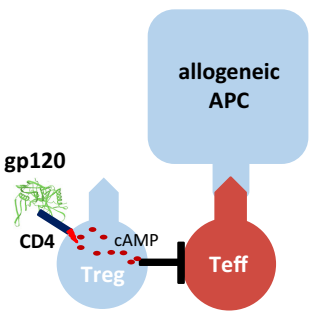

FIGURE 1 | Gp120-mediated activation of human regulatory T cells (Treg). Recombinant gp120 binds to CD4 molecules on the surface of Treg, induces cytosolic cyclic adenosine monophosphate (cAMP) production and thus activates the suppressive function of Treg. Gp120-mediated activation is independent of T cell receptor stimulation. Therefore, gp120 is a polyclonal Treg activator. Inhibition of the tyrosine kinase Lck or blockade of adenylate cyclase activity prevents gp120-mediated cAMP production in Treg and their functional activation.

SUMMARY

In the last years, cellular tTreg therapy has arrived in clinical testing and could be the first curative treatment form of GvHD in the future. In contrast to current immunosuppressive drugs, often combined with severe adverse side effects due to general immunosuppression, short-time tTreg therapy has the potential to induce long-lasting tolerance without further treatment. The study results published so far are impressive. Significant progress has also been made in the manufacture of cellular tTreg products in recent years. However, the *in vitro* expansion, which is difficult to standardize, is still a significant obstacle to a broad and reproducible clinical application of this cellular product. The

CD4-mediated functional activation of tTreg could provide an important contribution to avoid *in vitro* expansion since this activation significantly enhances the suppressive properties of tTreg and therefore reduces the necessary number of cells for efficient GvHD inhibition *in vivo*.

In the meantime, the first closed systems for the production of cell products are available. However, significant improvements still have to be made. The new technologies have to ensure a standardized and reproducible production of the cell products with the same quality and quantity outside cleanroom laboratories in specialized centers, comparable with the production of leukaphereses in transfusion centers. This attractive and promising therapy can only be offered to all transplant patients, when the cell therapeutic itself can be produced in the same quality, quantity, and functionality at different locations.

AUTHOR CONTRIBUTIONS

HJ and JS contributed to the conception, design, writing, and revision of the manuscript. CB and AT contributed to the writing and revision of the manuscript.

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The Enigma of Heat Shock Proteins in Immune Tolerance

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The fundamental problem of autoimmune diseases is the failure of the immune system to downregulate its own potentially dangerous cells, which leads to destruction of tissue expressing the relevant autoantigens. Current immunosuppressive therapies offer relief but fail to restore the basic condition of self-tolerance. They do not induce long-term physiological regulation resulting in medication-free disease remissions. Heat shock proteins (HSPs) have shown to possess the capacity of inducing lasting protective immune responses in models of experimental autoimmune diseases. Especially mycobacterial HSP60 and HSP70 were shown to induce disease inhibitory IL-10-producing regulatory T cells in many different models. This in itself may seem enigmatic, since based on earlier studies, HSPs were also coined sometimes as pro-inflammatory damage-associated molecular patterns. First clinical trials with HSPs in rheumatoid arthritis and type I diabetes have also indicated their potential to restore tolerance in autoimmune diseases. Data obtained from the models have suggested three aspects of HSP as being critical for this tolerance promoting potential: 1. evolutionary conservation, 2. most frequent cytosolic/nuclear MHC class II natural ligand source, and 3. upregulation under (inflammatory) stress. The combination of these three aspects, which are each relatively unique for HSP, may provide an explanation for the enigmatic immune tolerance promoting potential of HSP.

Keywords: tolerance mechanisms, regulatory T cells, heat shock proteins, arthritis, rheumatoid, cell stress

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INTRODUCTION

Initial observations concerning the significance of heat shock proteins (HSP) for immune tolerance were obtained in the model of heat-killed mycobacteria induced adjuvant arthritis (AA) in Lewis rats. In this model, T cell clones, having only one singular T cell receptor (TCR), had been established by limiting dilutions following repeated re-stimulations with mycobacterial antigens. Upon *in vivo* transfer, these clones had the capacity to produce induction and suppression of the disease (1). These T cell lines had been raised from immunizations and repeated *in vitro* re-stimulations of collected splenocytes with crude heat-killed *Mycobacterium tuberculosis*. These T cell lines were found to respond with specificity to some mycobacterial protein fractions. And interestingly, these same mycobacterial protein fractions were also capable of inducing T cell responses in peripheral blood mononuclear cells obtained from RA patients (2). Since then it became of interest to define the exact nature of the antigens involved. The antigen recognized by T cell clone A2c, the clone with the capacity to protect against induction of AA, was obtained by molecular cloning of *M. tuberculosis*-derived genes (3). It was a 60-kDa protein which was found upon immunization to

protect against AA. Based on sequence homologies with known HSPs, such as the 60-kDa GroEL of *Escherichia coli*, this protein was recognized as the mycobacterial HSP60. Subsequently, this molecule was found to protect in various experimentally induced animal models, including collagen and avridine arthritis, NOD diabetes, experimental allergic encephalomyelitis, some allergic disorders, and atherosclerosis [reviewed in Ref. (4)].

Further exploration of this recombinant mycobacterial HSP60 in the AA model revealed the presence of at least nine different T cell epitopes in this HSP60 recognized by T cells in Lewis rats (5). Of these T cell epitopes, the sequence at positions 256–265 was most conserved. When T cell lines were generated against all nine distinct epitopes, only the T cell line with specificity for this conserved epitope protected upon *in vivo* transfer against induction of AA. And immunizations with synthetic peptides spanning the nine different epitopes showed that only the 256–265 peptide protected against disease. These findings had suggested that the induction of T cell regulation in the AA model depended on the cross-recognition of host-tissue expressed HSP60 by the mycobacterial HSP60-specific T cells. In more general terms, T cell responses to conserved sequences of microbial HSPs seemed to become endowed with the capacity to restore tolerance and to act as regulatory T cells (Tregs). And above all, whichever the exact interpretation of these findings could be, experiments performed by various groups had indicated the capacity of microbial HSP, and besides HSP60 also other HSPs, to induce a disease suppressive T cell response.

THE CONTROVERSY AROUND HSP AND THEIR POSSIBLE DAMAGE-ASSOCIATED MOLECULAR PATTERN (DAMP) ACTIVITIES

Intracellular HSPs are upregulated in cells under stress. If, and if so how HSPs are exported out of the cell has remained enigmatic. HSPs have no signal sequence for transport over cell membranes. Nonetheless, the extracellular presence of HSPs has been documented in various experimental systems. The controversy arises when the extracellular soluble HSPs are said to act as pro-inflammatory molecules, the so-called DAMPs. Such DAMP activities are somewhat difficult to reconcile with the fact that intracellular HSPs and their MHC presented peptides were seen to have anti-inflammatory disease suppressive activities in experimental models of chronic inflammation and in first clinical trials (4, 6–9). Part of the demonstrated pro-inflammatory effects may have arisen from the fact that earlier work by many different groups was performed with recombinant mycobacterial HSPs produced in *E. coli*. Although attempts were made and reported to purify the recombinant protein, several published claims regarding the pro-inflammatory nature of HSPs may have been based on the activity of LPS and possibly other contaminants present in the proteins. In various instances, when pure HSP proteins were tested, no inflammatory activity was observed (10). This, in combination with the findings of the anti-inflammatory effects of HSP in experimental models, seems to indicate that HSPs are missing several of the qualities of the so-called true DAMPs. As we have argued before, HSP can be rather DAMPERs of the immune response instead of DAMPs (11, 12).

By their aggressive nature, true DAMPs necessarily are residing intracellularly. HSPs, however, are often reported to have activities as extracellular mediators. In addition to this, when DCs are cultured in the presence of purified HSP, the DCs are not activated. In the case of mycobacterial HSP70, it was shown that it inhibited DCs in their maturation from bone marrow-derived precursors, it induced production of IL-10 in DCs and the treated DC reduced T cell proliferation (13). In another experimental setup, mycobacterial HSP70 was also shown to modulate DCs and to produce DCs that upon *in vivo* transfer inhibited experimental arthritis in mice (14). All latter observations are difficult to reconcile with pro-inflammatory DAMP-like activities being a natural characteristic of HSPs.

HSP-DIRECTED IMMUNE RESPONSES PRESENT IN PATIENTS' DISEASE REMISSION

An extensive analysis of T cell responses to HSP60 was made in patients with juvenile idiopathic arthritis (JIA) (15–17). JIA is a heterogeneous disease with subtypes. A major subtype is self-limiting, known as persistent oligoarticular JIA, in which a maximum of four joints is affected. This self-limiting nature of JIA is regarded to result from adequate immune regulation, through which the immune response has managed to restore tolerance for self. Although self-limiting, OA-JIA often causes permanent joint damage with lifelong disability. On the other hand, polyarticular JIA, with more than four joints affected in the first half year of the disease must result from a failure to restore tolerance. Oligoarticular forms of arthritis have shown to feature T cell responses to HSP60, whereas polyarticular JIA has not or at least much less (16). And in addition, a longitudinal follow-up of these OA-JIA patients showed that phases of disease remission were proceeded by phases of enhanced HSP60-specific T cell responses (17). These observations suggested that in patients with OA-JIA, HSP60-specific T cells contributed to regulation of disease. The production of IL-10 in peripheral blood mononuclear cells of the patients was fully in line with this possibility (18, 19).

Similar observations were made in patients with juvenile dermatomyositis (DM). Muscle biopsy samples from juvenile DM patients showed upregulation of Hsp60 and peripheral blood mononuclear cells showed proliferative responses in the presence of HSP60. Production of pro-inflammatory cytokines by muscle-derived T cells in response to Hsp60 was associated with a poor clinical prognosis, whereas human Hsp60-specific induction of IL-10 was followed by clinical remission (20).

In multiple sclerosis (MS), some studies have profiled antibody repertoires. In one of the studies, it appeared that relapsing-remitting MS was characterized by HSP70 autoantibodies. And this was not observed in both primary and secondary progressive MS. In other words, immune responses to HSP70 were associated with disease that exhibited the intrinsic capacity to control, to some extent, inflammation. In this study, antigen microarrays defined unique serum immune signatures linked to different

stages and pathological processes in MS. In this case, immune responses to HSPs seemed associated with remitting forms of the disease (21).

INDUCTION OF Tregs BY HSP70 IN A MOUSE ARTHRITIS MODEL AND IN HUMANS

In the model of proteoglycan-induced arthritis (PGIA) in BALB/c mice, we have performed epitope mapping of mycobacterial HSP70. Similar to what we had done earlier for HSP60 (5), we now identified a very conserved epitope which had close sequence homologies with multiple members of the mammalian HSP70 family of molecules. The peptide based on this mycobacterial HSP70 was named HSP70-B29. Recently, we showed that the HSP70-B29 peptide induced Tregs, a CD4+ T cell population with the intrinsic capacity to control inflammation. These Tregs are CD4+CD25+Foxp3+ and upon *in vivo* transfer the cells suppressed PGIA in mice (22). Furthermore, *in vivo* depletion of transferred Tregs, with a depleting antibody specific for the congenic CD90.1 marker, abrogated disease suppression. Transferred cells exhibited a stable phenotype and were found in joints and draining lymph nodes up to 2 months after transfer. In humans, B29 was a promiscuous binder for all major HLA-II molecules, HLADRB1* 04:01 in particular. Also in humans, B29-specific Tregs were detected (23). And importantly, these B29-specific T cells were shown to cross-recognize the mammalian HSP70 homologs. Initial experiments with human B29-pulsed tolerizing dendritic cells showed the ability to activate B29-specific T cells and induce a regulatory phenotype in these human T cells, as based on the expression of CD49b, LAG-3, and GITR (Nicolic and Roep, unpublished).

MECHANISMS THROUGH WHICH HSPs MAY INDUCE TOLERANCE MEDIATING Tregs

There are three possible mechanistic explanations that may act in synergy, for a tolerance promoting anti-inflammatory effect of HSPs. It is along these possibilities that further mechanistic research could be undertaken.

HSPs Are Evolutionary Conserved

As mentioned earlier, evolutionary conservation of microbial HSPs has led to antigenic similarities with their mammalian self-homologs present in the host. Despite this, HSPs are quite immunogenic. The microbial molecule HSP60, for example, was known as the "common antigen of gram negatives" already before its molecular definition (24). In addition to this, T cells with specificity for the conserved parts of the molecules can be easily detected (5, 25). Therefore, in principle, the exposure to microbiota-associated HSP in the tolerizing gut mucosa (26–28) or exposure to commensal microbe-associated HSP in the skin (29) may trigger HSP-specific Tregs with focus on the relatively conserved—repeatedly encountered—parts of the molecules. When microbiota-associated bacteria are being sampled, such as in the case of the gut by macrophages or dendritic cells with their protrusions through the epithelial layer, and become transported to the mesenchymal lymph nodes, the intracellular presence will cause stress in these bacteria, leading to a further upregulation of bacterial HSP. By such means, the immune system will be familiar with the safe presence of microbiota-derived HSP and may well utilize this trustworthy set of antigens for maintaining mucosal tolerance at the level of T cells with respect to this safe set of microbial antigens. Various studies have documented the nature of the TCRs of gut Tregs. Based on the relatively unique nature of colonic Treg TCRs, it was postulated that Tregs in the colon may have differentiated extra-thymically due to contact with bacterial or food antigens (26). On the other hand, others have emphasized the presence of shared TCRs between thymic Tregs and colonic Tregs, suggesting the thymic selection on the basis of self-recognition and the expansion in the periphery through "recognition of cross-reactive microbial antigens in the intestines" (27, 28). Whichever scenario will be the dominant one, there is definite evidence for cognate interactions between gut Tregs and microbiota-associated antigens (30). By their nature, HSP may well constitute a set of microbiota-associated antigens that may dominate in this respect and that drive tolerance promoting Treg.

HSPs Are the Most Frequent Cytosolic/Nuclear MHC Class II Natural Ligand Source

MHC molecules that reach the cell surface are conformationally dependent on the presence of peptides in their peptide-binding grooves. This is the case for MHC class I molecules and also for the MHC class II molecules that interact with our CD4+ Tregs. MHC elution studies have shown that HSPs are among the most frequent cytosolic/nuclear MHC class II natural ligand sources (22). In the supplementary data set of the paper of Paludan et al. (31), the more relevant HSP70 family members are listed, next to GAPDH, with the MHC II molecules they were eluted from. Therefore, even in absence of inflammation, HSPs form a major part of the normal MHCII ligandome. When presented by tolerizing DC in the tissues, HSP-specific Tregs may become induced, contributing to the "tolerance promoting" default setting of the healthy immune system.

Also for the thymus, MHC elution studies have shown the presence of stress-associated molecule fragments in the MHC–peptide matrix. In this case, stress-associated molecule fragments were retrieved more from MHCII molecules than from MHCI molecules, and this especially in the DC depleted, and therefore positively selecting, thymocytes (32). Interestingly enough, also a fragment of HSP70 was eluted in this manner which contained our HSP70-B29 peptide. Another study has presented the immuno-phenotyping of HSP-expressing cells in fetal and adult thymus (33). There was shown to exist the complete concordance of Lu5 (pancytokeratin), a marker for thymic epithelial cells, and HSP70. In fact, a strong expression of HSP70 (and HSP27) was detected in medullary and cortical thymic epithelial cells. It was furthermore shown that thymic DCs and macrophages (CD68+ cells) were negative for HSP70 expression. Altogether, whereas the negatively selecting bone marrow-derived cells were negative

for HSP70, the positive selecting thymocytes were featuring a strong expression.

From this, it can be concluded that HSPs are well positioned to contribute to, thymus dependent, central tolerance, by positively selecting HSP-specific Tregs. In the periphery, the HSP ligands as bound in the MHCII peptide-binding clefts may furthermore assist to maintain such Tregs and to act as targets for the regulatory activity of the centrally selected Tregs. And in addition to this, also peripherally induced Tregs (Tr1?) may target such HSP ligands in the MHCII-binding clefts.

HSPs Are Upregulated in Tissues under (Inflammatory) Stress

Tissue stress resulting from inflammation ensures upregulated levels of intracellular HSPs (34). In this manner, upregulated HSP presented by MHC may act as a functional biomarker of inflammation. This is especially the case for HSP70, as HSP70 is as a key regulator, molecularly involved with the process of chaperone-mediated autophagy (CMA). Dengjel et al. have analyzed the MHCII ligandome obtained from nutrient-deprived HLA-DR4+ human B cells (35). The stress caused by nutrient deprivation had led to autophagy, which influenced the loading of the MHCII compartments of the cell. Possibly through the mechanism of CMA, a chaperone-dependent targeting of cytosolic proteins to lysosomes, a preferential loading of MHCII with HSP70 fragments takes place. In the cleft of the HLA-DR4 molecules, also our HSP70-B29 was present in this case. Given the known association of HLA-DR4 with RA, this finding is of interest. Apparently, also RA patients with disease predisposing HLA molecules have in principle the genetic capacity to present a proposed disease protective peptide to their T cells.

The enhanced expression of mammalian HSP60 in synovial tissues of JIA patients has been reported by Boog et al. (36). A more complete study covering the upregulated expression of HSP in the inflamed synovium was reported by Schett et al. (37). An immunochemical analysis with HSP70-specific antibodies revealed strong staining in synovial fibroblasts and macrophages in the synovial tissues of patients with RA and not in those of patients with osteoarthritis. Induction of hsp70 expression and nuclear translocation of HSF1 in synovial cells was shown by immunofluorescence microscopy after incubation of synovial cells at raised temperature or incubation with TNF-α.

Under (sterile) inflammatory conditions, tolerizing DCs will have their MHC II more heavily loaded with HSP, which may enable them to herewith induce, expand, and activate HSP-specific Tregs at the sites of inflammation. Tregs may have evolved by the need to control inflammation and may depend for their function on a repertoire of TCRs that enables the recognition of HSP, in line with the abundant presence of HSP in the T cell-selecting thymus (32). Together with IL-10 as a regulatory cytokine also produced by cells under stress (38), it seems that cell-stress and control of inflammation are naturally connected.

Also by other means, HSPs may contribute to the inhibition of the inflammatory process. In various studies and interestingly also by various mechanisms, intracellular HSP70 was found to inhibit the inflammatory stimuli-dependent activation of the pro-inflammatory NF-κB signaling pathway (39, 40). Moreover, extracellular HSP70 was shown to have anti-inflammatory effects through inhibition of MAPKs and NF-κB signaling pathways leading to a downregulated production of IL-6, IL-8, and MCP-1 upon TNF-α stimulation of synoviocytes obtained from RA patients (41).

CONCLUSION

Therefore, cell stress-associated intracellular HSPs have a tolerizing effect through T cells in combination with control of inflammatory mediator production. For these reasons, cell stress and the consequential expression of HSPs can be seen as a central element in the control of inflammation. As said, the "enigmatic" HSPs are no DAMPs, but rather tolerance promoting DAMPERs (11).

AUTHOR CONTRIBUTIONS

WE wrote the paper. The other authors edited the paper.

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The Future of Immunotherapy: A 20-Year Perspective

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Immunotherapy is the field of immunology that aims to identify treatments for diseases through induction, enhancement or suppression of an immune response. Immunotherapies designed to instigate or enhance an immune response are considered "activating immunotherapies" while those designed to repress an immune response are "suppressive immunotherapies." This perspective will focus on two areas of immunotherapy, activating immunotherapies for cancer and suppressive immunotherapies for autoimmunity both of which have seen a resurgence in interest in recent years and are likely to transform the treatment of many human diseases in the next 20 years. Effective immunotherapies for cancer, where the aim is to activate tumor-specific immune responses, will be totally different from those designed to suppress the immune response to self-antigens in autoimmune disease. Furthermore, the reader will appreciate that the degree to which side effects of immunotherapies are acceptable will differ drastically between life-threatening cancers and chronic, debilitating but not necessarily life-threatening autoimmune conditions.

Keywords: cancer, autoimmune disease, immunotherapy, cancer vaccines, multiple sclerosis

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THE GLOBAL HEALTH BURDEN

Improvements in sanitation and effective vaccination are gradually reducing the impact of infectious diseases across the world. The World Health Organisation (WHO) predicts a continuing decline in global mortality resulting from respiratory, perinatal, and other infections, excluding HIV/AIDS. While global mortality due to AIDS has declined rapidly, this rate is not predicted to change dramatically over the coming 20 years (1). By contrast, global mortality due to cardiovascular disease, cancer, non-infectious respiratory disease, and other inflammatory diseases will increase with cardiovascular disease predicted to be the greatest killer followed by malignant neoplasms and chronic obstructive pulmonary disorder. There is a disturbing increase in the incidence and prevalence of immune-mediated inflammatory diseases (IMIDs), including autoimmune and auto-inflammatory diseases. Among these, neurological conditions, such as multiple sclerosis (MS) and myasthenia gravis, are increasing at a rate of 3.7% per year while rates for gastrointestinal, endocrine, and rheumatic diseases are increasing by at least 6% per year (2). Type 1 diabetes is increasing rapidly across Europe and North America and, most disturbingly, the greatest rate of increase is in the 0- to 4-year age group (3). Similar rises in incidence rate are seen for a range of other autoimmune conditions. Crohn's disease and ulcerative colitis, examples of auto-inflammatory conditions, are increasing at a similar rate across the world, reflecting their emergence as global diseases (4).

IMMUNE-MEDIATED INFLAMMATORY DISEASES

The increasing prevalence of IMIDs demands a more precise classification and better fundamental understanding of the pathology underlying these diseases. This will lead to improved diagnosis through use of selective biomarkers, earlier detection and intervention thereby avoiding complications, identification of high-risk populations through better understanding of genetic and environmental influences enabling avoidance of contributory triggers or prevention through immunotherapy. McGonagle and McDermott proposed a classification of IMIDs based on the genetic factors involved in their etiology (5). They defined monogenic auto-inflammatory diseases, such as Blau syndrome, familial Mediterranean fever, and tumor necrosis factor receptor-associated periodic syndrome, as being one end of a spectrum of inflammatory diseases with monogenic autoimmune diseases, such as autoimmune lymphoproliferative syndrome, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, and certain complement deficiencies as the other end of the spectrum (**Figure 1**). The vast majority of both auto-inflammatory and autoimmune diseases, such as type 1 diabetes (6), are polygenic and, therefore, fall in between the two ends of the spectrum. Generally speaking auto-inflammatory diseases are associated with mutations influencing innate immunity, including the inflammasome genes, and are not associated with autoantibodies, autoreactive T cells or have MHC-disease associations. On the other hand, genetic polymorphisms associated with autoimmune diseases are found in genes regulating the adaptive immune system and together these permit the generation and subsequent lack of control of autoreactive T cells leading to production of autoantibodies. Strikingly, most classical autoimmune diseases have a strong association with genes in the MHC class II and are more common in women than men (7). Of the 5–10% of people in Western countries suffering from autoimmune diseases approximately 80% are women. This may be because X-chromosome inactivation or reactivation influences self-tolerance or the possibility that X-chromosome encoded miRNAs may influence susceptibility to autoimmune diseases. Furthermore, it is clear that both X-linked genes and the sex hormones produced influence innate and adaptive immunity, inflammation, and autoimmunity (7).

IMMUNOTHERAPY OF AUTOIMMUNE DISEASES

The Holy Grail for treatment of autoimmune diseases is to discover a means of selectively suppressing the specific autoimmune disease while leaving the rest of the immune system functionally active for control of infectious diseases and cancers. The aim is to develop treatments with increasing specificity for disease in order to decrease the risk of potential side effects. The ultimate aim is to provide a cure; however, the likelihood of success for this aim will depend on the particular autoimmune disease and associated pathology. For example, it may be sufficient to deplete autoreactive cells to correct the immune imbalance and reset homeostatic control of autoreactivity. In other cases, however, it may be necessary to continue treatment to arrest disease progression.

Currently, control of autoimmune diseases depends on the use of non-specific immunosuppressive drugs with associated side effects. Taking multiple sclerosis (MS) as an example, there are a variety of treatments that are being developed that aim to increase specificity for disease. Alemtuzumab is an antibody specific for CD52 that deletes all leukocytes and has

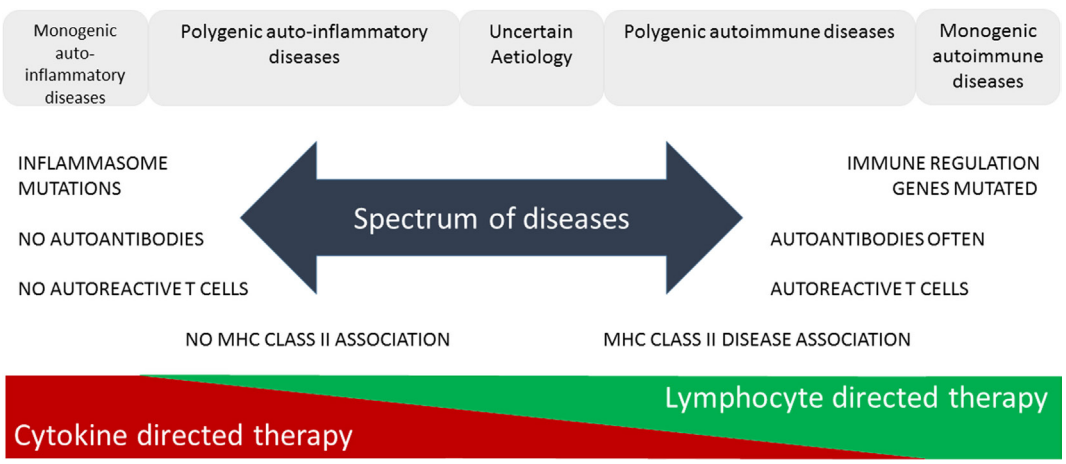

FIGURE 1 | Immune-mediated inflammatory diseases. This figure is based on "A proposed classification of the immunological diseases" by McGonagle and McDermott (5). The figure distinguishes the etiological basis of auto-inflammatory and autoimmune diseases and more clearly defines the definition of these diseases according to their monogenic or polygenic basis. The spectrum of diseases proposed are more associated with either innate (auto-inflammatory) or adaptive (autoimmune) immune responses and range from monogenic auto-inflammatory to monogenic autoimmune diseases. This classification not only helps us understand the genetic and immunological basis of the disease but also predicts more effective means of immunotherapy.

a dramatic effect on the inflammation in and progression of MS. A median seven-year follow-up of relapsing–remitting MS patients treated with Alemtuzumab revealed that up to 70% of trial participants had an improved or unchanged disability compared to baseline. However, treatment was associated with secondary autoimmunity in approximately 48% of the treated individuals, with Graves' disease being the most common complication (8).

Multiple sclerosis is considered to be a T-cell mediated disease; however, depletion of CD4 T cells alone was not an effective treatment (9). MS is characterized by the presence of oligoclonal bands of immunoglobulins in cerebrospinal fluid (10); however, it has been difficult to associate these antibodies with a clear target for autoimmune pathology. Nevertheless targeting CD20 on B cells is proving an effective means of controlling relapsing–remitting MS and even reducing disability in primary progressive disease (11). CD20 is expressed on pre-B, naïve, and memory B cells but not plasma cells. It is thought that depletion of these cells by rituximab or ocrelizumab will affect not only production of potentially pathogenic antibodies but also cytokine secretion by B cells and most likely their ability to present antigen to T cells (12, 13). Side effects due to B cell depletion appear limited to a higher than normal risk of herpes reactivation and breast cancer (11).

Drugs designed to reduce lymphocyte migration into the CNS have shown promising results. For example, fingolimod acts as a Sphingosine 1 phosphate (S1P) receptor agonist that results in S1P receptor downregulation thereby preventing lymphocyte migration from lymph nodes (14). This drug has a temporary effect on heart rate but otherwise has remarkably few side effects. A more targeted drug, preventing T cell migration into the CNS, is natalizumab. This drug targets the integrin VLA-4 required for lymphocytes to cross the blood brain barrier and reduces annual relapse rates and disability progression. However, there is a 1/300 chance of developing progressive multifocal leukoencephalopathy (PML) as a result of treatment (15). PML is caused by the human polyoma JC virus that infects and kills oligodendrocytes causing devastating damage when immune surveillance of the CNS is compromised.

It is difficult to see how non-specific manipulation of the immune response in autoimmune diseases such as MS will ever be completely safe or free of side effects. One increasingly promising approach is the use of low-dose interleukin 2 for treatment of autoimmune diseases (16). This relies on the fact that effector T cells respond weakly to low-dose IL-2 *in vivo* whereas Foxp3$^+$ Treg cells, which express the high-affinity IL-2 receptor (CD25), proliferate following low-dose IL-2 treatment *in vivo* (17). Low-dose IL-2 treatment is well tolerated; however, it is possible that non-specific expansion of the Foxp3$^+$ Treg population may influence susceptibility to infections and cancer in some individuals.

Many of the autoantigens associated with autoimmune diseases, such as MS, are known (18). In light of this, a number of groups have begun developing approaches designed to selectively target antigen-specific lymphocytes associated with autoimmune diseases. These range from injection of T-cell epitopes derived from self-antigens (19–22) through administration of tolerogenic dendritic cells carrying autoantigen peptides (23), the design of nanoparticles combined with peptide alone (24) or peptide and immunosuppressive drug (25) to the sophisticated construction of nanoparticles coated with complexes of MHC class II molecules and antigenic peptides (26, 27). Currently, the mechanisms by which these antigen-specific approaches protect against and treat autoimmune diseases are not clear. Work in preclinical models of autoimmune disease show that they function by either deleting autoreactive T cells, inducing anergy, or generating cells with a regulatory phenotype. Most importantly, results of clinical trials have not revealed significant side effects associated with antigen-specific immunotherapies.

In the next 20 years, we will discover that different regulatory T cell populations protect against different immune pathologies, including autoimmune diseases. Accordingly, we will design antigen-specific approaches optimized for induction of Foxp3$^+$, IL-10-secreting Tr1-like, or CD8$^+$ Treg all of which have been associated with protection from disease through antigen-specific immunotherapy. We will know how to administer antigens to selectively induce the relevant Treg population and will have tested the most effective delivery approach. Furthermore, we will have discovered drugs to co-administer with antigens in order to promote specific subsets of regulatory cells; for example, GSK-3 have been shown to promote IL-10 secreting Tr1-like cells (28) while PI3 Kinase inhibitors selectively support Foxp3$^+$ Treg cells (29). Most importantly, it will be essential to identify drugs that make it possible for regulatory cells to function in an inflammatory environment (30–32).

IMMUNOTHERAPY OF CANCER

Cytotoxic T cells are potent killers of cancer cells. However, both CD4 and CD8 tumor-infiltrating lymphocytes (TILs) tend to be suppressed and, hence, unable to control tumor growth. There are various mechanisms leading to suppression of TILs including the presence of Treg cells (33, 34) and the secretion of inhibitory mediators, such as adenosine, prostaglandins, and arginase (35–38). A universal feature of TILs is the upregulation of inhibitory receptors on those cells that are unable to control the cancer (39). Molecules currently under investigation include CTLA-4, PD-1, LAG-3, TIGIT, and Tim-3. The outcome of clinical trials reveals that antibodies to PD-1 and CTLA-4 are extremely powerful in reversing the suppression of TILs. Their use has shown great promise in different cancer types, prominently melanoma and small-cell lung carcinoma (40). However, the use of such "checkpoint inhibitors" does not work in all patients and we currently do not understand why. Furthermore, the use of checkpoint inhibitors, such as the combination of anti-PD-1 and anti-CTLA-4, causes severe toxicity in the majority of patients treated. Toxicity depends on the individual and ranges from inflammation of the GI tract, the most common complication, to autoimmune phenomena affecting the thyroid, skin, liver, joints, pancreas, and brain, i.e., common targets for organ-specific autoimmune diseases. At this time, we do not understand why treatment with the same combination of antibodies induces discrete autoimmune phenomena in different individuals; presumably, this reflects the presence of selective groups of

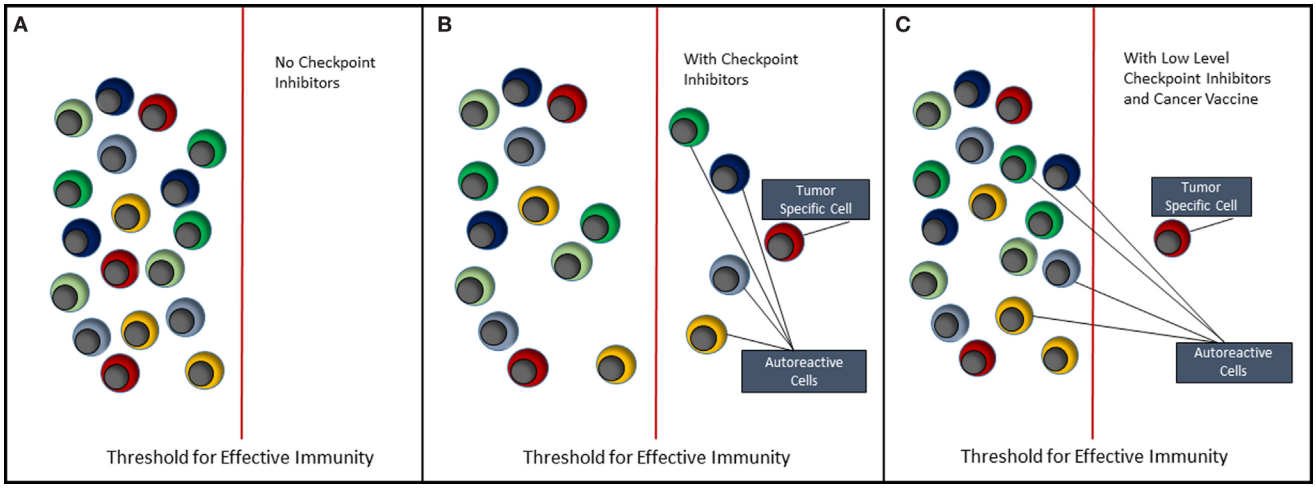

FIGURE 2 | Immunotherapy of cancer. This figure depicts the effect of immunotherapy with checkpoint inhibitors on the immune system. **(A)** It reflects the steady state in which both tumor-antigen reactive and self-antigen reactive T cells remain quiescent, i.e., in a state of tolerance. **(B)** When checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 are administered, both tumor-specific and autoreactive T cells break tolerance, respond to their antigens, clear the tumor, but allow expansion of autoreactive T cells resulting in autoimmune disease. **(C)** It reflects the situation where either the type of inhibitory receptor targeted by checkpoint inhibitor is changed or the amount of checkpoint inhibitor is reduced to a level that does not trigger autoreactive T cells. It is suggested, however, that coadministration of a selective cancer vaccine will lead to expansion of tumor-specific T cells and hence tumor clearance.

pre-disposing genes in these individuals. Much current research involves investigation of altered dosing regimens or combinations of checkpoint inhibitors in order to reduce the level of toxicity. Injection of checkpoint inhibitors directly into metastatic tumor sites could enhance their efficacy with less associated toxicity as shown for Treg depleting antibodies (41). However, breaking the tolerance of TILs may never be possible without causing some degree of induced self-reactivity unless there is a means of selectively activating tumor-specific cells while leaving other self-reactive cells dormant.

The future of cancer immunotherapy lies in the combination of selective cancer vaccines and checkpoint inhibitors or some other means of relieving immune suppression associated with the tumor. As shown in **Figure 2**, it is possible to lower the threshold for effective antitumor immunity by blocking inhibitory receptors such as PD-1 and CTLA-4. However, the use of checkpoint inhibitors alone (**Figure 2B**) will never selectively activate tumor-specific cells without coincidentally causing activation of self-reactive cells and, hence, causing some form of auto-inflammatory or autoimmune condition. Currently, we understand very little about how most of the inhibitor receptors targeted by checkpoint inhibitors actually function (42). These molecules downregulate cell signaling at the immune synapse; however, the mechanisms involved are largely unknown. Detailed knowledge of this would reveal common signaling and regulatory pathways that could provide more controlled targets for pharmaceutical intervention. Ultimately, it should be possible to reduce the level of or change the combination of checkpoint inhibitors, such that self-reactive cells are no longer activated. We then need a means of selectively immunizing for an antitumor response using a cancer vaccine.

Importantly, we are entering a revolutionary era for research into the neoantigens associated with tumors and the application of this knowledge in vaccine development. For example, the use of massively parallel sequencing for detection of mutations within tumors combined with machine learning approaches, to predict which of those mutated peptides bind with high affinity to HLA molecules, has allowed development of immunogenic vaccines targeting predicted neoantigens. A recent study described the application of this approach in melanoma whereby four of six vaccinated patients had no recurrence 2 years after vaccination while two with recurrent disease experienced complete tumor regression following treatment with anti-PD-1 (43). This outstanding achievement, combining molecular analysis and computer prediction, holds great promise for the future of cancer vaccination and shows the power of combination immunotherapies. The same combination approach could be applied to the tumor microenvironment whereby inhibition of suppressive molecules, such as adenosine, prostaglandin, or arginase, could be combined with a vaccine to boost the anti-cancer approach. The next 20 years will see a stream of breakthroughs in which immunotherapeutic approaches are combined to selectively target tumors while avoiding unnecessary toxicities.

CONCLUSION

The immune system has evolved to protect us from infection. The human immune system is immensely complex and the drawback of developing an immune system that may recognize and respond to all infections is the potential for hypersensitivity reactions. These manifest as allergic responses to environmental agents and autoimmune responses to self-antigens. Equally, the

immune system has developed sophisticated regulatory mechanisms to protect against rejection of the human allograft during pregnancy and reduce the risk of autoimmune diseases. These immune regulatory mechanisms serve as barriers to effective cancer immunity: the challenge to cancer control and eradication is how to have one without the other, i.e., how to promote effective cancer immunity without the toxic side effects of autoimmune diseases. Recent breakthroughs in the use of checkpoint inhibition, when combined with cancer vaccination, will make this feasible: the key factor is to target the relevant cancer antigen. For autoimmune diseases, we have depended on non-specific immunosuppressive drugs for far too long. We have failed to learn from the allergy field where effective immunotherapy is achieved by targeted desensitization using allergy associated antigens. The antigen-specific immunotherapies referred to in this perspective article herald a new era of immunotherapy for autoimmune diseases where again the key factor is to target the relevant antigen, in this case the self-antigen.

AUTHOR CONTRIBUTIONS

DW conceived and wrote the perspective article.

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REVIEW
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β_2 Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function

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Emerging evidence suggests that the β_2 integrin family of adhesion molecules have an important role in suppressing immune activation and inflammation. β_2 integrins are important adhesion and signaling molecules that are exclusively expressed on leukocytes. The four β_2 integrins (CD11a, CD11b, CD11c, and CD11d paired with the β_2 chain CD18) play important roles in regulating three key aspects of immune cell function: recruitment to sites of inflammation; cell–cell contact formation; and downstream effects on cellular signaling. Through these three processes, β_2 integrins both contribute to and regulate immune responses. This review explores the pro- and anti-inflammatory effects of β_2 integrins in monocytes, macrophages, and dendritic cells and how they influence the outcome of immune responses. We furthermore discuss how imbalances in β_2 integrin function can have far-reaching effects on mounting appropriate immune responses, potentially influencing the development and progression of autoimmune and inflammatory diseases. Therapeutic targeting of β_2 integrins, therefore, holds enormous potential in exploring treatment options for a variety of inflammatory conditions.

Keywords: β_2 integrins, CD11/CD18, dendritic cells monocytes and macrophages, immune regulation, autoimmunity

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INTRODUCTION

The integrin family of proteins is comprised of 24 heterodimeric transmembrane adhesion receptors. Each integrin is formed through the non-covalent association of 1 α-subunit and 1 β-subunit; currently, 16 α-subunits and 8 β-subunits have been identified. Their expression on virtually all human cells and their complex signaling mechanisms explain their wide variety of biological roles, including blood clotting, cell adhesion, and migration.

Due to their extensive importance in biological systems, elucidating integrin signaling and receptor function has been of great interest since their characterization as adhesion molecules over 30 years ago. Integrins are important signaling proteins that mediate interactions of the cell with extracellular matrix proteins and with other cells via cell-surface ligands. Integrins exist in a continuum between a folded inactive form with low affinity for their ligand and an extended high affinity conformation (1), although even bent integrins are able to bind ligand in rare instances (2). As immune cell adhesion and extravasation into lymph nodes and tissues forms part of initiating an effective immune response, β_2 integrin conformation on the surface of leukocytes needs to be tightly regulated. β_2 integrins on the surface of circulating leukocytes tend, therefore, to be largely inactive (2) until inside-out and outside-in signaling trigger integrin-mediated adhesion and extravasation into tissue (**Figure 1**).

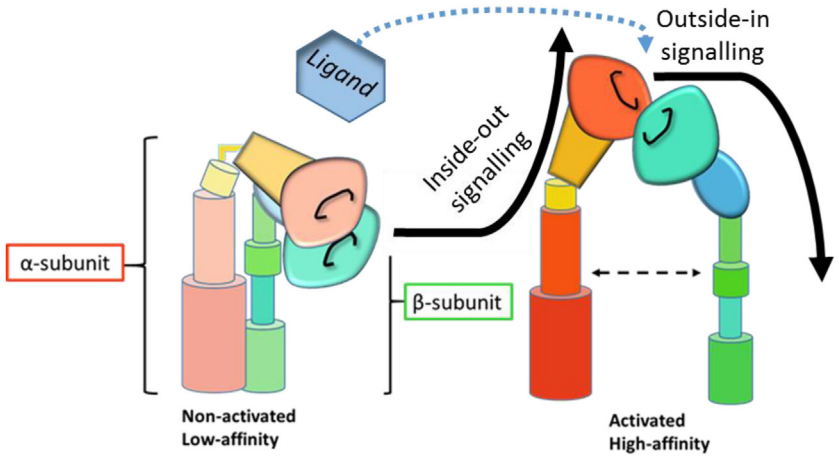

FIGURE 1 | Schematic representation of integrin activation and signaling. Inside-out signaling induces a conformational change in the integrin to the active, high affinity state. Upon ligand binding, active integrins then transmit outside-in signals and downstream signaling cascades. [Adapted from Byron et al. (3), with permission from the *Journal of Cell Science*].

Inside-out signaling modifies how cells interact *with* their environment by facilitating receptor affinity and avidity (4) to allow binding to extracellular ligands. Outside-in signaling, on the other hand, mediates intracellular events in response *to* their environment by eliciting downstream signaling cascades in response to receptor occupation. The complex details of integrin signaling are reviewed elsewhere (5, 6) and are beyond the scope of this review. Briefly, inside-out signaling is mediated by talin (7) and kindlin (8, 9) binding to the intracellular domain of the β_2 subunit, a process initiated by chemokine receptor or Toll-like receptor (TLR) engagement (10, 11), which results in a conformational change in the integrin from a low-affinity to a high-affinity state. Outside-in signaling is then initiated by ligand binding to high-affinity integrin receptors (**Figure 1**). Downstream signaling events mediate the formation of focal complexes and adhesions through rearrangement of the actin cytoskeleton. The relative importance of affinity and avidity on integrin signaling and function is heavily debated (12, 13), but dynamic interaction between these processes and both inside-out and outside-in signaling seems likely (14).

β_2 integrins are the focus of this review, as they are exclusively found on leukocytes and therefore of particular importance for the immune system. They mediate cell recruitment into lymphoid organs and inflamed tissues by facilitating firm leukocyte arrest on endothelial cells and extravasation after cell rolling (15); cellular interactions between leukocytes including immunological synapse formation (16); and intracellular signaling cascades that influence cytoskeletal rearrangement, activation, proliferation and impact on cellular responses to TLRs. Importantly, through these three processes, β_2 integrins can have either pro-inflammatory or anti-inflammatory outcomes. The β_2 integrin subunit (CD18) can pair with one of four α-subunits (α_L—CD11a, α_M—CD11b, α_X—CD11c, and α_D—CD11d), forming leukocyte function-associated antigen-1, Mac1/CR3 (macrophage-1 antigen, complement receptor 3), P150,95/CR4 (complement receptor 4), and CD18/CD11d, respectively (**Figure 2**). For consistency, this review will utilize only the CD nomenclature. Both function and cell-specific expression of β_2 integrins vary according to the α-subunit involved.

The main ligands for the β_2 integrin family members are outlined in **Figure 2**. Briefly, CD11a binds to intracellular adhesion molecule-1 (ICAM-1), -2, -3, and -5, which are expressed by a variety of cells including leukocytes and endothelial cells, thereby mediating leukocyte recruitment to lymph nodes and sites of inflammation as well as cell–cell adhesion. CD11b binds the complement proteins iC3b and C4b with high affinity, mediating phagocytosis of complement-coated particles but can also bind ICAM-1, fibrinogen, and more than 40 other ligands (17). The sequence of CD11c is very close to that of CD11b, and indeed CD11c binds several of the same ligands including iC3b, ICAM-1, and fibrinogen. Multi-ligand binding capacity of CD11d is proposed to largely overlap with CD11b and includes ECM-associated proteins fibronectin, fibrinogen, vitronectin, Cyr61, and plasminogen (18).

This review will provide an overview of β_2 integrin expression on monocytes, macrophages and DCs, before exploring the paradoxical pro-inflammatory and regulatory roles of β_2 integrins in immune regulation in three key aspects of immune function: recruitment and migration, cellular interactions, and downstream cell signaling (**Figure 3**). We will furthermore review how dysregulated integrin signaling could contribute to inflammatory and autoimmune conditions and introduce the therapeutic potential of targeting β_2 integrins.

EXPRESSION OF β_2 INTEGRIN SUBUNITS BY DENDRITIC CELLS (DCs), MONOCYTES, AND MACROPHAGES

The expression of β_2 integrin subunits varies in different leukocyte subsets and between mice and humans. In general terms, CD11a is expressed on all leukocytes at varying levels, while CD11b,

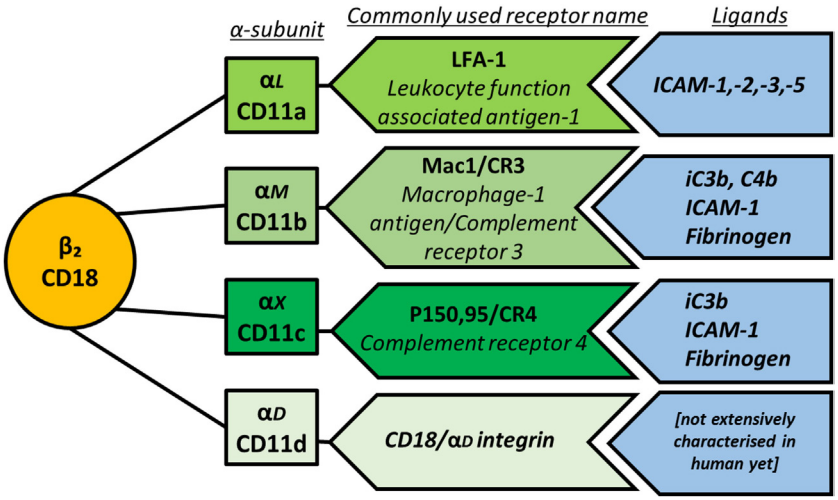

FIGURE 2 | Schematic representation of β_2 integrin subunit pairing, depicting the β-subunit CD18 as the common subunit non-covalently associating with one of four α-subunits. The main ligands for each integrin are also shown.

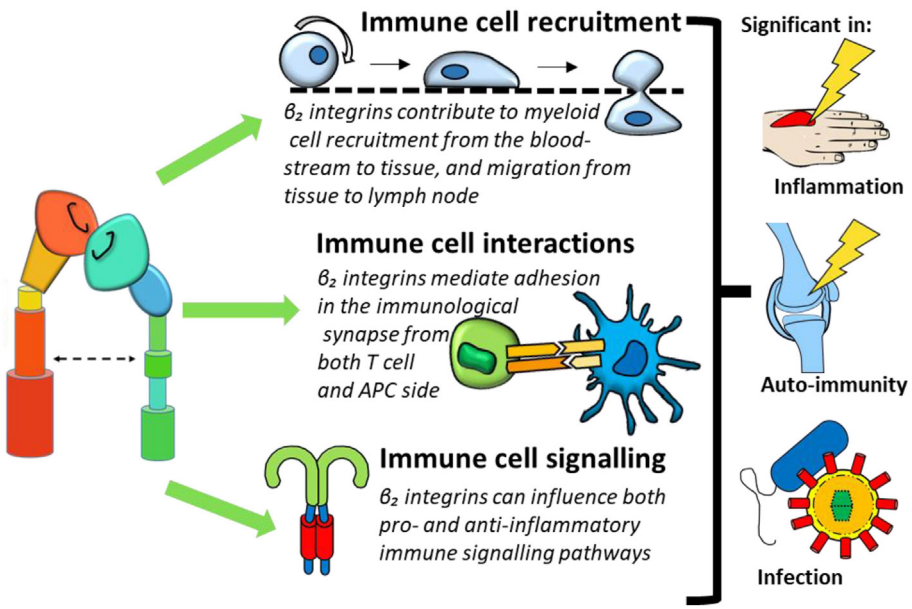

FIGURE 3 | β_2 integrin involvement in immune cell function can be categorized into three processes: immune cell recruitment, immune cell interactions, and immune cell signaling. Dysregulation of these functions could contribute to conditions such as inflammation, immunity, and infection.

CD11c, and CD11d are predominantly expressed by monocytes, macrophages and DCs. Specifically, in humans, monocytes express all four β_2 integrin-associated alpha subunits (CD11a, CD11b, CD11c, and CD11d) with CD11a and CD11b expression greater than CD11c (19, 20); macrophages express CD11a and CD11b at lower levels than monocytes together with CD11c at similar levels to monocytes (21); while DCs mainly express CD11c together with CD11a, though some DC subsets also express CD11b (22). While CD11d has received less attention than the other β_2 integrins due to the absence of commercially available human antibodies, Miyazaki and colleagues showed CD11d expression on monocyte-derived DCs and macrophages as well as most circulating monocytes (23). To complement the scarce available data, mRNA expression data for the CD11d subunit ITGAD were consulted. While Villani and colleagues (24) find monocytes to express highest levels of ITGAD mRNA, the Expression Atlas (25) reports highest expression in DCs, with ITGAD expression in monocytes remaining below detectable threshold. However, overall both RNAseq data sets show that CD11d mRNA expression is very low in monocytes, macrophages, and DCs. **Table 1**

TABLE 1 | β_2 integrin expression on dendritic cells (DCs), monocytes and macrophages—human and murine findings.

Cell type	CD11a/CD18 (α_L/β_2)	CD11b/CD18 (α_M/β_2)	CD11c/CD18 (α_X/β_2)	CD11d/CD18 (α_D/β_2)
DCs	*Human*: high levels of CD11a on monocyte-derived DCs (22, 27–29); plasmacytoid DCs (pDCs) also express CD11a (30); reduced CD11a/CD18 levels upon DC activation (31)	*Human*: CD11b present on monocyte-derived DCs (22, 27, 28); detected in cDCs, but not in pDCs (31–33); higher on cDC2 than cDC1s (33, 34); reduced CD11b/CD18 levels upon DC activation (31)	*Human*: pDCs lack CD11c (31); expressed on mature DCs (31); CD11c expression is higher on cDC2 than cDC1s (33, 34); monocyte-derived DCs also express CD11c (32); reduced CD11c/CD18 levels upon DC activation (31)	*Human*: expressed on monocyte-derived DCs (23), single-cell mRNA data suggests low gene expression in DCs (25)
	Mouse: expressed by cDCs, particularly the $CD8^+$ subset, and by pDCs (35); also highly expressed by bone marrow-derived DCs	*Mouse*: expression of CD11b in mouse cDCs is subset-specific: higher on $CD8^-$ than $CD8^+$ splenic DCs (35); expressed in sub-populations of gut DCs (36); absent from pDCs (37); expressed by bone marrow-derived DCs (38)	*Mouse*: CD11c highly expressed on cDCs and typically used as a DC marker (38); expressed by pDCs (39) and bone marrow-derived DCs (40)	*Mouse*: no protein expression data available, RNA-seq data suggest medium ITGAD gene expression in murine DCs (25)
Monocytes	*Human*: expressed by circulating monocytes (21, 29, 41)	*Human*: highly expressed by circulating monocytes (21, 34, 41); differentially expressed on osteoclast precursors (42)	*Human*: expressed on circulating monocytes (21, 34) and classical, non-classical, and intermediate monocytes (31)	*Human*: expressed on majority of circulating monocytes, higher on $CD16^-$ cells compared to $CD16^+$ cells (23)
	Mouse: expressed by circulating monocytes (43)	*Mouse*: high expression of CD11b on murine monocytes (44)	*Mouse*: thought to be absent from most monocytes (45); though may be upregulated upon stimulation/maturation (44)	*Mouse*: lowly expressed by circulating monocytes, upregulated upon differentiation into macrophages (46), low ITGAD mRNA expression (25)
Macrophages	*Human*: expressed by monocyte-derived macrophages (21, 43); reduced expression on monocyte-derived macrophages compared to blood monocytes (21)	*Human*: expressed on monocyte-derived macrophages (47–49); expressed on alveolar macrophages, though at lower levels compared to blood monocytes (21)	*Human*: lowly expressed by monocyte-derived macrophages (21, 48–50)	*Human*: expressed on monocyte-derived macrophages *in vitro* (23)
	Mouse: expression dependent on tissue: present on pulmonary, but not on microglia, spleen or peritoneal macrophages (51)	*Mouse*: abundantly expressed by peritoneal macrophages (52, 53); highly expressed on dermal macrophages (54)	*Mouse*: expressed on alveolar macrophages (55); absent from bone marrow-derived macrophages and dermal macrophages (54)	*Mouse*: expressed by peritoneal macrophages (56)

provides the details of expression of all β_2 integrin subunits in human and murine monocytes, macrophages, and DCs. Where available, expression analysis on DC subsets is given using the Guilliams nomenclature (26), which was recently confirmed and expanded by Villani and colleagues (24).

Animal studies have been instrumental in elucidating integrin function in monocytes, macrophages, and DCs. β_2 integrins are highly conserved across species, with mice, rats, and rabbits most commonly used as models. Importantly β_2 integrin-deficient mice are considered an appropriate model of the human condition leukocyte adhesion deficiency (LAD) where β_2 integrin expression or function is lost (57). However, while β_2 integrin structure is largely similar between species, cellular expression levels can vary significantly. A common example is CD11c, which in mice is predominantly expressed by conventional (cDCs) and plasmacytoid DCs (pDCs), although can also be expressed on lymphocyte subsets. In humans, on the other hand, CD11c is expressed not only on DCs but also monocytes, macrophages, granulocytes, and natural killer cells (19, 38). Animal and human studies therefore have to be compared with great care, and validation of concepts conceived in animal models in human cells remains a priority in elucidating the functions of β_2 integrins.

β_2 INTEGRINS AS REGULATORS OF IMMUNE FUNCTION

Evidence for β_2 Integrin Contribution to Immune Regulation

There is mounting evidence that puts β_2 integrins at the center of the balance between immune priming and tolerance. Integrin-deficient humans and mouse models show that β_2 integrins are important negative regulators of the immune system. LADs are genetic human disorders caused by the reduction or complete absence of β_2-integrins (LAD-I) (58) or by mutations in the integrin-activating protein kindlin-3 (LAD-III) (59). These disorders are characterized by profound impairment of leukocyte recruitment to peripheral sites of infection. Patients with LAD suffer from increased susceptibility to infection and impaired inflammatory responses (60), resulting in markedly reduced lifespan if no therapeutic measures are taken. Paradoxically LAD patients also suffer from chronic inflammatory diseases. Examples of conditions prevalent in LAD patients include intestinal colitis (61) and periodontitis (62) suggest that β_2 integrins have an important role in suppressing inflammation and promoting

immune tolerance. Supporting this, the presence of functional β_2 integrins improved symptoms in a model of skin inflammation by restricting DC-mediated T cell activation (63).

LAD pathology can be replicated in β_2 integrin knockout (KO) mouse models, underlining the importance of β_2 integrins for immune cell recruitment in both humans and murine models and the similarities between the species. From studies in KO mice and LAD patients, we know β_2 integrins are essential in mediating T cell recruitment to lymph nodes and leukocyte, particularly neutrophil and T cell, recruitment to sites of inflammation. Here, we will further explore the roles of these integrins in monocytes, macrophages, and DCs.

β_2 Integrins Regulate Recruitment and Migration of Mononuclear Phagocytes

Evidence suggests that leukocyte recruitment *to* tissues is dependent on β_2 integrins, because of the requirement for these adhesion molecules in the firm adhesion to the endothelial layer under shear flow conditions and for subsequent transendothelial migration (64). However, leukocyte migration *within* tissues is thought to occur independently of β_2 integrins, as cells use an actin-dependent flowing and squeezing mechanism of movement in three-dimensional environments (64).

Geissmann and colleagues showed that the adhesion of patrolling murine monocytes to blood vessel walls is significantly decreased when CD11a is blocked (45). Similarly, chemotactic migration of human monocytes *in vitro* is inhibited when CD18 function is blocked (65). However, murine monocyte recruitment to sites of inflammation was found to occur independently of CD11a and CD11b (66), suggesting that β_2 integrins are primarily involved in the homeostatic migration of monocytes and that their role is redundant during inflammation. On the other hand, increased expression levels of CD11d on macrophages mediates their retention at inflammatory sites in mice (56).

The role of β_2 integrins in DC and macrophage recruitment to secondary lymphoid organs and tissues seems to be dependent on the inflammatory state of the body. Bone marrow-derived DCs (BMDCs) from mice where all integrins, including β_2, are knocked out, migrated from the site of injection (ear) to the draining lymph node in similar numbers to their wild-type counterparts when activated with lipopolysaccharide (LPS). This suggests that DC migration during inflammation is not dependent on integrins. However, under steady-state conditions, the absence of functional β_2 integrins from murine BMDCs (using signaling-deficient β_2 integrin knock-in BMDCs) was found to increase migration from tissue (footpad) to draining lymph node, leading to the hypothesis that β_2 integrins function to restrict migration in the steady-state by anchoring DCs in the tissue site. As a consequence of increased DC migration to the draining lymph node, the same study showed an increase in Th1 cytokine production (67), further supporting a negative regulatory role for β_2 integrins on DCs. In addition, a murine model of skin inflammation also showed an increase in migratory DCs in the draining lymph node of β_2 integrin signaling-deficient mice, as well as at the site of inflammation, though whether this was dependent on the inflammation or not was not determined (63). Overall, the cellular environment seems to determine the requirement for functional β_2 integrins in the migration of both monocytes and DCs *in vivo*: integrins play a role in monocyte recruitment and DC migration under steady-state conditions, but are dispensable during inflammation.

β_2 Integrins Regulating DC–T Cell Interactions

In addition to their roles in leukocyte recruitment and migration, β_2 integrins are also important mediators of cellular interactions. Functional β_2 integrins are important in the formation of the immunological synapse between antigen-presenting cells (APCs) and T cells. The context and dynamics of this interaction determine whether T cells become activated or tolerized. β_2 integrins, and their ligand, ICAM-1, are expressed by both the T cell and the APC and are vital in immune synapse formation. Importantly, it is becoming increasingly clear that β_2 integrins expressed by the APC and T cell have opposing functions in the immune synapse, resulting in differential outcomes for the T cell response.

On the T cell side, CD11a clusters in the peripheral supramolecular activation cluster (P-SMAC) and binds to ICAM-1 on the APC (68). This molecular interaction stabilizes the connection made between T cell receptor and peptide:MHC on the APC in the central SMAC (16, 69), thereby enhancing TCR signal transduction (70). While T cell CD11a therefore has a largely pro-inflammatory effect, enhancing T cell activation, proliferation, and differentiation, a role for T cell integrins in regulation of activation, for example, in different T cell subsets, is not ruled out.

On the APC side of the immunological synapse, β_2 integrins have also been shown to be involved, likely binding to ICAM-1 on the T cell. Importantly, the integrins on the APC regulate the outcome of the T cell response. For example, in murine models, active CD11b on DC surfaces inhibits the DC–T cell interaction (71). The reduced antigen-presenting capabilities of murine bone marrow-derived macrophages compared to BMDCs were therefore proposed to be due to their comparably larger surface expression of activated CD11b (71, 72). This suppressive role for DC CD11b has also been shown in human cells. When CD11b on human monocyte-derived DCs binds its ligand ICAM-1, both CD86 expression on DCs and DC-induced T cell proliferation were reduced (73). Interestingly, ligation of CD11b/CD18 decreases the ability of murine BMDCs to stimulate T cells and elicit a downstream response (74), CD11b/CD18 interactions can suppress Th17 cell differentiation (75), suggesting a strong role for this specific β_2 integrin in immune regulation. This suggests that the activated conformation of CD11b/CD18 is extensively involved in regulating the immune system and has strong negative and positive regulatory functions depending on cell type they are expressed on.

Furthermore, the expression of activated β_2 integrins on murine DC surfaces significantly reduces T cell activation (71) and further studies actually demonstrated an inverse relationship between forced activation of murine BMDC CD11a and T cell activation (72), suggesting a directly limiting effect of active β_2 integrins on T cell activation by APCs.

Overall, the role of integrins as adhesion molecules carefully mediating and regulating cellular interactions is not to be underestimated for mounting an effective immune response.

β_2 Integrins Regulate Immune Cell Signaling

In addition to their roles in leukocyte recruitment and interactions, several studies show that integrin outside-in signaling following ligand binding can directly affect cell function. Chinese Hamster Ovarian cells transfected with CD11c acquire the ability to bind both LPS and Gram-negative bacteria, as well as the ability to initiate downstream activation signals (76). In contrast to their anti-inflammatory roles on DCs, CD11b or CD11c receptor occupation on the surface of human monocytes stimulates cell-specific pro-inflammatory pathways (77), such as secretion of IL-8, MIP1α, and MIP1β.

Generally, the interplay between TLR4- and β_2 integrin-mediated signaling is controversial. On the one hand, it has been shown that CD11b positively regulates TLR4 signaling (78), especially in murine BMDCs. Several studies report β_2 integrins act in synergy with LPS (79–81), therefore suggesting a potential pro-inflammatory role for CD11b. By contrast, other studies report that β_2 integrins negatively affect TLR signaling. Complete absence of β_2 integrins in mice (CD18 KO) was shown to result in a strong increase of TLR signaling (82) and the absence of CD11b specifically from murine macrophages causes exacerbated TLR-mediated inflammatory responses, resulting in increased susceptibility to endotoxin shock and *Escherichia coli* sepsis (83). Mechanistically, CD11b signaling has been shown to induce degradation of the key TLR signaling components, MyD88 and TRIF, directly dampening TLR responses in macrophages (83). Moreover, activation of CD11b on human inflammatory arthritis synovial macrophages *via* binding to its ligand ICAM was shown to indirectly inhibit TLR signaling (84) by inducing expression of IL-10 and the inhibitory factors SOCS3, ABIN-3, and A20. Integrins furthermore restrict TLR signaling on both murine macrophages and DCs (63). The role of β_2 integrins in modulating TLR signaling is, therefore, complex, although one could tentatively propose that CD11b specifically seems to have opposing TLR4-mediated roles in inflammation, depending on the APC surface it is expressed on. However, while this could hold true for TLR4 signaling, this might not be the case for all TLRs. CD11b deficiency in murine BMDCs, while negatively affecting TLR4-mediated pathways, actually leads to an increase in DC cross-priming of cytotoxic T cells, a process mediated by the microRNA-146a (85). β_2 integrin regulation of TLR-mediated responses therefore remains incompletely understood, with future studies hopefully elucidating the complex and intricate nature of these receptor interactions.

A variety of studies available suggest a significant immunoregulatory role for β_2 integrins, not only by their mediation of adhesive and migratory processes, but also by immunological signaling. However, other studies suggest that, given the right cellular environment or cell type, β_2 integrins can also have a strong pro-inflammatory effect (see **Table 2** for comparison). When considering these opposing functions of integrins, it seems likely that even slight disturbances in integrin expression, signaling or activation could result in significant immunological effects, thus potentially contributing to a variety of autoimmune, inflammatory, and infectious conditions.

β_2 INTEGRINS IN INFLAMMATION, INFECTION, AND AUTOIMMUNITY

Evidence for the role of β_2 integrins in contributing to the development and progression of inflammatory and autoimmune conditions is accumulating. Considering that β_2 integrin signaling can have opposing functions depending on subunit pairing and the immune cell type it is expressed on, it is not surprising that these receptors play important roles in both *contributing to* as well as *negatively regulating* inflammatory processes.

Human genetic studies point to a role of β_2 integrins in inflammation and autoimmunity. A polymorphism of *ITGAM*, the CD11b subunit, increases the risk for the autoimmune disease systemic lupus erythematosus (87) (SLE), which shares genetic risk factors with rheumatoid arthritis (RA) (88). Disease risk for inflammatory bowel disease, similarly characterized by dysregulation of immune function specifically in the intestine, increases with amplified expression of alleles for both *ITGAL*, encoding CD11a, and the β_2 integrin ligand *ICAM1* (89). Gene expression of CD11d in humans and mice was found to be increased in white adipose tissue in obesity, a condition characterized by an increase in systemic inflammation (90). Furthermore, CD11d activation led to increased IL-1β expression (23), which when overproduced

TABLE 2 | Summary of the roles for β_2 integrins in monocytes, macrophages, and dendritic cells (DCs).

Cell type	Recruitment and migration	Interactions with T cells	Signaling
Monocytes	β_2 integrins mediate recruitment of monocytes under homeostatic conditions (45, 65), but dispensable for recruitment during inflammation (66)	Yet to be determined	Yet to be determined
	Pro-inflammatory	*Unknown*	*Unknown*
Macrophages	β_2 integrins reported to mediate macrophage retention at inflammatory sites (56, 86)	Yet to be determined	β_2 integrin signaling dampens macrophage responses to Toll-like receptor (TLR) stimulation (82, 83)
	Pro-inflammatory	*Unknown*	*Regulatory*
DCs	Under homeostatic conditions β_2 integrins restrict DC migration from tissue to lymph nodes (67); Migration from tissue site to draining lymph nodes during inflammation occurs independently of integrins (64)	DC integrins contribute to contact formation with T cells—this role inhibits full T cell activation (71, 72, 74)	β_2 integrin signaling functions to restrict DC activation both in response to TLR stimulation and under homeostatic conditions (67)
	Regulatory	*Regulatory*	*Regulatory*

can contribute to a variety of autoinflammatory conditions (91). While dysregulation of β_2 integrin signaling seems likely to be involved in a variety of autoimmune diseases and inflammatory conditions, exact mechanisms are still unclear, and further investigation of both signaling pathways and genetic basis will be needed to fully elucidate their complex roles.

Recent studies have focused on β_2 integrin involvement in RA, which serves as an excellent example of the opposing roles β_2 integrins can take in disease. Expression of CD11a is increased in inflamed synovial tissue, where it is hypothesized to contribute to cell activation and on-going joint destruction (92, 93), but not in peripheral blood of RA patients. However, as CD11a is also involved in facilitating immune cell migration to sites of inflammation, clear-cut cause and effect of the presence of activated β_2 integrins in the synovium is difficult to establish. Blocking all β_2 integrins reduced inflammation in a rabbit RA model (94), while absence of CD11a led to complete resistance to disease induction in a KB × *N* serum transfer mouse model of arthritis (95). Furthermore, both a small molecule antagonist against CD11a and a CD11a-monoclonal antibody (mAb) proved to be similarly successful in reducing both inflammatory-mediated bone destruction and cytokine mRNA levels within the murine joint (96, 97). Mice with mutations in the β_2 integrin ligand ICAM-1 also show reduced susceptibility to the collagen-induced arthritis (CIA) model (98). Clearly, CD11a–ICAM-1 interactions are essential for leukocyte recruitment to the inflamed joint.

However, evidence is emerging that other β_2 integrins may function to control inflammation in arthritis. CD11b KO mice, for example, show exacerbated joint pathology in the KB x N serum transfer model of arthritis, underlining the starkly opposite roles different β_2 integrins can play (95). A recent study replicated these results in a CIA model and, furthermore, showed that exacerbated joint pathology resulted from elevated IL-6 levels and an increase in Th17 cell priming, which could be rescued by introducing a CD11b-expressing DC cell line (99). On the other hand, blocking CD11b immediately before onset of disease significantly reduced disease burden in two different models of arthritis (CIA and a DBA/1 to severe combined immunodeficiency transfer model of arthritis) (100), suggesting that the role of CD11b in inflammatory arthritis may differ depending on the cell type involved and the disease stage.

When considering the importance, as well as the obvious complexity, of β_2 integrin function in autoimmune diseases such as RA, therapeutically targeting β_2 integrins will have to be carefully balanced but also holds great promise to offer novel treatment options.

APPLICABILITY OF INTEGRIN-TARGETING THERAPIES

Modulating integrin function to improve mal-adaptation or excessive activation of the immune system is of great interest in a variety of autoimmune and inflammatory conditions. However, achieving efficacy without immunocompromising side effects might prove challenging. Here, we discuss the progress and failures in developing integrin-targeted therapies and speculate on the routes forward for success.

To date, targeting integrins therapeutically has had mixed success in the clinic. The only mAb targeting β_2 integrins, Efalizumab, which targets CD11a, was originally developed as a treatment for psoriasis (101). However, several patients presented with the potentially fatal disease progressive multifocal leukoencephalopathy (PML), caused by reactivation of the JC virus, which results in a white matter disorder of the brain (102). Although the mechanism of PML development in Efalizumab-treated patients was not investigated, we speculate that viral reactivation was likely either due to the loss of immune cell recruitment to the brain to control the virus (103) or due to the mAb itself crossing the blood–brain barrier (104). Due to the occurrence of PML, Efalizumab was withdrawn from European and American markets due to its associated safety issues in 2009.

Although targeting β_2 integrins has so far failed in the clinic, targeting other integrins for the treatment of colitis and Crohn's disease has proved successful. The mAb against the α_4 integrin, Natalizumab, was developed for the treatment of multiple sclerosis and Crohn's disease (105, 106). This mAb binds to $\alpha_4\beta_1$ and $\alpha_4\beta_7$. However, PML also occurs in some Natalizumab-treated patients (integrin $\alpha_4\beta_1$ is also involved in leukocyte recruitment to the brain) and so is no longer used widely (107). More recently, a specific $\alpha_4\beta_7$ targeting mAb Vedolizumab has shown success in safety efficacy in Crohn's disease and ulcerative colitis. This success story underlines the potential of targeting integrins for therapeutic purposes.

In order to realize the potential of targeting β_2 integrins therapeutically, it will be necessary to improve the strategy. As indicated by the success of Vedolizumab over Natalizumab, one way to do this is to target the right integrin subunit(s) in order to reduce the likelihood of side effects. Targeting CD11a, in the form of Efalizumab, proved unsuccessful in the clinic. As CD11a is expressed by almost all leukocytes, has vital roles in leukocyte recruitment and has immunoregulatory effects in mononuclear phagocytes, the resulting serious side effects from targeting this molecule therapeutically are, perhaps, not surprising. Targeting other CD11 subunits might be a more effective strategy. For example, CD11b, CD11c, and CD11d have a more restricted pattern of expression in leukocytes (predominantly on monocytes, macrophages, and DCs), which may make these molecules more suitable targets. Importantly, it is vital that we consider the pro- and anti-inflammatory functions of β_2 integrin subunits and design drugs to target them appropriately. CD11b, for example, has clear regulatory roles in macrophages and DCs, meaning that we could potentially exploit this immunosuppressive pathway by activating, rather than blocking, this integrin subunit. Such a strategy may have less risk of serious side effects. It is, therefore, essential that we fully understand the specific functions of individual integrin subunits in different leukocyte populations in order to target β_2 integrin subunits effectively in the clinic.

Another option to explore is blocking not the β_2 integrin itself, but the ligand of interest. Targeting the CD11a and CD11b ligand, ICAM-1, has shown beneficial results especially in early RA (108), although immunogenicity of the mAb in question restricts clinical use (109) and problems caused by impaired leukocyte recruitment prevail.

Further potential difficulties in developing integrin-targeting therapy include the close signaling relationships that exist in some integrins, potentially leading to complex downstream effects mediated even by an activating mAb highly specific for a β_2 integrin (110). Carefully elucidating downstream signaling pathways and further increasing drug specificity is therefore essential to bring more integrin therapeutics into the clinic.

Innovative avenues to explore include computationally designed integrin proteins with constitutively activated or inactivated subunits, which could find applications in both pharmacological testing and therapy (111). Furthermore, developing small molecular drugs targeting β_2 integrins viable for oral use remains a priority, as it could offer an alternative way to yield the same beneficial results without the dangerous side effects of mAbs. An example is the small molecule CD11b agonist, Leukadherin-1, which previous studies found to reduce monocyte-mediated TNF-release by mimicking natural ligand binding. When NK cells and monocytes were pre-treated with Leukadherin-1, innate inflammatory signaling in human *ex vivo* studies was suppressed (112). While the study noted some caveats, for example, the differences of CD11b function on different cell types (78), the drug is still being explored for the treatment of SLE. Another small molecule currently in development is the CD11a antagonist BMS-587101, which acts by reducing CD11a-mediated adhesion and to a lesser effect T cell proliferation. It significantly improved both murine models of lung inflammation and transplant viability (113).

Continuous effort to increase drug specificity and further understand their complex delicate signaling networks will be needed to bring β_2 integrin-targeting drugs into the clinic. But while the use of integrin-targeting drugs has been contentious in the past, their potential in treating a wide variety of immune diseases is enormous and should not be neglected.

CONCLUSION

This review explored the opposing nature of β_2 integrin pro- and anti-inflammatory functions in three main immune functions, making them prime candidates to be both important mediators and regulators of the immune system. The first is migration, which allows for targeted immune cell recruitment to sites of infection and tissue damage. The second is adhesion, not only preceding immune cell extravasation at sites of inflammation, but also an important factor in initiating the adaptive immune response by facilitating cellular interactions. Finally, immune cell signaling, which allows for fine-tuned cooperation between a wide variety of immune cells. Considering the fact that β_2 integrins play a complex role in three important areas of the immune system and their differential expression on monocytes, macrophages and DCs, it becomes clear that the variety of studies presented in this review is by no means exhaustive. The common message is evident: β_2 integrins are involved in complex immunoregulatory signaling pathways. However, in addition to their well-established pro-inflammatory roles in recruitment and activation, β_2 integrins also have essential immunoregulatory functions. Dysregulated integrin signaling, expression and surface activation is therefore likely to contribute to a variety of inflammatory and autoimmune conditions. Elucidating the function of β_2 integrins further therefore promises to provide novel therapeutic targets for various disorders, RA being just one example.

AUTHOR CONTRIBUTIONS

CH and VM designed the structure of the review. LS wrote the first draft. CH and VM revised the manuscript. LS composed the figures. All authors have seen and agreed on the finally submitted version of the manuscript.

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REVIEW
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The Role of Lipid Metabolism in T Lymphocyte Differentiation and Survival

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The differentiation and effector functions of both the innate and adaptive immune system are inextricably linked to cellular metabolism. The features of metabolism which affect both arms of the immune system include metabolic substrate availability, expression of enzymes, transport proteins, and transcription factors which control catabolism of these substrates, and the ability to perform anabolic metabolism. The control of lipid metabolism is central to the appropriate differentiation and functions of T lymphocytes, and ultimately to the maintenance of immune tolerance. This review will focus on the role of fatty acid (FA) metabolism in T cell differentiation, effector function, and survival. FAs are important sources of cellular energy, stored as triglycerides. They are also used as precursors to produce complex lipids such as cholesterol and membrane phospholipids. FA residues also become incorporated into hormones and signaling moieties. FAs signal *via* nuclear receptors and their channeling, between storage as triacyl glycerides or oxidation as fuel, may play a role in survival or death of the cell. In recent years, progress in the field of immunometabolism has highlighted diverse roles for FA metabolism in CD4 and CD8 T cell differentiation and function. This review will firstly describe the sensing and modulation of the environmental FAs and lipid intracellular signaling and will then explore the key role of lipid metabolism in regulating the balance between potentially damaging pro-inflammatory and anti-inflammatory regulatory responses. Finally the complex role of extracellular FAs in determining cell survival will be discussed.

Keywords: T cell, fatty acid, metabolism, regulatory T cell, tolerance, lipotoxicity, Th17, cholesterol

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Abbreviations: ACC1, acetyl-CoA carboxylase 1; AMPK, AMP-activated protein kinase; CD5L/AIM, CD5 molecule like/apoptosis inhibitor expressed by macrophages; CNS, central nervous system; CPT1, carnitine palmitate transferase; DAG, diacylglyceride; DCA, dichloro acetic acid; DGAT, diacylglycerol acyl transferase; EAE, experimental autoimmune encephalomyelitis; FA, fatty acid; FABP, fatty acid-binding protein; FAO, fatty acid oxidation; FATP, fatty acid transport protein; FFA, free fatty acid; GPR, G protein-coupled receptor; HDL, high-density lipoprotein; IL, interleukin; LAL, lysosomal acid lipase; GLUT1, glucose transporter 1; LCFA, long-chain fatty acid; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; MCFA, medium-chain fatty acid; MUFA, monounsaturated fatty acid; NFAT, nuclear factor of activated T cells; OXPHOS, oxidative phosphorylation; PDHK, pyruvate dehydrogenase kinase; SCAP, SREBP cleavage-activating protein; PPAR, peroxisome proliferator-activated receptor; RORγt, RAR-related orphan receptor gamma t; RXR, retinoid X receptor; SCFA, short-chain fatty acid; SFA, saturated fatty acid; SNP, single-nucleotide polymorphism; SREBP, sterol regulatory element-binding protein; TCA, trichloroacetic acid; TG, triglyceride; VLCFA, very long-chain fatty acid; VLDL, very low-density lipoprotein.

INTRODUCTION

How Are Dietary Lipids Sensed by Cells, How Do They Signal?

Free Fatty Acids (FFAs)

Free fatty acids are defined as those not bound to albumin or esterified into larger molecules such as triglycerides (TGs) or phospholipids. FFAs have a simple structure of an aliphatic chain of varying length linked to a carboxyl group (**Figure 1**). Fatty acids (FAs) are classified according to their length in carbon atoms, their degree of saturation and whether their double bonds are in *cis* or *trans* orientation. For example, oleic acid, an 18 carbon unsaturated long-chain fatty acid (LCFA), can be abbreviated c9-18:1 indicating it has one *cis* double bond at the ninth carbon atom counting from the carboxyl terminal. FAs with 2–6 carbon atoms are termed short-chain fatty acids (SCFAs), 6–12 as medium-chain fatty acids (MCFAs), 14–18 as LCFAs, and over 20 as very long-chain fatty acids (VLCFAs). Essential FAs (i.e., those which the human body cannot produce) are predominantly diet derived. SCFAs such as propionic acid (C3:0) and butanoic acid (C4:0) are produced by bacteria residing in the gut lumen as a result of fermentation of fiber or dietary carbohydrate (1–5). They have a role in Treg homeostasis as will be discussed later.

Signalling

CD4 and CD8 T cell subsets are heavily dependent on, and influenced by, extra and intracellular FA content for their functions. These cells discriminate between both quantity and quality of FAs. Depending on these parameters, cell fate decisions are made resulting in changes to memory, subset differentiation, pathogenicity, and survival. Before these FA-influenced cellular decisions are made the cells have to recognize FAs, transfer them from the extra- to intracellular environments, signal to nuclear receptors, and convert the FAs into storage TGs or use them as fuel. The mechanisms of FA transport and signaling are diverse. There are numerous binding proteins and receptors for FAs that enable them to remain soluble in the extracellular environment, signal at the plasma membrane, be transported within cells and enable promotion of transcription factor activity. These will be discussed in turn.

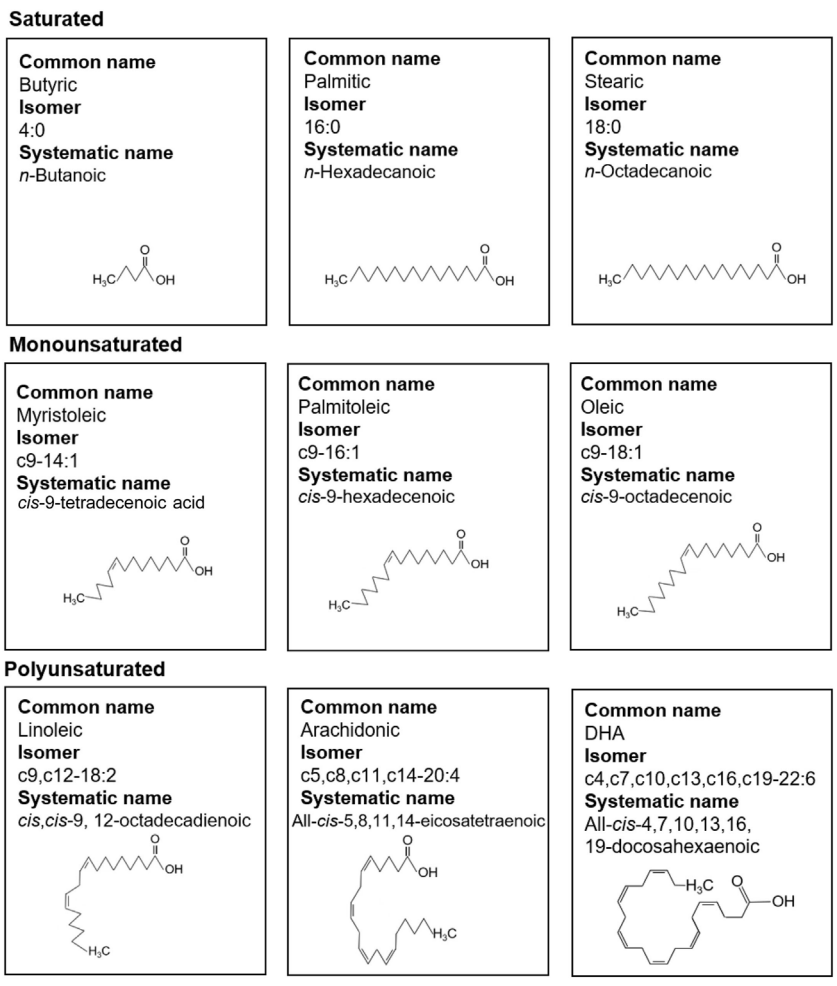

FIGURE 1 | Fatty acid (FA) nomenclature. Common names, isomer formulas, systematic names, and structure of common saturated, monounsaturated, and polyunsaturated FAs.

Extracellular Transport

The human body requires approximately 0.3 mol FA to be transported from adipose tissue to fat-consuming tissues every 24 h (6). This requires approximately 0.3 mM FA concentration in the blood plasma (6). However, FAs have a much lower solubility than this in aqueous solution (7). To enable the concentration in plasma to be elevated to the required level FAs are transported around the body *via* lymphatics and blood in two ways. First, they are made soluble as TGs associated with chylomicrons and very low-density lipoproteins and second, as non-esterified FAs non-covalently bound to albumin. Albumin is an abundant 585 amino acid globular protein (8) containing 17 disulfide bridges (9), imparting great stability to the molecule with a half-life of around 20 days (9). Around 40 g is produced by the liver per day, and one-third to two-thirds of total albumin is in the interstitial compartment (10). Albumin has around seven binding sites for FAs of moderate to high affinity (6). Albumin is the major fatty acid-binding protein (FABP) in blood and interstitial fluid. Binding of FAs to albumin increases their concentration by several orders of magnitude.

Plasma-Membrane FA Receptors

Fatty acids have pleiotropic effects on T cells that depend on the mode of T cell activation, length of the FA, and degree of saturation in addition to the degree of metabolic substrate availability in the cell's environment. In order for extracellular FAs to exert signaling or metabolic consequences on cells they first need to be recognized and/or taken up by the cell. T cell-surface receptors for FAs include G protein-coupled receptors (GPCRs), CD36, fatty acid-binding protein TM ($FABP_{TM}$), and members of the fatty acid transport protein (FATP) family.

G Protein-Coupled Receptors (GPCRs)

Five cell-surface GPRs specific for FAs have been described; GPR 40, 41, 43, 84, and 120. They all have different affinities for FAs of different lengths. GPR41 and 43 have specificity for SCFAs, GPR84 for MCFAs, and GPR40 and GPR120 for LCFAs. However, of these only GPR84, the medium-chain FA receptor has been shown to be expressed by CD4 and CD8 T cells (11). GPR43 has high affinity for SCFAs and has been reported to be expressed by colonic Treg (cTreg) (4). There is some uncertainty about the degree of expression of the SCFA-binding GPRs GPR41 and 43 on colonic T cells (12–14). Expression of SCFA-binding GPRs may be context- or T cell subset dependent.

CD36

Fatty acids may enter T cells through two basic processes. First, there is some evidence that they may enter the cell by passive diffusion, as T cells incorporate FAs into their membranes from their environment (15, 16). FA uptake at the plasma membrane is mostly controlled by membrane transport proteins such as CD36, plasma membrane-associated FABP, and FATPs. CD36 also known as fatty acid translocase is an integral plasma-membrane glycoprotein found on the surface of many cell types. It imports LCFAs inside cells and is a member of the class B scavenger receptor family of cell-surface proteins. CD36 binds many ligands in addition to FAs including oxidized phospholipids (17), oxidized low-density lipoprotein (LDL) (18, 19), native lipoproteins (20), and collagen (21). It has a hairpin membrane topology with two heavily glycosylated transmembrane regions (22). CD36 binds through its extracellular portion to the plasma-membrane FABP, $FABP_{TM}$ and through its cytoplasmic portion to cytoplasmic FABP. The concerted action of this complex of three transport/chaperone proteins is thought to facilitate the diffusion and stabilization of FAs into T cells (**Figure 2**) (22).

Fatty Acid-Binding Proteins

Once inside the cell FAs are bound by FABPs to increase their aqueous solubility in the cytoplasm and to chaperone them to

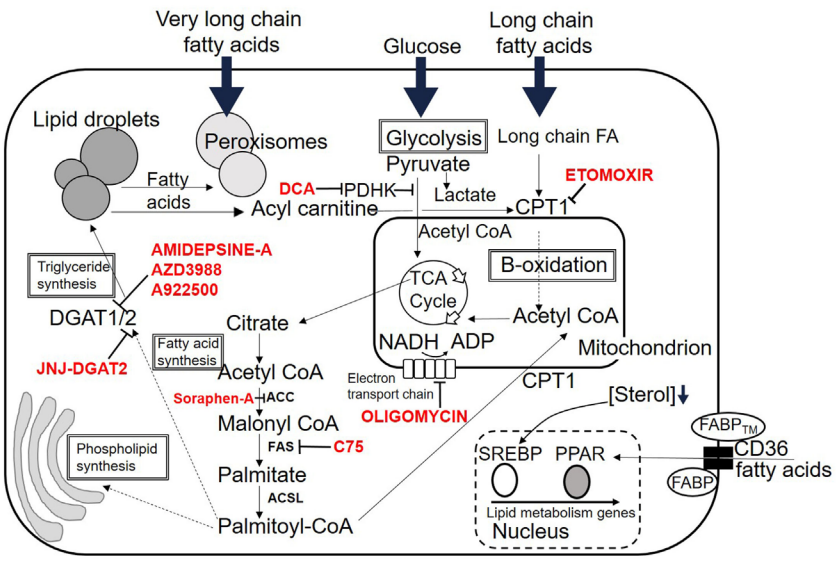

FIGURE 2 | Fates of lipid within the cell. Overview of possible intracellular destinations of fatty acids. Commonly used inhibitor drugs for key pathways are shown in red. Dotted lines indicate multiple intermediate steps not shown due to space. See text for details. Abbreviations are listed at the start of the review.

the correct cellular locations. FABPs comprise a family of nine proteins, most abundantly expressed in tissues involved in lipid metabolism. They can be divided into two groups, those associated with the plasma membrane ($FABP_{TM}$) and cytoplasmic FABPs ($FABP_c$). Each FABP has a different ligand specificity. For example, FABP1 and 5 bind to saturated, monounsaturated, and polyunsaturated FAs with no preference for any of these while FABP3 binds n6PUFAs such as arachidonic acid (23). FABPs have been proposed to coordinate FA uptake, stabilization, transport, and synthesis of FAs (24, 25). They may act as gatekeepers to the nucleus, regulating entry of FAs which signal *via* peroxisome proliferator-activated receptors (PPARs), discussed below. FABP4 and 5 are upregulated in a subpopulation of resident CD8 memory cells (**Figure 3**) and are critical for memory function (26), discussed later.

Fatty Acid Transport Proteins

The FATP family, also known as the solute carrier family 27, are a group of six transmembrane transporters with a heterogeneous tissue and cell distribution. These proteins transport VLCFAs into the cell where they are simultaneously converted into very long-chain acyl-CoA esters. This esterification results in "metabolic trapping" of the FA within the cell, a process also known as "vectorial acylation" (27). A similar process occurs with the hexokinase-mediated phosphorylation of glucose. Although a certain level of redundancy exists with FATP and CD36 and $FABP_{TM}$, single-nucleotide polymorphisms (SNPs) in FATPs may predispose carriers to elevated risk of metabolic disease. For example, SNPs in FATP1 are associated with increased plasma TG levels (28, 29).

FA-SIGNALING RECEPTORS

Peroxisome Proliferator-Activated Receptors

Once in the cell, FAs have multiple fates. In addition to fueling mitochondrial respiration, they also signal *via* nuclear receptors to alter transcription of genes important for lipid homeostasis. PPARs are a subset of the family of nuclear hormone receptors, transcription factors which are activated by lipophilic molecules (30) and control genes mostly involved with lipid metabolism. The PPARs consist of an N-terminal ligand-independent activation domain, a conserved DNA-binding domain, a C-terminal ligand-binding domain, and a C-terminal ligand-independent activation domain (31). PPARs bind to DNA targets on peroxisome proliferator response elements as obligate heterodimers with the retinoid X receptors (RXRs) independent of their ligands (32). They also bind to other transcription factors to either repress or enhance their activity. Binding to RXR and DNA target sequences is of greater affinity and stability when the PPARs are bound to their ligands (33, 34). Most FAs can activate and act as ligands for PPARs but in general, PPARs have a preference for long-chain polyunsaturated fatty acyls (PUFAs) (35). Three different PPAR forms have been cloned. PPAR α and β/δ are associated with highly oxidative metabolically active tissues such as cardiac muscle, brown adipose tissue, and liver whereas PPARγ is more ubiquitously distributed. PPARα is considered a master regulator of lipid catabolic processes and increases transcription of genes associated with lipid catabolism. PPARβ/δ increases metabolism of LCFAs in muscle and decreases glycolaysis during sustained exercise (36).

Peroxisome proliferator-activated receptor γ acts as a nutrient sensor for non-esterified LCFAs and alters transcription in cells to promote their storage as triacyl glycerides (37). PPARγ activates genes associated with transport of FAs across the plasma membrane (CD36 and FABP4). It also activates genes associated with storage of FAs as TGs such as the perilipins. PPARγ also controls metabolic shift from glucose oxidation to TG production by inhibition of pyruvate dehydrogenase and upregulation of enzymes involved in triose production necessary for FA esterification (38, 39).

All three of the PPAR family members have been shown to play a role in T cell activation, proliferation, and differentiation into Th1, Th2, Th17, and Treg lineages (40) PPARγ is thought to inhibit the activity of nuclear factor of activated T cells and subsequent interleukin (IL)-2 production by T cells (40–42). PPARγ

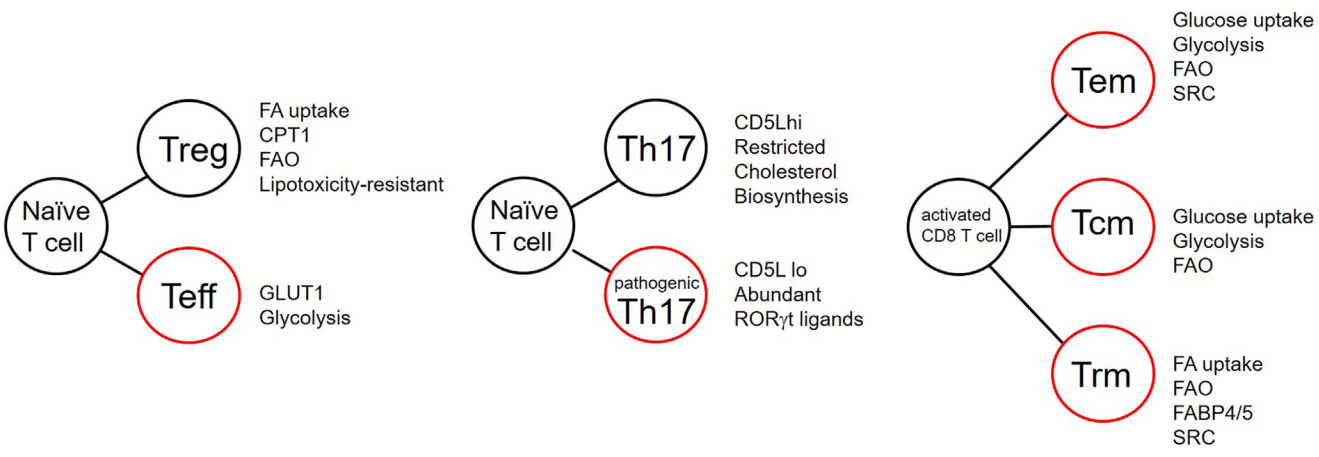

FIGURE 3 | T cell differentiation and the effects of lipid metabolism. Summary of reported metabolic differences between T cell subsets during differentiation. Listed attributes indicate equal or increased features. FA, fatty acid; CPT1, carnitine palmitate transferase; FAO, fatty acid oxidation; GLUT1, glucose transporter 1; SRC, spare respiratory capacity; FABP, fatty acid-binding protein. Cells outlined in red indicate potentially inflammatory subsets.

agonists have also been shown to potently inhibit the induction of inflammation in *in vivo* colitis models (43, 44). The decision of CD4 T cells to differentiate into Th17 or Treg is governed, in part, by PPARγ activity. Conversion of naïve effector T cells into TGFβ-induced Treg (iTreg) induction is enhanced in the presence of the PPARγ ligand ciglitazone (45). Conversely PPARγ deficiency in T cells results in elevated disease scores in the mouse model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), with greater numbers of central nervous system (CNS) infiltrating Th17 cells (46). PPARα and PPAR β/δ also have a potent anti-inflammatory role in EAE models. Treatment with gemfibrozil, a PPARα agonist inhibited EAE disease severity *via* skewing of T cells to a Th2 phenotype (47). Agonists of PPARβ/δ GW-0742 could also inhibit EAE severity partially *via* reduction of IL-1β production (48). PPARγ is upregulated in a specialized adipose tissue-resident Treg subset described in more detail below.

Sterol Regulatory Element-Binding Proteins (SREBPs)

Sterol regulatory element-binding proteins are transcription factors, which activate all genes necessary for FA synthesis (49). They have a basic helix-loop-helix leucine zipper structure (23) and exist in two forms produced by differential exon usage, resulting in two separate promoters (50). SREPB1 activates genes involved in *de novo* lipogenesis whereas SREBP2 activates genes necessary for cholesterol synthesis and uptake. SREBPs are made as cytoplasmic precursor molecules and must be cleaved and transported to the nucleus before binding to their target genes (51). When there is sufficient cholesterol in the cellular environment, SREBPs complex with SREBP cleavage-activating protein (SCAP) a cholesterol sensor and chaperone (52, 53), which keeps the complex tethered to the endoplasmic reticulum in an inactive state (51). Reduction in the cholesterol concentration within the cell results in a conformational change in SCAP allowing SCAP/SREBP translocation to the Golgi apparatus. Here, SREBPs are sequentially cleaved by site 1 and site 2 proteases (S1P and S2P) releasing the N-terminal portion to transfer into the cell nucleus (54, 55). SREBPs bind to consensus regions in the promoters of their target genes termed insulin response elements. Target genes include those involved in synthesis of cholesterol from acetyl-CoA and transfer of cholesterol into the cell (49). They are controlled by phosphorylation and degraded by ubiquitination. SREBs have been shown to be crucial to licensing blastogenesis and expansion of CD8 T cells in response to viral infection (56). In this context, SREBs are required for supplying sufficient lipids for membrane synthesis to allow expansion. An overview of lipid fates within T cells is shown in **Figure 2**.

A ROLE FOR LIPID METABOLISM IN T-CELL SUBSET DIFFERENTIATION

Metabolic Requirements Change during the Life of a T Cell

T cells change their metabolic mode to fulfill requirements placed upon them during development, activation, proliferation, and formation of memory. Activation signals *via* the T cell receptor induce a program of blast formation and extensive cell division. This is both energy-demanding and requires formation of new cellular components such as membranes, DNA, and proteins for increased cell size and mitosis. To meet these requirements activated T cells adapt to preferentially utilize glucose and aerobic glycolysis to fuel ATP production. Aerobic glycolysis is less efficient in production of ATP than oxidative phosphorylation (OXPHOS). Despite this T cells take advantage of the fact that products of the glycolytic, and linked pentose phosphate, and trichloroacetic acid pathways such as citrate and ribose-5-phosphate are precursors of membrane and nucleic acids, both required for organelle biogenesis during proliferation. Microenvironmental cues in the form of cytokines and co-stimulatory triggers guide T cells down different functional routes including multiple CD4 helper T cell subsets and regulatory T cells. In addition to these cues, it is becoming clear that metabolic substrate availability is also a driver of T cell fate, discussed below. At the culmination of an immune response, T cells either enter into apoptosis or revert to non-dividing memory T cells. Memory T cells revert to lipid oxidation to generate energy, being quiescent they are less dependent on organelle biogenesis.

CD8 T Cells

CD8 T cells have been shown to have distinct requirements for FAs to fuel memory differentiation, and subset specialization (**Figure 3**). Pearce and colleagues (57) generated CD8 effector and central memory cells (T_{CM}) *in vivo*, in a murine listeria monocytogenes-ovalbumin (OVA) infection model of OT-1 chicken egg OVA-specific TCR transgenic mice. T_{CM} have elevated fatty acid oxidation (FAO) when compared with effector CD8 T cells. Paradoxically, these cells also take up less FAs from their environment. Instead, CD8 T_{CM}, in this model, use extracellular-derived glucose to fuel FA synthesis and TG synthesis. The cells then hydrolyze these lysosomally stored TGs using the enzyme lysosomal acid lipase in a process termed "cell-intrinsic lipolysis." The reason why CD8 T_{CM} in this model engage this type of "futile cycle" is not currently understood.

Subset specialization in memory CD8 T cell metabolism has recently been reported (26). Kupper and colleagues demonstrated that tissue-resident memory CD8 T cells (T_{RM} cells) in human and mouse differ from T_{CM}. T_{RM} are a tissue-resident population of memory T cells, which may be CD4+ or CD8+, which reside in barrier epithelia, and persist for long periods to protect the host from pathogenic bacteria and viruses (58–60). CD8 T_{RM} are transcriptionally distinct from central memory cells (61). When OT-1 TCR transgenic mice were inoculated with recombinant vaccinia virus expressing OVA, they showed that the T_{RM} that this protocol generated have elevated expression of proteins involved with FA uptake and FAO compared with T_{CM}. This included expression of FABPs 4 and 5 (FABP4,5) (**Figure 3**). T_{RM} have an increased requirement for FA metabolism compared with central memory cells or effector CD8 T cells. T_{RM} lacking FABP4 and 5, or those treated with inhibitors of FAO have attenuated function and reduced persistence in skin epithelia (26).

Thus, both subset specialization and environmental localization have a role in determining the metabolic requirements of CD8 memory T cells, with central memory cells appearing to be

less dependent on environmental FAs than their tissue-resident counterparts for stability and functional competence.

CD4 T Cells

A fundamental question that remains to be answered in immunometabolism is whether environmental metabolic substrate availability drives T cell differentiation, or whether cell-intrinsic programs dictate metabolic requirements which are then selected by the environment. Several recent publications have provided evidence that CD4 cell fate determination is likely the combination of both processes. The choice of development into either iTreg or Th17 lineage is determined by cell extrinsic and intrinsic cues. These include cytokines TGFβ, IL-6, IL-23, metabolic substrate availability, transcription factor expression [Foxp3, RAR-related orphan receptor gamma t (RORγt)], and the activity of key metabolic enzymes (12, 26, 40–42, 46, 57, 62–69). CD4 Teff and iTreg have been reported to have different metabolic requirements (70). Rathmell and colleagues reported that Teff have elevated glucose transporter 1 expression, preferential requirement for glucose, and higher levels of glycolysis than iTreg (70, 71), which rely on FAs as their preferred metabolic substrates. In the absence of FAs or inhibited FAO, iTreg are unable to develop *in vitro*. iTreg have elevated AMP-activated protein kinase (AMPK) compared with effector T cells, and activation of AMPK is sufficient to skew differentiation toward the iTreg lineage both *in vitro* and *in vivo* (70). In this study, palmitate exposure induced apoptosis selectively in Teff, suggesting that availability of lipids may select for preexisting Treg in addition to imparting a metabolic advantage. Indeed, Foxp3 expression is sufficient to re-program cells to upregulate many proteins and enzymes associated with FAO and mitochondrial OXPHOS (65) including many components of the mitochondrial electron transport system. Expression of Foxp3 imparts selective survival of cells exposed to saturated LCFAs palmitate and stearate at moderately raised physiological concentrations. This effect is dependent on FAO in these cells as inhibitory drugs, targeting several enzymes of the FA β-oxidation pathway, reverse the protective effect (65).

Foxp3 may be required to protect Treg in environments high in FAs, but also in environments low in glucose and high in lactate, such as the intestinal tract and ischemic tissues (72). Foxp3 was reported to suppress Myc and glycolysis, thus enhancing OXPHOS and NAD regeneration, protecting Treg from lactate-mediated inhibition of proliferation (62). While favoring immune tolerance in ischemic tissues and the gut, these mechanisms may also be detrimental to immune defense against tumors.

There are several reported metabolic checkpoints involved in controlling whether CD4 T cells develop into Th17 effector cells or iTreg under identical environmental conditions. A recent report highlighted the role of the enzyme pyruvate dehydrogenase kinase (PDHK) in selective regulation of T cell differentiation and inflammation (64). Pyruvate dehydrogenase was identified as a bifurcation point in the choice between glycolytic and oxidative metabolism (**Figure 2**). Th17 cells express higher levels of PDHK than Th1 or Treg, and inhibition of PDHK resulted in preferential expansion of Treg. This effect was partly due to the effects of elevated reactive oxygen species generated following PDHK inhibition, to which Treg are resistant.

A selective requirement for *de novo* FA synthesis has been reported for Th17 cell development and functions (63). Th17 cells are thought to favor a glycolytic/lipogenic mode of metabolism for their development which requires acetyl-CoA-carboxylase 1 (ACC1). ACCs catabolize the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, essential for FA synthesis in the cytosol. Th17 cells use this pathway for production of cellular membrane phospholipids, whereas Treg preferentially take up exogenous FAs for this function. Inhibition of ACC1 in human and mouse T cells impairs the development of Th17 cells and preferentially allows development of Treg (63).

Fatty acid metabolism has also been reported to control pathogenicity within the Th17 compartment (68). Activation of T cells in the presence of the cytokines TGFβ and IL-6 promotes differentiation of IL-17-producing cells, which are poor at inducing EAE. Addition of IL-23 to these cultures induces cells which produce IL-17 and are also potent inducers of EAE pathology. These cells are termed "non-pathogenic" and "pathogenic," respectively. Kuchroo and colleagues identified CD5 molecule like/apoptosis inhibitor expressed by macrophages as a molecule expressed in non-pathogenic but not pathogenic Th17 cells (**Figure 3**). CD5L modulates the intracellular lipidome through modifying FA synthesis *via* binding to FA synthase. In this way, it inhibits FA synthesis. CD5L also alters the FA composition including the inhibiting the amount of PUFAs such that cholesterol biosynthesis is inhibited through inhibition of the enzymes sc4mol and cyp51 (**Figure 3**) (68). Consequently, the concentration of available RORγt ligands is reduced in the cell. They showed that saturated fatty acid (SFA) increased whereas PUFA decreased binding of RORγt to the *Il17* and *Il23r* loci. CD5L is a general inhibitor of Th17 pathogenicity as its removal converts non-pathogenic Th17 cells into pathogenic cells capable of causing inflammation *in vivo* (68).

Cholesterol Biosynthetic Intermediates and T-Cell Functions

Cholesterol and its biosynthetic intermediates have profound effects on multiple aspects of immunity. These include roles in B lymphocyte homing to lymph nodes (73), control of viral replication (74), macrophage phagocytosis (75), inflammasome activation (76), antitumor responses of CD8 T cells (77), and neutrophil traps (78). Cholesterol metabolites, particularly oxysterols are increasingly being shown to have roles in T cell development, function, and migration (**Figure 4**) (75). Cholesterol derivatives signal in T cells *via* the liver X receptor family (LXR) of transcription factors. LXRα and LXRβ transcription factors have multiple positive and negative effects on transcription in many cell types (75). LXRα is predominantly expressed in adipose tissues where it controls genes involved in catabolism of cholesterol while LXRβ is expressed ubiquitously including in lymphocytes. The ligands for LXRs *in vivo* include cholesterol precursors and oxysterols. These include desmosterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (79). LXRs not only control genes involved in cholesterol and FA biosynthesis but also suppress the activity of genes under control of NF-κB and AP-1 (80, 81). LXR ligation may have pro- or anti-inflammatory

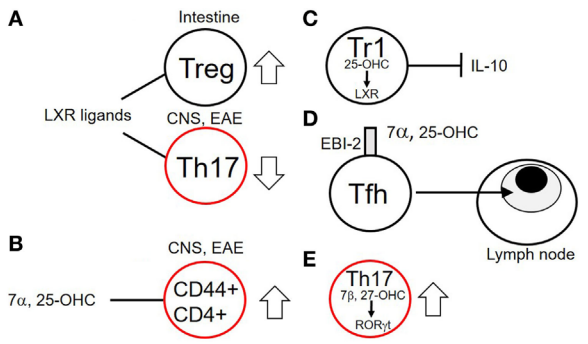

FIGURE 4 | Effects of cholesterol biosynthetic intermediates on T cell functions. **(A)** Liver X receptor family (LXR) ligands increase Treg numbers in the intestine while decreasing numbers of Th17 cells in the central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis (EAE). **(B)** The oxysterol 7α,25-OHC increases numbers of activated CD44+ CD4+ T cells in the CNS of mice with EAE. **(C)** Tr1 cells produce the sterol 25-OHC which *via* binding to the LXR inhibits production of interleukin (IL)-10 in a negative feedback loop. **(D)** 7α,25-OHC *via* its plasma-membrane receptor EBI-2 promotes migration of T follicular helper (Tfh) cells to the T cell zones proximal to B cell follicles. **(E)** Th17 cells produce 7β,27-OHC, an endogenous ligand for the RAR-related orphan receptor gamma t (RORγt) transcription factor, necessary for their function. Cells outlined in red indicate potentially inflammatory subsets.

roles depending on the cell type and sterol involved. In EAE, LXR ligation is protective (**Figure 4B**) (82). Mice deficient in LXR have increased infiltration of inflammatory cells into the spinal cord and more severe demyelination (83). LXR ligand treatment of the EAE model results in decreased disease severity, decreased Th17 polarization, and a reduction of IL-17 (84). Pharmacological LXR agonists have been reported to enhance Treg differentiation, increasing the number of gut Treg in mice (**Figure 4A**) (85). Certain oxysterols have also been reported to play pro-inflammatory roles in EAE (86). Chalmin et al. reported a pro-inflammatory role for 7α, 24-hydroxy cholesterol (7α, 25-OHC) in EAE (86). They showed that 7α, 25-OHC promoted increased migration into the CNS of activated CD44+ CD4+ T cells, *via* the cell-surface reporter EBI-2 (GPR183). Deletion of the enzyme responsible for production of 7α, 25-OHC, cholesterol 25 hydroxylase, reduced the severity of EAE by limiting trafficking of pathogenic CD44+ CD4+ cells into the CNS. A potentially pro-inflammatory role for 25-OHC has been described in IL-27-induced type-1 regulatory (Tr1) cells (**Figure 4C**) (87). These cells express 25-OHC and cholesterol 25 hydroxylase. 25-OHC inhibits IL-10 production *via* the LXR, inhibiting the regulatory potential of these cells. Cyster and colleagues described a mechanism for 7α, 25-OHC in mediating the correct migration of T follicular helper (Tfh) cells in the lymph node between the T cell zone and B cell follicle, *via* the receptor EBI-2 (**Figure 4D**) (88). A positive role for oxysterols has been established for Th17 function (**Figure 4E**) (89, 90). The transcription factor RORγt is expressed in lymphoid tissues and is crucial for development of thymocytes, lymph nodes, gut associated lymphoid tissue and Th17 cells (91–94). For optimal activity RORγt needs to bind to cholesterol derivatives *via* its ligand-binding domain. The oxysterols 7β, 27-dihydroxycholesterol (7β, 27-OHC) is the most potent oxysterol ligand for RORγt (89). Binding of 7β, 27-OHC enhances Th17 differentiation. Th17 endogenously produce both 7β,27-OHC and 7α,27-OHC, and it has been shown that mice lacking the enzyme responsible for production of 7β,27-OHC (CYP27A1) have a deficiency in Th17 cells (89). Inhibition of cholesterol esterification in CD8 T cells by inhibition of the enzyme ACAT1 has been shown to potentiate antiviral CD8 T cell functions (77). This was shown to be a result of elevated cholesterol in the plasma membrane, which enhances TCR signaling and formation of the immunological synapse. This result suggests that cholesterol metabolism may represent a novel target for cancer therapy.

SCFAs and Colonic cTreg

Fatty acids have a major role in shaping a population of regulatory T cells resident in the mucosal layer of the colon (1, 3–5). cTreg play an important role in maintenance of tolerance to antigens derived from food and bacterial flora. These cells depend on resident gut bacteria of the *Bacteroides* and *Clostridia* species for their induction and function (2, 66). Gut bacteria are required to break down indigestible dietary fiber and carbohydrates. SCFAs such as acetate, propionate, and butyrate are produced by bacteria as a result of fermentation of such dietary components. The gut lumen has between 50 and 100 mM SCFA content (95); however, the concentration of SCFA in the gut lumen of germ free mice is markedly reduced compared with mice housed in specific pathogen-free conditions (4). The size of the gut cTreg pool is thought to be controlled by the bacterially derived SCFA concentration. Addition of propionate to the drinking water of germ-free mice increases their cTreg numbers (4) but has no effect on numbers of splenic, mesenteric, or thymic Treg numbers. Conversely, inhibition of colonic bacterial numbers with vancomycin results in a reduction in cTreg numbers, which is reversible by addition of SCFA to the drinking water of mice. The SCFA butyrate is an inhibitor of class I and IIa histone, deacetylases and, as such, has a potent effect on histone 3 acetylation surrounding the promoter and conserved non-coding regions 1 and 3 of the Foxp3 locus, regions essential for induction of peripheral Treg (1). SCFA induction of cTreg depends on the Foxp3 enhancer conserved non-coding sequence-1 (CNS1), showing that this induction is *via de novo* induction of Treg locally, as thymic Treg do not require CNS1 for their development (69). Maintenance of immune tolerance *via* SCFA Treg induction requires the SCFA receptors GPR43 on colonic epithelial cells in addition to GPR109A on dendritic cells and, in addition to enhancing Treg numbers, increases the tolerogenic properties of CD103-expressing colonic dendritic cells (5).

Visceral Adipose Tissue Treg

Adaptations to preferential FA metabolism are seen in a specialized subset of Treg residing in lean (visceral) fat tissue, termed visceral adipose tissue Treg or VAT Treg (96–98). These cells accumulate in visceral fat early in life (98) and expand in an MHC/peptide and IL-33-dependent fashion (98). The cells differ from conventional Treg in several ways. They constitute a very high proportion of CD4+ T cells in adipose tissue (40–80%) (97) and have a transcriptional profile that is different from conventional Treg, overexpressing chemokine receptors CCR1 and CCR2 and

IL-10 (97). They also overexpress several transcripts associated with FA metabolism such as diacylglycerol acyl transferase 1, CD36, and low-density lipoprotein receptor. These cells have a distinct T cell receptor repertoire from conventional Treg. Many of these differences are due to expression of the adipocyte master regulator transcription factor PPARγ which, together with Foxp3, cooperates to program their specialized function (96). PPARγ is necessary for VAT Treg to accumulate in visceral fat and to inhibit inflammation within obese fat, restoring responsiveness to insulin. Treg-specific knock ut of PPARγ results in fewer VAT Treg, but no change in splenic Treg number or function. Pioglitazone, a synthetic PPARγ agonist, increases the number of VAT Treg in high fat diet-fed obese mice but has no effect on the numbers of splenic Treg (98).

FAs and Lipotoxicity

The increasing prevalence of obesity worldwide is leading to an epidemic of related health problems including diabetes and coronary artery disease. Much of the harm done to individuals with elevated body mass indices arises from raised plasma free fatty acid levels. This has been shown to trigger the metabolic syndrome (99). Adipocytes are adapted to store excess TGs as fat droplets, but non adipose cells such as pancreatic beta cells, hepatocytes and lymphocytes have a limited capacity to convert FFAs to TGs in fat droplets. In such cells exposure to elevated FFAs can result in cellular damage and ultimately cell death, a process called lipotoxicity (65, 100, 101).

Exposure of T cells to FAs and lipids in culture has varied effects depending on the type of FA and the concentration. Exposure of human Treg to high-density lipoproteins (HDL), but not LDLs significantly reduces these cells' apoptosis in response to serum starvation in *in vitro* cultures, but has little protective effect on naïve and memory CD4 T cell survival under the same conditions (102). This was reported to be due to HDL operating *via* the scavenger receptor class B type I, increasing spare respiratory capacity and basal respiration in Treg. Low doses of FAs may induce T cell activation with higher doses resulting in apoptosis (103–106). Moderately raised physiological levels of saturated FAs induce in primary T cells or T cell lines cytochrome-*c* release from mitochondria, loss of mitochondrial membrane potential, externalization of phosphatidyl serine (65), caspase activation, and DNA fragmentation (105) indicating an apoptotic mechanism. Loss of CD4 T cells in non-alcoholic fatty liver disease has been attributed to mitochondrial damage and apoptosis induced by reactive oxygen species released in response to linoleic acid (107).

T cells can convert excess exogenous FAs into neutral lipids such as triacyl glycerides and cholesterol esters (**Figure 2**). Channeling of dietary LCFAs to distinct metabolic routes has been shown to correlate with their propensity to induce lipotoxicity in many non-lymphoid cell types (100). This channeling of FAs into neutral lipids, stored as intracellular lipid droplets is protective to the cell. In general saturated LCFAs can induce cytotoxicity whereas monounsaturated FAs are non-toxic or are cytoprotective to cells (101). It has been shown in multiple cell types that addition of monounsaturated fatty acid (MUFA) to cells dose dependently protects against the cytotoxic effects of SFA by inhibiting FA synthesis and channeling FAs to TGs (100, 108–111). The protective effect of MUFAs in lipotoxicty occurs *via* a mechanism involving the endoplasmic reticulum MUFA sensor UBXD8. UBXD8 inhibits TG synthesis by blocking conversion of diacylglycerides to TGs. An excess of MUFAs relieves this inhibition, licensing production of inert TGs, and thus protecting the cell from lipotoxicity (108). It remains to be seen whether this mechanism of protection from lipotoxicity by MUFAs operates in T cell subsets; however, upregulation of FAO pathways by Foxp3 endows Treg with a selective survival advantage during exposure to raised SFA concentrations *in vitro* (65).

Potential Therapeutic Applications

Because the metabolism of T lymphocytes is so closely linked to their activation, differentiation, and survival, there is tremendous interest in manipulating metabolic processes for therapeutic purposes. This topic has been well reviewed recently (112–115) so only a brief summary of potential lipid metabolic drug targets will be described here. It is likely that many existing drugs used to normalize metabolic imbalances might be "repositioned" for use in other indications including treatment of autoimmunity and graft-versus-host disease (GVHD). Statins are drugs that inhibit cholesterol synthesis by inhibiting the action of the enzyme HMG-CoA-reductase, an enzyme that generates mevalonate, a key intermediate in this pathway. Prescribed for the treatment of raised plasma cholesterol, they are one of the most prescribed drugs in the world. Statins have a potent inhibitory effect on differentiation of Th17 cells, skewing differentiation toward Treg (116, 117). Simvastatin was shown to promote Treg differentiation and inhibit Th17 development under Th17 polarization conditions. These effects were dependent on inhibition of protein geranylgeranylation by the drug (116). Simvastatin may also inhibit the inhibitory SMADS; SMAD6 and SMAD7, the consequence being that the drug synergizes with low amounts of TGFβ to generate pTreg (117). Statins also reduce the intracellular concentration of desmosterol, a cholesterol precursor and potent endogenous RORgt ligand (118). It is likely that this property of statins may be exploited for inhibition of Th17-mediated inflammatory conditions in the future.

The PPARα agonists, gemfibrozil, and fenofibrate are oral drugs widely prescribed for the treatment of hypertriglyceridemia. Both are able to treat ongoing signs of EAE in mice (119). Inhibition of PPARα in T cells was shown to skew the immune response, promoting IL-4 production and inhibiting IFN-γ. These results suggest that the PPARα agonist family of drugs might be repositioned for use in autoimmune diseases such as MS.

Activated inflammatory T cells in GVHD have been shown to rely on OXPHOS for proliferation (120). These alloreactive effector T cells have a strong preference for FAs to fuel this metabolic mode, upregulating transcriptional coactivators for lipid catabolism and increasing their FA uptake. Inhibition of FA beta-oxidation with etomoxir reduced the survival of alloreactive effector T cells but has no effect on syngenic T cell expansion. These observations raise the prospect of modulating lipid metabolism to selectively inhibit alloreactive T cells in GVHD using drugs such as etomoxir or perhexiline (121).

CONCLUSION

T cell differentiation, functions, and survival are increasingly demonstrated to be linked to processes of metabolism, particularly lipid metabolism. CD4 and CD8 subset differentiation, memory, effector function, and survival are dependent on various aspects of lipid synthesis, catabolism, and storage. There is intense interest in revealing aspects of metabolism, which are uniquely required for particular T cell subsets, so as to identify opportunities for therapeutic manipulation. The challenges, as the field progresses, will be to identify those differences that are "programmed" by transcription factors as compared with those which result from environmental cues. The links between metabolic processes, cell signaling, genetic, and epigenetic control are just beginning to be identified and represent an exciting new dimension in the area of immune regulation.

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Minimum Information about T Regulatory Cells: A Step toward Reproducibility and Standardization

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Cellular therapies with CD4+ T regulatory cells (Tregs) hold promise of efficacious treatment for the variety of autoimmune and allergic diseases as well as posttransplant complications. Nevertheless, current manufacturing of Tregs as a cellular medicinal product varies between different laboratories, which in turn hampers precise comparisons of the results between the studies performed. While the number of clinical trials testing Tregs is already substantial, it seems to be crucial to provide some standardized characteristics of Treg products in order to minimize the problem. We have previously developed reporting guidelines called minimum information about tolerogenic antigen-presenting cells, which allows the comparison between different preparations of tolerance-inducing antigen-presenting cells. Having this experience, here we describe another minimum information about Tregs (MITREG). It is important to note that MITREG does not dictate how investigators should generate or characterize Tregs, but it does require investigators to report their Treg data in a consistent and transparent manner. We hope this will, therefore, be a useful tool facilitating standardized reporting on the manufacturing of Tregs, either for research purposes or for clinical application. This way MITREG might also be an important step toward more standardized and reproducible testing of the Tregs preparations in clinical applications.

Keywords: minimum information model, T regulatory cells, immunotherapy, good manufacturing practice, cell therapy, immune tolerance

INTRODUCTION

T regulatory cells (Tregs) are dominant cellular compounds of the immune system protecting the body from autoimmune reactions. These cells are also involved in imposing tolerance to alloantigens such as transplanted allogeneic cells and tissues (1–5). For all these reasons, several Treg-based therapeutics are being tested in clinical trials as a prophylaxis or treatment of autoimmune diseases, graft-versus-host disease after hematopoietic stem cell transplants or rejections after solid organ transplants (6). The list of potential applications in the future is even wider. At the same time, manufacturing of Tregs

for preclinical and clinical experiments varies considerably between different centers, which significantly diminishes possible comparisons between the trials. For this reason, future development of these therapies is hampered as it happens that the available results from different trials are contradictive. The specificity of cellular products makes it difficult to verify the results in huge multicentre trials and therefore better standardization of early-phase trials as well as cellular products themselves might facilitate the progress in this promising branch of medicine.

We propose here a tool for standardization of Tregs studies designed on the basis of so-called minimum information models (MIMs). These models have gained increasing popularity among scientists as they enable the interpretation of reported data, comparison between data from different studies and facilitate experimental reproducibility (7, 8). MIMs provide mechanisms that all laboratories report at least the key facts about their analysis in a clear and consistent manner, allowing a comparison across the whole field. Our consortium has already designed the MIM called minimum information about tolerogenic antigen-presenting cells (MITAP). This is a reporting framework that makes transparent differences and similarities of different tolerogenic antigen-presenting cells (tolAPC) (9). It provides minimum reporting guidelines for the production process of tolAPC used in preclinical and/or clinical studies. We have followed the MITAP experience and designed a MIM for the manufacture of Tregs. We call it minimum information about T regulatory cells (MITREG). MITREG will be a useful resource for investigators reporting their data on the use of *in vitro* expanded natural Tregs or induced Tregs in preclinical models or clinical trials.

METHODS

Setting Up MITREG: Community Building and Initial Analysis

The community was mainly built on the experience of our completed MITAP initiative. For several years now, we have been working together in the field of tolerogenic cellular therapies under the umbrella of the consortium AFACTT (action to focus and accelerate cell-based tolerance-inducing therapies—http://www.afactt.eu/). It brings together European scientists and clinicians with the aim of jointly addressing issues related to the translation and clinical application of these new treatments. Having the experience of MITAP, we used this document as a template to describe Treg therapies. For MITREG, we also tried to extend the initiative beyond Europe and invited scientists working on tolerogenic cellular therapies from around the world. This way we ensured a broadly reflective discussion taking into account various opinions and current practices of many laboratories within the discipline.

The work on this MITREG document covered a series of "exercises" that provided some initial data. Like for MITAP, the exercises aimed at gathering "terms" in order to acquire basic vocabulary in use within the community. The first, so-called "sticky-note" exercise performed at several AFACTT meetings assumed that each participant wrote a term on a sticky-note; these were then collated and clustered on a wall by the whole group, identifying synonyms and related terms. Second, we used the MITAP template to incorporate the collected terms and created an initial version of MITREG. This document underwent several rounds of face-to-face and online consultations with AFACTT members to improve its clarity. Internally agreed version was circulated to external specialists in the field. This external feedback was collected and implemented in the final version of the MITREG document. Finally, we used the existing literature to obtain a picture of how well the required information has been described in published articles.

RESULTS

Overview of the MITREG Document

The design of the MITREG document followed the concept of MITAP, which facilitated the whole process. It describes the manufacturing of Treg products in a chronological way. The document is divided into four sections highlighting critical points of the process and regulatory issues. The document describes the details that should be provided by investigators, which would allow other researchers to repeat the process. It also advises on the use of existing taxonomies and databases to provide the information in a uniform manner, and it suggests the use of other MIMs where appropriate. The full MITREG document can be found on archive.org (http://w3id.org/ontolink/mitreg) and it is also included in the Appendix A (MITREG document).

Section 1: Cells at the Start of the Procedure

This section describes characteristics of the biological material *before* it undergoes any manipulation. There are five subparts asking for (a) essential information about the donor, (b) source of the cells, (c) the methods used to separate Tregs, (d) the phenotype after separation, and (e) the number of Tregs after separation.

Section 2: Expansion/Differentiation

This section describes the protocol that has been used to expand or differentiate Tregs. The specificity of Tregs was a challenge here as different subsets can be obtained with a wide range of methods. Tregs can be either isolated and optionally expanded or can be induced from naive precursors. There are five subsections giving details on (a) preculture conditions, (b) culture conditions, (c) the protocol used to expand or differentiate cultured Tregs, (d) stimuli used during the process, and (e) the way Tregs are stored immediately after expansion/differentiation.

Section 3: Cells after Expansion/Differentiation

This section describes the characteristics of Tregs *after* the expansion or differentiation. It is mainly focused on the phenotype of the final Treg product as well as its suppressive activity verified in any form of functional assay. It also documents the cell yield from the entire process and, if the product is for clinical use or testing

of adoptive transfer in animals, the details on administration of the cells to the recipient.

Section 4: About the Protocol

This final section describes remaining details of the experimental or clinical protocol such as primary or secondary goals as well as regulatory issues such as adherence to particular acts or directives including compliance with good practice requirements (GCP, GLP, or GMP guidelines). Finally, the name and contact details of the corresponding author(s) must be provided.

The MITREG document is accompanied by a handy checklist to assist investigators in ensuring that all the relevant detail is provided before submitting their manuscripts for publication. The checklist can be found at archive.org (http://w3id.org/ontolink/mitreg) and is also included in the Appendix B (MITREG checklist).

Prevalence of MITREG Data in Extant Published Articles

The purpose of the MITREG document is to ensure that authors provide sufficient basic information about their production protocol. An implicit assumption is that currently some or all of this information is not being routinely described. To test this assumption, we reviewed a number of articles about Treg products and for each we determined whether it included data described in the MITREG document.

In detail, 19 Treg articles were selected (predominantly from members of AFACTT or from researchers well known in the field) and read in detail. The articles are given chronologically in the references but the order in **Figure 1** is different and anonymized (10–28). For each section of MITREG, we determined whether the information required was directly stated in the article (or referenced) (**Figure 1**: green squares), partly stated in the article (**Figure 1**: yellow triangles), not present at all (**Figure 1**: red circles), or whether information was not present due to lack of relevance for the publication (**Figure 1**: gray circles). For example, section 1-ai of MITREG describes the species used in the experimental setup. An article with the phrase "human" or "*Homo sapiens*" would fall into the first category (*included in the publication*). However, when mice are used and only the species is mentioned: "mouse" or "*Mus musculus*," but not the strain, it would fall into the second category (*included but details missing*). Many articles do not describe their experimental methodology, but instead refer to another article ("as described previously"); in this case, we checked the article up to two references deep and if found, the information was considered as "present" (**Figure 1**: green squares), if not it was considered as "not present" (**Figure 1**: red circles). This work was performed by four independent scientists with experience in the field.

Results are shown in **Figure 1**. This figure shows that in some sections like the species, characteristics, ethics, and cell dose transferred sections, reporting is good with almost all revised articles describing these. However, other sections are often very poorly reported. For example, storage of cells, anticoagulant used and the number/viability of cells after each separate step are not described in most articles. Moreover, important information (container type, concentration of cells) to repeat the performed experiments is missing in almost all articles.

Sustainability

We have taken particular care to consider the issues of digital sustainability for MITREG. A well-known problem with resources linked with URLs given in articles is that URLs are often lost over time: around a 25% loss 3 years after publication (29). We have, therefore, specifically addressed this issue by use of a stable identifier space; the MITREG document and checklist are hosted by archive.org, an organization committed to long-term digital preservation. In addition, we have used a permanent identifier (http://w3id.org/ontolink/mitreg) thereby providing a redirection-step.

Resources are available in a number of formats: both PDF and Word for manipulability, but also a simple HTML representation, ensuring vendor-neutrality and future-proofing, in so far as this is possible.

DISCUSSION

Minimum information models aid investigators by providing a specific guideline of what is required to interpret and compare experimental findings. Furthermore, reporting guidelines will facilitate independent validation of published results, a fundamental precept of scientific research. This is to our knowledge the first proposal of a minimum information standard on the description of experimental as well as clinical manufacturing and application of Tregs. The generation of MITREG was initiated by members of the European AFACTT consortium to fill a recognized gap in data reporting standards in the Treg community. MITREG was realized with the help of key international players in the Treg field.

Nine years after the first-in-man report, there are currently close to 30 recruiting or ongoing clinical trials administering Tregs in autoimmune settings, inflammatory diseases, transplantation and graft-versus-host disease (6). Clinical grade reagents for Treg isolation by magnetic activated cell sorting have become available to the growing community and off the shelf products and GMP-compatible fluorescence-based cell sorting is currently been available from multiple manufacturers of novel closed system devices, further increasing the diversity of isolation techniques (30). Given the low frequency of Tregs in the periphery, most clinical applications require an *in vitro* cell expansion culturing step classifying them as advanced therapy medicinal products. A growing number of culturing methods are being developed and published aiming at Treg induction, enhanced *ex vivo* expansion, alloreactivity and more recently, the implementation of specific T cell receptors or chimeric antigen receptors (17, 18, 25, 31–39). We are thus at a point where protocol diversity is growing exponentially, emphasizing the necessity to harmonize reporting regimens as a prerequisite of reproducibility and quality assurance. By analyzing extant articles according to the MITREG document (**Figure 1**), it also becomes clear that there is a big gap in what is currently being reported and what the community considers important and wants to receive in a Treg production/expansion

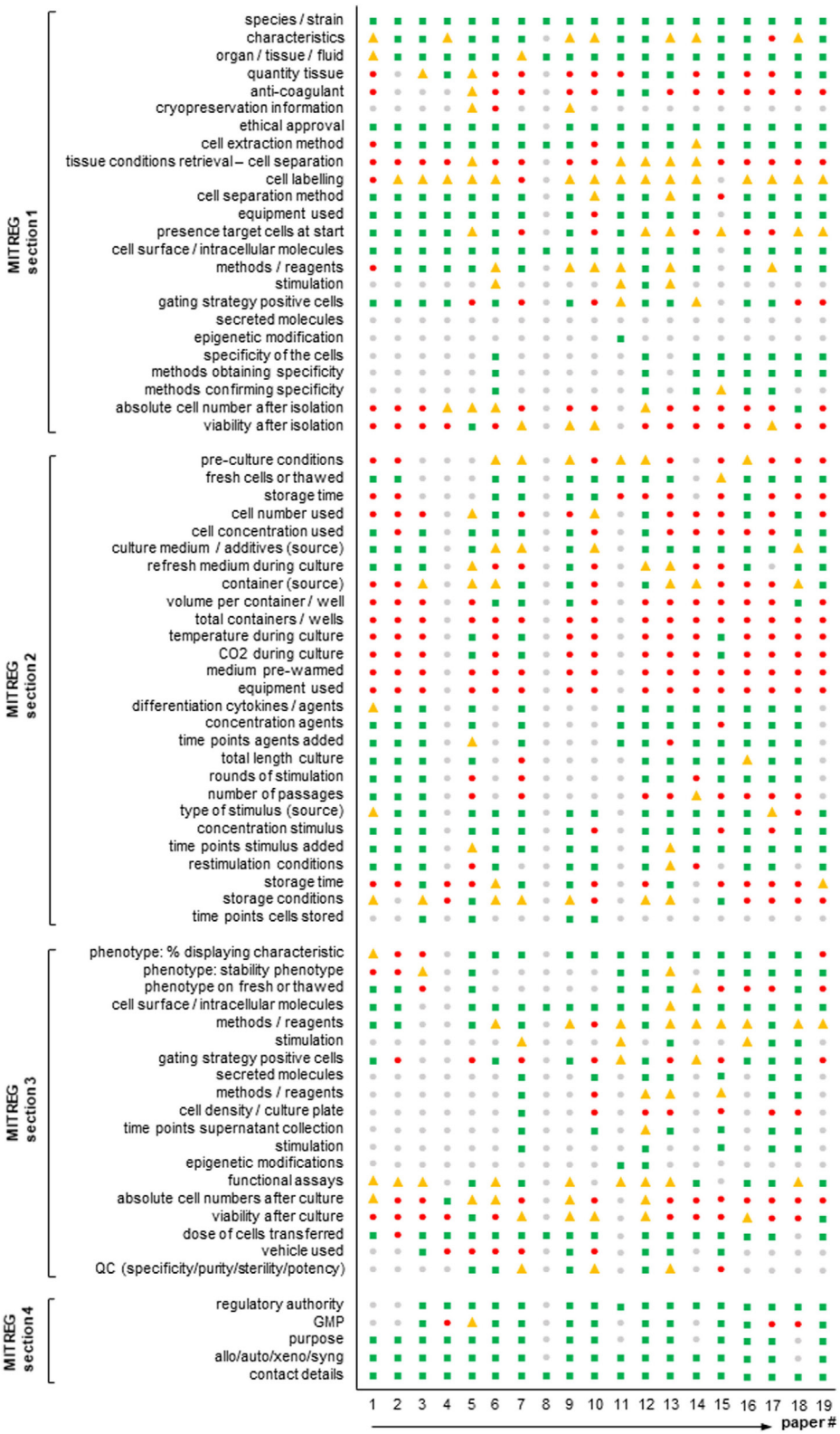

FIGURE 1 | Agreement of published T regulatory cell (Treg) articles with the minimum information about T regulatory cell (MITREG) document. Graph showing the results of a total of 19 Treg articles (10–28). The order in the figure is anonymized and different from that in the references. MITREG data directly stated in the article (■ green squares), partly stated in the article (▲ yellow triangles), not present at all (● red circles), or not present as it was not relevant for the publication (● gray circles).

protocol. For example, storage conditions, cell numbers and viability and anticoagulant used are almost never reported, but are most likely measured or known by the researcher. Moreover, essential information to allow experiments to be repeated is often missing.

Together with MITREG we provide a checklist that was designed with maximal flexibility to incorporate newly developed methodologies. While MITREG does not aim at uniform protocols or dictating quality checks, it is expected to enable a mere description of the growing diversity in production procedures. We expect it to mature as novel technologies arise and become a consensus guideline within the Treg community. Only by exact reporting we will be able to identify differences in Treg preparations that may help to understand results from clinical studies. We anticipate that MITREG will be a starting point for further joint efforts of the Treg community that will ultimately lead to optimized cellular therapy.

AUTHOR CONTRIBUTIONS

As described in the Section "Methods," these recommendations are the common effort of all the authors, who were involved in the design, acquisition, and interpretation of available data on Tregs as well as revised critically and approved final version of the MITREG document. In addition, AF, MG, NG, and RS were involved in collecting and analysis of the data sent by the contributors and SG, CH, GL, PL, EC, and PT supervised the work and edited the article.

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

The reviewer LH declared a shared affiliation, with no collaboration, with several of the authors RS, JI, CH, and PL to the handling editor.

APPENDIX A

MINIMUM INFORMATION FOR T REGULATORY CELLS (MITREG)

Introduction

The purpose of this document is to enable the description of the generation of T regulatory cell (Treg) products for therapeutic application or experimental usage. It was designed to suit reports using endogenous, induced, antigen-specific, and polyclonal freshly isolated and expanded Tregs.

This document is split into four sections, each describing a different aspect of the process. Not all sections will be relevant to all Treg products.

Information in some sections of this document may be covered by other Minimum Information documents, or defined vocabularies. For example, flow cytometry is described in MIFlowCyt,[1] microarray data by MIAME,[2] T-cell assays by MIATA,[3] and production of standardized tolerogenic antigen-presenting cells by MITAP,[4] Authors are encouraged to use these resources as appropriate.

Use of Terminology

The key words "**must**," "**should**," and "**may**" in this document are to be interpreted as follows:

must: this word means that the information is an absolute requirement. Failure to provide this information is in strict violation of the specification.

EXAMPLE: the species and the source of the cell material are required for all experiments.

should: this word means that there may exist valid reasons for particular protocols to not provide these data, but that these data need to be provided if it is relevant to the protocol.

EXAMPLE: if the Tregs were generated or enriched using an antigen then this must be described, although there may be protocols where polyclonal Tregs are applied.

may: this word means that the data are optional and do not need to be included, but can be provided.

EXAMPLE: the health or age of the organism can be provided, but there may be protocols where this is not assessed, even though it could be.

These definitions are modified from RFC 2119 (https://tools.ietf.org/html/rfc2119).

[1]http://flowcyt.sourceforge.net/miflowcyt/
[2]http://fged.org/projects/miame/
[3]http://miataproject.org
[4]https://doi.org/10.7717/peerj.2300

(1) Cells at the start of procedure

This section describes the characteristics and state of the cells used in the procedure prior to any form of cell manipulation processes such as cell expansion and/or differentiation.

(a) Essential information about the donor

(i) Species and strain

The taxonomy of the organism from which the cells originated. You **must** use names according to the NCBI Taxonomy.[5] If the strain of the species is known, you **should** indicate this.

EXAMPLE: Homo sapiens/human; Mus musculus, Rag$^{-/-}\gamma_c^-$ (B6, H-2b)

(ii) Characteristics of the organism

Include information about the organism from which the cells originated that is not adequately described by the species/strain information. This **may** include details of their health, age, sex, or any treatments or environmental conditions to which they have been exposed to (e.g., medication). You **may** also include information that is specific to your laboratory, such as an individual identifier number. If you have purchased experimental animals (e.g., BALB/c mice) or tissues (e.g., human bone marrow) you **should** indicate the source of purchase.

EXAMPLE: healthy/volunteer/male/6-weeks-old/male/BALB/c mice/purchased from Charles River (Margate England)

(b) Source of cell material

The organ, tissue, or fluid from which the cells have been isolated **must** be stated. If you use a blood product you **should** state the product and the source (e.g., hospital department, blood bank) from where it was obtained. You **should** use terminology from Uberon,[6] or the Foundational Model of Anatomy.[7] You **should** also indicate the quantity of the sample by mass or volume, and, if applicable, which anti-coagulant was used. Additional details **must** be included if the source material was derived from cryopreserved samples (e.g., umbilical cord blood). This would include the methods and duration of storage and initial cell counts. The statement on use/ethics committee approval/written informed consent **MUST** be included.

EXAMPLE: apheresis/buffy coat/bone marrow aspirate/peripheral blood, Sanquin blood supply; 250 ml; EDTA

(c) Cell separation process

(i) Cell handling and labeling

The methodology used to extract the cells from the source material **must** be stated. You **should** also indicate the time between cell material retrieval and start of the isolation process. You **should** indicate how the tissue was kept during this time, including the

[5]http://www.ncbi.nlm.nih.gov/taxonomy/
[6]http://www.uberon.org
[7]http://fme.biostr.washington.edu/FME

temperature and you **may** indicate the container and fluid. You **must** indicate cell labeling procedures, including characteristics and source of labeling buffers and reagents. Other details, such as cell suspension volume and concentration, incubation temperature and washing steps **should** be included.

EXAMPLE: apheresis products were stored overnight at 4°C; Tregs were enriched by magnetic-activated cell sorting (MACS® Technology); Cells were labeled with anti-CD8-coated magnetic beads (CliniMACS® CD8 Reagent, Miltenyi Biotec) in 95 ml of PBS containing 1 mmol/l EDTA and 0.5% human albumin (PBS/EDTA buffer, Miltenyi Biotec) for 30 min at room temperature on an orbital shaker.

(ii) Cell separation equipment and process

The equipment (e.g., AutoMACS®, CliniMACS®, Aria III™ Fluorescence Activated Cell Sorter) and process used to enrich for the cells of interest **should** be stated. The presence of the target population in the starting material should be described.

EXAMPLE: anti-CD8 bead-labeled cells were resuspended in 100 ml of PBS/EDTA/0.5% HA. $CD8^+$ cells were depleted with the use of the 2.1 depletion program on the CliniMACS® Cell Separation Device (Miltenyi Biotec).

(d) Phenotype

Characteristics of the cells that have been isolated **should** be described and how this has been determined. Where only a proportion of cells in the population display a characteristic, you **should** indicate the percentage.

(i) Cell surface and intracellular markers

Identifying molecules that are, or are not, expressed by the cells on their surface or intracellularly is useful. You **should** describe: (1) what you measured, (2) the methodology used for the measurement (including information on reagents; if using mAbs, information on clonotype, conjugate, and manufacturer **must** be provided), (3) whether the cells received a stimulus and for how long before the measurement was carried out, and (4) the method used to set marker or population positivity (e.g., fluorescence minus one method). You **should** use cluster of differentiation (CD) names when available (e.g., use CD62L instead of the alternative name L-selectin)—a full list of regularly updated CD numbers can be found on the website run by the HCDM[8] (human cell differentiation molecules). Otherwise, you **may** use databases, e.g., Uniprot[9] for proteins and ChEBI[10] for non-protein organic molecules.

EXAMPLE: FOXP3 (PE-Cy7, clone PCH101, eBioscience) expression was measured directly after cell isolation by intracellular staining using the Foxp3/Transcription Factor Staining Buffer Set from eBioscience. Percentage of $CD4^+CD25^{high}CD127^{-/low}FOXP3^+lin^-$ doublet$^-$ Treg cells was determined by flow cytometry (FACS Canto II™, Becton Dickinson). After the isolation, 98.0% (median, range 97–99.5%) of the cells presented this phenotype.

(ii) Secreted molecules

Molecules that are, or are not, secreted by the cells are useful to identify. These include cytokines (e.g., IL-10) and other soluble mediators. You **should** describe: (1) what you measured, (2) If using Abs, clone, conjugate and source of all antibodies and reagents used **must** be provided, (3) the methodology used for measurement, (4) cell density/milliliter of medium and plastic ware (e.g., 96 w round/flat bottom), (5) when supernatant was collected for cytokine concentration measurement, and (6) whether the cells received a stimulus and for how long before the measurement was carried out.

EXAMPLE: IFN-γ; ELISA; supernatant after 24 h of unstimulated cell culture.

(iii) Epigenetic modifications

Epigenetic modification relevant to the characteristics **should** be described if determined. Method of detection DNA demethylation **should** be clearly described.

EXAMPLE: the mean percentage of demethylated TSDR of the foxp3 gene in the Treg population was 7% (Epiontis, Berlin, Germany).

(iv) Specificity

Polyclonal or antigen-specific, especially genetic modifications to manipulate specificity **should** be described. You **should** describe: (1) what is the specificity of the cells, (2) the methodology used to obtain the specificity, and (3) the methodology used to confirm the specificity. To describe the specificity of your cells, you should use CD names when available (e.g., use CD19 instead of the alternative name B4)—a full list of regularly updated CD numbers can be found on the website run by the HCDM8 (human cell differentiation molecules). Otherwise, you may use databases, e.g., http://hla.alleles.org, for HLA alleles, Uniprot9 for proteins and ChEBI10 for non-protein organic molecules describing the targets for your cells.

EXAMPLE: HLA-A2-specific CAR (A2-CAR) Tregs were generated with lentiviral vectors encoding an HLA-A2-specific CAR by cloning and sequencing the heavy- and light-chain variable regions of the mAb and fusing the resulting scFv to portions of CD8, CD28, and CD3ζ in a second-generation CAR structure. Tetramers made from HLA-A2 were used to confirm the specificity of binding the cells to HLA-A2.

(e) Cell numbers

(i) Absolute cell number

You **should** indicate the total number of cells present after extraction, and how they have been counted.

EXAMPLE: 980×10^6 cells as determined by Coulter counting.

(ii) Viability

You **should** indicate the percentage of cells that are alive, and how this has been determined. The percentage of apoptotic cells

[8] http://www.hcdm.org/
[9] http://www.uniprot.org/
[10] https://www.ebi.ac.uk/chebi/

should be stated if determined (indicate whether the starting material is fresh or frozen).

EXAMPLE: 95% viability as determined by trypan blue exclusion. 5% of $CD3^+$ T-cells had a phenotype indicating early apoptosis ($7\text{-}AAD^-$, $AnnexinV^+$) as measured by flow cytometry.

(2) Expansion/differentiation

The section describes the protocol that has been used for expansion/differentiation of the isolated cells described in the previous section (Section 1). This process will hereafter be referred to as the expansion/differentiation process.

(a) Pre-culture conditions
The conditions under which the cells are kept after isolation but before starting the expansion/differentiation process (the fluid and type of container they are kept in, and at what temperature) **should** be described. The indication whether the starting material is fresh or thawed **must** be provided. You **should** also indicate the length of time between cell extraction and start of the expansion/differentiation process.

EXAMPLE: isolated cells were placed in PBS with1% human serum albumin in a Falcon tube and kept at room temperature for up to 30 min before starting the culture.

(b) Culture conditions
The conditions under which the cells are kept during the expansion/differentiation process **should** be stated.

(i) Cell number
The number of cells used for the expansion/differentiation process **should** be stated, if different from numbers stated in Section 1e*i.*

EXAMPLE: in total 5×10^6 cells were put into culture

(ii) Cell concentration
The concentration of cells in the medium at the start of and throughout the expansion/differentiation process **should** be stated as cells/milliliter.

EXAMPLE: cells were put into culture at a concentration of 1×10^6 cells/ml

(iii) Culture medium
The medium in which the cells are grown **must** be described, including its source, and whether it has any additives (e.g., antibiotics, inactivated serum), excluding the stimuli that are described later. If you use more than one type of medium, or refresh the medium during the culture, then you **should** describe that here.

EXAMPLE: X-VIVO15 (Lonza) supplemented with5% human male type AB-serum (Sigma)

(iv) Culture container
The physical container in which the culture is carried out. This can include tissue culture plates, tissue culture bags or flasks. You **should** state the type of container, size and manufacturer. You **should** also indicate the total cell culture volume per container or well, as well as the total number of containers used.

EXAMPLE: 20 ml of medium in a 100 ml MACS Good Manufacturing Practice (GMP) Cell Differentiation bag (Miltenyi Biotec); 1 bag

(v) Culture environment
Describe the physical environment in which the cells are kept during the expansion/differentiation process. This **should** include the temperature and CO_2 concentration. You **should** note whether medium has been pre-warmed. You **may** describe the equipment used to maintain the culture environment.

EXAMPLE: 37°C, 5% CO_2; Medium was pre-warmed to 37°C; Sanyo CO_2 incubator

(c) Expansion/Differentiation protocol
The protocol that is used to expand/differentiate the cells **should** be described. This **must** include the type and source of cytokine(s) or other agent(s) added into the medium, and at what time point and concentration **should** be included. You **should** also state the total length of the culture period as well as the rounds of stimulation, rounds of culture change, and the number of cell passages.

EXAMPLE: rapamycin (final concentration of 100 nM; Rapamune®, Pfizer) was added on day 0, 2, 5, 7, and 9. IL-2 (final concentration of 500 IU/ml; Proleukin®, Novartis) was added on day 2, 5, 7, and 9. Cells were harvested on day 12.

(d) Stimulus
It **should** be stated whether the cells are expanded/differentiated polyclonally or in an antigen-specific manner or against an alloantigen. The protein(s), antibody(ies), accessory cells or other preparation(s) (e.g., antigen-presenting cells; APCs) with which the cells are stimulated **must** be named. You **must** describe the source of the preparation, concentration, and time point(s) at which it/they are added to the cell culture. Restimulation conditions, if any, should also be stated.

EXAMPLE: cells were stimulated with CD3/CD28 MACS GMP ExpAct Treg Beads (Miltenyi Biotec) at a 4:1 bead:cell ratio. Cells were stimulated with CD40-activated allogeneic B cells (30 Gy-irradiated) at a ratio of 10 B cells per nTreg cell.

(e) Storage
The conditions in which the cells are kept after completion of the expansion/differentiation process, but before being used in any subsequent experimental assay or treatment **should** be described. You **should** indicate the fluid and temperature in/at what the cells are being kept, as well as the length of time. You **should** indicate if cells are being frozen, and give details on the freezing and thawing procedures, including cell recovery and viability after thawing. You **should** also indicate if cells are taken out of their culture environment for any length of time during the expansion/differentiation process (e.g., if cells are frozen

before completion of this process, with the aim to resume it at a later date).

EXAMPLE: cells were kept in PBS 1% human serum albumin (Sigma) in a 50 ml Falcon tube at room temperature for a maximum of 2 h; Cells were frozen in FCS/10% DMSO.

(3) Cells after expansion/differentiation

This section describes the characteristics and state of the cells at the end of the expansion/differentiation process described in the previous section (Section 2).

(a) Phenotype
Characteristics of the cells at the end of their expansion/differentiation, including their specificity and purity (e.g., as% of target cells) **must** be described. Where only a proportion of cells in the population display a characteristic, you **should** indicate the percentage. You **should** report on the stability of the phenotype and how you determined this. It **should** be indicated if the phenotype of the cells post-expansion was determined using fresh viable cells, or rather after a freeze–thaw cycle in a batched analysis.

(i) Cell surface and intracellular markers
A number of phenotypic markers help to define the Treg cellular phenotype and specificity and are associated with distinct expression levels of surface and intracellular proteins. These markers are often characteristic of the transcriptional program of a cellular lineage and provide important information regarding the phenotypic stability and function of resulting cell products. You **should** describe: (1) what you measured, (2) the methodology used for measurement (including information on reagents; if using mAbs, information on clonotype, conjugate and manufacturer) **must** be provided, (3) whether the cells received a stimulus and for how long before the measurement was carried out, and (4) the method used to set marker or population positivity (e.g., fluorescence minus one method). You **should** use CD names when available (e.g., use CD127 instead of the alternative name IL-7Rα)—a full list of regularly updated CD numbers can be found on the website run by the HCDM (see footnote 8) (human cell differentiation molecules). Otherwise, you **may** use databases, e.g., http://hla.alleles.org, for HLA alleles, Uniprot (see footnote 9) for proteins and ChEBI (see footnote 10) for non-protein organic molecules.

EXAMPLE: intracellular IFN-γ and IL-17 expression was measured by flow cytometry after 4 h incubation with 20 ng/ml PMA and 1 µg/ml Ionomycin in the presence of 1 µl/ml GolgiPlug™ using the BD Cytofix/Cytoperm™ buffer set.

(ii) Secreted molecules
Indicate molecules that are, or are not, secreted by the cells. These include cytokines (e.g., IL-10) and other soluble mediators. You **should** describe: (1) what you measured, (2) if using mAbs, clone, conjugate, and source of all antibodies and reagents used **must** be provided, (3) the methodology used for the measurement, (4) cell density/ml of medium and plastic ware (e.g., 96 w round/flat bottom), (5) when supernatant was collected for cytokine concentration measurement, and (6) whether the cells received a stimulus and for how long before the measurement was carried out.

EXAMPLE: soluble IFN-γ, TNF-α, IL-17, and IL-10 were measured in the cell culture supernatant at a cell density of 1×10^6 cells/ml by ELISA according to the manufacturers' instruction.

(iii) Epigenetic modifications
Epigenetic modification relevant to the characteristics **should** be described if determined. Method of detection DNA demethylation **should** be clearly described.

EXAMPLE: the mean percentage of demethylated TSDR of the foxp3 gene in the Treg population was 97% (Epiontis, Berlin, Germany).

(b) Functional assay
You **should** describe any characteristic of the cells that has been measured by a functional assay (type of assays). This could either be the response of the cells to some stimulus or the behavior of other biological entities after exposure to the cells. There should be a clear indication of how the percentage of suppression was calculated (i.e., include formula). Whenever accessory cells such as responder cells are included in the assay, source and phenotype should be described. Behavior such as expression/production of molecules (described in Section 3a) does not need to be included.

EXAMPLE: proliferation-based suppression assay using CFSE labeled autologous $CD4^+CD25^-$ responder cells; IFN-γ based suppression assay

(c) Cell numbers

(i) Absolute cell number
You **must** indicate the total number of cells present at the end of the expansion/differentiation process, and how they have been counted and fold expansion **should** be included.

EXAMPLE: cell numbers were microscopically determined using C-Chip disposable counting chambers from NanoEnTek and fold expansion to day 0 was calculated.

(ii) Viability
You **must** indicate the percentage of cells that are alive and how this has been determined **should** be included.

EXAMPLE: 83% viability as determined by trypan blue exclusion

(d) Dosing
Whenever cells are transferred into an organism, details about dosing **must** be given. For clinical applications, information on the vehicle (solvent/medium) as well as intermediate components (trace amounts possible) **must** be given.

EXAMPLE: a single dose of 1×10^7 total nucleated cells per kilogram of body weight in 50 ml 0.9% NaCl was transfused i.v.

(e) Quality control
If the cells were produced for a clinical trial, you **must** describe release criteria and any methods used to determine sterility, specificity, purity, and quality of the product.

(4) About the protocol

In this section, we describe the general features about the protocol as a whole.

(a) Regulatory authority
Information about whether the protocol being used has been validated or quality-controlled to standards agreed to by an external regulatory authority **must** be stated. You **should** state the name of this authority. Also you **should** state whether the protocol follows GMP.

EXAMPLE: Medicines and Health Regulatory Authority

(b) Purpose
You **must** describe the overall purpose of the production of the cells.

EXAMPLE: prevention of transplant rejection; Treatment of patients affected by Crohns' disease.

(c) The relationship between the organism of origin of the cells and the target organism
You **must** state if the cell product is autologous/allogeneic/xenogeneic/syngeneic to the recipient.

EXAMPLE: patients receiving allogeneic kidney transplants and autologous Tregs. B6 mice receiving allogeneic (BALB/c xB6) heart transplants and syngeneic (B6) Tregs.

(d) Contact details
You **must** provide the name and contact information of the corresponding author(s).

(e) Citation
You **should** add information that your paper was written in accordance with the Minimum Information for T Regulatory Cells reporting guidelines.

APPENDIX B

(MITREG) Checklist

Must	Should	May	
			(1) Cells at the start of procedure
			(a) Essential information about the donor
			(i) Species and strain
■			Species
	■		Strain (if applicable)
			(ii) Characteristics of the organism
		■	Health
		■	Age
		■	Treatment/Environment
		■	Individual identifier number
	■		Source of purchase (if applicable)
			(b) Source of cell material
■			Organ, tissue, fluid, or blood product
	■		Source (if applicable)
	■		Quantity (volume, size, or weight)
	■		Anti-coagulant (if applicable)
	■		If using cryopreserved sample
■			Method and duration of storage
■			Initial cell counts
■			Ethical committee approval/written informed consent
			(c) Cell separation process
			(i) Cell handling and labeling
■			Cell extraction method
			Tissue conditions between tissue retrieval and cell separation
	■		Duration
	■		Temperature
		■	Container
		■	Fluid
			Cell labeling
■			Buffers and reagents (incl. source)
	■		Cell suspension volume and concentration
	■		Incubation temperature and duration
	■		Washing steps
			(ii) Cell separation equipment and process
	■		Methodology
	■		Equipment
	■		Presence of target cells in starting material described
			(d) Phenotype
	■		For any of the below, indicate the percentage of cells displaying the characteristic (if known)
			(i) Cell surface and intracellular markers
	■		Molecules measured [using cluster of differentiation (CD) names]
■			Details of reagents used and source (incl. mAb clone, fluorochrome)
	■		Methodology
	■		Stimulus and time of stimulation (if applicable)
	■		Gating strategy to determine positive cells
			(ii) Secreted molecules
	■		Molecules measured
■			Details of reagents used (incl. mAb clone, conjugate) and source
	■		Methodology
	■		Cell density/ml of medium and type of tissue culture plate
	■		Time point of supernatant collection
	■		Stimulus and time of stimulation (if applicable)
			(iii) Epigenetic modifications
	■		Epigenetic modification relevant to the characteristics
			(iv) Specificity
■			Specificity of the cells (polyclonal or antigen-specific)
	■		Methodology used to obtain specificity
	■		Methodology used to confirm specificity
			(e) Cell numbers
			(i) Absolute cell number
	■		Total number of cells at the end of the isolation process
	■		Methodology
			(ii) Viability
	■		Percentage of viable cells
	■		Methodology
			(2) Expansion/differentiation
			(a) Pre-culture conditions
			Storage conditions
	■		Fluid
	■		Type of container
	■		Temperature
■			Fresh or thawed
	■		Storage time
			(b) Culture conditions
			(i) Cell number
	■		The total number of cells put into culture
			(ii) Cell concentration
	■		The number of cells per ml of medium at start of culture
			(iii) Culture medium
■			Type(s) of medium
■			Source(s)

Must	Should	May	
■			Additives (excluding agents to maintain/induce T regulatory cells)
	■		Refreshment of the medium
			(iv) Culture container
	■		Type of container
	■		Size
	■		Manufacturer
	■		Cell culture volume per container or well
	■		Total number of containers or wells
			(v) Culture environment
	■		Temperature and CO_2 concentration
	■		Use of pre-warmed medium
		□	Equipment
			(c) Differentiation/tolerization protocol
■			Name of cytokine(s) or other agent(s) used
	■		Concentrations
	■		Time point(s) added to cell culture
	■		Total length of the culture period
	■		Rounds of stimulation
	■		Number of cell splitting
			(d) Stimulus
	■		Polyclonal/antigen-specific/alloantigen
■			Stimulus (agent and/or accessory cell)
■			Source
	■		Concentration
	■		Time point(s) added to culture
	■		Restimulation conditions (if applicable)
			(e) Storage
	■		Storage time
			Storage conditions
			If fresh
	■		Fluid
	■		Container
	■		Temperature
			If cryopreserved
	■		Freezing/thawing process
	■		Freezing medium
	■		Cell recovery and viability after thawing
	■		Time point at which cells are stored if different to the end of the culture process
			(3) Cells after expansion/differentiation
			(a) Phenotype
■			For any of the below, indicate the percentage of cells displaying the characteristic (if known)
	■		Stability of the phenotype (if tested)
	■		Phenotype tested on fresh or thawed cells
			(i) Cell surface and intracellular markers
	■		Molecules measured (using CD names)
■			Details of reagents used and source
	■		Methodology
	■		Stimulus and time of stimulation (if applicable)
	■		Gating strategy to determine positive cells
			(ii) Secreted molecules
	■		Molecules measured
■			Details of reagents used and source
	■		Methodology
	■		Cell density/milliliter of medium and type of tissue culture plate
	■		Time point of supernatant collection
	■		Stimulus and time of stimulation (if applicable)
			(iii) Epigenetic modifications
	■		Epigenetic modification relevant to the characteristics
			(b) Functional assay
	■		Response of the cells to a defined stimulus
	■		Behaviour of other biological entities after exposure to the cells
	■		If using accessory cells, describe phenotype and source
			(c) Cell numbers
			(i) Absolute cell number
■			Total number of cells at the end of the expansion process
	■		Methodology
			(ii) Viability
■			Percentage of viable cells
	■		Methodology
			(d) Dosing
■			Dose of cells transferred into organism (if applicable)
■			Vehicle (solvent/medium) and intermediate components (for clinical trials only)
			(e) Quality control (for clinical trial only)
■			Specificity
■			Purity
■			Sterility
■			Potency
			(4) About the protocol
			(a) Regulatory authority
■			External authority that approved the protocol
	■		Does protocol follow Good Manufacturing Practice?

Must	Should	May	
■			**(b) Purpose** The disorder for which the cell treatment has been manufactured
■			**(c) Relationship between the source organism for the cells and the target organism** Allogeneic/autologous/ xenogeneic/syngeneic

Must	Should	May	
■			**(d) Contact details** Name and contact information of the corresponding author(s)
		■	**(e) Citation** Acknowledge the MITREG reporting guidelines

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METHODS
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Flow Cytometric Clinical Immunomonitoring Using Peptide–MHC Class II Tetramers: Optimization of Methods and Protocol Development

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With the advent of novel strategies to induce tolerance in autoimmune and autoimmune-like conditions, clinical trials of antigen-specific tolerizing immunotherapy have become a reality. Besides safety, it will be essential to gather mechanistic data on responding CD4$^+$ T cells to assess the effects of various immunomodulatory approaches in early-phase trials. Peptide–MHC class II (pMHCII) multimers are an ideal tool for monitoring antigen-specific CD4$^+$ T cell responses in unmanipulated cells directly *ex vivo*. Various protocols have been published but there are reagent and assay limitations across laboratories that could hinder their global application to immune monitoring. In this methodological analysis, we compare protocols and test available reagents to identify sources of variability and to determine the limitations of the tetramer binding assay. We describe a robust pMHCII flow cytometry-based assay to quantify and phenotype antigen-specific CD4$^+$ T cells directly *ex vivo* from frozen peripheral blood mononuclear cell samples, which we suggest should be tested across various laboratories to standardize immune-monitoring results.

Keywords: antigen-specific T cells, tetramers, tetramerization, protocol standardization, flow cytometry

INTRODUCTION

Autoimmune diseases result from chronic T cell and B cell autoimmune responses leading to tissue inflammation and damage. Helper CD4$^+$ T cells play a central role in autoimmune responses because they orchestrate the function of other immune cells including cytotoxic T cells and B cells. Current treatment strategies for autoimmune diseases target inflammation, the whole immune system, or the total T cell or B cell populations, modulating the immune response in antigen non-specific approaches. Antigen-specific tolerizing immunotherapy specifically targets the autoimmune response leaving the rest of the immune system unimpaired. Several different approaches to induce antigen-specific tolerance have been developed over recent years, and the first clinical trials are completed and are in progress (1, 2). These immunomodulatory approaches require the development of tools to assess the efficacy of antigen-specific tolerizing immunotherapy. To monitor antigen-specific CD4$^+$ T cell responses, antigen-specific CD4$^+$ T cells can be restimulated with peptide *ex vivo* for analysis of proliferation and cytokine production. However, responses to autoantigenic peptides in human

PB are typically much lower and more variable than in mouse models of autoimmune disease. Autoantibody titers provide an indirect measure of $CD4^+$ autoreactive T cell function. Such assays are robust and often already standardized and available as qualified assays in clinical laboratories. In recent years, peptide–MHC class II (pMHCII) multimers have emerged as a tool for analysis of antigen reactive, including autoreactive $CD4^+$ T cells in blood or other accessible tissue sites directly *ex vivo* or after a period of *in vitro* restimulation with peptide (3–6). They consist of biotinylated MHC class II molecules with bound peptide multimerized with fluorochrome-labeled streptavidin (7). The peptide presented by the MHC class II molecule is the self- or autoantigen that is targeted by the tolerizing immunotherapy. Hence, antigen-specific T cell receptors (TCRs) on $CD4^+$ T cells can be detected in flow cytometric assays to enumerate the number and, with cell surface-specific monoclonal antibodies, the phenotype of antigen-specific $CD4^+$ T cells restricted to a particular MHC class II molecule.

Various protocols on the use of pMHCI and pMHCII tetramers and surface antibody staining to enumerate and phenotype unmanipulated cells directly *ex vivo* or *in vitro* peptide-stimulated T cells have been published in recent years (8–10). However, autoantigen-specific $CD4^+$ T cells are rare in the circulation (generally less than $100/10^6$ CD4 T cells), the TCR is of low affinity and the pMHCII have a high off-rate (3, 11, 12), thus optimization of staining for consistent identification of TCR reactive with pMHCII is technically challenging. Several methodologies have been described to enhance detection, including staining cells with tetramers labeled with phycoerythrin (PE)-based fluorochromes followed by enrichment using PE-beads and magnetic-activated cell sorting (13). Alternative published approaches include the tyrosine kinase inhibitor dasatinib, to reduce TCR internalization and maximize TCR surface detection, and amplification of the tetramer signal using an antibody sandwich (7, 8, 14). There are also various options for the procurement or generation of multimer reagents, including specialized research laboratories, the NIH tetramer core facility, and several commercial suppliers, including MBL, ProImmune (PI), and Immudex.

In this methodological analysis, we compared several approaches to the staining of antigen-specific $CD4^+$ T cells using PE-labeled tetramers directly *ex vivo* and tested reagents from two commercial suppliers and one specialized research laboratory to identify sources of variability and limitations of the assay. We optimized a pMHCII tetramer flow cytometry-based protocol to quantify and phenotype unmanipulated antigen-specific $CD4^+$ T cells in the circulation of individuals to enable the visualization of cellular changes *in vivo*.

METHODS

Patient Samples

Peripheral blood mononuclear cells (PBMC) were isolated from PB of rheumatoid arthritis (RA) patients visiting the outpatient clinic of the Princess Alexandra Hospital in Brisbane, Australia. The protocol was approved by the Metro South and UQ HRECs, and informed consent was obtained from all participants.

pMHCII Tetramers

HLA-DRB1*0401 or HLA-DRB1*0101 hemagglutinin$_{306–318}$ and collagen type II$_{259–273}$ monomers were generated as previously described (14) and tetramerized using streptavidin-PE (BD Biosciences) or purchased either as monomers or tetramers from commercial suppliers (PI and MBL).

Monoclonal Antibodies

The following monoclonal antibodies were used in this study: antihuman CD3 evolve™ 655 (eBioscience), antihuman CD3 BUV737 (BD Biosciences), antihuman CD4 BUV395 (BD Biosciences), antihuman CD4 PerCP/efluor710 (eBioscience), antihuman CD4 AlexaFluor 700 (BD Biosciences), antihuman CD11c FITC (BioLegend), antihuman CD14 FITC (BioLegend), antihuman CD16 FITC (BioLegend), and antihuman CD19 FITC (BioLegend).

Flow Cytometric Analysis

Peripheral blood mononuclear cells were either stained directly after isolation or after storage in liquid nitrogen. Frozen PBMCs were thawed in RPMI [in the presence of 12.5 μg/ml DNase I (Sigma) until the first centrifugation step and with 6.25 μg/ml DNase I until the second centrifugation step]. Thawed cells were rested in a 37°C incubator for 15–20 min, with clumps of dead cells subsequently removed using a cell strainer (Corning). To prevent non-specific binding, FcR Blocking reagent (Miltenyi Biotech) was used according to the manufacturer's protocol (2 μl up to 10 million cells). When a dasatinib step was included during the staining procedure, cells were incubated with 50 nM dasatinib (Selleck Chemicals) in RPMI containing 10% human AB serum, 50 U/ml IL-2 and 25 mM glucose in a 37°C waterbath for 30 min. Tetramer was then added at a concentration of 4.2 μg/ml and incubated for 1 h at 4°C. The cells were then washed once in FACS Buffer (0.1% BSA and 2 mM EDTA in PBS) and incubated with a mix of surface antibodies. Where sandwich staining was included, cells were stained with anti-PE biotin (BioLegend) at 1:1,000 or rabbit anti-PE (MyBiosource) at 1:4,000 for 20 min at 4°C after the tetramer staining. Subsequently cells were washed once in 1× FACS Buffer and incubated together with surface antibodies and streptavidin-PE (BD Biosciences) at 1:1,000 or AlexaFluor 555 goat anti-rabbit (Life Technologies) for 20 min at 4°C. To discriminate live from dead cells, cells were washed in PBS and stained using the LIVE/DEAD fixable Dead Cell Stain Kit (Invitrogen) according to the manufacturer's protocol. All samples were acquired on a BD LSR Fortessa X20 (BD Biosciences).

RESULTS

The Numbers of pMHCII Tetramer-Positive Cells Were Comparable in Frozen and Freshly Isolated Cell Samples

In clinical trials, PBMC samples are normally collected at several time points to monitor mechanism or efficacy of treatment. To internally control for experimental variation, PBMC collected from a single donor at different time points are ideally frozen, then thawed, stained and analyzed using an internally controlled,

qualified assay. Collagen $II_{259–273}$ is the dominant epitope in the murine collagen-induced arthritis model in HLA-DRB1*04:01 or HLA-DRB1*01:01 transgenic mice and the collagen $II_{259–273}$ specific TCR repertoire in these mice is highly restricted (15). A functional T cell stimulation assay identified T cells responding to the same epitope in HLA-DRB1*04:01$^+$ RA patients and healthy controls (16). Furthermore, the dominant influenza epitope $HA_{306–318}$ can be presented by both HLA-DRB1*04:01 and HLA-DRB1*01:01 (17). HLA-DRB1*04:01-$HA_{306–318}$ tetramers were shown to identify antigen-specific T cells in RA patients (14, 18). Thus, HA-specific CD4$^+$ T cells represent an internal control for tetramer staining, independent of RA.

We first tested variability in the capacity to detect CD4$^+$ antigen-specific T cells from RA patients from freshly isolated or frozen PBMC using a published pMHCII tetramer-staining protocol (7, 14). We generated tetramers specific for HLA-DRB1*04:01-collagen $II_{259–273}$, HLA-DRB1*01:01-collagen $II_{259–273}$, HLA-DRB1*04:01-$HA_{306–318}$, and HLA-DRB1*01:01-$HA_{306–318}$ and assessed antigen-specific CD4$^+$ T cells in patients with an appropriate HLA type. In the first set of experiments, we endeavored to increase tetramer detection sensitivity by incubation with dasatinib and secondary antibody sandwich. Dasatinib is a tyrosine kinase inhibitor, which prevents internalization of the TCR and therefore increases the number of cell surface TCRs that can bind the tetramer and hence the detection signal. In some experiments, we included a sandwich step of biotinylated anti-PE antibody followed by streptavidin-PE or rabbit anti-PE followed by goat anti-rabbit AlexaFluor 555 to amplify the signal (7).

Fresh samples from RA patients were collected and stained immediately or frozen. Frozen cells were thawed and stained 3–8 days later with HLA-DRB1*04:01-collagen $II_{259–273}$ or HLA-DRB1*01:01-collagen $II_{259–273}$ tetramers. Collagen II-specific CD4$^+$ T cells were detected in both fresh and frozen samples (**Table 1**). There was no consistent increase or decrease in tetramer frequency in frozen and thawed cells relative to freshly isolated cells from the same donor. Furthermore, in the two patients in which the sandwich step was included, the tetramer frequency was the same or increased relative to samples stained without sandwich amplification (**Table 1**). These results indicate that while frozen samples can be used for the detection of antigen-specific CD4$^+$ T cells using pMHCII tetramers with or without sandwich amplification, the frequency of autoantigen-specific cells can vary from assay to assay in the same patient.

A Shorter Staining Process Resulted in Higher Yield of Cells for Analysis

PBMC, including activated PB T cells, from RA patients are fragile and have a high propensity for apoptosis in culture, which is amplified after thawing. Furthermore, we and others have shown that CD4$^+$ tetramer$^+$ T cells include activated memory T cells (6, 14, 18), which are particularly sensitive to manipulation *in vitro*. Given the variability in tetramer$^+$ cells from assay to assay, and our observations that cell death occurred during the prolonged staining procedure required for dasatinib, sandwich amplification and multiple washes ("long protocol"), we developed an alternative protocol with minimal cell handling, i.e., short tetramer incubation step at 4°C, followed by addition of cell surface antibodies without washing in between ("short protocol"). We compared the two protocols directly in HLA-DRB1*01:01$^+$ RA patients using HLA-DRB1*01:01-$HA_{306–318}$ and HLA-DRB1*01:01-collagen $II_{259–273}$ tetramers. The number of HLA-DRB1*01:01-$HA_{306–318}$-specific cells was fourfold higher using the short protocol than the long protocol (**Figure 1A**). Where these protocols were compared head to head in two further patients, the number of HLA-DRB1*01:01-collagen $II_{259–273}$-specific cells was twofold higher using the short protocol than the long protocol (**Figures 1B,C**; **Table 2**). We therefore compared cell yields and frequency of tetramer$^+$ cells across 20 HLA-DRB1*04:01$^+$ or *01:01$^+$ frozen PBMC from RA donors, 10 of which were stained with the long protocol and 10 with the short protocol (**Table 3**). For the long protocol, the number of PBMC included per tetramer stain was 3–18 × 10^6 cells, all of which had to be acquired on the flow cytometer to achieve sufficient frequency of tetramer$^+$ CD3$^+$ CD4$^+$ T cells (mean 0.002%). By contrast, for the short protocol ≤5 × 10^6 PBMC were included per tetramer stain, and of these, acquisition of 1–2 × 10^6 cells (mean 1.42 × 10^6, which did not use the entire tube of cells) was sufficient to achieve an improved frequency of tetramer$^+$ CD3$^+$ CD4$^+$ T cells (mean 0.012%). Thus, the short protocol not only increased the yield of PBMC that could be acquired on the flow cytometer for analysis but also increased the efficiency of detection of tetramer$^+$ cells without any increase in background staining in the FMO control (**Figure 1**). As a result, the number of cells required for acquisition dropped to 1–2 × 10^6 cells, which greatly decreased acquisition time and thus reduced assay cost. These data indicate that shorter processing time without wash steps reduces loss of cells during processing, preserves viability, and increases tetramer$^+$ T cell detection efficiency.

TABLE 1 | Overview number of detected collagen II-specific CD4$^+$ T cell in fresh and frozen samples.

Donor	Tetramer employed	# tetramer$^+$ cells/10^6 CD4$^+$ T cells, fresh	# tetramer$^+$ cells/10^6 CD4$^+$ T cells, frozen	# tetramer$^+$ cells/10^6 CD4$^+$ CD4$^+$ T cells, fresh$^+$ sandwich	# tetramer$^+$ cells/10^6 CD4$^+$ CD4$^+$ T cells, frozen$^+$ sandwich
1	HLA-DRB1*01:01-collagen $II_{259–273}$	54.36	53.44		
2	HLA-DRB1*01:01-collagen $II_{259–273}$	141.19	47.27		
3	HLA-DRB1*04:01-collagen $II_{259–273}$	25.59	66.70	80.64	108.64
4	HLA-DRB1*04:01-collagen $II_{259–273}$	55.82	20.05	56.72	55.81
5	HLA-DRB1*01:01-collagen $II_{259–273}$	1.26	12.51		
6	HLA-DRB1*01:01-collagen $II_{259–273}$	12.85	10.61		

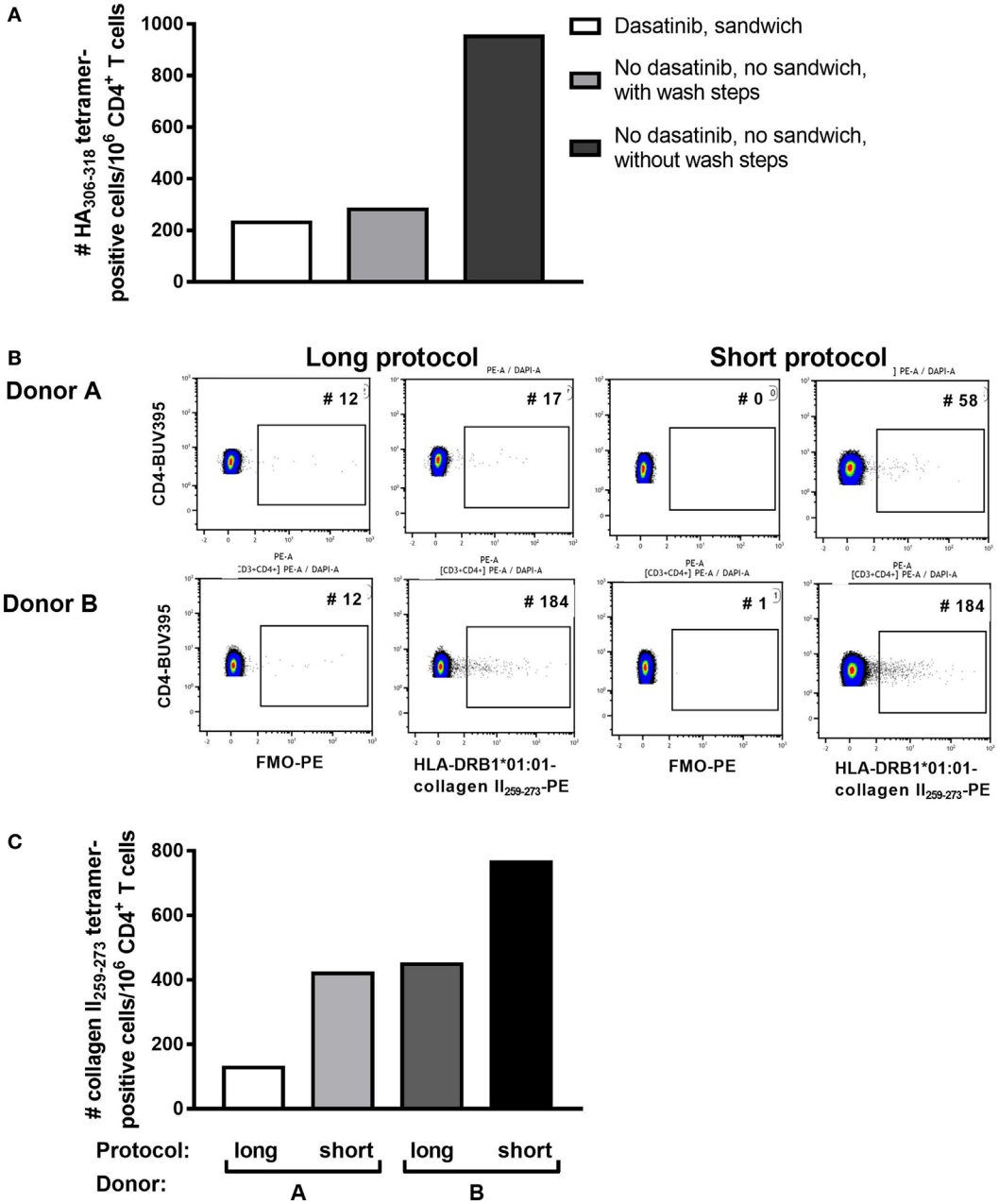

FIGURE 1 | Shorter staining protocol resulted in higher numbers of tetramer-positive CD4+ T cells. Three staining protocols were compared as indicated. **(A)** The number of HLA-DRB1*0101 $HA_{306-318}$-positive CD4+ T cells per million CD4+ T cells is depicted for the three protocols in peripheral blood mononuclear cells of one representative individual. **(B)** Flow cytometry plots for two patients stained with HLA-DRB1*0101-collagen $II_{259-273}$, using either the short or the long protocol. The negative control FMO plots are shown. **(C)** The number of HLA-DRB1*0101-collagen $II_{259-273}$+ CD4+ T cells per million CD4+ T cells using the short versus the long staining protocol in Donors A and B, as depicted in panel **(B)**.

Overcoming Challenges of Cell Yield after Thawing

Given that viability appears to be critical for identification of antigen-specific CD4+ T cells in RA PB, we compared different approaches to minimize the cell loss after thawing and to maximize the yield of cells for analysis of antigen-specific CD4+ T cells. First, we noted that after addition of DNase during the thawing process, free-floating DNA fragments and cell clump formation were minimized, increasing yield of cells in suspension. Second, resting thawed cells at 37°C for 20 min allowed cells to recover, and cell clumps could be removed from the cell suspension before staining, preventing clump formation during staining. We observed that clumps forming during staining are difficult to remove, often resulting in a further loss of cells. Third,

TABLE 2 | Recovery and identification of tetramer$^+$ cells after short and long staining protocols.

Figure	Protocol	# Cells stained × 10^6	# Cells acquired × 10^6	% Live CD3$^+$ CD4$^+$ cells	% Live tetramer$^+$ CD3$^+$ CD4$^+$ cells	Fold increase over long protocol	# tetramer$^+$/10^6 CD3$^+$ CD4$^+$ cells
1A	Long	2	1.04	NA	0.0048		128
	Short + wash	2	0.82	NA	0.0076	1.6	55
	Short – wash	2	0.9	NA	0.0140	2.9	413
1B Donor A	Long	2.5	0.8	40.1	0.0051		17
	Short	2.5	0.95	24.7	0.0104	2	58
Donor B	Long	2.5	1.1	57.0	0.0256		449
	Short	2.5	1.3	58.3	0.0447	1.74	783

*In the first experiment (**Figure 1A**), 2 × 10^6 peripheral blood mononuclear cells were stained per protocol, and in the second (**Figure 1B**) 2.5 × 10^6 cells were stained per protocol for each of 2 donors. The percentage (%) live tetramer$^+$ CD3$^+$ CD4$^+$ T cells identified using the long protocol was improved twofold to threefold by use of the short protocol without washes.*

TABLE 3 | Identification of tetramer$^+$ cells after short and long staining protocols.

HLA-DRB1*	Protocol	# Cells stained × 10^{-6}	# Cells acquired × 10^{-6}	% Gated live CD3$^+$ CD4$^+$ cells*	% Live tetramer$^+$ CD3$^+$ CD4$^+$ cells	# tetramer$^+$/10^6 CD3$^+$ CD4$^+$ cells
04:01	Long	17	4.1	55.8	0.0029	52.9
04:01	Long	9	2.3	49.4	0.0019	39.8
04:01/01:01	Long	9	3.2	74.5	0.0005	7.32
04:01	Long	14	5.0	61.8	0.0038	62.5
04:01	Long	13	5.1	68.5	0.0016	23.3
04:01	Long	15	4.3	78.1	0.0013	16.4
04:01	Long	3.2	1.4	71.0	0.0056	79.5
04:01	Long	7.5	2.1	71.2	0.0024	32.7
04:01	Long	7	2.9	51.5	0.0017	33.1
Mean (SD)		10.5 (4.5)	3.4 (1.3)	64.6 (10.4)	0.0024 (0.002)	38.6 (23.0)
04:01	Short	5	1.2	71.9	0.0208	289
01:01	Short	5	1.5	77.6	0.0165	213
04:01	Short	5	1.4	71.3	0.0176	248
01:01	Short	5	2.0	72.7	0.0279	384
04:01	Short	3	1.6	70.2	0.0074	106
01:01	Short	3	1.9	76.3	0.0146	191
01:01	Short	3	1.0	51.1	0.0064	126
04:01	Short	3	1.0	63.1	0.0077	123
04:01	Short	2	1.2	75.2	0.0029	38
01:01	Short	2	1.4	51.8	0.0023	44
Mean (SD)		3.6 (1.3)	1.4 (0.3)	68.1 (9.6)	0.0124 (0.008)	176 (110)

Peripheral blood mononuclear cells from 20 rheumatoid arthritis donors were stained with collagen $II_{257–273}$ tetramers according to HLA-DRB1 type (column 1) using the long or the short protocols. The number of cells stained and acquired as well as the percentage (%) and number (#) of tetramer$^+$ T cells are shown. Whereas all cells stained were subsequently acquired for the long protocol, in the short protocol 1–2 × 10^6 cells were collected per sample. * for the long protocol, CD3$^+$ CD4$^+$ T cells were gated based on exclusion of FITC$^+$ lineage$^-$ dump channel in combination with Aqua$^+$ live/dead discriminator. For the short protocol, green live/dead discriminator was included in the FITC dump channel, which was used for exclusion.*
NA, not available.

as noted earlier, cells are lost with every wash step, and reducing the number of wash steps increased cell yield (**Table 2**; **Figure 1**).

Tetramers from Different Sources Result in Staining Variability

For a clinical trial, monomers or tetramers qualified by a commercial supplier are preferred to research reagents. To test variability between reagents we compared staining with custom HLA-DRB1*04:01-collagen $II_{259–273}$ and HLA-DRB1*01:01-collagen $II_{259–273}$ tetramers purchased from two commercial suppliers, MBL and PI, with research tetramers produced as previously described (14). Cells were stained according to the manufacturer's protocol, which was very similar to the short protocol described earlier except that PI and MBL recommended staining cells with tetramer for 2 h at 37°C. Since the above tests using research tetramers were optimized at 4°C, we compared the manufacturer's protocol staining cells with tetramers for 2 h at 37°C with our protocol staining cells with tetramers at 4°C for 1 h. Compared with the FMO and research tetramers, the commercial tetramers had an overall spreading of the CD3$^+$ CD4$^+$ tetramer-negative PE signal and a reduction in fluorescence intensity of the cloud of HLA-DRB1*04:01-collagen $II_{259–273}^+$ T cells (**Figure 2A**). Staining at 37°C increased cell clumping and reduced tetramer staining using either research or MBL tetramers. HLA-DRB1*01:01-collagen $II_{259–273}$ T cells were virtually undetectable when staining with MBL tetramers (**Figure 2B**).

Commercially supplied tetramers have been tetramerized from biotinylated monomers using undisclosed methodology and reagents, and a volume/test rather than concentration is provided by

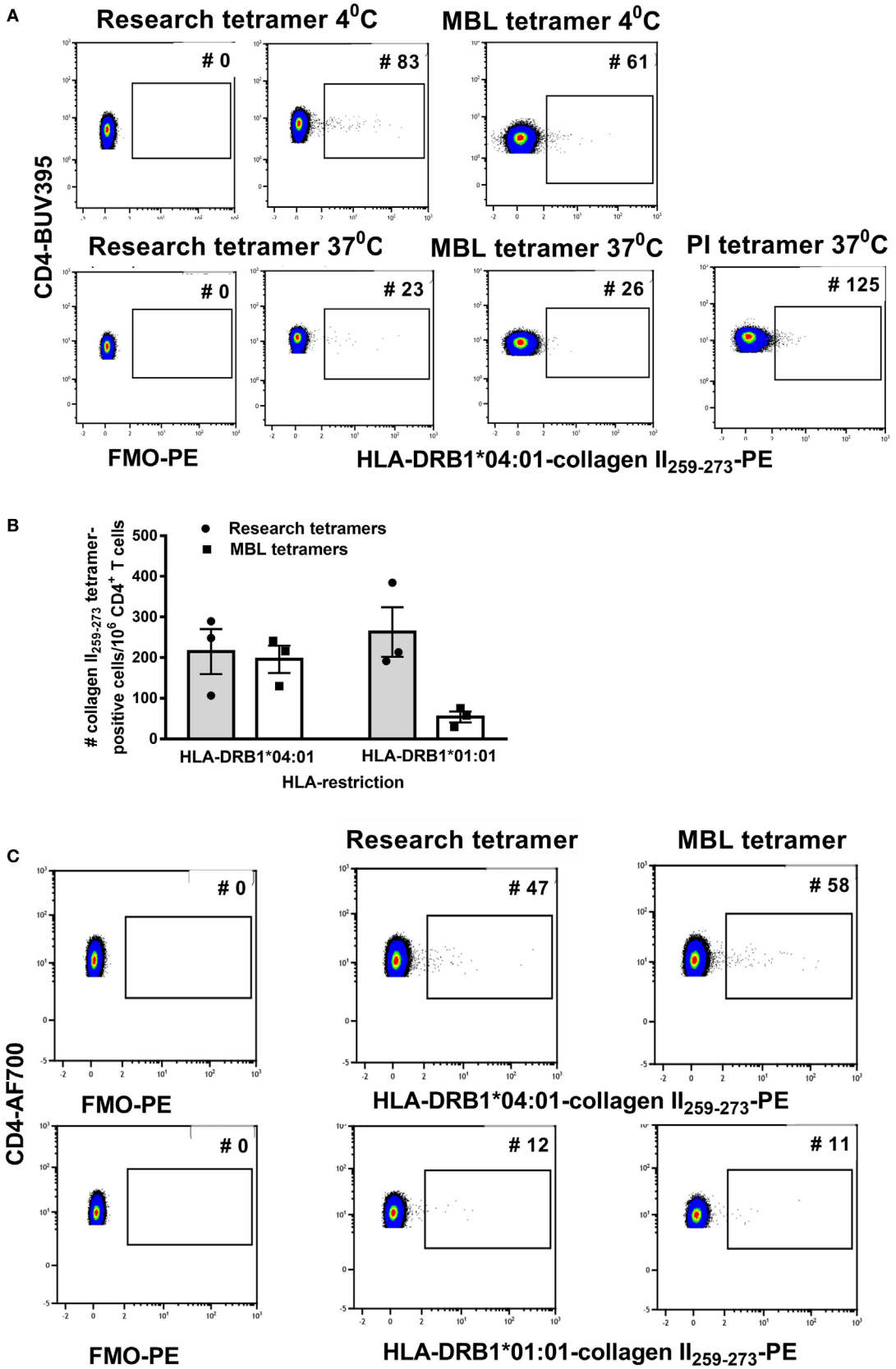

FIGURE 2 | Continued

FIGURE 2 | The streptavidin–biotin ratio is important for optimal tetramer formation. Research tetramers were compared with tetramers purchased from MBL and ProImmune (PI). **(A)** Flow cytometry plots of a representative donor depicting FMO-PE and staining with HLA-DRB1*0401-collagen $II_{259–273}$ tetramers using the short staining protocol (representative of three HLA-DRB1*04:01$^+$ and three HLA-DRB1*01:01$^+$ donors with rheumatoid arthritis). **(B)** The number of tetramer-positive cells per million CD4$^+$ T cells identified using HLA-DRB1*0401-collagen $II_{259–273}$ and HLA-DRB1*0101-collagen $II_{259–273}$ tetramers from two sources (n = 3 replicates per donor), staining at 4°C. **(C)** Flow cytometry dot plots depicting the number of tetramer-positive cells using tetramers generated from monomers as depicted, from two sources using the same formula to calculate the streptavidin–biotin ratio and each stained at a concentration of 4.2 μg/ml (representative of three individual donors). In panels **(A,B)**, staining with research and MBL tetramers was carried out at 4 and 37°C and with PI tetramers at 37°C. In panel **(C)**, all tetramers were stained at 4°C.

the manufacturer. The observed difference in PE signal between research and commercial tetramers suggested differences in staining may be related to the streptavidin–biotin ratio used for tetramerization and/or tetramer concentration when staining. To be able to standardize across comparisons, we purchased DRB1*04:01-collagen $II_{259–273}$ and HLA-DRB1*01:01-collagen $II_{259–273}$ biotinylated monomers from MBL, requested details of % biotinylation and monomer concentration and tetramerized each, based on the percentage of monomer biotinylation according to the formula in **Table 4**. The resulting tetramers generated in our laboratory from MBL or research biotinylated monomers gave comparable staining on the same patient cell samples (**Figure 2C**) when stained at 4.2 μg/ml at 4°C. These results indicate that for optimal reproducibility between tetramer production facilities and laboratories receiving different batches of tetramers, it is important to standardize the procedures for tetramerization from biotinylated monomers, maintaining a consistent source of streptavidin fluorochrome, a consistent streptavidin–biotin ratio, and a consistent tetramer concentration in the staining reaction.

Optimizing Fluorochromes and Gating Strategy

Tetramer intensity varies with the tetramerization procedure (**Figure 2A**), and streptavidin-PE intensity varies somewhat depending on the manufacturer (data not shown). Therefore, when comparisons are required, it is important to standardize these parameters for the entire study and for all laboratories involved. Furthermore, after construction from monomers, tetramers will lose integrity after 2–3 months. Therefore, it is important to calculate the assay time required for a batch of clinical samples so that they can be analyzed with a single monomer batch. The brightest signal relative to background will be obtained with streptavidin conjugated to fluorochromes such as PE, APC, or Brilliant Violet (BV) 421. We have successfully combined tetramers of different pMHC specificities conjugated to BV421 and PE, respectively to identify single or double labeling, i.e., to determine TCR cross-reactivity (6). Reduction in myeloid cell background is avoided with the use of Fc block, live/dead and lineage exclusion gating, and optimization of CD3 and CD4 staining to ensure tight T cell gating, with, minimal background staining of CD3$^+$ CD4$^-$ T cells. **Figure 3** is an example of the gating strategy. For cell surface staining, we avoid the use of tandem dyes based on PE, which could degrade and give false positive PE signal in the tetramer-PE channel. Staining and tetramerization protocols are provided in Supplementary Material.

TABLE 4 | Formula for streptavidin-PE volume calculation for tetramerization of biotinylated monomers.

Volume Strep-PE to add 10 times (μl) = ((amount monomer (μg) × 0.74285714285714)/8)/5
Volume monomer needed = amount monomer (μg)/concentration biotin (mg/ml)
Concentration biotin = concentration monomer × percentage biotinylation

The rationale is as follows: Mass of biotinylated monomer used/70,000 (M_w of Class II HLA-DR) = Volume of Strep-PE/52,000 (M_w of Strep-PE) × 8 (1:8 ratio of Strep-PE to biotinylated monomer needed) × 0.5 (concentration Strep-PE in mg/ml).

DISCUSSION

As a result of advances in antigen-specific immunotherapeutic approaches to induce tolerance, such as the delivery of antigen-exposed tolerogenic dendritic cells or other forms of antigen delivery designed to promote tolerance, early-stage translation to clinical trials in autoimmune disease has begun (1, 19–22). While animal data are promising, it is likely that for consistent demonstration of robust clinical outcomes from antigen-specific approaches, ongoing basic and clinical development from a number of angles will be required. During the current exploratory phase in this field, it is essential to gather as much information as possible on the CD4$^+$ T cells responding to delivered antigen to assess and to improve on the outcomes of clinical trials of antigen-specific immunomodulatory approaches. pMHCII multimers represent an excellent tool to monitor antigen-specific CD4$^+$ T cell responses, as they can be used to quantify the frequency and assess the phenotype of antigen-specific T cells (6, 14). In addition, as staining reagents, they can facilitate in-depth, exploratory single cell analyses, such as TCR sequencing, transcriptomics, assessment of oligoclonal expansion, and cloning (6, 23). Furthermore, because they can be applied to flow cytometry or cell sorting, multimers are much more sensitive and versatile than *ex vivo* analyses such as ELISPOT, which struggle to detect low level cytokine responses above background produced by autoantigen-specific memory T cells (24–26).

However, due to the low avidity and high off-rate between pMHCII and the TCR, these assays are technically challenging for use in clinical trials. Here, we investigated reagent and assay limitations that could impact the use of peptide–HLA–DR tetramers in clinical trial settings for immunomonitoring. We describe a peptide–MHC class II flow cytometry-based assay to quantify and phenotype antigen-specific CD4$^+$ T cells. We show that this assay can be used in frozen PBMC, but that optimal cell yield for identification of antigen-specific CD4$^+$ T cells after thawing is best achieved by reducing staining time before analysis on the flow

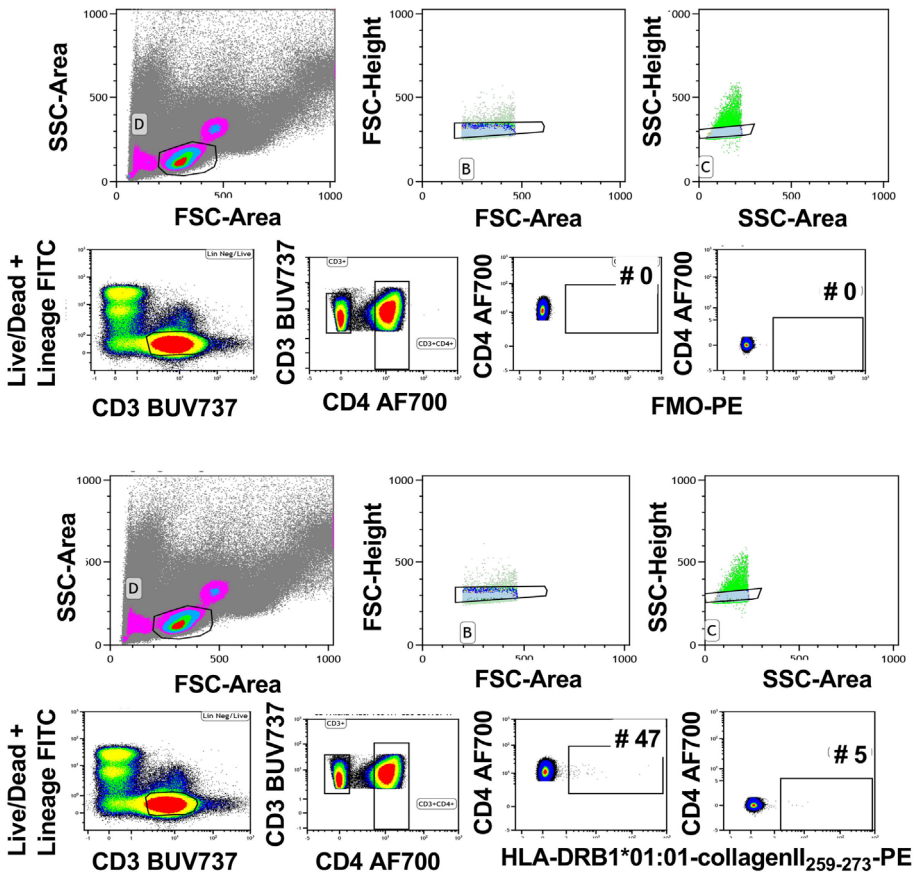

FIGURE 3 | Gating strategy for tetramer analysis. Live cells were gated using forward and side scatter. Subsequently, single cells were gated using forward scatter height and area followed by side scatter height and area (upper panels). Live CD3$^+$ cells were gated using Lineage/LIVE/DEAD marker negative and CD3-positive cells. Next, CD4$^+$ T cells were gated by gating on the CD3$^+$ CD4$^+$ double-positive cells. The FMO sample was used to determine the tetramer-positive gate. The gate was set on the CD4-postive cells that are negative in the tetramer channel (middle panels). This gate was used to identify the CD4$^+$ tetramer$^+$ cells in the rest of the samples (bottom panels). Representative of 40 experiments.

cytometer. Elimination of dasatinib and sandwich amplification steps, combining staining steps, reducing the number of washes, adding DNAse during thawing, and resting of the cells after thawing all improved efficiency of identification of tetramer$^+$ CD4$^+$ T cells. As a result of these modifications, replicates of the same tetramer stain on the same sample showed good reproducibility of cell frequency (**Figure 2B**). Furthermore, to optimize the number of tetramer-positive cells detected, the source of monomers and the tetramerization formula must also be taken into account, as this will affect the staining outcome. When tetramerized similarly from biotinylated monomers and stained at the same concentration, replicates of the same samples stained with tetramers from different sources also showed good reproducibility. Furthermore, we provide an example of gating strategy and tips on fluorochrome use. Previously we have demonstrated by single cell sorting using pMHCII multimers that HLA-DRB1*14:02-Vimentin$_{59-71}^+$ and HLA-DRB1*14:02-Vimentin$_{59-71}$Cit64$^+$ autoreactive T cells are oligoclonally expanded in the blood of RA patients, and that a representative HLA-DRB1*14:02-Vimentin$_{59-71}$-reactive TCR cloned from the identified sequences could be restimulated with HLA-DRB1*14:02-Vimentin$_{59-71}$ or HLA-DRB1*14:02-Vimentin$_{59-71}$Cit64, but not HLA-DRB1*14:02-CLIP (6). These studies indicate that autoantigen-specific cells can be identified, cloned and characterized for peptide specificity and cross-reactivity using the methods described here for pMHCII staining.

Even though the described modifications resulted in reproducible identification of self-antigen-specific CD4$^+$ T cells, there are limitations to tetramer assays apart from the low cell frequencies identified by staining. Due to the low avidity between self-antigen–MHCII complexes and TCRs, the mean fluorescence intensity of tetramer staining is low. As a result background and positive staining tend to merge—hence attempts to amplify staining signal with dasatinib and sandwich stains—which creates difficulty in setting the cutoff for positive staining. This is much less of a problem for tetramers of higher avidity, e.g., pMHCI tetramers, HLA–DQ–gliadin peptide tetramers, or tetramers for B cell receptor staining. To determine the cutoff between background and positive staining of autoantigen-specific CD4 tetramers, we have used FMO gating. This is because control peptide tetramers typically identify T cells with low-avidity staining to the control peptide and mismatched HLA tetramers typically

identify low numbers of alloreactive T cells. While it is useful to compare frequency of T cells with these reactivities relative to the antigen-specific T cells of interest, these controls are unhelpful for discriminating between background and positive staining. An alternate strategy is to check background tetramer staining in the $CD3^+CD4^-$ T cell gate. Using the strategies outlined here, this was demonstrated to be minimal (**Figure 3**). However, it is important that the antigen-specificity of $tetramer^+$ T cells be verified by other means, i.e., transduction of paired alpha-beta TCR derived from $tetramer^+$ T cells and restimulation with the appropriate antigen presenting cells and peptides. Finally, general availability of a range of positive control reagents (antigen-specific, HLA-restricted CD4 T cell clones or hybridomas) would be helpful to validate tetramers produced by research labs and facilities, as well as those sold by companies. Although some of these autoantigen-specific reagents exist, these have been challenging to produce on a broad scale so far.

Since the development of technologies to produce pMHCII tetramers, methodologies for effective, specific and reproducible identification of antigen-specific $CD4^+$ T cells from blood and other tissues of mice and humans have been improving. While these methods represent our current optimized techniques for reproducibility in clinical studies, we anticipate that research methods will continue to improve with advances in reagents and analytical technologies, such as mass cytometry. This evolution will likely be rapid due to a strong drive to understand mechanisms and responses to antigens in autoimmune diseases and the outcomes of immunotherapies designed to modify those responses.

AUTHOR CONTRIBUTIONS

Contributed and/or interpreted data: DJ, NR, and HN. Provided reagents: JR, KL and HR. Wrote paper and study design: DJ, NR, and RT. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00008/full#supplementary-material.

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Conflict of Interest Statement: RT holds and has filed patents surrounding technology for targeting DCs for antigen-specific tolerance and is a director of the spin-off company, Dendright, which is commercializing antigen-specific immunotherapy in collaboration with Janssen Biotech Inc. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Cell Therapy in Organ Transplantation: Our Experience on the Clinical Translation of Regulatory T Cells

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Solid organ transplantation is the treatment of choice for patients with end-stage organ dysfunction. Despite improvements in short-term outcome, long-term outcome is suboptimal due to the increased morbidity and mortality associated with the toxicity of immunosuppressive regimens and chronic rejection (1–5). As such, the attention of the transplant community has focused on the development of novel therapeutic strategies to achieve allograft tolerance, a state whereby the immune system of the recipient can be re-educated to accept the allograft, averting the need for long-term immunosuppression. Indeed, reports of "operational" tolerance, whereby the recipient is off all immunosuppressive drugs and maintaining good graft function, is well documented in the literature for both liver and kidney transplantations (6–8). However, this phenomenon is rare and in the setting of liver transplantation has been shown to occur late after transplantation, with the majority of patients maintained on life-long immunosupression to prevent allograft rejection (9). As such, significant research has focused on immune regulation in the context of organ transplantation with regulatory T cells (Tregs) identified as cells holding considerable promise in this endeavor. This review will provide a brief introduction to human Tregs, their phenotypic and functional characterization and focuses on our experience to date at the clinical translation of Treg immunotherapy in the setting of solid organ transplantation.

Keywords: transplantation, regulatory T cells, clinical trials, good manufacturing practice, cell therapy, technical transfer

Tregs: PHENOTYPE AND HETEROGENEITY

Tregs are characterized by the expression of CD4 and CD25 molecules and more specifically the transcription factor FOXP3, a master control gene underpinning Treg development and function. More recently, the inverse expression of the α-chain of IL-7R, CD127, combined with the expression of CD4 and CD25 has been shown to demarcate a pure population of Tregs demonstrating stability and optimal function (10, 11). Tregs are far from homogenous and over the years have been characterized into several subsets, most of which have been associated with various facets of Treg function (12).

Treg subsets can firstly be stratified according to their origin: thymus-derived and peripherally derived Tregs (tTregs and pTregs, respectively). While there have been phenotypic markers which have suggested differentiation between the two subsets, such as Helios and Neuropilin-1, to date these are still contentious (13–16). The only way to reliably differentiate tTregs and pTregs has been to interrogate the Treg-specific demethylated region (TSDR). Here, tTregs display a fully demethylated TSDR, whereas pTregs are only partially demethylated (17–20). Furthermore, Tregs have also been delineated on account of their antigen naivety in relation to their differential expression of CD45RA and FOXP3. The seminal work of Miyara et al., described the heterogeneity of the Treg population in three phenotypically and functionally distinct subpopulations: Population I (P1) naive or resting Tregs ($CD45RA^{+}FOXP3^{Lo}$); population II (P2) effector Tregs ($CD45RA^{-}FOXP3^{Hi}$), both of which are suppressive *in vitro*; and population III (P3) the "non-suppressive," cytokine secreting non-Tregs ($CD45RA^{-}FOXP3^{Lo}$) (21). However, we and others have previously shown that population III is indeed suppressive, and within this population, identified a subpopulation of Tregs that express the C-type lectin CD161 and produce the proinflammatory cytokine, IL-17 (22–24).

In support of the heterogeneity of the Treg compartment, using a new technology, the single-cell mass cytometry (cytometry by time-of-flight) we have conducted an in-depth characterization of Tregs, further demonstrating the true extent of their heterogeneity, with 22 different clusters identified (25). In the clinical setting and through utilizing the same principles and technology, Kordasti et al. identified the Treg subset that predicted response to immunosuppressive therapy in patients with aplastic anemia (26). Additionally, we have recently extended the analysis of T helper-like subpopulations of Tregs, demonstrating that Th2-like Tregs are enriched in the tumor sites (27). There is no doubt that the future will see the discovery of many more markers heralding Treg purity. For an in-depth review into Treg phenotype subsets the reader is directed to the following reviews (12, 28, 29).

Treg MECHANISM OF ACTION

Tregs are defined by their immunoregulatory suppressive qualities. However, no one mechanism defines Tregs. Instead, it is believed that several mechanisms behave in concert, which promote immune regulation. All T lymphocytes rely on IL-2 for their survival and proliferation. By their expression of the interleukin 2 receptor, CD25, Tregs deplete stores of IL-2, curbing the survival of surrounding T lymphocytes (28). In a more active mechanism of suppression, the Treg surface molecule, cytotoxic T lymphocyte antigen 4 (CTLA-4), is known to bind the costimulatory molecules CD80/86 with a higher affinity than its proinflammatory competitor CD28, expressed on conventional T effector cells, thus preventing T effector activation. This negative costimulatory molecule has also been proposed to upregulate indoleamine 2, 3-dioxygenase expression on dendritic cells, responsible for the catabolism of tryptophan, which in turn suppresses immune responses through the generation of the immunosuppressive molecules, in particular kynurenine (30). Further in-depth investigation into the mechanism of action of CTLA-4 has revealed that CD80/86 ligands on antigen-presenting cells (APCs) are captured by a process of trans-endocytosis, in turn impairing further T cell activation (31–33). Deficiencies in CTLA-4 have been associated with lymphoproliferative disorders and the development of severe T-cell-mediated autoimmune diseases, which is why CTLA-4 is recognized as a key molecular target governing Treg-mediated suppression (33, 34).

The ectoenzyme, CD39, is abundantly expressed on Tregs, its expression allows Tregs to hydrolyze the proinflammatory danger signal, adenosine triphosphate, to the anti-inflammatory mediator, adenosine, which following interaction with the adenosine A2A receptor, has been reported to have immunosuppressive and anti-proliferative effects (35). $CD39^{+}$ Tregs have also been found to suppress the release of IL-17, alongside IFN-γ and IL-2, from Th17 cells, while $CD39^{-}$ T cells had an increased propensity to produce IL-17 (36). Furthermore, the reconstitution of positively selected CD39-null mouse models of colitis with soluble apyrase, a mediator with enzymatic activity identical to CD39, reversed their increased susceptibility to develop auto-immune diseases and prevented a Th-1 skewed immune response. Further studies by Gibson et al., have highlighted the importance of CD39 expression for Treg mechanism of action in a T cell transfer model of colitis (37). Additionally, we have established a further mechanism by which Tregs function through the release of exosomes, expressing ectoenzyme CD73, which regulate target cells through the purinergic generation of adenosine (38).

Reports have also suggested a cytotoxic role of Tregs in depleting T effector numbers through a perforin-dependent and granzyme-dependent manner (39). Similarly, there have been reports of Tregs expressing Galectin-9, which following binding with the T-cell immunoglobulin and mucin-domain containing-3 receptor CD44 on effector T cells, has been shown to induce apoptosis (40). More recently, this mechanism of suppression has been proposed to be limited to pTregs (41). It has also been postulated that Tregs have the capacity for cytokine-mediated suppression involving the regulatory cytokines: IL-10 (42), TGF-β (43, 44), and more recently IL-35 (45).

Treg ISOLATION AND EXPANSION

Clinical trials of Treg therapy in transplantation are focused on tipping the balance of immune homeostasis in favor of regulation. However, in order for this to occur the *in vivo* Treg pool needs to be expanded significantly in order to drive immune tolerance (46). As such, there has been huge interest in either the *in vivo* expansion of these cells or their adoptive transfer. Here, we focus on the prerequisites that need to be fulfilled in order to permit the adoptive transfer of these cells.

Firstly, Tregs need to be isolated from the peripheral blood and this is by no means an easy feat. As mentioned earlier, Tregs are highly heterogenous and as such, the debate of which population of Tregs would serve as optimal cell product in adoptive transfers is a highly discussed topic. Isolation to date has largely been governed by magnetic bead isolation, a process whereby depletion of $CD8^{+}$ T cells and positive selection of $CD25^{+}$ T cells using magnetic

beads allows isolation of Tregs (47, 48). This process results in the separation of a consistent number of cells from whole blood using an approach that has been utilized by us in the two clinical trials aimed at testing the safety of expanded autologous Tregs in patients receiving either kidney (ONE Study, NCT02129881) or liver (ThRIL, NCT02166177) transplants. However, this process does not allow for enrichment based on multiple parameters and isolating Tregs based upon $CD25^+$ expression alone results in contaminating T effector cells which express low levels of CD25 molecules. Methods to overcome this have been explored in Treg expansion. One such strategy is the *ex vivo* expansion of these cells in the presence of the mTOR-inhibitor, rapamycin (RAPA) (49).

We and others have shown that RAPA is an ideal treatment strategy for preferential expansion of Tregs (50–52). RAPA confers a proliferative advantage to Tregs by affecting the Akt/mTor pathway. Indeed, the molecular signal controlled by this pathway is not essential for Tregs, but crucial for the activation and proliferation of conventional T cells (51, 53). Using this approach, we have shown that large-scale expansion of functionally potent Tregs is possible when starting from a population of cells with low purity (48). In our studies, another potential candidate drug for the expansion of Tregs is the vitamin A metabolite, all-trans retinoic acid (ATRA). Some studies have shown that ATRA can be a good treatment for the induction of adaptive Tregs (54) or very pure Tregs (55). Our data showed that ATRA favors the *ex vivo* expansion of a population of highly suppressive Tregs from magnetic bead purified Tregs, although these cells produce a significant amount of IL-17 and IFN-γ following stimulation. However, further characterization of Tregs cultured with ATRA has shown the expansion of $FOXP3^+CD161^+$ Tregs (51), which is encouraging based on our previous work reporting that CD161 identifies a specific sub-population of IL-17-producing $FOXP3^+$ Tregs with a strong capacity to suppress conventional T cell proliferation (22). In contrast, Tregs expanded in the presence of RAPA show decreased expression of CD161, as well as reduced production of pro-inflammatory cytokines. The combined treatment of Tregs with RAPA and ATRA demonstrated that the suppressive function and stability of Tregs is maintained (decreased CD161 expression and lack of IL-17 production).

This analysis was extended to the subtypes of Tregs and while P2 Tregs did not expand *in vitro*, P1 Tregs expanded well in the presence of RAPA, ATRA or the combination of the two drugs. In contrast, RAPA (alone or in combination with ATRA) strongly reduced P3 Treg proliferation, while the treatment with ATRA alone showed a negligible inhibitory effect on the expansion of this subset (51). In support of this observation, we have unpublished data showing that when P1 and P3 were cultured together at 1:1 ratio, the presence of RAPA, but not ATRA, gave a much stronger proliferative advantage to P1 on P3 Tregs. At the end of culture, P1 could overgrow P3 Tregs. Additional experiments showed that RAPA treatment could inhibit the production of proinflammatory cytokines in the P3 subset and negatively affect the expansion of $FOXP3^+CD161^+$ Tregs (51). Finally, we have also demonstrated that these treatments can influence the migratory ability of expanded Tregs. Our findings showed that the treatment of Tregs with RAPA led to the expression of skin-homing receptors cutaneous lymphocyte-associated antigen and CCR4 (51), as well as CXCR3, a chemokine receptor which enables homing to the liver (48). Instead, Treg cultures in the presence of ATRA resulted in a high percentage of cells coexpressing gut-homing receptors such as CCR9 and α4β7 (51).

TECHNICAL TRANSFER OF THE Treg MANUFACTURING PROCESS INTO THE GOOD MANUFACTURING PROCESS (GMP) UNIT

One of the most difficult and time-consuming aspects of translating the extensive research carried out in the laboratory to the clinical setting is the transfer of research protocols into a GMP unit. This process, referred to as technical transfer, requires careful management to ensure the resulting product maintains a consistently high level of quality, while achieving the aim of the researcher. In this pursuit, a manufacturing process was developed with the aim of creating a Treg Investigational Medicinal Product (IMP) that could be used in the ONE Study (NCT02129881) or ThRIL (NCT02166177) clinical trials.

There are many factors to consider during the technical transfer (**Figure 1**), and important milestones include: the sharing of technical information from the research department to the GMP facility, process development, preparation of the product specifications, process validation and finally authorization of the process by the Medicines and Healthcare Products Regulatory Agency (MHRA) through the submission of a Clinical Trials Authorization (CTA). The process initially starts with the sharing of a detailed description of all process raw materials, manufacturing methods, equipment used, specifications, and test methods from the transferring research department to the GMP unit. It is important to ensure that all raw materials, reagents and consumables that were used in the research laboratory are GMP-compatible. During the technical transfer stage of these Treg trials, different GMP-compatible reagents and culture conditions were compared, ensuring that the final protocol resulted in phenotypically stable Tregs that could be isolated and expanded consistently while maintaining their suppressive function (**Table 1**).

The transfer of the Treg process into the GMP unit also provided the opportunity to optimize the manufacturing method and introduce automated processes to increase the levels of consistency between batches. The original research process included a peripheral blood mononuclear cells (PBMCs) isolation using density gradient centrifugation with Ficoll® Paque Plus (GE Healthcare, Switzerland) and 50 ml centrifuge tubes. In order to close out and automate this process, the SEPAX system (Biosafe, Switzerland) was adopted. The SEPAX device is an automated cell separation system which processes within closed single-use disposable tubing sets. The maximum blood input volume to run the NeatCell Programme (SEPAX density gradient cell separation using Ficoll® Paque Plus) is 120 ml, therefore the SEPAX volume reduction programme (SmartRedux) was introduced to ensure the full initial blood sample could be processed. The data generated from these initial runs identified that the volume reduction

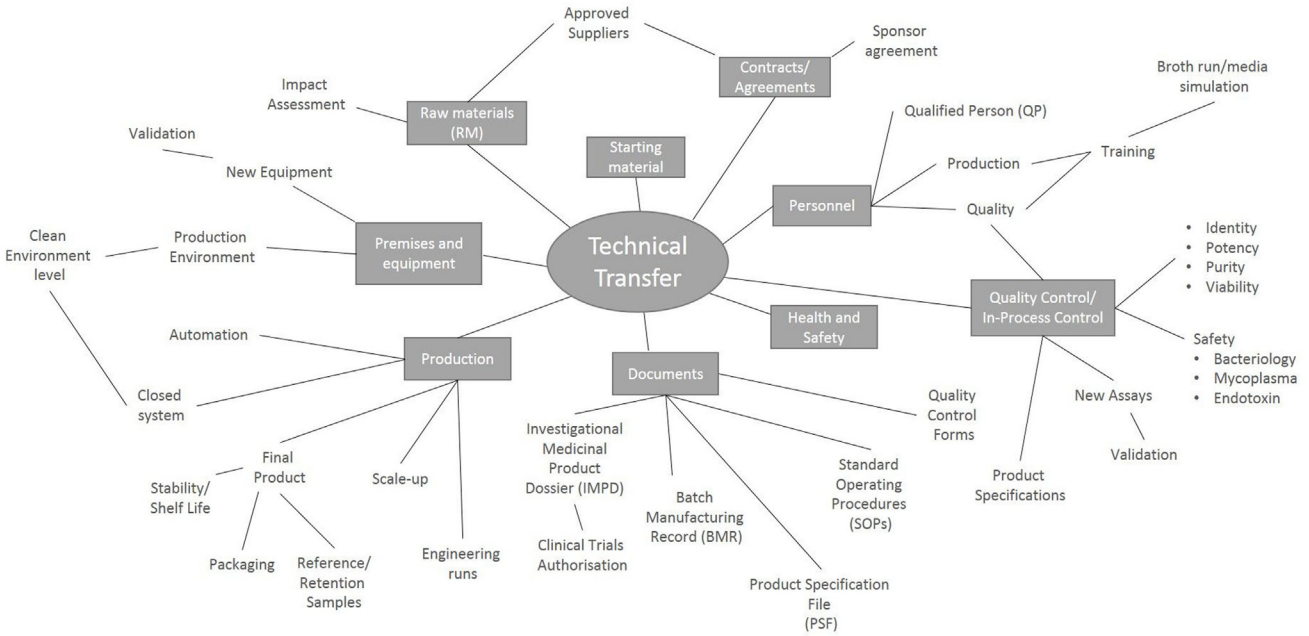

FIGURE 1 | Key points to consider during a technical transfer process. Schematic representation of the processes involved during the transfer of a manufacturing process from the research laboratory into a Good Manufacturing Process unit.

TABLE 1 | Comparison of research- and GMP-grade reagents.

Reagent	Research lab		GMP unit	
CD8 MicroBeads	CD8 MicroBeads	Miltenyi Biotec	CliniMACS CD8 Reagent	Miltenyi Biotec
CD25 MicroBeads	CD25 MicroBeads	Miltenyi Biotec	CliniMACS CD25 Reagent	Miltenyi Biotec
αCD3/CD28 Beads	Dynabeads™	Invitrogen	GMP ExpAct Treg Beads	Miltenyi Biotec
Interleukin-2	Proleukin	Novartis Pharmaceuticals	Proleukin	Novartis Pharmaceuticals
Rapamycin	Rapamycin	LC Laboratories	Rapamune	Pfizer
Cell culture medium	X-Vivo 15 with Phenol Red	Lonza	TexMACS	Miltenyi Biotec
Human AB serum	Research grade	Biowest	Premium grade	Seralab
Expansion device	Culture plates and flasks	VWR	Culture bags	Miltenyi Biotec

step alone was sufficient, as the erythrocytes did not interfere with the CliniMACS Plus (Miltenyi Biotec, Germany) magnetic cell selections.

The specifications of the final product are set by the research team and from this information, a product specification file (PSF) can be written. The PSF contains, or refers to files containing, all the information required to draft the standard operating procedures, batch manufacturing records, and the quality control assay forms. These documents ensure standardization and traceability of the process for every batch manufactured.

With these documents in place, scale-up runs can be performed allowing the manufacture process to be tested within the GMP unit and ensuring enough Tregs could be generated to produce the dose required for the patients, perform all QC assays and to create the required reference and retention samples. During the scale-up runs, the opportunity to further close out the system and minimize the risk of contamination was taken. For example, Tregs were cultured using plates and T-flasks in the research laboratory. During the scale-up process, this method was adapted to utilize closed-system culture bags. However, it was found that transferring the process into bags reduced the expansion rate of the Tregs. In spite of this, the required numbers of Tregs were still achieved and so this closed system modification was incorporated into the final manufacturing process.

With the manufacturing methods finalized during the scale-up runs and following the validation of the QC assays, the entire process was validated by performing six engineering runs. These engineering runs provided documented evidence that the GMP unit could routinely and consistently produce the Treg product that met the required specifications.

The data, from both the research experiments and GMP unit, was compiled to create the IMP Dossier (IMDP). This document contains information on the quality, manufacture and control of the IMP. This is one of the essential documents, along with a EudraCT number and approval from an ethics committee, that form the CTA submission to the MHRA.

RECRUITMENT AND MANUFACTURE OF Treg BATCHES IN THE ONE STUDY AND ThRIL TRIAL

The ONE Study (NCT02129881) initiated in 2014, a dose-escalation phase I/II trial carried out under a large EU consortium. The aim of The ONE Study was to evaluate different regulatory cells in kidney transplant recipients allowing the direct comparison of the safety, practicality and therapeutic effect of each cell type. In the UK, in collaboration with The University of Oxford, our group manufactured sixteen batches of polyclonal Tregs, of which 12 were certified for administration. A dose escalation design was implemented with four escalating doses of 1×10^6, 3×10^6, 6×10^6, and 10×10^6/kg, with the last patient being treated with the maximum dose in January 2016. Four of the batches could not be certified and dosed, three of which were due to insufficient cell numbers to formulate the dose and one batch failing the release criteria due to bacterial contamination. High levels of variability were observed in the expansion capability of the Tregs obtained from different patients. Due to this, a substantial amendment was made to the IMPD allowing flexibility in setting a date for the harvesting of Treg culture. This amendment reduced the risk of failing batches during the manufacture of an expensive and time-consuming product.

The last patient in the ThRIL trial (NCT02166177) was dosed in July 2017. This was a Phase I/IIa clinical trial of Treg immunotherapy in the setting of liver transplantation initiated at King's College London. Here, the safety, tolerability and efficacy of polyclonally expanded Tregs in combination with depletion of alloreactive T cells (ATG) and short-term immunosuppression was assessed. For this trial, patients awaiting a liver transplant were recruited with the aim of administering the cell therapy 2 months after transplantation. Out of an initial 23 patients enrolled, 7 of these patients received a transplant and the manufacture of the Treg IMP was initiated. Of these seven batches, three were completed and certified by a qualified person for administration. Two patients were removed during manufacturing, one due to proteinuria and the other due to death unrelated to the transplant. The other two batches did not meet the specifications (cell number and purity). The complications in patient retention lead to a substantial amendment to the MHRA to allow the recruitment of patients 6-month posttransplantation rather than while they were on the waiting list. This delay in recruitment was made as the transplanted patients were deemed to be more stable, and hence more amenable for cell therapy application. After this amendment was approved, a further six batches of the Treg IMP were manufactured and these patients were successfully treated.

Although these two trials are still in the patient follow-up stage, no serious adverse events have been observed suggesting that polyclonal Treg therapies are safe. We are now in the preparation stages of Phase II clinical trials in renal, liver, islet and heart transplant patients.

THE RELEVANCE OF SPECIFICITY FOR Treg THERAPY

While the polyclonal expansion of Tregs using anti-CD3/antiCD28 beads is relatively straightforward and readily translatable into the clinic, extensive data from preclinical animal studies have demonstrated that the adoptive transfer of Tregs with direct or indirect allospecificity are superior to polyclonal Tregs at reducing graft rejection (56–58). However, the expansion of antigen-specific Tregs presents an additional set of parameters that need to be addressed, including the origin of APCs and the dose required. Initial studies by Taylor et al., used allogeneic splenocytes to enrich for murine allo-specific Tregs, which were more efficient at reducing graft versus host disease in a murine model compared to anti-CD3 stimulated Tregs (59). More recently, we have shown in a murine transplant model that Tregs need to have both direct and indirect allospecificities to induce indefinite survival of heart transplants (57). Furthermore, using a humanized transplant mouse model, in which immunodeficient mice were reconstituted with PBMCs, we show that human Tregs with direct allospecificity significantly reduced alloimmune-mediated injury of human skin grafts, when compared with polyclonal Tregs. As part of the ONE Study Consortium, Tregs with direct allospecificity are currently being evaluated at two different sites in the USA (NCT02244801 and NCT02091232). GMP regulations are far more rigorous in Europe compared to USA and the investigators have been able to use a standard cell sorter in their manufacturing process to purify Tregs, which is not possible in Europe. Few patients have been treated to date, but the completion of the trials will undoubtedly be very informative with regards to the safety of allospecific Tregs and provide possible clues on how well they compare against treatment with polyclonal Tregs.

In recent years, the transduction of chimeric antigen receptors (CAR) on T cells has shown great promise in the field of cancer cell therapy, particularly for B cell lymphomas where there is a clear target antigen such as the B lymphocyte antigens, CD20, and CD19. This has paved the way for its potential use in Tregs. We and others have shown that the expression of CAR in Tregs can potentially be used for the treatment of xeno-GVHD and allo-graft rejection (60–62). In a human skin xenograft transplant model, the adoptive transfer of CAR Tregs were more effective at alleviating the alloimmune-mediated skin injury caused by transferring allogeneic PBMCs compared to polyclonal Tregs. Recently, the US Food and Drug administration approved two CAR T cell therapies, the first one, Kymriah (Tisagenlecleucel) was developed for the treatment of patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia and Yescarta (axicabtagene ciloleucel) for the treatment of adults with refractory large B cell lymphomas. These approvals signify an important development and no doubt will pave the way for continued commercialization of cell therapies.

CONCLUSION

A key breakthrough in the translational potential of Treg cell therapy was the demonstration that human Tregs could be successfully isolated and expanded *ex vivo* while maintaining immunoregulatory function. This has enabled the application of these cells in the clinic, leading to Treg adoptive transfer in phase I clinical trials of bone marrow transplantation and type I diabetes (63–66) and more recently in the setting of solid organ transplantation (67, 68). The success of these trials is reliant on

a highly reproducible process for the sustained manufacture of autologous patient-derived Tregs. In the setting of solid organ transplantation, we are faced with the challenge of a more targeted approach to suppress the immune system, and as such efforts have focused on the expansion of allo-antigen specific Tregs for cell therapy application. Here, we have highlighted our experience to date. However, whether a generalized effect of immunosuppression by the adoptive transfer of polyclonal Tregs could potentially be diminished by more targeted Treg therapy requires further investigation. The major drawback of the two phase I clinical trials completed by us is that the isolation technique for regulatory T cells relies on first generation magnetic bead isolation. The inability of this technique to select cells based on stricter criteria ($CD25^{hi}$) or multiple parameters (low expression of CD127) has led to the development of a GMP compliant FACS cell sorter (MACSquant Tyto cell sorter, Miltenyi Biotech). The validation of the MACSQuant Tyto and its GMP accreditation has now meant that we can isolate Tregs based on several markers, further enhancing the purity and quality of the infused product. The high level of purity achieved with the MACSQuant Tyto will allow us to generate donor-specific Tregs either by using donor-derived APC or by transduction with CAR. Finally, the selection of the most favorable Treg population, which will give the best therapeutic advantage, will likely be further enhanced by the advent of new technologies.

With scientific knowledge and technology rapidly advancing in the field, the future of Treg cell therapy is set to only progress further. As such, our ultimate aim of immune tolerance in transplantation is soon to become a reality.

AUTHOR CONTRIBUTIONS

GL, NS, and NG contributed to the conception, design, writing, and revision of the manuscript. ST, CS, LF, TV, and RL contributed to the writing, and revision of the manuscript.

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frontiers in Immunology

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Peptide Immunotherapy for Type 1 Diabetes—Clinical Advances

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Autoimmune and allergic diseases occur when an individual mounts an inappropriate immune response to a self-antigen or an innocuous environmental antigen. This triggers a pathogenic T-cell response resulting in damage to specific tissues and organs. In type 1 diabetes (T1D), this manifests as destruction of the insulin-secreting β cells, resulting in a life-long dependency on recombinant insulin. Modulation of the pathogenic T-cell response with antigen-specific peptide immunotherapy offers the potential to restore the immune homeostasis and prevent further tissue destruction. Recent clinical advances with peptide therapy approaches in both T1D and other diseases are beginning to show encouraging results. New technologies targeting the peptides to specific cell types are also moving from pre-clinical development to the clinic. While many challenges remain in clinical development, not least selection of the optimal dose and dosing frequency, this is clearly becoming a very active field of drug development.

Keywords: autoimmunity, type 1 diabetes, peptide immunotherapy, tolerance, antigen specific

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INTRODUCTION

Type 1 diabetes (T1D) is an organ-specific autoimmune disease. Auto-reactive T cells attack the insulin-producing beta (β) cells of the pancreatic islets, leading to a loss of endogenous insulin production and subsequent impaired glucose metabolism. Management of the disease requires daily administration of exogenous insulin and frequent monitoring of blood glucose levels. While there have been significant advances in recombinant human insulins and technologies both to deliver insulin and monitor blood glucose levels, many patients do not achieve optimal glycemic control. This is particularly apparent in children and young adults, in whom blood glucose control, measured by glycated hemoglobin (HbA1c) levels, is typically poor, reaching greater than 8% in the majority when the desired levels are below 7.5% (1). The longer-term complications associated with elevated levels of HbA1c are significant and include retinopathy, nephropathy, and neuropathy. Clearly, there is a need for new therapies that either preserve or restore β-cell function to improve glycemic control and patient outcomes.

Preservation of an individual's own endogenous insulin secretion by modulation of the immune system would be the optimal solution. However, the major challenge for this therapeutic approach is that at the time of diagnosis, a considerable loss of β cells has already occurred (2). Encouragingly, there are now supportive data to suggest that preservation of residual β-cell function at the time of diagnosis may indeed lead to improvements in glycemic control (3, 4) and thereby impact upon long-term outcomes. Preventing immune-mediated attack on β cells would also open the possibility for β-cell regeneration and/or replacement therapies to be more effective and durable.

PEPTIDE IMMUNOTHERAPY

Autoimmune and allergic diseases arise when an individual mounts an inappropriate immunological response to a self-antigen or an innocuous environmental antigen (5). Antigen-reactive T cells become activated and expand in number, and the regulatory T-cell pool is no longer capable of controlling the immune response, leading to the loss of immune homeostasis. Peptide immunotherapy offers the potential to restore immune homeostasis *via* the expansion of the regulatory T-cell pool and the deletion and/or anergy of the pathogenic T-cell population (**Figure 1**).

Clinical proof of concept that peptide therapy can restore immune homeostasis has been championed by studies of allergic disease. Cat-PAD is a peptide cocktail consisting of seven peptides from Feld1, the major cat allergen. A recent clinical study has shown that a short course of intra-dermal treatment with these peptides resulted in a clinically meaningful reduction in rhino-conjunctivitis symptoms, 2 years post-treatment (6). Disappointingly, a large phase III study failed to confirm the earlier positive clinical data (ClinicalTrials.gov: NCT01620762). This may in part be explained by a large placebo effect that was observed and the transition to field-based studies, which inherently introduce greater variability. An ongoing clinical study focusing on the phenotype of the allergen-specific T cells following administration of Cat-PAD will confirm whether peptide therapy can modulate the pathogenic T-cell response (ClinicalTrials.gov: NCT02311413).

Clinical studies with peptides derived from auto-antigens for the treatment of autoimmune disease are also now emerging. A phase IIa study with a peptide immunotherapy treatment for relapsing remitting multiple sclerosis demonstrated statistically significant reductions in total and new T1 Gadolinium enhancing brain lesions (representing sites of inflammation and damage) measured using magnetic resonance imaging during treatment. This therapy consists of a cocktail of four peptides derived from myelin basic protein.[1] Two recent phase I studies with a peptide immunotherapy treatment for celiac disease have also shown encouraging data suggesting that the antigen-specific T-cell responses can be modulated following peptide treatment (7). Further development of NexVax2, a mix of three peptides derived from gluten, is planned.

PEPTIDE IMMUNOTHERAPY FOR T1D

For peptide therapy to be successful in treating autoimmune or allergic disease, it is essential that the major auto-antigens responsible for driving the disease are known. Using the presence of auto-antibodies to the same antigens as a guide increases the probability that appropriate antigens have been selected. The ability to detect antigen-specific T cells is also important not only to aid the initial epitope discovery of disease-relevant peptides but also to track modulation of the T-cell phenotype following clinical administration.

Type 1 diabetes emerges as an ideal autoimmune disease for trialing treatment with peptide therapy. A number of the major antigens have been identified, including proinsulin, GAD65, IA-2, and ZnT8. Auto-antibodies to these antigens can be detected both in the sera of patients at the time of diagnosis and in individuals who are at high risk of future disease, whether they are entirely

[1]https://apitope.com/apitope-announces-positive-atx-ms-1467-phase-iia-data-relapsing-multiple-sclerosis/

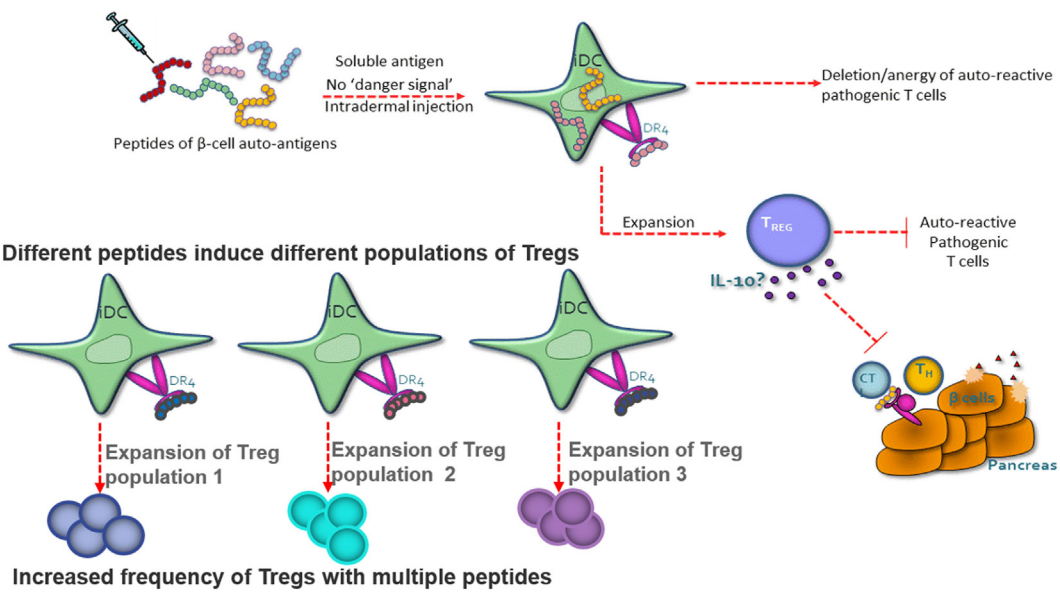

FIGURE 1 | Peptide therapy restores immune homeostasis *via* natural antigen-specific immunoregulatory pathways. Highly soluble peptides are taken up by immature dendritic cells and presented to antigen-specific CD4+ T cells. The CD4+ T cells have one of the three potential fates; death, anergy, or the expansion/generation of a regulatory T-cell phenotype. The regulatory T cells suppress the pathogenic T cell *via* IL-10 secretion. Each peptide has the capacity to induce a different population of regulatory T cells.

asymptomatic with no signs of disease, or have early indications of impaired glucose tolerance (8). Antigen-specific T cells from the blood of patients and at-risk subjects can be detected and, indeed, exhibit a proinflammatory cytokine response upon antigen stimulation *ex vivo* (9, 10). It is appealing to propose that progression of disease toward diabetes in high-risk subjects will be more tractable to therapies such as peptide administration than the scenario of waiting for the disease to become established. In the future, this will need to be tested in multiple antibody-positive non-diabetic subjects, whose risk of disease development over a lifetime approaches 100% (**Figure 2**).

An early clinical study in T1D patients administered an altered peptide ligand (APL) derived from the insulin B9-23 epitope (NBI-6024) showed some initial promise (11). Modulation of the antigen-specific T-cell response from a predominantly IFNγ response to a more Th2 bias was detected. However, a subsequent clinical study powered to assess the effect of repeated administrations of NBI-6024 on endogenous insulin production, as measured by C-peptide levels in adult and adolescent patients with new-onset T1D, failed to demonstrate any clinical benefit (12).

In parallel to the clinical development of NBI-6024, a natural peptide sequence derived from proinsulin was also under evaluation. An initial phase 1 safety study in patients with long-standing diabetes and low c-peptide levels (<200 pmol/L) compared three monthly intra-dermal injections of 10 versus 100 µg of the C19-A3 peptide (13). The peptide was shown to be well tolerated and safe. In addition, as was observed in the initial NBI-6024 study, there was a trend toward modulation of the antigen-specific T-cell response with an apparently increased frequency of IL-10 producing cells in the low-dose treatment group.

Subsequently, a second clinical study with the single proinsulin peptide was conducted in newly diagnosed patients and the results have recently been reported (14). In this study, monthly versus fortnightly intra-dermal injections of 10 µg of peptide were compared, over a period of 6 months. The peptide was shown to be well tolerated and safe. Although the study was not powered to examine efficacy at the level of preservation of β-cell function, there was evidence in some patients on the active treatment that the rate of decline in secreted c-peptide slowed and daily insulin use and HbA1c stabilized. Intriguingly, these subjects who could be labeled "treated c-peptide responders" also showed immunological changes compared with non-responders on active treatment, including greater propensity for CD4 T-cell production of IL-10 upon *ex vivo* stimulation and enhanced expression of the canonical regulatory T cell transcription factor FoxP3 on peripherally generated, adaptive regulatory T cells post-therapy. These findings, linking clinical and immunological outcome provide an encouraging platform on which to build strategies for biomarkers of peptide immunotherapy.

The clinical studies described thus far, both in T1D and the other indications, have focused on either a single peptide to modulate the pathogenic T cell response or multiple peptides from the same antigen. A different approach, using multiple peptides from two different auto-antigens linked to the pathogenesis of T1D, is currently being evaluated as a treatment for new-onset T1D patients (ClinicalTrials.gov: NCT02620332). This phase Ib tolerability and safety study evaluating monthly intra-dermal injections of a cocktail of multiple peptides should provide new insights when data become available in 2018.

A slightly different peptide therapy approach to those previously described is also under clinical evaluation. The approach is to take a natural peptide derived from one of the major autoantigens linked to T1D and modify it slightly to improve its affinity (15). These modified peptides are known as Imotopes™.

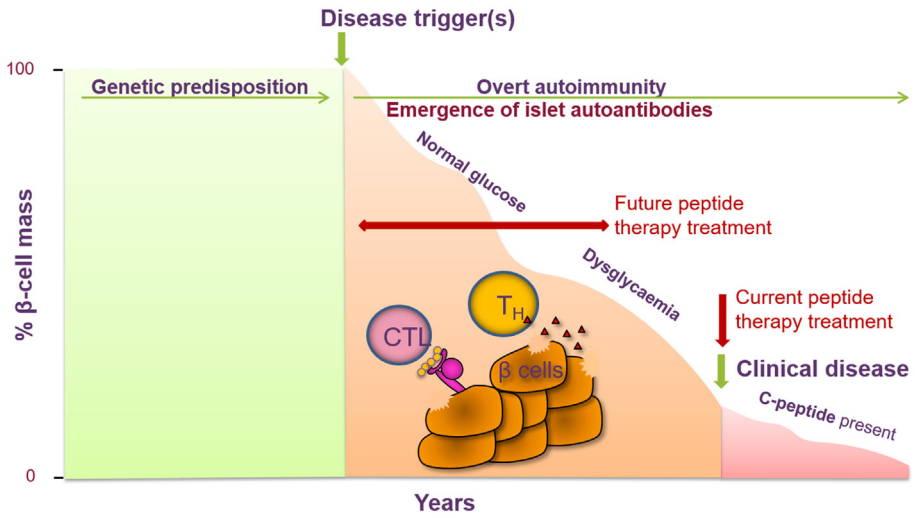

FIGURE 2 | Peptide therapy for type 1 diabetes (T1D) offers the opportunity to halt further progression of disease in newly diagnosed individuals and to prevent clinical symptoms of disease in at-risk individuals. T1D is a T-cell-mediated autoimmune disease characterized by the destruction of the pancreatic β cells, resulting in a decrease in insulin secretion (measured *via* c-peptide). Genetic factors have been identified as contributing to the risk of developing T1D as well as certain environmental factors. Prior to the clinical symptoms of T1D, auto-antibodies to one or more islet cell antibodies are detected and individuals become dysglycaemic. Current intervention with peptide therapy is focused on treating newly diagnosed patients. Future invention studies aim to treat individuals prior to clinical diagnosis in the prevention setting.

Injection of the Imotopes™ is believed to elicit antigen-specific cytolytic CD4 T cells that induce lysis of antigen-presenting cells (APCs) with which a synapse is formed, as well as the auto-antigen-specific bystander T cells, activated on the surface of the same APC. A phase Ib safety and tolerability study is currently underway, exploring three different doses administered sub-cutaneously in alum, with study completion expected December 2018 (ClinicalTrials.gov: NCT03272269).

PROMISING PRE-CLINICAL PEPTIDE THERAPY APPROACHES

The peptide therapy approaches leading the way in clinical development for both T1D and other autoimmune diseases are relatively simple. The peptides are administered either intra-dermally or sub-cutaneously in the absence of any additional substantial modifications. Whether these approaches are sufficient to bring significant clinical benefit in large phase III clinical studies remains to be seen, but certainly, the promise peptide therapy holds for restoration of immune regulation has led a number of pharmaceutical companies to develop novel and innovative ways to target the peptides in a more specific manner for potentially enhanced efficacy. These include targeting of peptides to the erythrocyte (16), encapsulating peptides into nanoparticles (17–19) and encapsulating peptides into liposomes with α-GalCer.[2] A highly novel approach being developed involves coating peptide-MHC complexes onto nanoparticles to generate "navacims" (20). As yet, there are no data to indicate which method of peptide delivery will be optimal and bring the greatest clinical benefit, but clearly this is a fast evolving field.

FUTURE CHALLENGES AND OPPORTUNITIES

While peptide therapy for T1D is clearly entering an exciting phase of drug development, there are still a number of unanswered questions and challenges, not least whether the approach will bring real clinical benefit. Selecting the optimal dose of peptide and optimal dosing interval is one key challenge. This is in part because it is difficult to study both the kinetics of peptide uptake and the bio-distribution of peptides. Access to better immunological tools to track the antigen-specific T cells will undoubtedly help our understanding; this includes further development of both tetramers and *ex vivo* assays that selectively stimulate antigen-specific cells (21). The ability to sample T cells at the site of active disease and/or site of peptide administration may also provide greater insight into the mechanism of action of peptide therapy. It is generally believed that peptide therapy induces a population of IL-10 secreting regulatory T cells. However, this is primarily based on *in vivo* data from mouse studies that have clearly demonstrated expansion of an IL-10 secreting, regulatory T-cell population following sub-cutaneous administration of peptide (22). Translation of this mechanism into the clinical setting is still to be proven and may well depend on the route of administration of the peptides. Nonetheless, as discussed earlier, circulating IL-10 producing, antigen-specific CD4 T cells have been observed post-intra-dermal peptide administration in studies in T1D patients and this mode of action remains a major focus of attention.

Once the technologies and assays have been further refined so that tracking of antigen-specific T cells, both pathogenic and regulatory, can be accomplished, additional questions can start to be addressed. Importantly, degrees of clinical efficacy can begin to be correlated with both the frequency of regulatory T-cell expansion and the phenotype of the expanded regulatory T cells. Is better efficacy achieved *via* the generation of IL-10 producing regulatory T cells, the Foxp3+ regulatory T cell, or a combination of both? It will also be important to understand the longevity of the effect and whether this is dependent on the phenotype of the regulatory T cell. It may be that the different routes of administration of peptides and the newly emerging targeted technologies mentioned previously lead to the generation of different populations of regulatory T cells. It is not inconceivable to think that different diseases may be better treated by induction of one particular population of regulatory T cell versus another. In addition, different stages of progression within the same disease may be more amenable to treatment by one subset of regulatory T cell over another. This may be particularly true for diseases where there is significant epitope spreading. In this situation, one might envisage early in disease using a peptide treatment that induces the Foxp3+ regulatory T cell and later in the disease pathogenesis, using a peptide treatment that induces the IL-10 secreting regulatory T cell.

One must also acknowledge that administration of peptide therapy alone may not be sufficient to prevent disease in an inflammatory setting. In this setting, the peptide therapy may indeed be inducing a population of regulatory T cells, but the inflammatory environment may also be driving a pathogenic T-cell population, which is not adequately suppressed by the regulatory cells. Therapies that directly inhibit the pathogenic T cell while sparing the regulatory T cell would be optimal under these circumstances. These could include therapies that target the APC to prevent full activation, achieved for example using antibodies against CD40. Alternatively, therapies that inhibit effector T-cell function directly could be beneficial. This could be achieved either *via* inhibition of T-cell co-stimulatory receptors such as CD40L, OX40, and ICOS or *via* engagement of negative regulators such as PD-1 and TIGIT. Modulation of the cytokine milieu may also synergize with the efficacy of peptide therapy. This could be achieved *via* direct inhibition of the proinflammatory cytokines secreted by the pathogenic T cell or inhibition of cytokines responsible for maturation of the APC.

While the challenges are undoubtedly there, peptide therapy does offer a significant opportunity to restore immune regulation in T1D and other autoimmune diseases. Further clinical studies and technological advances will hopefully translate peptide therapy into a safe, effective, and highly specific novel class of therapeutics.

AUTHOR CONTRIBUTIONS

ES wrote the review and was involved in designing the figures. MP was involved in designing the figures and supervised the writing and editing of the manuscript.

[2]http://www.regimmune.com/product-pipeline/tid/

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Conflict of Interest Statement: ES is an employee of UCB Pharma Ltd. who are developing peptide immunotherapy for type 1 diabetes. MP is employed by King's College London, which has a licence agreement in place with UCB Pharma to develop peptide immunotherapy. MP receives research funds and honoraria from UCB.

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ORIGINAL RESEARCH
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Tolerogenic Nanoparticles Induce Antigen-Specific Regulatory T Cells and Provide Therapeutic Efficacy and Transferrable Tolerance against Experimental Autoimmune Encephalomyelitis

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T cells reacting to self-components can promote tissue damage when escaping tolerogenic control mechanisms which may result in autoimmune disease. The current treatments for these disorders are not antigen (Ag) specific and can compromise host immunity through chronic suppression. We have previously demonstrated that co-administration of encapsulated or free Ag with tolerogenic nanoparticles (tNPs) comprised of biodegradable polymers that encapsulate rapamycin are capable of inhibiting Ag-specific transgenic T cell proliferation and inducing Ag-specific regulatory T cells (Tregs). Here, we further show that tNPs can trigger the expansion of endogenous Tregs specific to a target Ag. The proportion of Ag-specific Treg to total Ag-specific T cells remains constant even after subsequent Ag challenge in combination with a potent TLR7/8 agonist or complete Freund's adjuvant. tNP-treated mice do not develop experimental autoimmune encephalomyelitis (EAE) after adoptive transfer of encephalitogenic T cells; furthermore, tNP treatment provided therapeutic protection in relapsing EAE that was transferred to naïve animals. These findings describe a potent therapy to expand Ag-specific Tregs *in vivo* and suppress T cell-mediated autoimmunity.

Keywords: nanoparticles, immunological tolerance, rapamycin, regulatory T cells, experimental autoimmune encephalomyelitis

INTRODUCTION

Maintenance of peripheral immunological tolerance is a dynamic and continuous process. Most self-reactive T lymphocytes are deleted in the thymus or differentiate into natural T regulatory cells (Tregs), but some can also enter the pool of naive circulating cells. Self-reactive naive T cells that escape the thymus and encounter their cognate antigen (Ag) in the periphery can differentiate into induced Tregs (1, 2). Tregs maintain immune homeostasis *in vivo*, and their dysfunction, caused by the loss of expression of the master transcription factor Foxp3, leads to the development of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans characterized by systemic T cell activation and multiorgan autoimmunity (3). In most autoimmune diseases, genetic and environmental factors result in the dysregulated expansion of autoreactive lymphocytes that mediate damage to self-tissue (4, 5). For example, in multiple sclerosis, a chronic

neuroinflammatory disease, myelin proteins are actively targeted by immune cells resulting in myelin degradation, loss of neuronal function, and progressive paralysis (6). There has been substantial progress in the identification of small molecule and biological therapies that ameliorate disease, but there is no cure (7, 8).

Antigen-specific immune tolerance has been a long-standing goal in the treatment of autoimmune diseases. Dendritic cells (DCs) and other Ag-presenting cells are at the crossroads of immunity and tolerance. The context in which DCs encounter Ag can determine the nature of the T cell response (9). Danger signals, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), induce DC maturation resulting in the expression of co-stimulatory molecules and cytokines that drive effector T cell activation and differentiation (10). Vaccines often employ an adjuvant to provide this "danger" context to induce an adaptive, Ag-specific effector response (11). Recently, there has been interest in the identification of "tolerogenic adjuvants" that would enable the induction of Ag-specific Tregs rather than effector T cells (12). Rapamycin, an inhibitor of the mTOR signaling pathway, has been shown to induce tolerogenic DCs (itDCs) *in vitro*, which are capable of inducing regulatory T cells and suppressing disease when adoptively transferred *in vivo* (13–15). Our group and others have demonstrated that tolerogenic nanoparticles (tNPs; also known as synthetic vaccine particles or SVPs) and microparticles encapsulating rapamycin induced tolerogenic DCs *in vivo* causing the differentiation of Ag-specific regulatory T cells (16–20).

In this study, we further characterize the induction of Ag-specific endogenous Tregs by acute treatment with tNPs composed of polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA) polymers encapsulating peptide Ag and rapamycin. We demonstrate therapeutic efficacy of tNPs in a model of relapsing experimental autoimmune encephalomyelitis (rEAE) and show that tolerance can be adoptively transferred from a tNP-treated animal to a naive animal. Furthermore, mice treated with tNPs were protected against EAE following transfer of encephalitogenic T cells.

MATERIALS AND METHODS

Mouse Models

The following animals were used: female C57BL/6nTac (RRID:IMSR_TAC:b6), B6.Cg-Tg(TcraTcrb)425Cbn/J (RRID:IMSR_JAX:004194), B6.129S6-*Rag2*^{tm1Fwa} N12 (RRID:IMSR_TAC:1329), B6.SJL-*Ptprc*^{a}/BoyAiTac (RRID:IMSR_TAC:4007), and SJL/J (RRID:IMSR_JAX:000686). Experiments involving animals were performed in compliance with state and federal regulations and approved by the Institutional Animal Care and Use Committee of Selecta Biosciences or Hooke Laboratories.

Nanoparticles (NPs)

Manufacture of NPs has been previously described (18). PLGA, pegylated PLA (PLA-PEG), and rapamycin were dissolved in dichloromethane to form the oil phase. An aqueous solution of Ag ($OVA_{323–339}$ peptide, 2W1S peptide, or $PLP_{139–151}$ peptide) was then added to the oil phase and emulsified by sonication (Branson Digital Sonifier 250A). Following emulsification of the Ag solution into the oil phase, a double emulsion was created by adding an aqueous solution of polyvinylalcohol and sonicating a second time. The double emulsion was added to a beaker containing phosphate buffer solution and stirred at room temperature for 2 h to allow the dichloromethane to evaporate. When creating NPs containing rapamycin but no Ag, or NPs without any encapsulated agents, a similar oil-in-water single-emulsion process was used. The resulting NPs were washed twice by centrifuging at 75,600 × *g* and 4°C followed by resuspension of the pellet in phosphate-buffered saline (PBS). MHC class II (MHCII) peptides used were 2W1S (2W, EAWGALANWAVDSA, CSBio), $OVA_{323-339}$ (OVA_{323}, ISQAVHAAHAEINEAGR, Bachem B06481), or $PLP_{139-151}$ (PLP_{139}, HCLGKWLGHPDKF, Genemed Synthesis). NPs containing peptide alone are denoted as follows: NP[2W], NP[OVA_{323}], and NP[PLP_{139}]. NPs containing peptide and rapamycin are denoted as follows: NP[2W-Rapa], NP[OVA_{323}-Rapa], and NP[PLP_{139}-Rapa]. NPs containing peptide and rapamycin are referred herein as tNPs. Empty NPs (NP[Empty]) were used as controls.

EAE Models

Relapsing EAE was induced by injection of SJL mice subcutaneously (s.c.) at four sites in the back with PLP_{139} emulsified in complete Freund's adjuvant (CFA) followed by intraperitoneal (i.p.) injection of 154ng of pertussis toxin (PTx) 2 h later (Hooke Laboratories EK-2120). Pathogenic cells used for adoptive transfer models of EAE were propagated by immunizing SJL mice with PLP_{139}/CFA (Hooke Laboratories EK-0120). Seven days later, spleens were excised from immunized mice and single-cell splenocyte suspensions were isolated through mechanical dissociation. Red blood cells were lysed (Sigma R7757) and splenocytes were restimulated *in vitro* in RPMI 1640 containing HEPES (Life Technologies 15630080), L-glutamine–penicillin–streptomycin (Sigma G6784), MEM Non-Essential Amino Acids Solution (Life Technologies 11140-050), MEM Sodium Pyruvate Solution (Life Technologies 11360-070), and 2-Mercaptoethanol (1000X, Life Technologies 21985-023) with Hooke PLP_{139} in TC Media, 100× (Hooke Labs DS-0121) for 3 days before being injected i.p. into recipient mice. Regulatory cell adoptive transfer studies were carried out in a similar manner. After s.c. treatment of donor mice with NPs, their spleens were excised, and single-cell splenocyte suspensions were isolated through mechanical dissociation. *In vitro* culture was carried out as done with pathogenic cells with the modification that splenocytes were restimulated with PLP_{139} in the presence of 100 U/ml IL-2. Sickness scoring assessments were carried out as previously described (18). EAE was scored on a 0–5 scale as follows: 0, no obvious changes in motor functions of the mouse in comparison with non-immunized mice; 1, limp tail; 2, limp tail and weakness of hind legs; 3, limp tail and complete paralysis of hind legs (most common) or limp tail with paralysis of one front and one hind leg; 4, complete hind leg and partial front leg paralysis; 5, death or euthanized because of severe paralysis. Demyelination was scored by H&E staining of central nervous system (CNS) sections with the NP[Empty] group used as the baseline for tissue disruption.

Immunizations and Treatments

100μg of 2W peptide admixed with 20μg R848 (Selecta Biosciences) or emulsified 1:1 with CFA (Sigma F5881) was injected i.p. or s.c. as an immunization. NPs containing peptide alone were injected at a 4–5μg dose of peptide i.v. or s.c. NPs containing rapamycin alone (NP[Rapa]) were injected at a 50μg dose of rapamycin i.v. or s.c. tNPs were injected at a 4 to 5μg dose of peptide and a 50μg dose of rapamycin i.v. or s.c.

Endogenous 2W1S:IA^b + T Cell Enrichment

2W1S-specific CD4 T cells were enriched and enumerated as previously described (21). Briefly, mice were sacrificed, and splenocytes were isolated by mechanical dissociation. 2W1S:IA^b tetramers conjugated to allophycocyanin (APC) or phycoerythrin (PE) (NIH Tetramer Core Facility, mouse 2W1S) were incubated at room temperature with splenocytes for 45 min. Cells were washed then incubated with anti-APC or anti-PE microbeads (Miltenyi Biotec, 130-090-855, 130-048-801) for 20 min at 4°C. Cells were washed, resuspended, and eluted over a magnetized bead-packed LS column (Miltenyi Biotec, 130-042-401). Positively selected 2W1S:IA^b cells were expelled from columns and phenotyped by flow cytometry.

Flow Cytometry

Samples were analyzed on a Becton Dickinson FACSCanto II with the following conjugated antibodies: TCRβ (BD Biosciences 553171, RRID:AB_394683), CD45R (BD Biosciences 561226, RRID:AB_10563910), CD45.2 (BioLegend 109830, RRID:AB_1186098), CD4 (BioLegend 100433, RRID:AB_893330), CD44 (BioLegend 103029, RRID:AB_830786), CD11b (BioLegend 101245, RRID:AB_2561390), CD11c (BioLegend, 117338, RRID:AB_2562016), Foxp3 (Thermo Fisher 12-5773-82, RRID:AB_465936), TCRVa2 (Thermo Fisher 17-5812-82, RRID:AB_1659733), and Live/Dead Fixable Viability Stain Aqua (Thermo L34957).

IFNγ ELISpot

Sterile, white 96-well filter plates with 0.45- μm pore size Hydrophobic PVDF membrane (EMD Millipore, Billerica, MA, USA, Cat#MSIPS4W10) were coated with 5μg/ml of purified anti-mouse IFNγ capture antibody (BD Pharmingen 551881) in dilution buffer (DB) (PBS 1× Corning cellgro 21-040CV) with 2% fetal bovine serum (FBS; heat inactivated, Medsupply partners MSP-1003-3/Hi) overnight at 4°C. Unbound antibodies were discarded by emptying the wells followed by blocking with complete medium (CM) [RPMI 1640 (1× with Corning glutagro, Mediatech 10-104-CV) supplemented with FBS (FBS, heat inactivated, Medsupply partners MSP-1003-3/Hi), Pen/Strep and L-glutamine (100×), Gibco by Life technologies 10378-016, 2-Mercaptoethanol (55 mM), Gibco by Life technologies 21985] for 90 min at 37°C. 1×10^5 lymph node cells per well were incubated in the IFNγ capture antibody-coated plates in CM as a test condition overnight at 37°C. The plates were washed three times with wash buffer (WB) [PBS containing 0.05% Tween20 (Sigma P1379)] after which the plates were incubated for 90 min at room temperature with 2μg/ml biotinylated anti-mouse IFNγ detection antibody (BD Pharmingen 551881) diluted in DB. The plates were washed three times with WB followed by Stretavidin-HRP (BD Pharmingen 557630) addition diluted 1:100 in DB and incubated for 1 h at room temperature. The plates were washed three times with WB and three times with PBS followed by addition of AEC substrate (BD Pharmingen, 551951) for spot development. The plates were read on a Zeiss KS Elispot reader system, using KS Elispot software version 4.9.16.

RESULTS

tNPs Inhibit the Proliferation of Ag-Specific Effector CD4$^+$ T Cells

We evaluated the activation of OTII transgenic T cells that recognize the 323–339 peptide of chicken ovalbumin (OVA_{323}) after adoptive transfer into mice that were previously treated with NPs containing the OVA_{323} peptide alone (NP[OVA_{323}]) or tNPs containing both OVA_{323} and rapamycin. Rag2$^{-/-}$-recipient mice were used to ensure that no endogenous lymphocytes would compete with the OTII T cells for binding to OVA_{323} peptide presented by Ag-presenting cells. NP[OVA_{323}] or tNPs were administered to the recipient animals 1, 3, or 5 days before OTII CD4$^+$ T cell transfer (**Figure 1A**). Recipient animals were sacrificed 3 days after cell transfer, and splenic OTII T cells were assayed for cell proliferation and Foxp3 expression. No differences in total numbers of OTII cells were observed when NPs were administered 3 and 5 days prior to cell transfer (**Figure 1D**); however, reduced proliferation capacity, a reduction in the total numbers of OTII cells and an increase in the proportion of Foxp3$^+$ OTII T cells were observed after tNP treatment administered 1 day prior to cell transfer compared to treatment with NP[OVA_{323}] (**Figures 1B–E**). These results corroborate our previous findings showing a greater proportion of Foxp3$^+$ Tregs after tNP treatment compared to NP[OVA_{323}] treatment. A smaller proportion of OTII T cells enter division after tNP treatment compared to NP[OVA_{323}], and the extent of cell proliferation was diminished.

tNPs Increase the Total Number and Proportion of Endogenous T Cells Expressing Foxp3 in an Ag-Specific Fashion

Next we utilized MHCII tetramers loaded with the 2W1S (2W) peptide to evaluate the effects of tNPs on endogenous wild-type Ag-specific CD4$^+$ T cells. This tetramer system has been validated to study T helper cell responses (22), T follicular helper cell differentiation (23), and Treg-mediated tolerance (24). We queried 2W:MHCII$^+$ endogenous cells from naive mice and found 18.2% were Foxp3 positive (**Figure 2A**). This served as our baseline proportion of 2W-specific Foxp3$^+$ cells in naive animals. We then compared this percentage to that from mice treated i.v. with three weekly injections of PBS, NPs containing 2W peptide alone (NP[2W]), NP[Rapa], or tNPs containing 2W peptide and rapamycin (**Figure 2B**). Two weeks following treatment, all mice were challenged i.v. with 50μg free 2W peptide, and their splenocytes were assayed for 2W-specific CD4 T cells 2 h after challenge. MACS-enriched

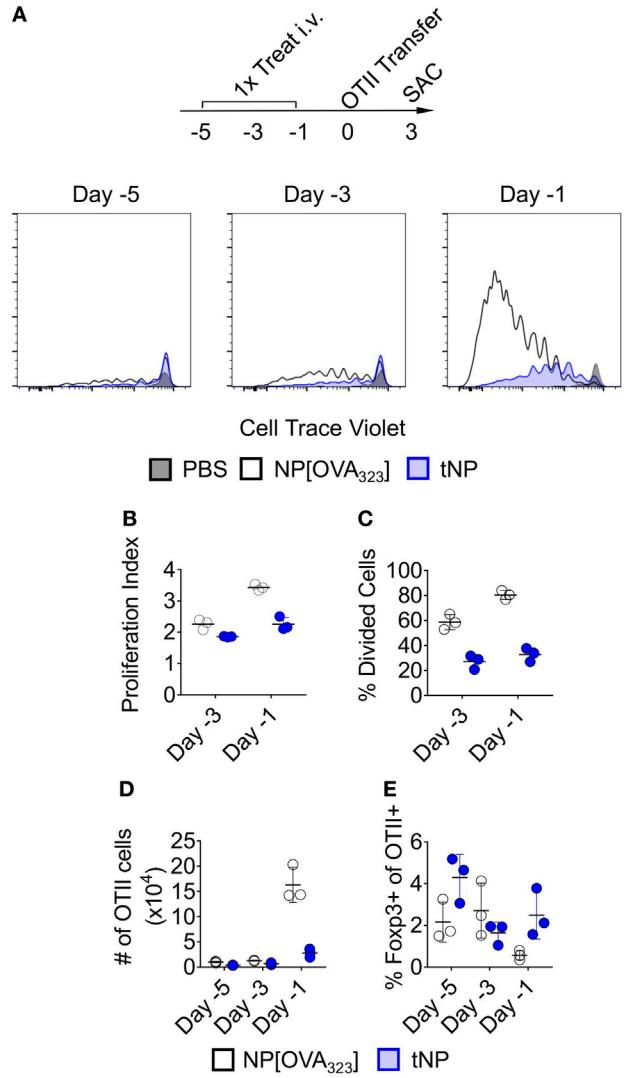

FIGURE 1 | Tolerogenic nanoparticles (tNPs) inhibit the proliferation of antigen (Ag)-specific effector CD4+ T cells. **(A–E)** Rag2$^{-/-}$ mice were treated i.v. with PBS, NP[OVA_{323}] at a 5μg dose of OVA_{323} peptide, or tNP (NP[OVA_{323}-Rapa]) at a 5μg dose of OVA_{323} peptide and a 50μg dose of rapamycin on days −5, −3, or −1. OTII cells were transferred to treated Rag$^{-/-}$ mice on day 0 and their spleens assayed for OTII cells on day 3. **(A)** Cell Trace Violet flow cytometry histograms of TCRVα2^{+} CD4^{+} gated OTII splenocytes, **(B)** OTII proliferation index, **(C)** % divided cells, **(D)** total cell number (#), and **(E)** % Foxp3 expression. The results represent an $N = 3$ from one experiment.

2W-specific T cells were gated *via* the following gating scheme: Live/Dead Fixable Aqua^{-}, B220^{-}, CD11c^{-} CD11b^{-} CD4^{+}, CD44^{+}, 2W:MHCII^{+}, and then probed for Foxp3 expression (**Figure 2B**). When comparing across all groups, only those mice treated with tNP or NP[2W] showed a significant proliferation of 2W-specific CD4^{+} T cells (**Figure 2B**). The total number of 2W-specific cells was significantly higher with tNP treatment compared to NP[2W] alone (**Figure 2C**). This result indicates that neither NP[2W] nor tNP treatment caused depletion of Ag-specific CD4 T cells. Importantly, the proportion and total number of 2W-specific Foxp3^{+}CD4^{+} cells was significantly higher in the tNP-treated group compared to NP[2W] treatment alone (**Figures 2D–F**). This level of increase in Foxp3^{+} cells was specific to 2W:MHCII^{+}CD4^{+} T cells, as it was not observed within the 2W:MHCII-negative population of CD4^{+} T cells (**Figures 2G,H**). These results indicate that tNP treatment selectively increased Ag-specific endogenous Tregs. In contrast, there was no difference in the proportion of 2W-specific Foxp3^{+} T cells between the naive and NP[2W]-treated animals. Another cohort of tNP-treated mice also had fewer IFNγ spot-forming units (SFUs) from draining lymph node cells than NP[2W]-treated animals 5 days after s.c. challenge with 2W and R848, a TLR7/8 agonist (**Figure 2I**). Neither NP[2W] nor tNP treatment induced Tbet, Gata3, IFNγ, or IL-4 in 2W-specific CD4^{+} T cells after peptide restimulation *in vivo* (Figure S1 in Supplementary Material).

tNP Treatment Increases Ag-Specific Endogenous Foxp3^{+} Tregs That Withstand Ag Challenge in the Presence of a TLR Agonist or CFA

To assess the stability of Foxp3 expression on endogenous 2W-specific CD4 T cells, mice were injected i.p. with 100μg of 2W peptide admixed with 20μg of R848, a potent TLR7/8 agonist (**Figure 3A**). A single tNP treatment administered before challenge induced an increase in the total number of 2W-specific CD4^{+} T cells compared to PBS and single-component NP controls (NP[2W] and NP[Rapa], **Figure 3B**). The average proportion of 2W-specific Foxp3^{+}CD4^{+} T cells within the 2W:MHCII^{+} population was higher in the tNP-treated (24%) and NP[2W]-treated (14%) groups compared to PBS and NP[Rapa]-treated controls (6.6 and 3.3%, respectively) (**Figure 3C**). However, the total number of 2W-specific Foxp3^{+}CD4^{+} T cells found after tNP treatment (3.2×10^3 cells) was at least ninefold higher compared to all other groups (0.34×10^3, 0.96×10^3, and 0.09×10^3 cells in PBS, NP[2W], and NP[Rapa] treated animals, respectively) (**Figure 3D**). These results suggest that the expression of Foxp3 in Ag-specific endogenous CD4 T cells after tNP treatment was increased compared to all single-component NP controls, even after Ag challenge in the presence of a potent TLR agonist. Similar results were observed in a three treatment model followed by challenge with Ag in CFA (**Figure 3E**). The total number of 2W-specific CD4^{+} T cells (3.23×10^4) along with the proportion (21.5%) and total number of Foxp3^{+}2W^{+}CD4^{+} T cells (6.8×10^3) was higher in mice treated with tNP compared to all other single-component NP controls (**Figures 3F–H**). The increased proportion and total number of 2W-specific Foxp3^{+} T cells, compared to PBS controls, was only observed in mice that received tNP alone (**Figures 2E,F**) or tNP followed by Ag challenge (**Figures 3G,H**). Treatment with NP[2W] did not show an increase in the proportion or number of 2W-specific Foxp3^{+}CD4^{+} T cells compared to PBS controls after challenge (**Figures 3G,H**). Neither one nor three NP[Rapa]-alone treatments increased Ag-specific Treg numbers above PBS controls (**Figures 3C,D,G,H**). These results further underscore the Ag-specific and pro-tolerogenic nature of tNP treatment.

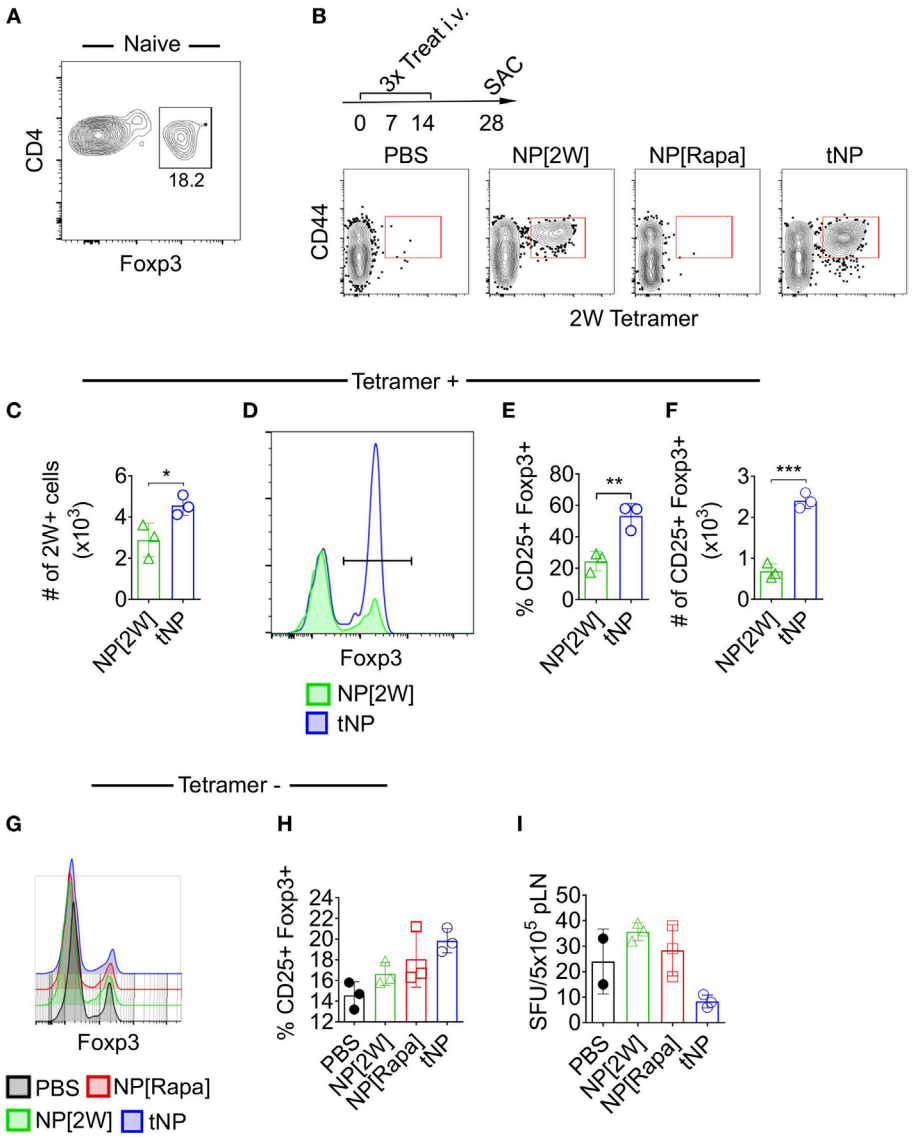

FIGURE 2 | Tolerogenic nanoparticles (tNPs) increase the total number and proportion of endogenous T cells expressing Foxp3$^+$ in an antigen-specific fashion. **(A)** Splenocytes from five naive mice were pooled and assayed with 2W:I-A^b tetramers after MACS enrichment to identify 2W-specific CD4 T cells (2W$^+$CD4$^+$). Their expression of Foxp3 was quantified. **(B)** WT mice were treated with PBS, NP[2W] at a 4μg dose of 2W peptide, nanoparticles containing rapamycin alone (NP[Rapa]) at a 50μg dose of rapamycin, or tNP (NP[2W-Rapa]) at a 4μg dose of 2W peptide and 50μg dose of rapamycin on days 0, 7, and 14, and their spleens were assayed with 2W:I-A^b tetramers to identify 2W$^+$CD4$^+$ cells. **(B)** Dot plots of 2W:I-A^b-PE MACS-enriched splenocytes stained as live/dead$^-$, CD11b$^-$, CD11c$^-$, B220$^-$, CD4$^+$. **(C)** # of 2W:I-A^b tetramer-positive cells from NP[2W] and tNP-treated groups. **(D)** Expression of Foxp3$^+$ on 2W$^+$CD4$^+$ T cells. **(E)** % and **(F)** # of 2W$^+$CD4$^+$ cells that are Foxp3$^+$. **(G)** Expression of Foxp3$^+$ on 2W-CD4$^+$ T cells. **(H)** % of 2W-CD4$^+$ cells that are Foxp3$^+$. **(I)** # of IFNγ spot-forming units (SFUs) from draining lymph nodes of mice treated and challenged 2W/R848 subcutaneously in **(B)**. Error bars indicate SD. The results from **(C–F)** represent an $N = 3$ from one experiment of two representative experiments. The results from **(A,B,G–I)** represent an $N = 3$ from one experiment. Statistics are derived from a one-way ANOVA with Tukey Multiple Comparison test for **(C,E,F)**. Significance: $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

tNP Treatment Confers Therapeutic Efficacy in a Relapsing Remitting Model of Experimental Autoimmune Encephalomyelitis (rEAE)

We next assessed the ability of tNPs to therapeutically treat disease in a model of rEAE. In this system, we generated tNPs containing myelin proteolipid protein peptide fragment 139–151 (PLP_{139}) with rapamycin or NPs containing PLP_{139} alone (NP[PLP_{139}]) for i.v. administration. Across all groups, 5 or 50μg of rapamycin and 0.5μg PLP_{139} were dosed alone or together as NP[PLP_{139}], NP[Rapa], or tNP. SJL mice were immunized s.c. with PLP_{139} emulsified in CFA followed by PTx i.p. to induce rEAE. In this model, animals start developing ascending paralysis from day 10 after immunization. In the initial study, mice were administered two doses of NPs on the third day and tenth day after the onset

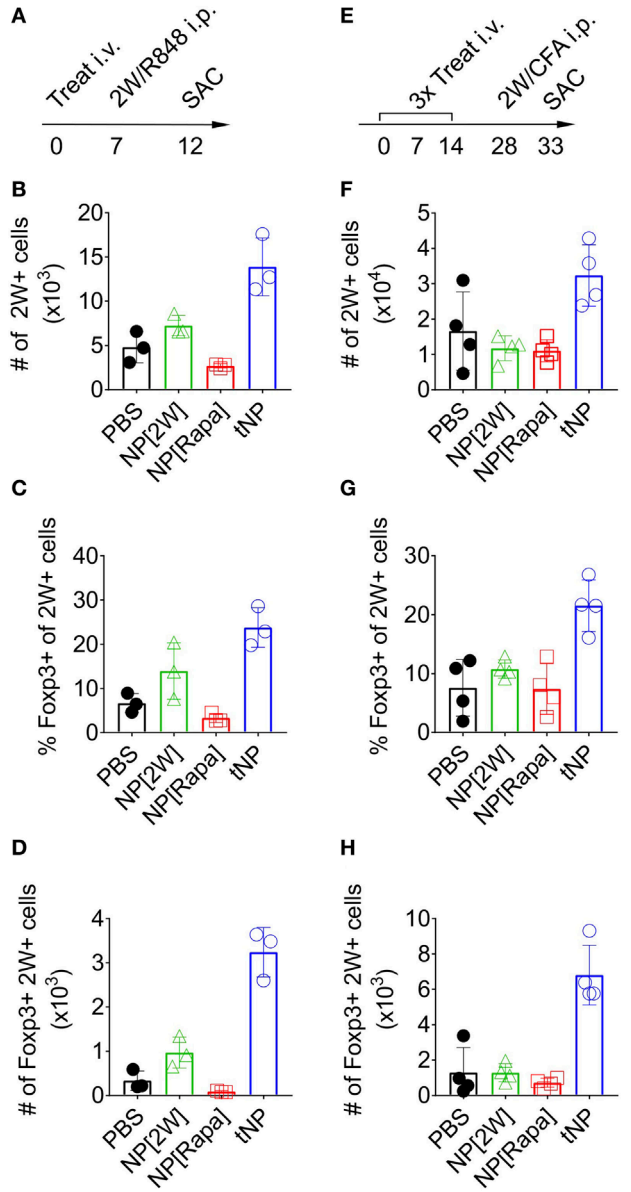

FIGURE 3 | Tolerogenic nanoparticle (tNP) treatment increases antigen-specific endogenous Foxp3$^+$ Tregs that withstand antigen challenge in the presence of a TLR agonist or complete Freund's adjuvant (CFA). **(A)** WT mice were treated once i.v on day 0 with PBS, NP[2W] at a 5μg dose of 2W peptide, nanoparticles containing rapamycin alone (NP[Rapa]) at a 50μg dose of rapamycin and tNP at a 5μg dose of 2W peptide and 50μg dose of rapamycin. Mice were then challenged i.p. with 100μg 2W peptide and 20μg R848 on day 7, and spleens were assessed for 2W$^+$CD4$^+$ T cells on day 12. MACS-enriched 2W$^+$ cells were Live/Dead Fixable Aqua$^-$, B220$^-$, CD11c$^-$, CD11b$^-$, CD4$^+$, CD44$^+$, 2W:Ia^{b+} and then probed for Foxp3 expression. **(B)** Total numbers (#) of 2W$^+$CD4$^+$ T cells from the spleen. **(C)** % and **(D)** total # of 2W$^+$CD4$^+$ T cells that are Foxp3$^+$. **(E)** WT mice were treated three times weekly, i.v. on days 0, 7, and 14 with PBS, NP[2W] at a 5μg dose of 2W peptide, NP[Rapa] at a 50μg dose of rapamycin, and tNP at a 5μg dose of 2W peptide and 50μg dose of rapamycin. Mice were then challenged i.p. with 100μg 2W peptide admixed 1:1 with CFA on day 28, and spleens were assessed for 2W$^+$CD4$^+$ T cells on day 33. **(F)** total # of 2W$^+$CD4$^+$ cells from the spleen. **(G)** % and **(H)** total # of 2W$^+$CD4$^+$ cells that are Foxp3$^+$. The results from **(B–D)** represent an $N = 3$ from one experiment. The results from **(F–H)** represent an $N = 4$ from one experiment.

of clinical symptoms. Therapeutic treatment with tNP completely inhibited disease relapse (Figure S3 in Supplementary Material). Next, we evaluated whether a single dose of tNP, administered at the peak of disease, could reverse disease relapse. All animals showed a typical complete remission after the first flare of paralysis with most animals becoming symptomless by day 19. Untreated control animals relapsed 7–8 days after initial disease peak reaching an average EAE disease score of 2 and maintained sickness until the end of the experiment. Relapse was controlled by tNP treatment, as shown by significantly diminished EAE scores compared to all other groups (**Figures 4A,B**; $p < 0.0001$). The disease was suppressed by tNP for the entirety of the relapsing period, while NP[PLP$_{139}$] reduced the length of relapse and attenuated disease after day 35 (**Figures 4A,B**). NP[Rapa] did not affect disease relapse at rapamycin doses that matched those administered in the tNP groups. tNP treatment also significantly

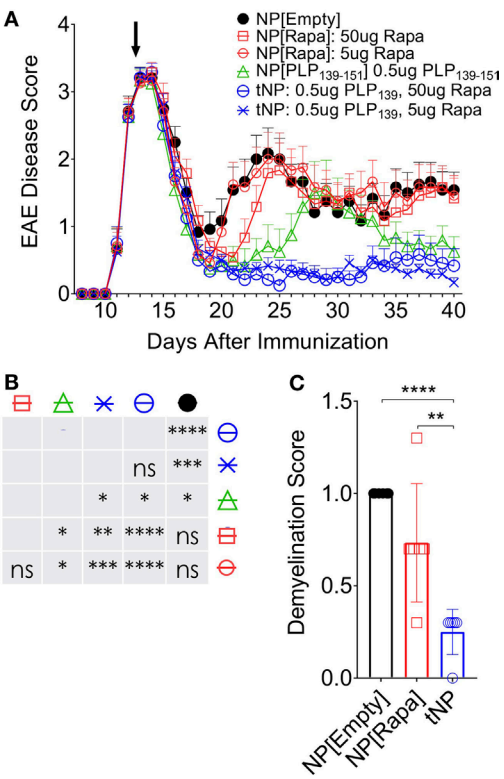

FIGURE 4 | Tolerogenic nanoparticle (tNP) treatment confers therapeutic efficacy in a model of relapsing experimental autoimmune encephalomyelitis (rEAE). A therapeutic treatment model of rEAE in SJL mice. Mice were immunized on day 0 with PLP$_{139}$/complete Freund's adjuvant (CFA) and pertussis toxin, and their EAE score was monitored from day 7 to the end of the study. Mice were treated once, i.v., on day 14 with empty nanoparticles NP[Empty], nanoparticles containing rapamycin alone (NP[Rapa]) at 5 or 50μg of rapamycin, NP[PLP$_{139}$] at 0.5μg PLP$_{139}$, or tNP at 0.5μg PLP$_{139}$ and 5 or 50μg of rapamycin. **(A)** PLP$_{139}$ and rapamycin contained in tNPs synergize to suppress disease to a greater degree than nanoparticles containing PLP$_{139}$ alone. **(B)** Table of statistical significance from **(A)**. **(C)** tNPs reduce demyelination in the central nervous system compared to NP[Empty] and NP[Rapa]. $N = 12$ for **(A)**. $N = 6$ for **(C)**. Error bars indicate SEM. Statistics are derived from a one-way ANOVA with Tukey Multiple Comparison test. Significance: $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$; and $^{****}p < 0.0001$.

reduced demyelination in the CNS compared to NP[Empty] and NP[Rapa] (**Figure 4C**; Figure S2 in Supplementary Material).

Efficacy of tNP Treatment in an Encephalitogenic T Cell Transfer Model of EAE

Encephalitogenic T cells were transferred into recipient mice that had been previously treated with NPs to test whether disease pathogenesis could be contained by an endogenous regulatory response induced by tNPs in the recipient mice. Donor animals were immunized with PLP_{139}/CFA on day −10, their spleens were harvested on day −3, and the splenocytes were restimulated *ex vivo* with PLP_{139} and transferred into recipient mice on day 0. The recipients were treated s.c. twice with NP[Empty], NP[Rapa], or tNP on days −14 and −21 prior to cell transfer (**Figure 5A**). Disease was completely abrogated by tNP prophylaxis, while the NP[Rapa] control had little effect (**Figure 5B**). These results indicate that treatment with tNPs containing both rapamycin and PLP_{139} induced a durable regulatory response capable of inhibiting pathogenesis mediated by the transferred encephalitogenic T cells.

tNP Treatment Induces Transferrable Tolerance in a Model of EAE

We next evaluated whether cells from tNP-treated animals could prevent disease in naive recipients immunized with PLP_{139}/CFA. Donor mice were treated s.c. with NPs on days −25 and −18. On day −4, their splenocytes were harvested and incubated with PLP_{139} and IL-2 for 72 h, a protocol that has been shown to expand Tregs *in vitro* and used in clinical protocols for Treg immunotherapy of organ transplant and type 1 diabetes (25–28). On day 0, the cells were transferred into naive animals that were then immunized with PLP_{139}/CFA (**Figure 5C**). Transferred splenocytes from tNP-treated mice significantly attenuated and delayed disease compared to splenocytes from both NP[Empty]- and NP[Rapa]-treated donors (**Figure 5D**). Disease was not suppressed by splenocytes transferred from NP[Rapa]-treated mice. These results indicate that tNP treatment induced a population of regulatory cells capable of conferring protection to naive recipients.

DISCUSSION

We previously demonstrated that administration of tNP after adoptive transfer of OTII T cells into wild-type mice was capable of inducing OTII Treg and inhibiting their expansion (16, 18). Here, we extend these findings by showing that a single injection of tNP in $Rag^{-/-}$ mice administered 1 day, but not 3 or 5 days, prior to OTII cell transfer into $Rag^{-/-}$ inhibits total Ag-specific T cell proliferation while expanding Ag-specific Tregs (**Figures 1A–E**). We further demonstrate that tNP treatment increases the proportion and total number of endogenous 2W-specific $Foxp3^{+}CD4^{+}$

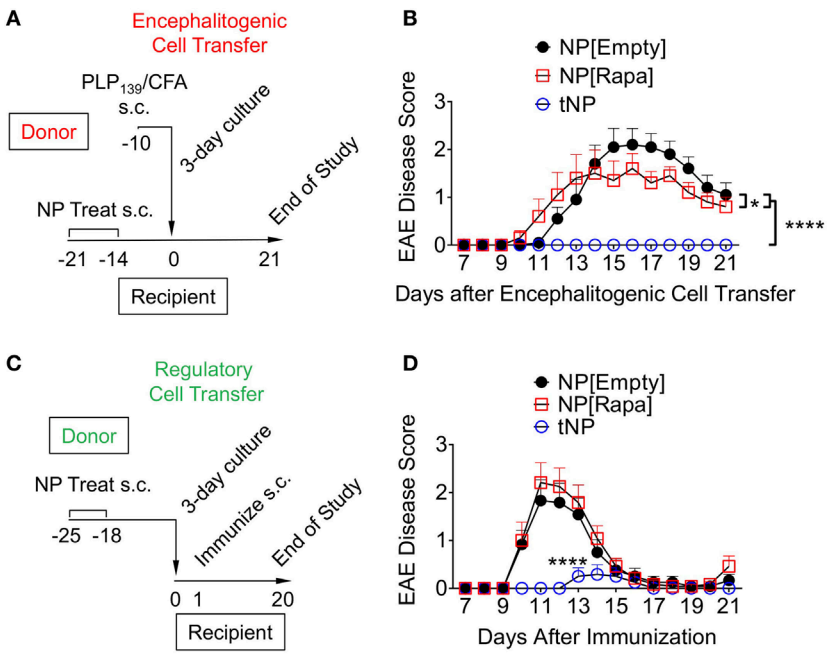

FIGURE 5 | Tolerogenic nanoparticles (tNP) induce prophylactic protection and transferrable tolerance in T cell transfer and direct immunization models of EAE. **(A)** Encephalitogenic cell transfer experimental design. Encephalitogenic cell donors were immunized on day −10, their splenocytes harvested on day −3 and incubated with PLP_{139} for 72 h before transfer into recipients treated on days −21 and −14 with empty nanoparticle (NP[Empty]), nanoparticles containing rapamycin alone (NP[Rapa]) at a 50μg dose of rapamycin, or tNP at a 0.5μg dose of PLP_{139} peptide and 50μg dose of rapamycin. **(B)** EAE scores from **(A)**. **(C)** Regulatory cell transfer experimental design. Donors were treated on days −25 and −18 with NP[Empty], NP[Rapa] at a 50μg dose of rapamycin, or tNP at a 0.5μg dose of PLP_{139} peptide and 50μg dose of rapamycin. Their splenocytes were harvested on day −4 and incubated with PLP_{139} and 100 U/ml of IL-2 for 72 h before transfer into naive recipients. **(D)** EAE scores from **(C)**. The results from **(B,D)** represent an $N = 12$ from one experiment. Error bars indicate SEM. Statistics are derived from a one-way ANOVA with Tukey Multiple Comparison test. Significance: $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$; $^{****}p < 0.0001$.

T cells using 2W:MHCII tetramers in a multiple treatment model (**Figures 2D–F**), illustrating that tNPs provide a greater tolerogenic stimulus for Foxp3 expression than NP[2W] alone, *in vivo*. This difference was not caused by rapamycin administration alone, as T cells not specific for 2W peptide (2W:MHCII-CD4$^+$) did not increase their Foxp3 expression to the same degree as 2W:MHCII$^+$CD4$^+$ T cells (**Figures 2G,H**). Upon s.c. challenge with 2W peptide and R848, tNP-treated mice showed fewer IFNγ SFUs from draining LN cells than mice treated with NP[2W] alone or NP[Rapa] alone (**Figure 2I**). Single (**Figures 3A–D**) and triple (**Figures 3E–H**) treatment models show that tNP increased 2W-specific Foxp3$^+$CD4$^+$ T cells after systemic challenge with 2W peptide co-administered with a potent TLR7/8 agonist or CFA compared to single-component NP controls. In additon, tNPs containing PLP_{139} and rapamycin prevent the pathological impact of encephalitogenic T cell transfer in a model of EAE (**Figure 5B**). tNPs show greater therapeutic benefit than NP[PLP_{139}] in a rEAE model (**Figure 4A**), while NP[Rapa] alone did not suppress EAE or demyelination at rapamycin doses equal to those in the tNP-treated groups (**Figures 4A–C**). Finally, EAE was suppressed by adoptive transfer of splenocytes from tNP-treated, but not NP[Rapa]-treated donors (**Figure 5D**). Together, these results indicate that tNP encapsulating rapamycin with Ag promotes Ag-specific Tregs that persist after Ag challenge in the presence of TLR agonists or CFA. Regulatory cells within the splenic milieu are induced by tNP and capable of transferring tolerance to naive recipients.

The macrolide compound rapamycin is a known inhibitor of mTOR. Previous work has shown the necessity of mTOR signaling to promote T cell expansion (29), differentiation (30), and resistance to anergy (31). The mTOR pathway drives anabolism when activated (32) and autophagy when blocked (33) while continually sensing nutrient levels and stress to confer specific control of those metabolic processes. Pharmacological inhibition of mTOR by chronic dosing with free rapamycin is used to prevent kidney transplant rejection (34). We previously demonstrated that only NP-encapsulated rapamycin, not free rapamycin, is capable of inducing immune tolerance when co-administered with Ag (16). Indeed, while a single dose of tNP containing rapamycin + OVA_{323} peptide inhibited OTII cell expansion, and enhanced the percentage of induced Foxp3$^+$ T cells, free rapamycin co-administered with OVA_{323} peptide had the opposite effect, namely enhancing expansion of OTII T cells and reducing the proportion of Foxp3$^+$ cells (18). We attribute these findings to tNPs being selectively taken up by APCs in the spleen following i.v. injection, whereas free rapamycin will biodistribute broadly and affect all cell types, including T cells (18).

Antigen-specific therapies for autoimmune diseases would reduce or eliminate the need for chronic immunosuppressant therapy (35). *Ex vivo* expansion of Tregs has been a significant clinical focus to treat autoimmune diseases (36–38); however, current techniques broadly expand polyclonal Tregs, not just Ag-specific cells, require personalized therapies that involve costly and complex manufacturing processes and carry the risk of expanding "unstable" Tregs that lose their regulatory function and can exacerbate disease. "Off the shelf" approaches include strategies to induce tolerogenic DC subsets *in vivo*, which can induce and expand Ag-specific Tregs (13, 39–42). Free peptide Ags may directly bind cell surface MHCII without processing and promote T cell anergy by presenting Ag in the absence of a co-stimulatory signal. Ag can also be targeted directly to APCs through antibody fusion proteins, such as anti-DEC205-PLP_{139} (43), or indirectly by targeting apoptotic red blood cells (44).

Synthetic NPs are an attractive strategy to target DCs and other APCs as these cells are very efficacious at capturing nanoparticulates. NPs have been shown to selectively traffic and accumulate in lymphoid tissues, such as lymph nodes following s.c. injection and the spleen and liver following i.v. injection, where they are selectively endocytosed by Ag-presenting cells (18, 45, 46).

Nanoparticles carrying peptides in the absence of an immunomodulator have been shown to be protective in EAE (47, 48) by targeting MARCO$^+$ macrophages or liver sinusoidal cells. A potential concern is that endocytosis of NPs containing Ag alone by activated APCs in an inflammatory microenvironment could present the Ag in a stimulatory context and inadvertently exacerbate disease. Moreover, this approach is limited to peptide Ags, as protein Ags encapsulated in particles are likely to be immunogenic. In our hands, NPs encapsulating peptide alone showed efficacy in EAE, but the protection was incomplete compared to tNP containing both rapamycin and peptide Ag (**Figure 4A**). Differences in NP size and surface properties may target different populations of cells (49).

Nanoparticles can be engineered to carry an immunomodulator payload that forces DCs to present Ag in a tolerogenic manner, even in a pro-inflammatory environment. Importantly, tNP containing Ag and rapamycin induced Treg populations that were maintained even after Ag challenge administered with a potent TLR agonist (**Figures 3C,D**). Moreover, tNPs were equally effective with both peptide and protein Ag to prevent antibody responses and have shown therapeutic efficacy in EAE following s.c. or i.v. administration [**Figure 4** (18)]. Therapeutic efficacy has also been shown in EAE with polyclonal expansion of Tregs from spleen and CNS after intranodal injection of microparticles encapsulating peptide and rapamycin (20). In addition to rapamycin, NPs delivering Ag with other immunomodulators, such as aryl hydrocarbon receptor ligands (50) and NFκB inhibitors (51), have also been shown to be effective in treating animal models of autoimmune disease.

While many promising preclinical approaches for Ag-specific immune tolerance have been described, few have reached clinical trials, and even fewer have shown evidence of efficacy in humans. There are several fundamental challenges in translating data from mice to humans; (1) selection of the appropriate Ag, (2) human genetic variation, and (3) achieving therapeutic efficacy in a well-established disease. Ag selection is simple in contrived animal models such as EAE where disease is induced by immunization with a specific Ag. In some diseases, candidate Ags have been identified; however, the specific pathogenic Ags may vary from patient to patient and evolve through epitope spreading. Peptide-specific approaches are relatively straightforward using inbred

strains of mice. However, MHC heterogeneity in humans poses a challenge to create a manageable set of peptides providing coverage for all major Ags for all patients. Finally, it is difficult to assess efficacy of therapeutic candidates in well-established disease in mice due to their short lifespan and the limitations of available models. Clinical trials with a cocktail of free peptide Ags (52) or peptides conjugated to autologous leukocytes (53) have reported initial biomarker evidence for Ag-specific immune modulation. Further studies are required to determine the level of efficacy and durability of therapy.

To mitigate some of the difficulties in establishing and evaluating immune tolerance induction in humans, we have chosen to focus initially on mitigation of antidrug antibodies (ADAs) to biologic therapies. The advantages from a drug development stand point are the elimination of Ag risk, as the Ag is the biologic drug, the ability to first assess tolerance in a prophylactic treatment setting and clear biomarker readout (i.e., ADAs). We have demonstrated the ability of NPs encapsulating rapamycin to inhibit the formation of ADAs against a variety of biologic drugs in preclinical studies, including coagulation factor VIII in hemophilia A mice (19), myozyme (or acid alpha glucosidase) in a murine model of Pompe disease (17), humira in a spontaneous model of inflammatory arthritis, and pegylated uricase enzyme in both Urox-deficient mice and non-human primates (16). The safety and efficacy of SEL-212, a combination of NP-encapsulated rapamycin co-administered with pegylated uricase, is currently being evaluated in an ongoing multidose Phase 2 clinical study in symptomatic gout patients with hyperuricemia (NCT02959918). Initial data from the single ascending dose Phase I clinical trial of SEL-212 (NCT02648269) showed dose-dependent inhibition of anti-uricase antibodies with a corresponding sustained reduction of serum uric acid (54). The ongoing Phase 2 study will assess the ability of tNPs to induce immune tolerance in patients.

ETHICS STATEMENT

Experiments involving animals were performed in compliance with state and federal regulations and approved by the Institutional Animal Care and Use Committee (IACUC) of Selecta Biosciences or Hooke Laboratories.

AUTHOR CONTRIBUTIONS

RL, RM, JF, and TK designed and executed the experiments. RM and TK reviewed and edited the manuscript. TV and TG formulated the nanoparticles. VC, PD, AS, and SA analyzed the small molecule and peptide content of the nanoparticles. RL performed OTII and 2W cell analysis assays. PK and JW performed IFNγ ELISpots. Hooke Laboratories performed the rEAE experiments. RL wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00281/full#supplementary-material.

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Resetting the T Cell Compartment in Autoimmune Diseases With Autologous Hematopoietic Stem Cell Transplantation: An Update

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Autologous hematopoietic stem cell transplantation (aHSCT) for autoimmune diseases has been applied for two decades as a treatment for refractory patients with progressive disease. The rationale behind aHSCT is that high-dose immunosuppression eliminates autoreactive T and B cells, thereby resetting the immune system. Post-aHSCT the cytotoxic $CD8^+$ T cells normalize *via* clonal expansion due to homeostatic proliferation within a few months. $CD4^+$ T cells recover primarily *via* thymopoiesis resulting in complete renewal of the T cell receptor (TCR) repertoire which requires years or never normalize completely. The increase in naïve T cells inducing immune tolerance, renewal of especially the regulatory TCR repertoire, and a less pro-inflammatory functional profile of the $CD4^+$ T cells seem essential for successful immune reconstitution inducing long-term remission. There is currently a knowledge gap regarding the immune response in tissue sites post-aHSCT, as well as disease-specific factors that may determine remission or relapse. Future studies on lymphocyte dynamics and function may pave the way for optimized conditioning regimens with a more individualized approach.

Keywords: autologous hematopoietic stem cell transplantation, autoimmune disease, T cell reconstitution, T cell receptor repertoire, regulatory T cell

INTRODUCTION

Autoimmune diseases in general are characterized by a loss of immune tolerance. This results in generation and activation of autoreactive T and B cells leading to inflammation and consequently tissue damage. Autologous hematopoietic stem cell transplantation (aHSCT) for autoimmune diseases aims to eliminate autoreactive T and B cells and regenerate an immune system which is self-tolerant (1). The first reported aHSCT for an autoimmune disease, systemic sclerosis (SSc), was in 1996 by Tamm et al. (2). Since then, aHSCT has also been increasingly applied for treatment-refractory patients with other progressive autoimmune diseases, including multiple sclerosis (MS), Crohn's disease, and juvenile idiopathic arthritis (JIA). Especially for SSc and MS, the clinical efficacy has been demonstrated in phase II and III clinical trials (3–6), whereas for Crohn's disease

Abbreviations: aHSCT, autologous hematopoietic stem cell transplantation; ATG, anti-thymocyte globulin; CLTA-4, cytotoxic T lymphocyte-associated protein 4; CYC, cyclophosphamide; DM type I, type I diabetes mellitus; GITR, glucocorticoid-induced TNFR-related protein; IL, interleukin; MS, multiple sclerosis; PD, pharmacodynamic; PD-1, programmed cell death-1 protein; PK, pharmacokinetic; SSc, systemic sclerosis; TCR, T cell receptor; Th, T helper; Treg, regulatory T-cell.

the clinical efficacy is much more variable (5, 7). In JIA, the need for transplantation has declined since the appearance of effective therapies (8). Though international clinical guidelines have acknowledged the value of aHSCT for selected patients with refractory disease, it generally remains a rescue therapy due to the invasive nature and risk of treatment-related mortality, as well as the continuous development of new therapeutics (9). Although aHSCT is not a curative treatment, long-term remission can be achieved, and patients with relapses are usually responsive to conventional treatment again (10).

Autologous HSCT treatment starts with mobilization of hematopoietic stem cells into the peripheral circulation using cyclophosphamide (CYC) and recombinant G-CSF. This enables apheresis of stem cells. $CD34^+$ selection, to purge the collected stem cells of T cells can be performed, although it is unclear whether it has clinical benefits. The next step is the immunoablative conditioning phase. In autoimmune disease high-dose CYC and occasionally, *in vivo* purging is performed by systemic administration of antibodies such as anti-thymocyte globulin (ATG) or rituximab. Finally, the hematopoietic stem cells are reinfused, which accelerates hematopoietic reconstitution (1). Exactly how aHSCT rewires a faulty immune system is still unknown. It is unclear which cells need to be depleted and which ones are important to keep. Additionally, not all cells are depleted by aHSCT and residing cells may pose a risk of early disease relapse. Understanding the quantitative and qualitative lymphocyte dynamics in relation to clinical outcome is therefore crucial to design less toxic but efficacious targeted therapies aimed at resetting the immune balance. Here, we will discuss the latest findings on T cell reconstitution post-aHSCT for autoimmune diseases, including T cell receptor (TCR) repertoire changes, and how these findings relate to clinical efficacy.

T CELL RECONSTITUTION

The innate immune system recovers within weeks post-aHSCT, in contrast to the reconstitution of the adaptive immune system which can take years [for recent in-depth reviews, see Ref. (10–14)].

Generally, the peripheral lymphocyte count and subsets at baseline, before aHSCT, are similar to healthy controls. Patients with MS that clinically responded to aHSCT in a phase II clinical trial, had higher memory $CD4^+$ and $CD8^+$ T cell counts pre-aHSCT compared with non-responders (15) and for SSc the same trend in higher complete $CD4^+$ and $CD8^+$ T cell counts pre-aHSCT for the responders was observed (16). This might suggest that patients with increased peripheral $CD4^+$ T cell activation pre-aHSCT may respond better to aHSCT.

$CD8^+$ T Cells

Following aHSCT, the lymphopenic environment drives lymphopenia-induced proliferation. Cytotoxic $CD8^+$ T cells are the first T cells to normalize and the ratio of naïve to memory $CD8^+$ T cells remains constant post-aHSCT. In patients with MS early expression (within 6 months) of the inhibitory molecule programmed cell death-1 protein (PD-1) on $CD8^+$ T cells correlated with a good clinical response post-aHSCT (17). Early PD-1 expression is likely protective by maintaining peripheral immune tolerance (18).

$CD4^+$ T Cells

$CD4^+$ T cell reconstitution is more dependent on thymopoiesis, and $CD4^+$ T cell numbers often requires years to normalize. As a consequence, there is an inversed CD4/CD8 T cell ratio. Furthermore, following aHSCT the residual naive T cells disappear, seemingly due to rapid maturation to effector memory T cells, resulting in decreased naive and increased effector memory T cells in the first 3 months post-aHSCT (17). Naive $CD4^+$ T cells increase upon thymic reactivation after several months, which results in a relative decrease of central memory $CD4^+$ T cells. The $CD4^+$ T cell compartment also reshapes post-aHSCT compared with baseline. Unfortunately, correlations with clinical outcomes were ambiguous. In a single arm study of 11 SSc patients receiving aHSCT, naive and memory $CD4^+$ T cells remained decreased during the follow-up period of 3 years (19). All patients had a good response to treatment. Decreased $CD4^+$ T cells after 9 months in both responders and non-responders was reported in another study in SSc patients (20). Faster increase of $CD4^+$ T cells in non-responders was seen in two studies in SSc patients (16, 20). Furthermore, while T helper (Th) 1 and 2 cells remain unaltered in frequency, Th17 cells diminish below baseline post-aHSCT, but normalize after 6 months. Functionally, post-aHSCT the Th1 and Th17 cells show a reduced interferon-γ and interleukin (IL)-17 response, respectively (12, 15, 17, 21–25). Above mentioned changes are also observed on transcriptional level, with the transcriptional program of $CD8^+$ T cells normalizing within 2 years post-aHSCT, whereas the transcriptional program of $CD4^+$ T cells significantly changes post-aHSCT but does not normalize (26).

Regulatory T Cells

Data regarding regulatory T cells is contradicting, with most studies observing an increase of regulatory T cells following transplantation, usually temporarily, although in some studies no changes or decreased relative frequencies are found (12, 15, 17, 21–25, 27, 28). Regulatory T cells of clinically responding SSc patients had increased levels of the immune regulators cytotoxic T lymphocyte-associated protein 4 (CTLA-4), a negative regulator of T cell function, and glucocorticoid-induced TNFR-related protein compared with non-responders (28). Upregulation of CTLA-4 on regulatory T cells post-aHSCT in both MS and type 1 diabetes mellitus (DM type 1) patients, and increased regulatory T cell functional marker expression such as IL-10 and transforming growth factor-β in DM type 1 patients post-aHSCT has also been observed, but in these cases without positive association with the clinical outcome (17, 24). In mice, graft-derived regulatory T cells were shown to have superior suppressive function compared with regulatory T cells that survived conditioning (29). In conclusion, renewal of the regulatory T cell compartment seems essential for long-term restoration of immune homeostasis, with qualitative changes of regulatory T cells having a more profound impact than quantitative changes, although this may differ per autoimmune disease.

TCR REPERTOIRE POST-TRANSPLANTATION

Initially, lymphopenia-induced proliferation following transplantation results in clonal expansion of residual T cells. Restoration of a fully competent T cell compartment with a diverse TCR repertoire is depending on thymic output that starts 3–6 months following HSCT. In SSc, the total TCR repertoire diversifies post-aHSCT, irrespective of the clinical outcome (16), although with a trend to a more diverse repertoire in patients without a relapse (20). However, a recent study in SSc patients did show increased TCR diversity at 1 year post-aHSCT in responders compared with non-responders. Furthermore, low overlap in TCRβ (CDR3) clonotype before and after transplantation was observed in responders (25% overlap) but not in non-responders (60% overlap) (28). This suggests that renewal of the TCR repertoire is important for the re-establishment of immune homeostasis. The difference in TCR repertoire outcome might be explained by use of ATG in the latter study, whereas this was not used or optional in addition to CYC in the first two studies (16, 20, 28).

In MS patients renewal of the $CD4^+$ T cell repertoire was observed, with the dominant $CD4^+$ T cell clones present at baseline not detectable following transplantation. In contrast, dominant $CD8^+$ T cell clones detectable pre-aHSCT remained present post-aHSCT, and were clonally expanded (21, 22). Especially early on, non-responding MS patients had a less diverse TCR repertoire than patients without relapse, though this difference had disappeared at 1 year post-transplantation (22). In JIA and juvenile dermatomyositis patients, the $CD4^+$ T cell and FOXP3 regulatory TCR repertoire were studied separately. Compared with baseline, responders had a far more diverse regulatory T cell repertoire post-aHSCT. In contrast, patients that experienced a relapse showed an even more oligoclonal repertoire compared with baseline. The $CD4^+$ TCR repertoire also diversified in responders, but less pronounced than in the regulatory T cell compartment. Similarly to MS patients, no overlapping $CD4^+$ TCRs were found pre- and post-aHSCT (29).

In conclusion, even though these studies were performed in patients with different autoimmune diseases there is a consistent pattern. The TCR repertoire of $CD8^+$ T cells remains relatively oligoclonal, and the same dominant TCR clones can be observed pre- and post-aHSCT without noticeable clinical consequences. This suggests these $CD8^+$ T cells are not self-reactive, or are but unable to induce disease activity following transplantation. The $CD4^+$ T cells are characterized by a complete renewal of the TCR repertoire, and especially an increased TCR diversity for regulatory T cells seems important for a successful induction of remission post-transplantation.

TISSUE T CELL DEPLETION

Almost all data regarding the immune reconstitution following aHSCT are based on cells in circulation. However, the aberrant inflammation in autoimmune diseases is primarily located in tissue sites. Recently, it was shown that in intestinal tissue of patients with refractory Crohn's disease the TCR repertoire diversifies post-aHSCT. Approximately 20% of TCR sequences were detected pre- and 6 months to 1 year post-aHSCT, demonstrating a vast resetting of the TCR repertoire (30). The clinical impact of a local expanding TCR repertoire has not been established, but these preliminary data show a local immune response occurs following transplantation. In SSc, the TCR repertoire in affected skin is strongly oligoclonal (31). Following aHSCT, there is a reduction of skin fibrosis in responding patients (follow-up time of approximately 6 years) (16), but whether this is associated with the generation of a polyclonal TCR repertoire in the skin is unknown.

Of note, the extent of tissue penetration of ATG and other immunosuppressive agents is unclear. In mice, depletion of T cells by ATG is less efficient in peripheral lymphoid organs compared with the blood (32). It is not unlikely that tissue resident memory T cells, antigen-experienced T cells permanently residing in barrier tissues (33), are not fully depleted. Is there a need for (more) profound tissue immune cell depletion, or does maintenance of the tissue immune cells protect patients after conditioning to not succumb to infection? Future studies may shed light on the extend of tissue lymphocyte depletion following conditioning and whether this affects clinical outcome.

THE CONDITIONING REGIMEN AND T CELL RECONSTITUTION

The conditioning regimen is an important factor in immune reconstitution post-aHSCT. In a study in 13 MS patients, using CYC and ATG for conditioning, the CD4/CD8 ratio remained lower than in healthy controls, and naïve $CD4^+$ T cells normalized 2 years post-aHSCT (23). In contrast, in another study in MS patients with CYC and total body irradiation employed for conditioning the CD4/CD8 ratio normalized at 2 years post-aHSCT, and an overshoot of naïve $CD4^+$ T cells was observed (21). In the latter study, all patients remained in remission during the follow-up of 2–3 years, whereas in the first study 30% experienced a relapse within 3 years post-aHSCT. However, these differences might also be partially explained by differential patient selection. ATG is often implemented in the conditioning regimen (13) and can severely affect immune reconstitution. As a polyclonal antibody it targets a plethora of immune cells and induces both direct and indirect cytotoxicity. Interestingly, a recent study on ATG exposure and clinical outcome in an allogeneic HSCT setting suggests that ATG dosing should be based on lymphocyte count rather than body-weight (34). Currently, ATG dosing in aHSCT patients is not individualized and monitoring both ATG levels and lymphocytes counts could be a helpful addition to define the optimal dosing strategy.

Long-term impact of an incomplete T (and B) cell reconstitution is unknown. While reliable data regarding incomplete immunological reconstitution post-HSCT is lacking, there are indications that this is not associated with long-term morbidity. A for instance, a study assessed outcomes in rheumatoid arthritis patients that received Alemtuzumab, an anti-CD52 antibody targeting primarily T and B cells. Follow-up for 20–25 years shows an incomplete reconstitution of T and B cells, especially central memory $CD4^+$ and $CD8^+$ T cells and naïve B cell numbers

remain reduced. The incomplete reconstitution, however, was not associated with differences in mortality, morbidity, or vaccine response when compared with rheumatoid arthritis patients that have received standard care (35). These data emphasize the need to understand the normal immune reconstitution to establish optimal conditioning regimens, as well as to compare different autoimmune diseases to identify disease-specific factors that might predict long-term remission or a relapse.

CLINICAL HETEROGENEITY INFLUENCING IMMUNE RECONSTITUTION

Unfortunately, interpretation of changes in T cell compartment after aHSCT is complicated by heterogeneity in type of autoimmune disease, conditioning regimens, patient selection, graft manipulation, post-transplantation treatment, and age- or treatment-dependent thymic involution. For instance, in some studies patients have severe immune dysregulation resulting in severe and/or progressive, treatment resistant disease. These patients have often received most available immunosuppressive therapies including biologicals before aHSCT, while in other settings (e.g., SSc) aHSCT is performed relatively early in disease. Another possible confounding factor is graft manipulation by selection of $CD34^+$ stem cells. The influence of this selection on immune reconstitution is reported in several studies, and although broadly used in treatments, there is conflicting evidence of its benefits (36–38). Finally, the numbers of patients included in clinical studies generally are too small to compare outcome between responders and non-responders. Despite all confounding factors, standardized (extensive) phenotypic characterization of T cell reconstitution would be an important step forward in elucidating T cell reconstitution and relating it to treatment regimens and outcome.

CONCLUSION AND FUTURE PERSPECTIVES

In the past 20 years, aHSCT has been applied for severe refractory autoimmune diseases but a comprehensive understanding of the immune reconstitution and the link with clinical outcome is still missing. Data suggest that the increase in naïve T cells, renewal of especially the regulatory TCR repertoire, and a less pro-inflammatory functional profile of the $CD4^+$ T cells are essential for successful immune reconstitution and the induction of long-term remission. Future studies on lymphocyte dynamics and function may pave the way for optimized conditioning regimens with a more individualized approach.

Important outstanding questions regarding the immune reconstitution following transplantation include: (1) What is the most effective conditioning regime for each autoimmune disease? This possibly depends on which immune cells are important in the pathogenesis, and the tissue(s) where the disease manifests, thus differ per autoimmune disease. (2) Is a personalized approach to the conditioning regime, depending on the immune status pre-aHSCT, needed to improve the clinical outcome? The recent observation that in a hematologic malignancy setting ATG dosing should be based on lymphocyte count may also apply to autoimmune settings. Extensive monitoring of immune depletion/reconstitution in combination with pharmacokinetic/pharmacodynamic modeling can contribute to the development of pre- and post-transplantation precision treatment. (3) What is the local tissue effect of the conditioning regimen? It is not clear to which extent for example ATG penetrates all tissues to deplete tissue T cells, and it is unclear if and to what extent residual tissue T cells contribute to relapses of disease. Together, future studies may shed light on the fine balance between effectively destroying and renewing the immune system, but with limited toxicity.

AUTHOR CONTRIBUTIONS

LL, JS, FR-B, JL, and FW have discussed, written, and edited the manuscript.

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MINI REVIEW
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Further Advances in Cancer Immunotherapy: Going Beyond Checkpoint Blockade

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Significant advances have been made to identify effective therapies that either restore or generate *de novo* a patient's immune response to cancer, so-called immunotherapy or immuno-oncology (IO) therapies. Some tumors overcome immune surveillance by promoting mechanisms to evade or suppress the immune system. This conference report highlights the clinical promise and current challenges of IO therapy, including the use of immune-checkpoint antagonist monoclonal antibodies. Furthermore, this report investigates advances in preclinical modeling of cancer immunobiology and how this is helping our understanding of which patients will receive clinical benefits from current immune-checkpoint treatment. Looking to the future, the report looks at emerging IO approaches, which aim to specifically target the tumor microenvironment. This includes the use of toll-like receptors (TLRs) agonists that link the activation of innate immune, cells to the priming of T cells and an adaptive memory anti-tumor immune response through to the reversal of local immunosuppression using adenosinergic and indoleamine 2,3-dioxygenase (IDO) inhibitors.

Keywords: immuno-oncology, toll-like receptors, oncology, Immunotherapy, tumour microenvironment

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INTRODUCTION

On 27th–30th June 2017, the 4th International Therapeutic Tolerance Workshop: First-in-Human Data was hosted by Newcastle University Institute of Cellular Medicine, UK. Session 2, Breaking Tolerance in Cancer, was Chaired by Andrew L. Mellor (Newcastle University). In this session, Robert W. Wilkinson (MedImmune Ltd., Cambridge, UK) gave a talk entitled "Immunological targets to combat Cancer," a synopsis of his talk is described here.

The most clinically advanced immuno-oncology (IO) therapies are monoclonal antibodies (mAbs) that modulate the activity of T cells, by blocking inhibitory pathways that act as immunological checkpoints. The promising anti-tumor activity of mAbs targeting the immune-checkpoint proteins, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), and the PD-1 ligand (PD-L1), led to regulatory approvals of these agents for the treatment of a variety of malignancies. The first of these drugs to be approved in 2011 was the anti-CTLA-4 antibody Ipilimumab (Yervoy®, Bristol-Myers Squibb) for the treatment of unresectable or metastatic melanoma (1). Subsequently, the anti-PD-1 mAbs, nivolumab (Opdivo®, Bristol-Myers Squibb) and pembrolizumab (Keytruda®, Merck & Co.) have gained regulatory approvals for the treatment of different cancers. More recently, clinical data with anti-PD-L1 antibody, durvalumab (Imfinzi®, MEDI4736), led to the approval for this drug in 2017 for the treatment of previously treated patients with locally advanced or metastatic urothelial carcinoma (2); further highlighting the potential of therapies that target immune evasion pathways.

CURRENT UNDERSTANDING OF RESPONSES TO IO THERAPY

Immuno-oncology therapy has created a paradigm shift in the treatment of some advanced-stage cancers, where it is now the standard of care. However, while these agents can produce long-lasting responses in some cancer patients, the response rate as monotherapies tend to be low. A key goal now is to develop a deeper understanding of why some patients respond to IO therapies while others exhibit pre-existing immunological resistance, and may therefore be non-responsive to treatment, or become refractory ("acquire" resistance) to IO therapy with time. The immunological contexture of a patients' tumor, the so-called "Immunoscore," has been shown to be prognostic for outcome in several malignancies, including melanoma and colorectal cancer (3–5). These histological studies advance our understanding of how the immunological microenvironment of the tumor may impact patient outcome. Indeed, based on the wealth of data, there is now an argument for inclusion of immunoscore and immunoprofiling in standard disease staging, which is currently based on anatomical site, histopathology, and the characterization of defined genetic features, and by the incidence of local/distal metastasis. At a very basic level, tumors can be broadly described as "hot, cold, or immunosuppressive," as determined by their profile of immune infiltrates. Tumors defined as "hot" are those with pre-existing tumor-infiltrating $CD8^+$ cytotoxic T cells and natural killer (NK) cells. By contrast, "cold" tumors are poorly infiltrated by T cells, and "immunosuppressive" tumors, harbored high proportions of suppressive myeloid cells, such as myeloid-derived suppressor cells. Tumeh et al. recently reported a greater tumor infiltration with $CD8^+$ cytotoxic T cells correlated with clinical responses to mAb's targeting an immune checkpoint (6). Furthermore, Higgs et al. have showed high tumoural IFNγ mRNA and PD-L1 protein expression associates with response to durvalumab (anti-PD-L1 blocking mAb) monotherapy in NSCLC patients (7). Going forward, it is likely that a range of determinates and biomarkers will be incorporated to fully understand and predict responses to IO therapy, including the cancer patient's somatic mutations and burden, tumor microenvironment (TME), and immune system characteristics.

RECENT LEARNING FROM PRECLINICAL MOUSE MODELS

To continue to advance the IO field, it will be important to use well-characterized and translationally relevant preclinical models. Currently, most IO therapies are tested in syngeneic transplanted mouse models of cancer, which means that the mice share a similar genetic background with the transplanted cells. The models are created by implanting a cancer cell line derived from a spontaneous, carcinogen-induced, or genetically engineered mouse tumor into an immunocompetent wild-type recipient. A survey of current literature points toward a lack of information about syngeneic tumor models, which potentially limits how well researchers can connect an IO therapy agent's effects to its predicated impact in patients. MedImmune recently reported that they have built a large panel of murine syngeneic tumor models and profiled them in detail using readouts including copy number variation, exome mutations, transcriptomics, cytokine levels, and immune cell profiles within tumors and lymphoid organs (8). They went on to select six commonly used syngeneic mouse models and measured responses to anti-CTLA-4 or anti-PD-1 mAbs. While there was heterogeneity among the models they found, the strongest determinants of checkpoint inhibitor responses were the profiles of immune cells within the tumors, which broadly determined whether a model was "hot, cold, or immunosuppressive." The "hot" cancer models (including, CT26 colorectal and RENCA kidney cancer models) were most responsive to anti-CTLA-4 and anti-PD-1 mAbs, a result that aligns with clinical evidence. Having a deeper understanding of the phenotype of preclinical models and how they relate to their human counterparts is helping to select optimal models to test preclinical hypotheses. For instance, the "cold" and "immunosuppressive" models will be valuable resources for groups developing IO therapies to overcome immunosuppression in the TME, such as cancer vaccines.

GOING BEYOND IMMUNE-CHECKPOINT BLOCKADE

In addition to immune-checkpoint mAbs, there are a number of novel IO therapeutic approaches being developed to treat cancer patients. Toll-like receptors (TLRs) are expressed on a broad range of myeloid cells and function to recognize conserved pathogen-associated molecular patterns. Signaling through TLRs leads to the activation of antigen-presenting cells and to expression of inflammatory cytokines. MEDI9197 is a potent TLR7 and TLR8 agonist and induces pro-inflammatory cytokines through activation of myeloid and lymphoid cells (**Figure 1**). Preclinical mouse studies indicate that intratumoural injection of MEDI9197 induces a local inflammatory response, characterized by upregulation of genes associated with the activation of innate and adaptive immunity in the tumor (9). Importantly, in mouse syngeneic models that respond poorly to mAbs targeting either PD-L1 or CTLA-4, combination with MEDI9197 significantly improved anti-tumor activity when compared to either monotherapy alone. MEDI9197 is currently being evaluated in human clinical trials as a monotherapy in subjects with solid tumors and in combination with durvalumab and/or palliative radiation in subjects with solid tumors (NCT02556463). Preliminary data in patients indicate that MEDI9197 induces pharmacodynamic effects consistent with its expected mechanism of action (10).

Other IO therapeutic approaches aimed at reversing immunosuppression in the TME include blocking generation of the immune suppressive factor adenosine and its associated pathway. CD73 is an ectoenzyme that generates adenosine *via* adenosine monophosphate (AMP) hydrolysis. MEDI9447 is an example of an anti-CD73 mAb capable of relieving AMP-mediated lymphocyte suppression *in vitro* and inhibition of mouse syngeneic tumor growth *in vivo* (11) and is currently being evaluated in the clinic

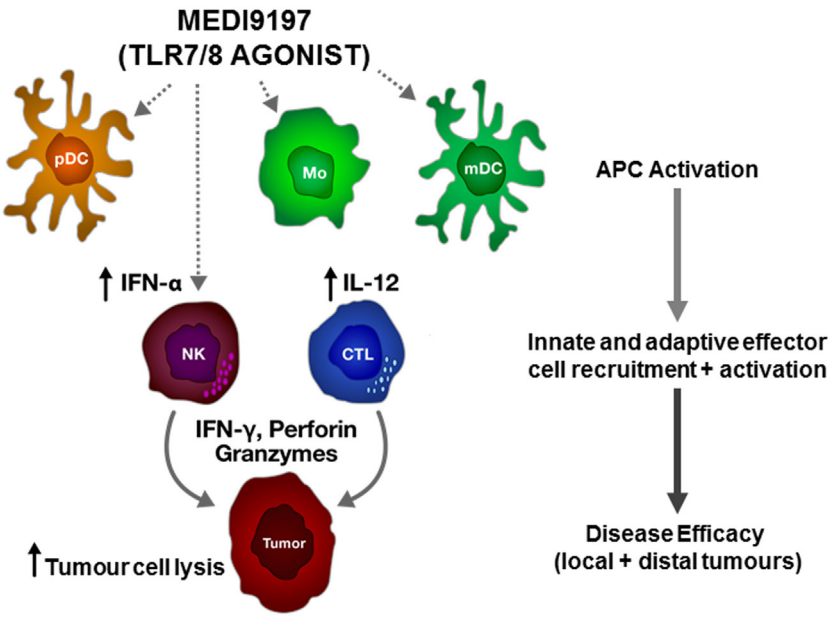

FIGURE 1 | Proposed mechanism of action of MEDI9197 following intratumoural administration. MEDI9197 activates toll-like receptor (TLR) 7 and 8 expressing cells, such as plasmacytoid dendritic cells (pDC), myeloid dendritic cells (mDC), and monocytes (Mo), which release type I interferons and pro-inflammatory cytokines, such as interleukin-12 (IL-12); leading to recruitment and activation of effector cells, including natural killer (NK) cells and cytotoxic T lymphocytes (CTL) to the tumor. The activated effector cells release interferon gamma (IFN-γ), perforin, and granzymes to kill the tumor cells.

(NCT02503774). Interestingly, preclinical studies targeting the adenosinergic pathway by co-inhibition of CD73 and A2A adenosine receptor signaling improves anti-tumor immune responses, including limiting metastasis (12). Another metabolic pathway implicated in immunosuppression is indoleamine 2,3-dioxygenase (IDO), which promotes tolerance by catabolizing the amino acid tryptophan and other indole compounds (13). Indeed, preclinical studies targeting the IDO pathway have gained much attention for their clinical potential, as an immune-checkpoint inhibitor, in overcoming tumor-induced immunosuppression (14).

SUMMARY

Significant advances have taken place in our understanding of the interplay between cancer and the immune system, including therapeutic intervention using IO therapies. Our understanding of which patients will benefit from IO therapy continues to evolve, alongside our understanding of how best to modulate the anti-cancer immune response through combinations with other IO therapies and/or standard of care treatments.

AUTHOR NOTE

4th International Therapeutic Tolerance Workshop, 27th–30th June 2017 at Newcastle University, UK; Session 2—Breaking Tolerance in Cancer. Chaired by Prof. Andrew Mellor, Newcastle University.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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